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Twenty-first century molecular methods for analyzing antimicrobial resistance in surface waters to support One Health assessments

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Abstract

Antimicrobial resistance (AMR) in the environment is a growing global health concern, especially the dissemination of AMR into surface waters due to human and agricultural inputs. Within recent years, research has focused on trying to understand the impact of AMR in surface waters on human, agricultural and ecological health (One Health). While surface water quality assessments and surveillance of AMR have historically utilized culture-based methods, culturing bacteria has limitations due to difficulty in isolating environmental bacteria and the need for a priori information about the bacteria for selective isolation. The use of molecular techniques to analyze AMR at the genetic level has helped to overcome the difficulties with culture-based techniques since they do not require advance knowledge of the bacterial population and can analyze uncultivable environmental bacteria. The aim of this review is to provide an overview of common contemporary molecular methods available for analyzing AMR in surface waters, which include high throughput real-time polymerase chain reaction (HT-qPCR), metagenomics, and whole genome sequencing. This review will also feature how these methods may provide information on human and animal health risks. HT-qPCR works at the nanoliter scale, requires only a small amount of DNA, and can analyze numerous gene targets simultaneously, but may lack in analytical sensitivity and the ability to optimize individual assays compared to conventional qPCR. Metagenomics offers more detailed genomic information and taxonomic resolution than PCR by sequencing all the microbial genomes within a sample. Its open format allows for the discovery of new antibiotic resistance genes; however, the quantity of DNA necessary for this technique can be a limiting factor for surface water samples that typically have low numbers of bacteria per sample volume. Whole genome sequencing provides the complete genomic profile of a single environmental isolate and can identify all genetic elements that may confer AMR. However, a

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main disadvantage of this technique is that it only provides information about one bacterial isolate and is challenging to utilize for community analysis. While these contemporary techniques can quickly provide a vast array of information about AMR in surface waters, one technique does not fully characterize AMR nor its potential risks to human, animal, or ecological health. Rather, a combination of techniques (including both molecular- and culture-based) are necessary to fully understand AMR in surface waters from a One Health perspective.

Keywords

molecular methods; surface waters; antimicrobial resistance; high throughput quantitative PCR; metagenomics; whole genome sequencing; One Health; environmental resistome; antibiotic resistance genes; mobile genetic elements

1. Introduction

Antimicrobial resistance (AMR) is not a new development within the world of microorganisms; it is naturally found in the environment, even in relatively pristine settings (Rothrock et al., 2016; van Goethem et al., 2018). However, with the advent of antibiotic usage to treat bacterial infections and the ensuing overuse of antimicrobial compounds in medicine and agriculture, AMR is on the rise in clinical health settings (O'Neill, 2016). In fact, the U.S. Centers for Disease Control and Prevention (CDC) has estimated that more than 70% of bacteria that lead to infections are resistant to at least one of the antibiotics commonly used for treatment (IDSA, 2004). Concurrently, AMR is increasing in the environment, primarily due to release of wastewater treatment plant effluent and agricultural runoff into surface waterways (Berkner et al., 2014). Of specific concern is the presence of AMR in surface waters, which poses a direct health threat to human populations through drinking water, recreational use, and irrigation of cropped lands. Furthermore, the environment plays a major role in the development, maintenance, and cycling of AMR.

Over the last decade, research has focused on trying to understand the importance of AMR in surface waters and how it may impact human, animal, and ecological health, a One Health approach to AMR (Franklin et al., 2016; Banerji et al., 2019; White and Hughes, 2019). While AMR can be well characterized in clinical health due to well-established standardized methods, these methods are not always useful for environmental samples due to the limited ability for environmental bacteria to grow under laboratory conditions (Roszak and Colwell, 1987). Studies of AMR in the environment are further confounded by intrinsic (background) environmental resistance, the large diversity of relevant bacterial and AMR targets, low concentrations of these targets, and the potential presence of previously uncharacterized targets or target combinations (Vaz-Moreira et al., 2013). Therefore, analyzing AMR in environmental samples, including surface water, remains a challenging problem. In addition, the ability to link AMR from the environment to humans and animals and vice versa is limited and hinders a One Health assessment of AMR.

Traditional water quality monitoring and assessments include culture-dependent analysis to screen specific indicator microbial species, such as Escherichia coli (E. coli), fecal coliforms, and pathogens. These culture-dependent techniques are still used to date

throughout the world. While standardized methods are not in place for analyzing AMR within surface waters, culture-based work has been the foundation of international surveillance efforts to monitor AMR (McLain et al., 2016). The WHO Global Action Plan for AMR developed a guide for AMR surveillance and research and recommended extended-spectrum beta-lactamase producing (ESBL)-E. coli as a potential candidate for AMR monitoring (WHO, 2015). This bacterium is readily detected using established methods that allows for comparisons of environmental samples with food and medical community samples. However, a major issue with utilizing culture-based methods is that non-pathogenic, environmental bacteria can represent important resistance reservoirs that are not easily cultured and thus avoid detection. Furthermore, these methods require a priori selection of study targets, which confines their scope to limited types of bacteria and AMR.

To overcome the difficulties in cultivating environmental bacteria, AMR research in surface waters can utilize molecular methods that analyze the DNA of a bacterial population for antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs) associated with AMR (e.g., plasmids, transposons, integrons, etc.) (Harbottle et al., 2006). However, knowing that a gene is present is insufficient for analyzing human health risks, as the presence of a gene does not necessarily mean that it is expressed within that population or that it is possessed by a human microbial pathogen. Various molecular approaches, e.g. polymerase chain reaction (PCR), metagenomics, and whole genome sequencing (WGS), also differ in their ability to characterize individual bacteria versus entire communities, targeted versus non-targeted natures, and ability to produce quantitative results. Consequently, analytical methods and combinations thereof must be carefully selected in accordance with specific research objectives, e.g., characterization of contamination sources, assessment of surface water exposures, determination of potential health risks (direct or indirect), etc.

The purpose of this review is to provide an overview of the three most common contemporary molecular methods that are available for analyzing AMR in surface waters: high throughput real time PCR (HT-qPCR), metagenomics, and WGS. While other recent reviews have overviewed these molecular methods and/or compared and contrasted a subset of these molecular techniques for AMR analysis in the environment (Nowrotek et al., 2019; Waseem et al., 2019; Gupta et al., 2020; Ishii, 2020), this review is specifically focused on surface waters and how these contemporary molecular techniques can be employed to analyze AMR in various surface water environments, characterize health risks, and move toward a One Health assessment of AMR. Each molecular method section will highlight the main advantages and disadvantages of each technique (Table 1) and recent studies as examples of how these techniques can be employed to analyze AMR in diverse types of surface water environments. This review will also discuss how these molecular methods can provide practical information about direct and indirect health risks in surface waters, while also referencing the applicability of older technologies in combination with these newer methods. Finally, how the selection of analytical methods could answer a variety of research questions about AMR in surface water environments will be addressed as well as how different methods could provide diverse information about human health risks and be able to offer a One Health assessment of AMR.

