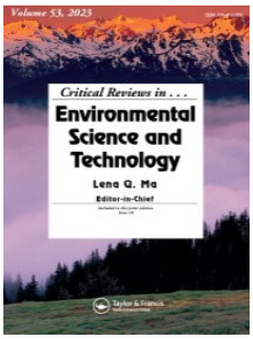

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Critical Reviews in Environmental Science and Technology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/best20

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To cite this article: Benjamin C. Davis, Connor Brown, Suraj Gupta, Jeannette Calarco, Krista Liguori, Erin Milligan, Valerie J. Harwood, Amy Pruden & Ishi Keenum (2023): Recommendations for the use of metagenomics for routine monitoring of antibiotic resistance in wastewater and impacted aquatic environments, Critical Reviews in Environmental Science and Technology, DOI: 10.1080/10643389.2023.2181620

To link to this article: https://doi.org/10.1080/10643389.2023.2181620

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Recommendations for the use of metagenomics for routine monitoring of antibiotic resistance in wastewater and impacted aquatic environments

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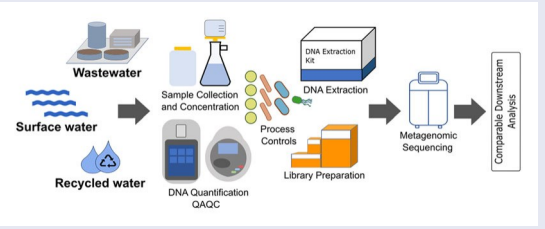
ABSTRACT



Shotgun metagenomic sequencing of the collective genomic information carried across microbial communities is emerging as a powerful approach for monitoring antibiotic resistance in environmental matrices. Metagenomics is advantageous in that known and putative antibiotic resistance genes (ARGs) (i.e., the resistome) can be screened simultaneously without a priori selection of targets. Additionally, as new ARGs are discovered and catalogued, stored sequencing data can be reanalyzed to assess the prevalence of emerging genes or pathogens. However, best practices for metagenomic data generation and processing are needed to support comparability across space and time. To support reproducible downstream analysis, guidance is first needed with respect to sampling design, sample preservation and storage, DNA extraction, library preparation, sequencing depth, and experimental controls. Here we conducted a systematic review to assess current practices for the application of metagenomics for AR profiling of wastewater, recycled water, and surface water and to offer recommendations to support comparability in the collection, production, and analysis of resulting data. Based on integrated analysis of findings and data reported across 95 articles identified, a field to benchtop metagenomic workflow is discussed for optimizing the representativeness and comparability of generated data. Through the reanalysis of 1474 publicly-available metagenomes, appropriate sequencing depths per environment and uniform normalization strategies are provided. Further, there is opportunity to harness the quantitative capacity of metagenomics more overtly through inclusion of sequencing controls. The recommendations will amplify the overall value of the metagenomic data generated to support within and between study comparisons, now and in the future.

KEYWORDS Antibiotic resistance; metagenomics; next-generation sequencing; standardization; wastewater; surface water

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/10643389.2023.2181620.

Introduction

Antibiotic resistance (AR) is a growing global health threat (O'Neill, 2016; World Health Organization, 2015) and, increasingly, the importance of environmental dimensions to its transmission and evolution are being recognized (European Commission, 2017; United Nations, 2017). Correspondingly, the need for unified approaches to assessing AR in the environment is evident (Huijbers et al., 2019; JPIAMR, 2019). Environmental monitoring can help to assess baselines of AR in pristine and anthropogenically-influenced environments, as well as local human and animal populations, and further aid in identifying high-risk areas for the evolution, selection, and transmission of antibiotic resistant bacteria (ARB) (Berendonk et al., 2015; Jin et al., 2022; Larsson et al. 2018; Pruden et al., 2021). Such information promises to be especially valuable toward informing specific policy/mitigation measures (Aarestrup & Woolhouse, 2020). Monitoring of influent sewage to wastewater treatment plants (WWTPs) has especially garnered attention as a means to capture collective antibiotic resistance genes (ARGs) circulating amongst the corresponding human population (Hendriksen et al. 2019; Prieto Riquelme et al. 2022) and has been shown to reflect local clinical prevalence of ARB (Pärnänen et al. 2019). The WWTP itself also represents a significant barrier to the dissemination of ARB and ARGs via reuse or to receiving surface waters and therefore removal efficiencies are of inter-est (Majeed et al., 2021). Furthermore, wastewater reuse and impacted surface waters represent poten-tial transmission pathways into and out of human populations (Garner et al., 2018; Keely et al., 2022).

Next-generation sequencing (NGS) is a powerful and promising tool for monitoring of aquatic environments (Garner et al., 2021a). Shotgun metagenomics applies NGS for the sequencing of DNA extracted across microbial populations inhabiting the sampled environment. The resulting metagenome (i.e., the collection of NGS reads captured from a sample) can be analyzed to characterize the resistome (i.e., the collective ARGs carried across a microbial community). The most common approach is to align the metagenome against publicly-available databases to compare metagenome-derived sequences to those of functionally verified ARGs, which currently number in the thousands (Alcock et al., 2020). The number and types of ARGs can then be compared across samples of inter-est. Detected ARGs can be classified and ranked by various means; this includes the antibiotics to which they encode resistance, the mechanism of resistance, and their degree of clinical relevance (i.e., extent to which they are found to interfere with treatment of human infections). The genetic context of various ARGs can further be explored to determine more information about the ARG of interest (chromosomally-bound or inter/intra-cellularly mobile), what kinds of mobile genetic elements (MGEs, e.g., plasmids, integrons, transposons) they are carried on, or whether they occur in known human pathogens or the commensal environmental flora. Metagenomics is also being utilized to mine putative and/or uncharacterized ARGs from public repositories to expand our knowledge of the known, emerging, and latent resistome (Arango-Argoty et al., 2018; Berglund et al., 2019).

The primary motivation for this critical review is to assess the state of sampling, sequencing, and analysis of shotgun metagenomics for AR monitoring in wastewater and impacted aquatic environments. Through a systematic analysis of data reported in relevant scientific literature, we provide guidance on the implementation of metagenomic workflows for routine monitoring purposes that support the generation of meaningful and comparable data. In part, our efforts reflect those in clinical and human microbiome domains such as STORMs, which proposes guidelines for improved reproducibility in metagenomic analysis (Mirzayi et al. 2021). However, these guidelines do not account for unique challenges related to environmental sampling, sequencing, and analysis of corresponding resistomes. Best practices guidelines for the inclusion of standards have been developed in the EMMI guidelines for qPCR (Borchardt et al., 2021); however there has been no such effort for metagenomics-based resistome monitoring. The specific objectives of this review were to:

1. Evaluate strengths and weaknesses of existing workflows for routine metagenomic analysis of resistomes characteristic of wastewater and impacted water environments

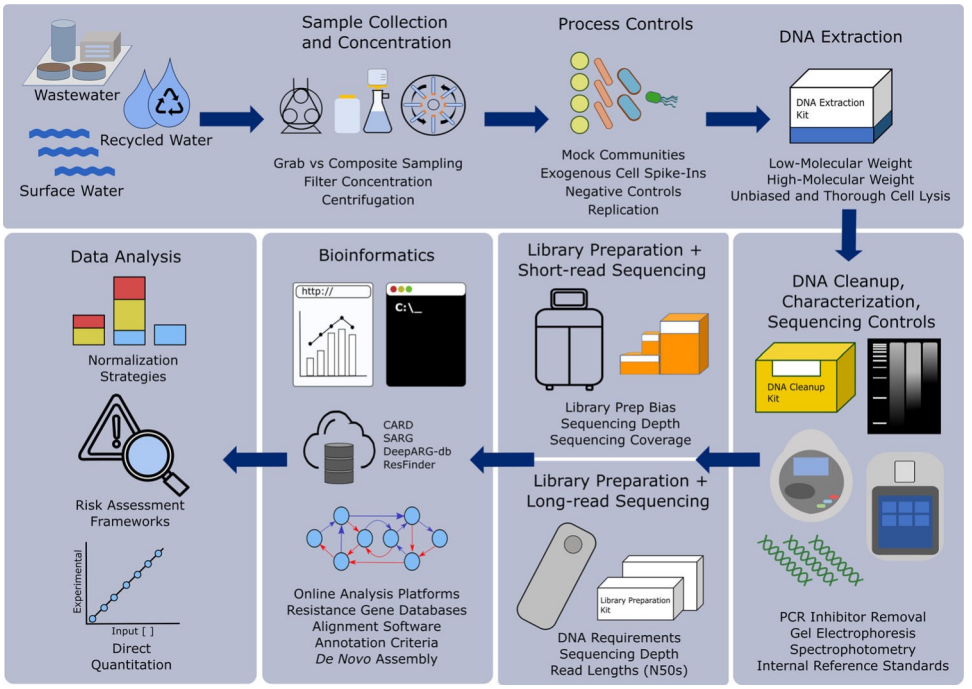

Figure 1. Overview of key decision points to consider in developing a workflow for metagenomic-based monitoring of ARGs in aquatic environments that are highlighted in this review.