2. High Throughput Real-Time PCR

Conventional PCR is recognized as one of the most important scientific advances in the 20th century that revolutionized molecular biology by providing a quick and easy method to isolate a gene of interest (e.g., ARG) in a test tube and create unlimited copies of that gene within hours (Powledge, 2004). With the advent of quantitative or real-time PCR (qPCR), gene targets could then be quantified, and this technology has become a standard practice for analyzing ARGs in surface waters. However, a comprehensive analysis of AMR via conventional PCR or qPCR is a tedious and time-consuming process. While these molecular techniques are suitable for small-scale studies analyzing a limited number of ARGs and other genes associated with AMR, this technology becomes cost ineffective and burdensome when a large suite of genetic targets needs to be analyzed in order to provide a thorough analysis of AMR within a surface water system. HT-qPCR is a newer technology that offers a rapid and convenient method for both evaluating many ARGs simultaneously and performing numerous assays per sample while using a smaller sample volume compared to conventional PCR.

HT-qPCR works at the nanoliter scale and only requires a limited amount of DNA per sample, which is a major advantage when working with samples from aquatic environments where the amount of DNA per sample volume is typically lower than other environmental matrices (e.g., soil, sediment, etc.) (Sander and Kalff, 1993). Frequently, the volume of sample and quantity of DNA per sample volume can be limiting factors for conventional PCR. Therefore, HT-qPCR creates an opportunity to analyze surface water samples containing lower quantities of DNA while also analyzing for a suite of AMR gene targets simultaneously. Since all gene targets are analyzed in one run, consistency may be increased due to fewer batches, which lessens the chance of pipetting errors and/or contamination. In addition to its advantages, HT-qPCR does have some disadvantages, such as the inability to optimize the assays for each gene of interest during a single run because all assays must utilize the same PCR cycling conditions. However, the use of probes instead of Sybr Green or other fluorescent detection methods does improve the specificity of HT-qPCR. Another disadvantage is the limited sample volumes that may lead to higher limits of detection (LOD) and limits of quantification (LOQ), which are of concern for detecting low level environmental contaminants like ARGs. Furthermore, reactions at a nanoliter scale are difficult or even impossible to recover to sequence the amplified products, which is frequently possible in conventional PCR and qPCR. Finally, another major disadvantage of this approach (and any type of PCR) is the need to know the sequences of the genes of interest ahead of time for appropriate selection and/or design of primers and probes, unlike metagenomic work that does not require a priori knowledge. On the other hand, HT-qPCR offers certain advantages over metagenomic sequencing by having lower limits of detection with reported detection limits of 10–4 ARGs/16S rRNA gene (Muziasari et al., 2016 & Muziasari et al., 2017) and the ability to be fully quantitative.

Currently, three major platforms for performing HT-qPCR have been used predominately in studies profiling ARGs in surface waters: Takara Smartchip (formerly Wafergen), Applied Biosystems OpenArray, and BioMark Dynamic Array (Waseem et al., 2019). Each of these

three platforms has distinctive high throughput capabilities and utilize different volumes for assay reactions.

2.1 SmartChip Real-Time PCR

The SmartChip HT-qPCR system has a 5184 reaction well platform providing a 54× high throughput capacity (e.g., 54 multiples of the 96 well format) and allows for flexible assay and sample formats, which is ideal for analyzing a large suite of AMR-associated genes. A recent review by Waseem et al., 2019 looked at 51 studies over the previous 7 years that evaluated ARG abundance and found that SmartChip was more frequently used than other HT-qPCR platforms for the purpose of profiling ARGs, especially in environmental samples. The first research paper published using SmartChip was in 2014, and of the studies analyzed in that review, SmartChip was used 75% of the time. SmartChip may be more frequently utilized for studying environmental AMR because it allows for a customized number of sample versus assay formats, which can be selected based on the design of each experiment. Most studies analyzing for AMR-associated targets have utilized the 18 versus 296 and 12 versus 384 Sample-Assay formats. Because SmartChip utilizes a larger (100nL) reaction volume, it also has improved analytical sensitivity when compared to other microfluidic chips like Applied Biosystems OpenArray and BioMark Dynamic Array that use reaction volumes of 33 nL and 7–9 nL, respectively (Lamas et al., 2016).

Numerous research studies focusing on AMR in surface waters have utilized the SmartChip technology to analyze suites of ARGs and genes related to AMR. A recent study investigated the patterns of ARGs in 42 natural waterbodies, specifically reservoirs and lakes, throughout China (Liu et al., 2018). The SmartChip HT-qPCR approach was used to target 285 ARGs (covering major classes of antibiotic resistance), eight transposase genes, one universal class I integron-integrase gene (intI1), and one clinical class 1 integron-integrase gene (cintI1). The SmartChip detected 167 unique ARGs and 9 MGEs. A spatial comparison showed that the relative abundances of ARGs (ARGs normalized to 16S rRNA gene copy numbers) were elevated in the south-central waters compared to the northern waters. A significant correlation was found between the relative abundance of ARGs and human activities. Notably, when looking at the absolute abundances of ARGs (gene copies/L not normalized to 16S rRNA), no differences were found between south-central and northern waterbodies. These differing results for relative versus absolute abundances in gene copy numbers suggest that normalization of ARG data should be carefully considered based on the study design. Overall, the patterns of ARG composition and spatial distribution found in this study were significantly affected by anthropogenic (highest explanation factor), spatial, and physico-chemical factors.

A watershed scale study of a subtropical river-reservoir system used the high-throughput capacity of the SmartChip system to analyze the ARG variations by season and environmental gradient (Chen et al., 2019). The river-reservoir system had two primary tributaries that received heavy non-point source pollution from untreated wastewater discharge, livestock wastewater, and agricultural runoff. The SmartChip PCR was used to profile 285 ARGs conferring resistance to the major classes of antibiotics, eight transposase genes, cintI1, and intI1. Of these gene targets, 242 ARGs and 9 MGEs were detected with

significantly higher copy numbers in the river water system than in the reservoir system. While absolute abundance of ARGs and MGEs increased in the springtime, the relative abundance of ARGs and MGEs (copies of ARG/MGE per 16S rRNA) did not vary by season. Therefore, the increased absolute abundance of ARGs in the springtime was most likely due to increased bacterial numbers. These results again demonstrate that the use of relative (normalized) abundance versus absolute abundances for ARG data should be carefully selected based on the design of the study. For example, relative ARG data would be useful to differentiate when changes in ARG numbers within a bacterial community are due to factors other than alterations in bacterial numbers. However, irrespective of absolute or relative ARG abundances, this study did determine that 204 ARGs were shared between the river system and the reservoir system, indicating that river system input may be responsible for the presence of ARGs in the reservoir system.