2. Identify sources of variability introduced by different data generation and processing techniques
3. Provide recommendations with respect to best practices for supporting generation of findable, accessible, interoperable, and reusable data (FAIR) (Wilkinson et al., 2016)

We further assess the current trajectory of the field as it moves toward standardized data reporting, NGS process controls, and datatypes for integration into future risk assessment models. The overall recommendations provide a framework to amplify the value of metagenomic data and analysis for the purpose of AR monitoring of aquatic environments (Fig. 1).

Literature review protocol

To generate a systematic review, search terms were applied in a three-tiered approach (Table S1 and Fig. S1). Tier 1 established topic level keywords that identified studies that were relevant to wastewater, water reuse, and surface water environments. Tier 2 ensured that the studies were relevant to AR, while Tier 3 established keywords to identify studies focusing on shotgun metagenomics. Literature returned between 2010–Dec 2020 via this search strategy were manually screened by two independent researchers to ensure that all included articles met the inclusion criteria. Articles focused on aquaculture, biosolids and biosolid treatment (anaerobic digestion, composting, etc.), treated drinking water, or laboratory-scale experiments were excluded. Studies involving only the use of secondary data (i.e., a collection of metagenomic datasets from other studies) were also excluded. Any disagreements between the two screeners on relevance were presented to a broader team of five researchers to reach a consensus on the applicability of the study toward informing a workflow that includes sampling, DNA sequencing, and data analysis. This approach produced 95 articles. Studies that met eligibility criteria were subjected to data extraction for the parameters outlined in Table S2.

Data extraction and analysis

All publicly-available metagenomes from the 95 included articles were downloaded from the Sequence Read Archive (SRA) and European Nucleotide Archive (ENA) (n ¼ 1775) for depth, coverage, and normalization analysis. Samples were then filtered for paired-end Illumina datasets pertaining to wastewater, recycled water, and surface water matrices using available metadata (n ¼ 1474). Briefly, we cleaned paired-end data using Trimmomatic (Bolger et al., 2014) (leading:3, tailing:3, slidingwindow:4:15, minlen:36) and ran forward reads through Nonpareil (Rodriguez-R et al., 2018) with option "-T kmer" to determine the relative metagenomic library coverage as function of dataset size. Reads were then merged with Vsearch, aligned against the CARD database (v3.3.1; 80% identity, 80% coverage, min 25 aa), and normalized to RPKM, PPM, 16S rRNA genes, ARGs/cell (rpoB), and an ARG density metric to assess their individual performances in mitigating sequencing bias. The full list of SRA and ENA accessions used, and details of the data analysis can be found in the supplemental materials (Supp Section 1). Data were analyzed in R (v 4.1.2) and visualized using ggplot2 (Wickam, 2009).

Sampling frequency, replication, and controls

A growing body of research is providing insight into baseline variability of WWTPs and other aquatic system resistomes (Majeed et al., 2021; Yin et al., 2019). In a landmark study of a Hong Kong WWTP, monthly sampling of the activated sludge basin was performed over a nine-year span and the resistome composition was found to turn over every 2-to-3-years (Yin et al., 2019). However, it is unclear the extent to which the observed patterns are generalizable across all WWTPs or how such dynamics vary with each stage and type of treatment. Coordinated surveillance is needed to help inform the sampling frequency and number of replicates needed to achieve metagenomic monitoring objectives. For example, if influent sewage resistomes are relatively stable across WWTPs with time, as observed in conventional WWTPs in the US (Majeed et al., 2021) and Hong Kong (Prieto Riquelme et al., 2022), then less frequent sampling may be sufficient when the purpose is broader comparison across WWTPs. However, if the purpose is to determine if anomalous ARGs of clinical concern are present in the influent and escaping into surface waters, then much more frequent sampling with replication and deep sequencing or target enrichment may be necessary (Lanza et al., 2018; Majeed et al., 2021). Shallower sequencing may be sufficient when the aim is to assess removal of dominant ARGs.