More recently, the presence of AMR in a semi-urban subtropical riverine ecosystem was analyzed by season under low and high human impacts (Peng et al., 2019) and over a 5 year time period (Peng et al., 2020). Using the SmartChip HT-qPCR system, 285 ARGs, eight transposase genes, and two integrase genes were quantified providing an analysis of spatial and seasonal distribution patterns of AMR associated genes over time. While 245 ARGs were detected in samples, only 19 ARG subtypes contributed significantly to total abundance (>70%) with the dominant subtypes conferring resistance to aminoglycosides, chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB), sulfonamide, tetracycline, and trimethoprim or multidrug resistance. Similar to the findings of Chen et al. (2019), the spatial distribution of these ARGs and MGEs from a pristine upstream area to downstream urbanized areas played a larger role in the richness and abundance of these genes in the riverine system than seasonal variation with highest diversity and abundance in downstream samples. In addition, only tetracycline resistance genes were found to be closely linked with MGEs. These studies determined that both human-dominated environments and MGEs can play important roles in shaping the ARG profiles and dynamics within a riverine system.

Animal agriculture can impact AMR in surface waters and was the focus of a catchment-scale study of ARGs and antibiotic resistant bacteria (ARB) from a watershed in central Iowa (Neher et al., 2020). The watershed was delineated into different catchments impacted by different types of manure application practices and urban influences. Unlike most other studies that have utilized the SmartChip system to analyze a large suite of ARGs, this study employed HT-qPCR to analyze a larger set of 144 samples and a smaller set of 12 ARGs. The ARG targets included those for aminoglycosides (aadA2), macrolides (ermB and ermF), pleuromutilin (vgaA and vgaB), sulfonamides (sul1 and sul2), and tetracyclines (tetA, tetM, tetO, and tetW). Standard curves were also run for four out of the twelve gene targets (16S rRNA, ermB, ermF, and tetM). Due to the ability to analyze a larger number of samples within the watershed, this study determined that manure applications had a greater impact on AMR within the catchments compared to urban influences. However, both manure and urban inputs significantly contributed to AMR in the watershed compared to the control site.

Several studies have used the SmartChip system to analyze AMR in rivers receiving effluents from treatment plants, which are known contributors to AMR contamination in

surface waters. One study analyzed how the spatial ecology of a wastewater treatment plant (WWTP) system receiving waste from both hospitals and communities may define the ARGs in surface waters downstream of treated effluent inputs (Quintela-Baluja et al., 2019. To determine the effects of the wastewater on downstream environmental resistomes (e.g., all ARGs in an environmental community), this study analyzed sewage inputs, the WWTP system, and surface waters upstream and downstream of effluent inputs. With the SmartChip's high throughput capacity, 285 ARGs, eight transposase genes, and three integrase genes were analyzed. The targeted ARGs conferred resistance to eight antibiotic classes or modes of resistance with aminoglycoside, beta-lactam, multidrug efflux pump, tetracycline, and MLSB resistance being the most prevalent. This study found that the WWTP effluent may contribute to ARG abundance and richness in the downstream river with 155 ARGs detected downstream compared to only 80 ARGs detected upstream of the WWTP. Furthermore, based on the ARGs analyzed, hospital wastewater appeared to contribute more unique ARGs to surface waters than community wastewater.

Another study analyzed AMR in a river receiving leachate effluent from a landfill treatment plant (Wang et al., 2020a). This study used SmartChip to examine the resistome in the leachate and surface water near the landfill treatment plant, and 16S rRNA-based Illumina sequencing was used to analyze the bacterial community composition. To assess AMR, 285 ARGs, 10 MGEs containing two integron integrase genes (cintI1 and intI1), and eight transposase genes were analyzed. Based on genes quantified (91 ARGs and 5 MGEs) and bacterial community analysis, the effluent from the landfill appeared to impact the downstream river due to similar bacterial community structure and ARG profiles. Furthermore, vancomycin (vanXD and vanSB) and carbapenem (cphA and blaGES) resistance genes were found in the effluent and downstream river, but not in the upstream river. The presence of vancomycin and carbapenem resistance is important for understanding the potential human health impacts and risk factors of this leachate going into the surface water system, since these antibiotics are listed as critically important to human medicine (WHO, 2019).

2.2 BioMark Dynamic Array PCR with Integrated Fluidic Circuit

The Fluidigm BioMark HD and associated Integrated Fluidic Circuit (IFC) has been used in several studies to investigate ARGs in environmental water sources. This high throughput microfluidic qPCR and digital PCR (dPCR) system is a 96× high throughput capacity platform (9216 reaction wells) with three PCR sample-assay combinations of 192.24, 48.48, 96.96 (sample.assay). The IFC microfluidic device facilitates HT-qPCR by integrating samples and PCR assays into individual reaction chambers using a series of channels that are controlled by pressurization of Nanoflex™ valves. This IFC microfluidic system provides precise reaction volumes, which eliminates the variability typically associated with dispensing liquids, and results in high rates of reproducibility.

The flexibility of this platform allows customization of PCR assays and scalability to run 144 to 9216 simultaneous reactions in approximately 4 h. The nanoliter reaction volumes (7–9 nL) conserve sample volumes for other purposes/analyses and reduce reagent usage, which could potentially save overall costs, particularly when labor is included. However, the

use of high-throughput customized assays requires identical cycling conditions for each primer pair utilized and the reduced volume (nanoliter) reactions could negatively affect analytical detection sensitivity. In fact, Sandberg et al. (2018) showed that when analyzing 39 genes (ARGs, heavy metal resistance and integrase genes) in 12 wastewater and drinking water samples, a preamplification step was required prior to performing the 48.48 Dynamic Array IFC PCR. This preamplification step was necessary to have adequate quantities of DNA to allow detection of the genes of interest. This preamplification step could impact the accuracy of analysis if preamplification results in gene copy numbers that are outside the range of values for the standard curve (e.g. unable to accurately quantify). Finally, the products of the reactions may not be recoverable after the assays are completed, so sequencing the gene targets may not be possible; therefore, it's typically best practice to utilize probes to ensure that the correct gene target is amplified.

Microfludic qPCR has been used to investigate the role of land use on the dissemination of ARGs into watersheds. Uyaguari-Diaz et al. (2018) sampled watershed sites characterized as urban, agricultural, or protected. Utilization of the Fluidigm BioMark 96.96 Dynamic Array IFC allowed for 96 samples that were collected across watersheds to be analyzed with 14 PCR assays (13 ARGs). The results showed that different suites of ARGs are present in higher concentrations dependent upon different land usage associated with specific anthropogenic activity. For example, aacA1, aadA1, intI2, strA, strB, sul1, and sul2 were present in higher concentrations at agricultural sites, whereas tet32 and tetQ were more prevalent at sites under the influence of urban activities. However, intI1 and tetW were prevalent in both agricultural and urban sites.

Several studies have utilized microfluidic PCR to attribute specific sources in the dissemination of ARGs in the environment. Stormwater drain outfalls were examined as a source of pollution in Tampa Bay, FL by using two different Dynamic Array IFCs to analyze 24 samples from 12 outfalls under dry and wet weather events (Ahmed et al., 2018). The 48.48 format was employed to quantify 47 ARGs, heavy metal resistance genes, and three integrase genes (Sandberg et al., 2018), and the 192.24 format was utilized to detect 22 fecal indicator bacteria and bacterial pathogens (Ishii et al., 2013). The concentrations of most of the targeted genes were higher during wet weather conditions, suggesting that fecal pollution from the nonpoint sources of storm drain outfalls contribute to microbial pollution in the study area.

Since antibiotics are often used to treat and control bacterial disease outbreaks in fish farms, Bueno et al. (2019) used microfluidic qPCR to examine the role of aquaculture fish farms in dissemination of ARGs in the rivers in Chile. A total of 44 ARGs and 3 integrase genes were analyzed using a 96.96 Dynamic Array in samples taken from retention ponds at five freshwater rainbow trout farms as well as upstream and downstream locations within the associated rivers. Sixteen ARGs were quantified and the most abundant genes were sul1, tetA and tetC. For most genes, the abundance was significantly higher in the retention pond than the upstream sites, and ARG abundance was higher at sites downstream from the farms than upstream sites. The farms utilized florfenicol and oxytetracycline to control pathogens, and nine tet genes and floR, which confer resistance to these antibiotics, were more abundant at downstream sites compared to upstream sites. Interestingly, a third (1/3) of the

genes showed a decrease in abundance at the furthest downstream site (approximately 132.7 m), suggesting a rapid decay of ARG input from the farms. However, two-thirds (2/3) of the genes showed higher abundances at the furthest downstream site, suggesting other sources of ARGs in the river system.

Bueno et al. (2020) examined whether WWTP and associated wild birds are a source of ARG dissemination into surface water environments. Sediment samples were collected longitudinally from upstream and downstream sites and discharge points for three WWTPs in Chile, along with fecal samples from wild birds at one of the WWTP. Extracted samples were interrogated for 44 ARGs and 3 integrase genes using the 96.96 Dynamic Array IFC. The most abundant genes detected among the WWTPs were sul1, qnrS, sul2 and strB, while blaKPC, blaSHV, blaTEM, sul1 and sul2 were most abundant in migratory and resident birds. These genes also correlated with the most frequently detected and abundant ARGs in surface water samples and the greatest abundance was found at the discharge points for WWTPs relative to upstream sites. Of the ARGs detected in both water and birds, significantly greater concentrations were observed in water. Utilizing the Dynamic Array IFC to analyze a wide range of samples, the authors were able to conclude that both WWTPs and wild birds play a role in disseminating ARGs into the environment. However, the extent of the input from either WWTPs or wild birds is unclear.

Hospital wastewater has been suggested as a source of high levels of ARGs into the sewer network and ultimately, to the environment and surface waters. Buelow et al. (2018) utilized microfluidic PCR with the 96.96 Dynamic Array IFC to determine how much hospital wastewater contributes to ARG abundance by quantifying 67 ARGs that confer resistance to 13 classes of antimicrobials in 24 samples, including hospital sewage, residential sewage, WWTP influent (urban and suburban), WWTP effluent (urban) and river water. Significantly higher relative abundances of genes that confer resistance to aminoglycosides, β-lactams and vancomycin were measured in hospital sewage relative to other samples, but this sewage appeared to contribute minimally to the overall sewer network as demonstrated by comparable relative abundances of ARGs among different WWTP influents with and without hospital sewage inputs. Furthermore, the authors observed a significant decrease in the relative abundance of ARGs for nine classes of antibiotics in WWTP effluent compared to influent and the quantities of ARGs in the effluent-influenced river water were similar to the WWTP effluent. Collectively, the authors concluded that hospital wastewater contributes minimally to WWTP influent and that wastewater treatment reduces ARG dissemination into the environment.

2.3 Applied Biosystems OpenArray

The OpenArray from Applied Biosystems is another HT-qPCR system that has reasonable capacity with a 32× high throughput format with 3072 reaction wells. It utilizes a reaction volume of 33 nL, which means it may have better analytical sensitivity than the Fluidigm system yet still offers the benefits of using less sample volume and laboratory reagents than other high throughput systems like SmartChip. A major drawback of this system is that the primers (and probes, if used) are pre-dispensed into the array by the company leading to limited run to run customization. Given this limitation in the ability to customize the array,

few studies have been performed analyzing ARGs in surface waters utilizing this system. However, this OpenArray has been recently proposed as a system for monitoring recreation waters, specifically for assessing beach water safety (Shahraki et al., 2019). This system was utilized to develop a rapid molecular genetic method for monitoring fecal indicator bacteria and human virulent pathogens. The goal was to quickly detect and quantify fecal indicator bacteria, identify contaminant sources (microbial source tracking), and detect human virulent pathogens. The effectiveness of the designed OpenArray plate was evaluated using five different environmental sample types (beach water, beach sand, beach pore water, stream water, and pond water), sewage samples (known contaminated samples), and fecal samples from various sources (Canada goose, dog, humans, pig, and seagull). The OpenArray platform was shown to reliably detect as low as two template copies per well with some markers being detected with as low as a single copy. Taqman probes were utilized to help ensure specificity of the assays for the gene targets.

Co-selection and the stability of bacterial AMR in source waters for drinking water following arsenic pollution accidents was analyzed utilizing the OpenArray system (Zhang et al., 2020). Bacteria were collected from the sand at a drinking water treatment plant and exposed to different levels of arsenic to simulate a pollution event. Before and after exposure to the different levels of arsenic, 295 ARGs were analyzed. Of the genes targeted, 164 resistance related genes were detected and quantified, which included ARGs that confer resistance to all major classes of antibiotics, class I and III integrons, and transposons. Most of the genes were beta-lactam resistance genes and efflux pump genes. An enrichment of ARGs was seen after 6 h of exposure of arsenic (III) at all levels. Genes were significantly increased in every detected class, in particular resistance genes for aminoglycosides, beta-lactams, and MLSB. Some of the enriched ARGs (cmlA, floR, sul1, and sul2) were located on plasmids or MGEs. Commonly, genes that confer resistance to aminoglycosides, beta-lactams, macrolides, sulfonamides, and tetracyclines co-occur with arsenic resistance genes in the same bacteria or on multidrug resistance (MDR) plasmids.

3. Metagenomics

Metagenomics is the molecular technique of sequencing all microbial genomes within an environmental sample yielding greater detail of genomic information and increased taxonomic resolution compared to PCR (Knight et al., 2018). Metagenomic approaches are a valuable source of information during the analysis of AMR in the environment and allows for determination of full characteristics of the environmental resistome and related impacts within surface waters. It is considered an open format approach that is suitable for exploratory discovery studies, since it allows for discovery of novel ARGs (Zhou et al., 2015). However, to perform metagenomic work on environmental samples, the quantity of DNA extracted from a sample must be high enough to obtain a library that yields enough coverage to fully characterize the microbiome. Given the relatively low numbers of bacteria per sample volume in surface waters, larger sample volumes (e.g. >1 L) may be necessary to perform metagenomic work. This review will focus on how metagenomics has been employed to analyze AMR in surface waters. A recent review by Gupta et al. (2020) goes into more detail about the specific bioinformatics platforms, ARG databases, and workflows that can be utilized to analyze ARGs in environmental microbiomes.

Characterization of the resistome within surface waters has typically focused on waters impacted by WWTP effluent and drinking water sources, like lakes and reservoirs, which are a public health concern. Ng and coworkers characterized the metagenomes in urban surface waters receiving WWTP effluent in Singapore to study the occurrence of clinically relevant ARGs (Ng et al., 2017). The metagenomes of wastewater and surface water were compared to determine the impact of wastewater within the surface water compartment. For library preparation, the DNA sequences were trimmed to ~450 base pairs. BBDuk from the BBTools suite of bioinformatic programs was utilized to assess the quality of the sequenced library. Using BLASTP, ARG-like open reading frames (ORFs) were identified by comparing with an Antimicrobial Resistance Protein Database, which was a combination of protein sequences from the Comprehensive Antibiotic Resistance Database (CARD) and Resfam Antibiotic Resistance Proteins database v1.2. This study found that a resistome of 21 ARGs encoding resistance to aminoglycoside, MLSB, quinolones, sulfonamides, and tetracyclines or multidrug resistant efflux pumps was shared across wastewater, treated effluent, and surface water samples suggesting that these genes are prevalent within diverse environments.

Another study that analyzed the impact of WWTP effluent on the resistome within receiving surface water bodies used metagenomics to determine the taxonomic composition and functional gene pool (including ARGs) within the natural microbial communities (Corno et al., 2019). The impact of WWTP effluent was determined by looking at short term exposures and different concentrations of effluent. Metagenomic approaches were used to analyze the response of the native bacterial communities in waters exposed to 5 different dilutions of wastewater effluent (0 to 100%). Wastewater was sourced from several WWTPs, including urban WWTPs (with and without industrial wastewaters) and different disinfection treatments (UV radiation, chlorination, or peracetic acid). DNA from each sample underwent shotgun metagenomic sequencing, and sequence libraries were prepared and sequenced using Illumina NextSeq 500 V2. ARG-like sequences were identified using the ARGs-OAP database. This study found that as the proportion of WWTP effluent increased, the resistome of the receiving waters had stabilization of ARG composition irrespective of the independent microbiome of that system. However, the taxonomic composition did not follow a similar trend of increased stabilization with increased WWTP effluent. Therefore, the assumption was that the increased perturbance by WWTP effluent caused the stabilization of the resistome and that the release of effluent into surface waters could lead to the global propagation of AMR.

Recently, metagenomics was utilized to compare the diversity of microbiomes and resistomes in beef feedlots, urban WWTP influent, and downstream environments (Zaheer et al., 2019). Water samples were analyzed from a catchment basin associated with a cattle feed lot and compared with cattle feces, manured agricultural soil, and urban sewage. The 35 samples underwent Illumina HiSeq2000 sequencing, resulting in approximately 54 million reads per sample. The MEGAREs database, a hand curated antibiotic resistance database, and a customized metal and biocide resistance (MBR) database were used to map ARG and MBR sequences. Approximately 0.12% of the reads aligned with 35 AMR mechanisms, and about 0.04% of the reads were linked to 15 MBR classes, including 32 resistance mechanisms. The abundance of AMR-MBR genes in different sample types from highest to

lowest was fecal, influent, catchment basin water, and soil. The catchment basin resistome consisted of 84 ARG and MBR gene groups conferring resistance to tetracyclines, macrolides, aminoglycosides, beta-lactams, sulfonamides, mercury, and MDR. Since the three most abundant ARG groups in the catchment basin were similar to fecal samples, this similarity may demonstrate the impact of runoff from the feedlot pen floors, especially tetracycline resistance. Feedlots shared anywhere from 24 to 38 ARG groups from tetracycline, macrolide, and aminoglycoside resistance classes with their associated catchment basins.

Metagenomics was also utilized to not only analyze the source, prevalence, and risk of ARGs in the sediments of a lake in China, but to also compare with other global lakes (Chen et al., 2019). The reservoir of ARGs in lakes was the focal point because these reservoirs could lead to ARGs persisting in the environment and create opportunities for their emergence in resistant pathogens. Samples were collected from the sediment of Lake Tai in China, and 36 metagenomic data sets were obtained representing lake sediments from five countries (Australia – 3, Canada – 6, Indonesia – 4, Rwanda – 5, and U.S.A – 18). The samples collected from Lake Tai underwent shotgun sequencing using the Illumina HiSeq platform resulting in >11.8 billion paired-end reads (average of 79.0 million reads per sample). Clean reads were de novo assembled using MEGAHIT, a fast single-node solution de novo assembler, with default parameters. The 36 public metagenomic data sets of sediments globally were assembled, predicted, and clustered with the same pipeline to make results comparable. DeepARG-DB, a recent release of a manually curated ARG resource, was used as the ARG reference database. Overall, 21 ARG types were detected in Lake Tai sediment with 82.5% of the total ARG coverage belonging to 6 dominant ARG types: multidrug, MLSB, bacitracin, quinolone, mupirocin, and trimethoprim. Diversity analysis of ARGs showed a total of 321 ARG subtypes. Of these subtypes, 63 core subtypes belonging to 14 ARG types that were shared by all samples and contributed to 90.9–93.7% of the total ARG coverage. Compared to the other lakes world-wide, Lake Tai in China had increased occurrences of ARGs, which were attributed to direct contamination with human and animal fecal material and residual antibiotics, metals, biocides, and other environmental contaminants. Notably, many of the subtypes detected in this study had not been reported in previous studies of lakes in China, or worldwide when using culture-dependent or qPCR approaches.

Lastly, Dang et al. (2020) used metagenomics to profile the resistome of the Danjiangkou Reservoir, the largest drinking water reservoir in China. Due to the long-term storage and residence time of water in reservoirs, the storage and accumulation of ARGs may be increased. However, resistomes in reservoirs have not been fully characterized, and potential health risks are not known. During different seasons, the resistomes of the reservoir water and sediment were investigated using metagenomic sequencing approaches. Bacterial community composition was determined using the Illumina MiSeq platform to amplify and sequence the 16S rRNA V3-V4 hypervariable regions. Shotgun metagenomic sequencing was performed simultaneously to identify ARGs. A library was constructed and sequenced using the Illumina HiSeq 4000 platform. Only 42 samples were subjected to sequencing, because four samples failed to construct libraries due to low DNA concentrations. ARG reads were identified against the Structured Antibiotic Resistance Gene (SARG) database. In

total, 436 ARG subtypes from 20 different ARG types were found in 24 water and 18 sediment samples. In general, the abundance of ARGs in sediment samples was higher than in water samples, and ARGs for vancomycin and bacitracin were most predominant in sediment and water, respectively. The number of ARGs was higher during the dry season, and the composition of ARG subtypes between seasons was significantly different in water samples but not in sediment samples. The assembled metagenome classified 14 contigs as pathogens carrying ARGs (E. coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa) with three possessing virulence factors. Therefore, the risk to public health may be higher during the dry season and strategies should be implemented to control sediment and monitor pathogens.

4. Whole Genome Sequencing

While culture-based work utilizing break point concentrations of antibiotic compounds has been the gold standard for determining AMR in human pathogens and clinical isolates, that work is time intensive and may not provide information about the entire collection of AMR present within a single isolate. Recent research has examined whether WGS can be utilized to provide a better understanding of the total resistance present within a bacterial isolate (Collineau et al., 2019; Hendriksen et al., 2019). WGS can theoretically identify all the AMR-associated genes present within one bacterial isolate without any restrictions on the class of genes and/or antibiotics, which is normally a constraint when only using phenotypic antimicrobial susceptibility testing (AST). In fact, WGS has been shown to predict phenotypic resistance well (McDermott et al., 2016), and multi-drug resistance within a single isolate could be defined with greater accuracy than phenotypic analysis (Ellington et al., 2017). Furthermore, new, unknown AMR-associated genes can be discovered to add to AMR reference gene databases and make them more comprehensive and up to date. Finally, WGS minimizes the common issues and disadvantages associated with culturing environmental bacteria to determine AMR.

Whole genome sequencing is anticipated to improve the surveillance of AMR in surface waters because it can provide a better understanding of the transmission of ARB and ARGs throughout the environment and then, into human and animal populations, which will assist risk assessment activities. Within the bacterial genome, AMR genes can be located on the chromosome or MGEs, which allow the spread of these genes either clonally (e.g. vertically) or by horizontal gene transfer, respectively. Knowledge of where a gene is located within a bacterial genome allows for a better understanding of the mode of transmission and potential risks associated with the spread of that gene. Plasmids appear to contribute to the spread of AMR genes (Madec and Haenni, 2018) but sequencing them is challenging. Using short read sequencing technologies is problematic due to repeated and/or redundant sequences within plasmids, which make it difficult to accurately construct the plasmid. As a result, it is challenging to discern where an AMR gene would be located (e.g. on the chromosome versus MGE). However, if long read technologies are utilized, the plasmid structure can be accurately constructed. If AMR genes located on plasmids can be differentiated from those found on the chromosome, then, WGS could be used to determine how these AMR genes may be spread (Orlek et al., 2017). Furthermore, if plasmid structures and other mobile

elements can be accurately constructed, co-selection of AMR could be determined for AMR genes that are located on the same genetic element (Wales and Davies, 2015).

Compared to conventional subtyping techniques, WGS can be employed to subtype bacteria using in silico techniques, which provide lower costs, higher resolution, increased robustness, and faster results. The discriminatory power of WGS far exceeds conventional subtyping (e.g. multi-locus sequence typing) and can distinguish between two bacterial isolates of the same species and suggest or refute an epidemiological relationship between them (Tassios and Moran-Gilad, 2018; Schürch et al., 2018). Furthermore, by having the ability to look at the entire genome, genes that increase the likelihood of growth or survival in the presence of antibiotic compounds and/or under other stress conditions (e.g. extreme temperatures, acidity, high osmolality, desiccation, detergents, or disinfectants) can be identified within bacteria of interest, specifically pathogens. Identifying genes that would provide an advantage during stress conditions, particularly in the presence of antibiotic compounds, is important to validate the survival of the bacteria. A major question about using WGS for identification of AMR and potential risks, especially without culturing, is whether the presence of a gene or genes within the bacteria will allow that bacteria to survive and maintain that resistance or pass it along in the environment (Collineau et al., 2019).

Recent research has begun to incorporate WGS when analyzing AMR in surface waters in addition to phenotypic and other genotypic techniques. Gomi et al. (2016) performed WGS analysis on 152 selected strains of 531 E. coli isolates from the Yamato River in Japan that first underwent phenotypic resistance analysis. Initial phenotypic analysis found that 14.3% of the isolates were MDR, 12.4% were non-susceptible to one to two classes of antimicrobials, and 73.3% were pan-susceptible. When WGS was performed, AMR in these isolates was confirmed by the discovery of more than 50 different resistance determinants, including chromosomal resistance mutations as well as acquired ARGs. In addition, among the 66 MDR strains that were sequenced, clinically important clonal groups were found including sequence type (ST) complexes that differed from one another by no more than two of the seven loci used for MLST genotyping. Within extraintestinal pathogenic E. coli (ExPEC) strains, clonal groups that were clinically important were prevalent. Finally, the analysis of phylogenic and virulence genes for a major clonal ExPEC group (ST95) that is associated with community onset infections showed that environmental strains were not significantly different than clinical strains. These results indicated that surface waters were contaminated by E. coli strains from clonal groups of clinical importance.

In Germany, ESBL/carbapenemase-producing bacteria in surface waters and sediment were analyzed for multidrug resistance using culture-based approaches and WGS (Falgenhauer et al., 2019). Bacteria were isolated using selective culture techniques (CHROMagar ESBL plates) with speciation determined on a subset that then underwent AST. The presence of MDR was evaluated in seven species of clinical relevance (Acinetobacter baumannii, Citrobacter freundii, Enterobacter cloacae complex, E. coli, Klebsiella oxytoca, Klebsiella pneumoniae, and Pseudomonas aeruginosa). WGS was used to analyze for ARGs and virulence determinants on a subset of 33 isolates, including the seven clinically relevant species. Of the isolates analyzed, 89% were clinically relevant species, 58% exhibited an

MDR phenotype, two E. coli isolates had STs linked to human infections (ST131 and ST1485), two isolates possessed mobile colistin resistance (mcr-1), and an isolate of Klebsiella pneumoniae was classified as hypervirulent.

Another recent study analyzing waterborne E. coli for AMR in surface waters and wastewaters of Northern Colorado utilized culture-based techniques coupled with WGS to validate the presence of AMR, specifically for ESBL and Klebsiella pseumoniae carbapenemase (KPC) mechanisms (Haberecht et al., 2019). Seventy E. coli isolates had AMR validated for a panel of 17 antibiotics using the broth microdilution technique. PCR was then used to further confirm common ESBL genes. Functional similarity was determined via dendrograms generated by the PhenePlate system assay that used patterns in metabolism of carbon source to determine clonal relatedness of bacterial isolates. WGS was performed on 21 isolates from wastewater to screen for 185 ARGs and confirmed diverse ARG classes in the genomes of those isolates. Of note, while KPC screening detected positive isolates, carbapenemase-encoding genes (ndm-1, oxa-48, and vim) were not found in those isolates. However, E. coli did possess genes for multiple efflux pumps. The presence of efflux pumps is one mechanism of resistance to carbapenem drugs and supports why isolates tested positive for KPC even though they did not possess known carbapenemase-encoding genes. This demonstrates the usefulness of WGS not only for in depth analysis of AMR within isolates, but also to increase resistance gene indexing. Based on the findings of this study, the authors suggested integration of phenotypic and genotypic profiling beyond the typical mechanisms associated with ESBL and KPC. Utilizing multiple approaches to screen isolates for AMR in surface waters may help prevent drug-resistant E. coli from spreading from waters to human and animals.

While the use of WGS in surface waters for analyzing AMR is still limited, the wide-spread utilization of this technique could lead to major advances in resistome surveillance where AMR could be predicted from genomic data alone. Studies, including those investigating Enterobacteriaceae and food-borne pathogens, have demonstrated that a high agreement (>96%) can be shown between the occurrence of AMR (genes or mutations) and the minimum inhibitory concentrations (MIC) of certain antibiotics at or above the clinical breakpoint or epidemiological cut-off values for AMR (McDermott et al., 2016; Mason et al., 2018; Tyson et al., 2018; Lauener et al., 2019). Whole genome sequencing has been shown to possess high analytical sensitivity or a true positive rate of >87% in accurately classifying AMR determinants in bacterial isolates with an AMR phenotype. The analytical specificity or true negative rate of WGS has been found to be even higher at >98% in accurately determining the absence of AMR determinants within phenotypically determined AMR susceptible isolates (Gordon et al., 2014; McDermott et al., 2016; Shelburne et al., 2017).

Additionally, recent research has employed machine or deep learning to produce sequence data from bacterial genomes, which demonstrates the possibility of using WGS techniques to predict AMR and perhaps the MICs of antibiotics (Eyre et al., 2017; Nguyen et al., 2018; Nguyen et al., 2019). Bioinformatic analysis can provide a frame of reference for co-carriage or selection of specific genes that are responsible for different multidrug resistance patterns and temporal allelic frequency and trends. It could also provide other genetic information

such as potential for horizontal gene transfer and distribution of AMR by bacterial or contaminant sources. WGS can also determine the presence of co-resistances that may not have been assayed or not easy to assay (e.g. disinfectant and/or heavy metal resistance). Finally, utilizing WGS techniques to detect and track AMR offers an opportunity to develop a One Health surveillance framework for AMR across and between human, food, animal, and environment systems.

5. Characterization of Antimicrobial Resistance Risks

While all of the presented methods provide valuable insight regarding sources and fates of AMR in the environment, the primary purpose for analyzing AMR in surface waters is often to determine what type of hazard environmental AMR poses and link environmental AMR to human and animal health, a One Health perspective. Water creates a conduit for the spread of AMR to human and animal populations and can present both direct and indirect health risks (Finley et al., 2013). The direct risk is exposure to clinically relevant resistant bacterial pathogens via surface waters, and closely linked environments, that lead to increased AMR in human or animal populations. Indirect risks are increases or alterations of AMR in the environment that will lead to the amplification of AMR traits or transfer of ARGs from non-pathogenic environmental bacteria to pathogenic or commensal bacteria (Bürgmann et al., 2018; Vikesland et al., 2017). Such gene transfer events need only happen once to present significant new public health threats (Ashbolt et al., 2013).

The type of hazard that is analyzed and the specific questions that are being posed will determine which analytical methods to employ. Each of the above approaches has unique advantages and disadvantages that allow them to address various types of risk (Huijbers et al., 2019). For the analysis of direct risks, AMR in surface waters needs to be directly linked to infectious capacity among human or animal populations (e.g. exposure to pathogenic AMR bacteria) (Amarasiri et al., 2019). This type of analysis has traditionally relied on culture work, which identifies and characterizes viable bacteria capable of expressing AMR traits of interest. However, as pointed out previously, culturing environmental bacteria is problematic and may not be able to assess certain types of AMR, including novel or emerging modes of resistance (Bengtsson-Palme et al., 2018). As an alternative to phenotypic testing, WGS can offer a new avenue for identifying environmental bacteria, including pathogens and commensals, that pose direct health risks by analyzing and surveilling the resistome of bacterial isolates and providing insight into their antimicrobial susceptibility. While initial culture to generate isolates may be required, WGS has been found to predict phenotypic resistance in a rapid, high-throughput, non-targeted format compared to conventional MIC testing (McDermott et al., 2016; Walker et al., 2019). WGS also has the potential to directly identify high-priority strains of interest, including both strains of known concern and those with similar genetic profiles. In theory, observed variability in AMR, virulence, and survival traits through WGS could be used to tailor quantitative microbial risk assessment (Collineau et al., 2019).

Metagenomics can identify diverse ARGs in environmental samples, which can then be ranked in terms of direct risk to human health based on their mobility (e.g. location on an MGE), resistance mechanism, and association with pathogens (Martínez et al., 2015; Oh et

al., 2018). In environmental metagenomic studies, the ARGs that pose the highest risk are those known to contribute to clinical treatment failures and have been shown to reside on MGEs hosted by human bacterial pathogens (Martínez et al., 2015). Likewise, metagenomic approaches that link ARGs to MGEs and host genomes can be used to understand whether these traits are mobile or housed by pathogenic bacterial strains without the need for initial cultivation or a priori target selection (Oh et al., 2018). Oh et al. (2018) assembled shotgun sequencing data from environmental samples (WWTP effluent, dairy lagoon, and hospital sewage) to determine whether contigs containing ARGs also contain sequences like MGEs and human pathogens. The co-occurrence of these traits among contigs were mapped into a three-dimensional hazard space. The three dimensions were defined as (i) contigs with ARG-like sequences, (ii) contigs annotated with ARGs and MGE-like sequences, and (iii) contigs annotated with pathogen genomes as well as ARGs and MGEs. Using this three-dimensional hazard space, the “resistome risk” of samples was compared, demonstrating clustering of sources and anticipated trends (WWTP effluent < dairy lagoon < hospital sewage).

While targeted molecular analyses such as qPCR can provide a conservative indicator of potential human health risk, gene concentrations alone are difficult to interpret in a quantitative risk framework, since it is unknown whether the genes are within live bacteria, notably pathogens (Bürgmann et al., 2018). Therefore, ideally, a combination of culture-based work along with WGS or metagenomics may provide the most comprehensive analysis of direct threats from AMR in surface waters (Graham et al., 2019). Exposure to ARGs, which may later result in hard-to-treat infections when established among commensal bacteria and transferred to pathogens, is a potential endpoint of interest in this work. For example, Leonard et al. (2018) applied metagenomic methods to characterize ARGs in composited E. coli isolates from recreation waters in England, using results to estimate national-level exposure to these genes during various water activities. The authors predicted that 2.5 million water sports sessions resulted in user ingestion of at least 100 E. coli borne ARGs.

For analysis of indirect risks, molecular methods are perhaps best suited. Quantitative methods such as HT-qPCR can be employed to assess or compare the prevalence of ARGs and related genes within a study system, while non-targeted sequencing approaches such as metagenomics and WGS are advantageous for their ability to characterize a broad range of potential determinants, including novel ones (Bürgmann et al., 2018). While culture work can be used to determine the phenotypic expression of resistance, it does not capture the entire bacterial community or a full diversity of resistance. This may result in the omission of uncultivable environmental bacteria, which can serve as reservoirs of ARGs and MGEs in the environment, and emerging resistance types or host-gene combinations that are relevant to human health (Bengtsson-Palme et al., 2018; Vikesland et al., 2017).

WGS of environmental isolates can be used to study the evolution and transmission of AMR as well as to stratify potential risks based on their genetic profiles (Collineau et al., 2019). Similarly, ARGs and resistomes identified by metagenomics can be compared in their likelihood to present human health concerns (Martínez et al., 2015; Oh et al., 2018). For example, Chen et al. (2019) used metagenomic identification of ARGs, MGEs, and pathogen

genes in lake sediments to project their co-occurrence into a three-dimensional exposure space. While sequencing methods are generally limited to semi-quantitative results, HT-qPCR assays can then be employed to target a suite of identified genes for quantitative study and additional monitoring in environmental samples (Amarasiri et al., 2019). HT-qPCR provides a quantitative abundance measurement that is critical to many study designs, such as AMR amplification or transport in environmental systems, while canvasing the entire microbial community and avoiding the limitations of cell culture discussed above (Rocha et al., 2019). Coupling sequencing methods with quantification of ARGs and MGEs provides a thorough assessment of AMR within the environment and valuable insight into potential health risks from a One Health perspective.

6. Future Needs and Directions

This review has provided an overview of contemporary molecular methods and how they can be utilized in surface waters to characterize AMR. In addition, this review presented information on how best to characterize risk utilizing a variety of methods. Over the past century, molecular methods have improved remarkably and provide large amounts of data quickly. However, while molecular methods possess many advantages compared to culture-based analysis, a single molecular tool by itself does not provide adequate information to fully characterize AMR within surface water systems. Using a combination of various molecular techniques as well as incorporating culture-based techniques would provide the most comprehensive analysis of AMR, specifically if risk assessment is a priority.

In addition to the contemporary molecular approaches presented in this review, the inclusion of metatranscriptomics in the analysis of AMR in surface waters would provide information about ARG expression, the dynamics of its expression within a microbial community, and even functional information about that microbial community. To date only a handful of studies have utilized metatranscriptomics for analysis of ARGs in the environment (WWTPs, activated sludge, animal feces, soil, and wild birds). These studies have shown that metatranscriptomics can be utilized to confirm that ARGs are in fact transcriptionally active within a microbial community. The metatranscriptome can also show how expression of the ARGs can differ, sometimes significantly, from the ARG abundance determined through other molecular methods. The use of metatranscriptomics in combination with the molecular approaches featured in this review for the analysis of AMR in surface waters would provide better insight into the movement and expression of ARGs (Wang et al., 2020b).

Furthermore, standard molecular approaches and reporting methods are necessary for analyzing AMR in surface waters so that direct comparisons can be made between studies and laboratories. Standardized approaches would allow a greater understanding of AMR not only at the local/study scale, but at a global surveillance scale. Standardization would also facilitate linking environmental AMR with human and animal populations across individual studies to support broad One Health assessments. Currently, standard approaches and methods for studying AMR in the environment via molecular techniques have not been developed and/or verified across laboratories to determine consistency and comparability. For example, in metagenomics, standardization of the number of reads necessary to fully

characterize the metagenome would create reliable and comparable data across studies. Likewise, not having a standard approach for reporting ARGs that are quantified via PCR techniques is problematic for comparison purposes if absolute abundance versus relative abundance is reported, especially since quantification of ARGs is a main way that AMR is measured and monitored in the environment.

Overall, the implementation of standard approaches and methods for analysis of AMR in surface waters and the environment would provide a way to gather more accurate information about the flow of resistance between humans and animals and the environment (McEwen and Collignon, 2018). In order to fully understand and control AMR, the human, animal, and environmental dimensions that are interdependent must be characterized, since they are the basis of the One Health Approach to AMR (Collignon and McEwen, 2019). Monitoring systems are currently in place that enable extensive surveillance efforts to track antibiotic use and AMR in human and animals (e.g. National Healthcare Safety Network, National AMR Monitoring System, and National Animal Health Monitoring System). However, similar surveillance systems are not in place for tracking and monitoring AMR in surface waters and the environment. As a result, standardization of approaches and reporting methods, which would involve incorporation of different techniques (molecular and/or culture-based), is necessary to accurately characterize and track AMR in the environment. This toolbox of methods would produce data to support monitoring systems for AMR in the environment and provide a foundation for a One Health approach to studying AMR and its risks to humans, animals, and the environment.

Acronyms

AMR	Antimicrobial resistance
ARG	Antibiotic resistance genes
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
HT-qPCR	High throughput quantitative polymerase chain reaction
WGS	Whole genome sequencing
MGE	Mobile genetic elements
MIC	minimum inhibitory concentration

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Highlights

- Molecular methods offer extra insight into antimicrobial resistance in surface waters
- Methods for detecting antimicrobial resistance have advantages and disadvantages
- Combination of culture- and molecular-based methods is needed to determine risk
- Characterizing environmental antimicrobial resistance offers a One Health perspective

Table 1.

Advantages and disadvantages of current methods utilized to characterize antimicrobial resistance in surface waters.

Method	Advantages	Disadvantages
Culture-based	Feasibility of implementation Low technical requirements Potential for global data comparability Ability to determine MIC of culturable bacteria and phenotypic changes under selection pressure of antibiotics Consistent with existing water quality regulations and monitoring programs	Labor- and time-consuming Lacks representation of full microbial community Inherent cultivation bias with easily cultivated bacteria being cultured the most (i.e. selects for fast growing bacteria with overnight cultures whereas many environmental bacteria may need more culture time) Neglects unculturable bacteria Lack of bench-marking against culture-independent methods
qPCR	More precise quantification of target gene Ability to detect low-abundance genes Quantify ARGs in different environments. With newer technologies, able to analyze a large suite of target genes	Inability to ascertain gene expression qPCR inhibition (Of note, ddPCR is not as sensitive to inhibitors) Inability to directly discriminate extracellular from intracellular DNA Limited by design of primers and known antibiotic resistance genes
Metagenomics	Analyze all genes in environmental samples Carry out bacterial taxonomy and functional gene (ARGs, MGEs, etc.) analysis simultaneously Eliminates problems with unsuitable primer design and PCR biases Discover new antibiotic resistance genes	PCR-dependent and PCR biases can affect analytical sensitivity and accuracy (i.e. exaggerations of dominant taxa or omitting low number abundance taxa) Doesn’t provide enough sequencing depth to enrich and assemble genomes of a single strain (esp. in complex matrices), however is platform dependent Poor repeatability, high cost, and laborious with complex sample preparation and analysis No live, dead, or active discrimination when not culturing first Results dependent on library preparation and bioinformatic workflows
Whole Genome Sequencing – Antimicrobial Susceptibility Testing (WGS-AST)	Antimicrobial resistant bacteria can be typed and traced by specific allele profiles. Determine co-carriage of specific genes causing different multi-drug resistance patterns. Directly link phenotypic antibiotic resistance with the presence or absence of certain genes.	Limited to the individual bacterial cells that can be cultured and sequenced. Requires accurate and up to date reference databases