Biological replication, i.e., independently processing and sequencing multiple samples representing a given condition/site, helps to account for variability introduced by incomplete homogeneity, or minor spatiotemporal variation in complex biological systems. Technical replication, where singular biological samples are sequenced multiple times, can control and account for batch effects across sequencing lanes and flow cells (Borchardt et al., 2021). Among the articles identified in this study, biological replication was infrequent: 19/95 studies (Table S3). This is likely due to the high per-sample cost of metagenomics as well as the tendency for ecological sur-vey designs to favor sampling a larger number of sites/conditions at shallow sequencing depths rather than a few sites/conditions with deeper sequencing and replication (i.e., breadth over depth) (Filazzola & Cahill, 2021). We found appreciable levels of variability between biological replicates across studies and environments (Jia et al., 2021; Roy et al., 2018; Petrovich et al., 2018; Wang 2018). For example, Petrovich et al. (2018) documented standard errors of ARG 'abundance per genome equivalents' between biological triplicates of influent samples as high as 0.45 for specific antibiotic resistance classes, although the overall resistome compositions did not fluctuate on ordination plots. These standard errors decreased two to three orders of magnitude in downstream activated sludge and effluent samples, suggesting greater spatiotemporal biological stability in subsequent engineered systems. Based on these observations, we suggest that if the

expected biological variability of ARG abundance in a system is high, emphasis should be placed on implementing biological replication, but if it is low, then emphasis can be placed on less repli-cation at greater sequencing depths.

Including at least one technical replicate per flow cell is also good practice to help reveal biases in library preparation and sequencing by sequencing cores. Negative controls serve as a check for any contamination events that occur during sampling, processing, and DNA extraction that may contribute to background detection of microbes and ARGs (Fig. 2), however only 15/95 studies reported the inclusion of any negative controls (Table S4). A field blank, in which a sampling bottle is filled with molecular-grade water and processed together with samples, is useful for capturing all possible sources of contamination in a workflow. If contamination is found, further controls (e.g., DNA extraction kit/filter blanks) can be analyzed to identify the source and improve the workflow. The inclusion of negative controls is also good practice and can be especially useful in differentiating low abundance taxa or ARGs from technical noise or laboratory contamination (Borchardt et al., 2021). Unfortunately, only a single study included negative controls in sequencing runs.

In the studies examined in this review, samples were almost exclusively collected as grab samples. Composite samples, which may be spatially-, flow-, or time-weighted, may be more appropriate where replicate grab samples are infeasible (Centers for Disease Control and Prevention, 2020). Studies evaluating time-sensitive wastewater-based surveillance of illicit drugs (Rodayan et al., 2014), total phosphorus and nutrients (Johannessen et al., 2012), and SARS-CoV-2 (Kopperi et al., 2021) demonstrated that time-weighted composite and grab samples yielded highly comparable results. This suggests the relative stability of wastewater compositions and treatment efficacy over diurnal timescales such that a reasonable degree of replication should be able to capture signals of interest. This stability may not be the case with more variable sample types, such as river water, where diurnal variation is more accentuated. For routine monitoring purposes, ideal samples would be equal flow or equal time composite samples, although a grab sample is better than no sample.

Sample preservation and storage affect sample representativeness

Appropriate preservation and storage ensure that subsequent analysis is representative of the sam-ple at the time it was collected. This is particularly critical for time series data and comparisons across systems. A recent comprehensive analysis of storage conditions of raw pig feces and domestic wastewater samples revealed systematic biases that impacted downstream metagenomic analysis (Poulsen et al., 2021). The authors found that both storage time (immediate processing, 16 hrs, 64 hrs; and long-term storage at 4, 8, and 12 months) and temperature (deep freezer, 80 C; freezer, 20 C; refrigerator, 5 C; room temperature, 22 C) resulted in significant fluctu-ations in taxonomic and resistome composition; although if immediately frozen (at either 20 C or 80 C), batch effects were minimized. If freezer storage is not possible, the authors stressed that samples should be processed immediately. The need to immediately freeze or analyze the sample poses a challenge during field work or when seeking to include low income countries in global-scale studies (Hendriksen et al. 2019; Roy et al., 2018). Where the shipping of samples is necessary, fixing samples in 50–100% ethanol, freezing at 20 C, and shipping on ice has shown to both prevent significant fluctuation of resistomes and preserve the integrity of DNA (Li et al., 2018). Sample preservation reagents have been shown to preserve the integrity of soil micro-biomes (Pavlovska et al., 2021), human microbiomes (Bartolomaeus et al., 2021), and fecal SARS-CoV-2 RNA (Natarajan et al., 2021), even at room temperature; although these techniques have not been systematically assessed for analysis of aquatic resistomes. The addition of preservation reagents may also preclude sub-sampling the same sample for multiple analyses (e.g., transcrip-tomics, metabolomics, cultivation, pharmaceuticals analysis) (Poulsen et al., 2021). To minimize

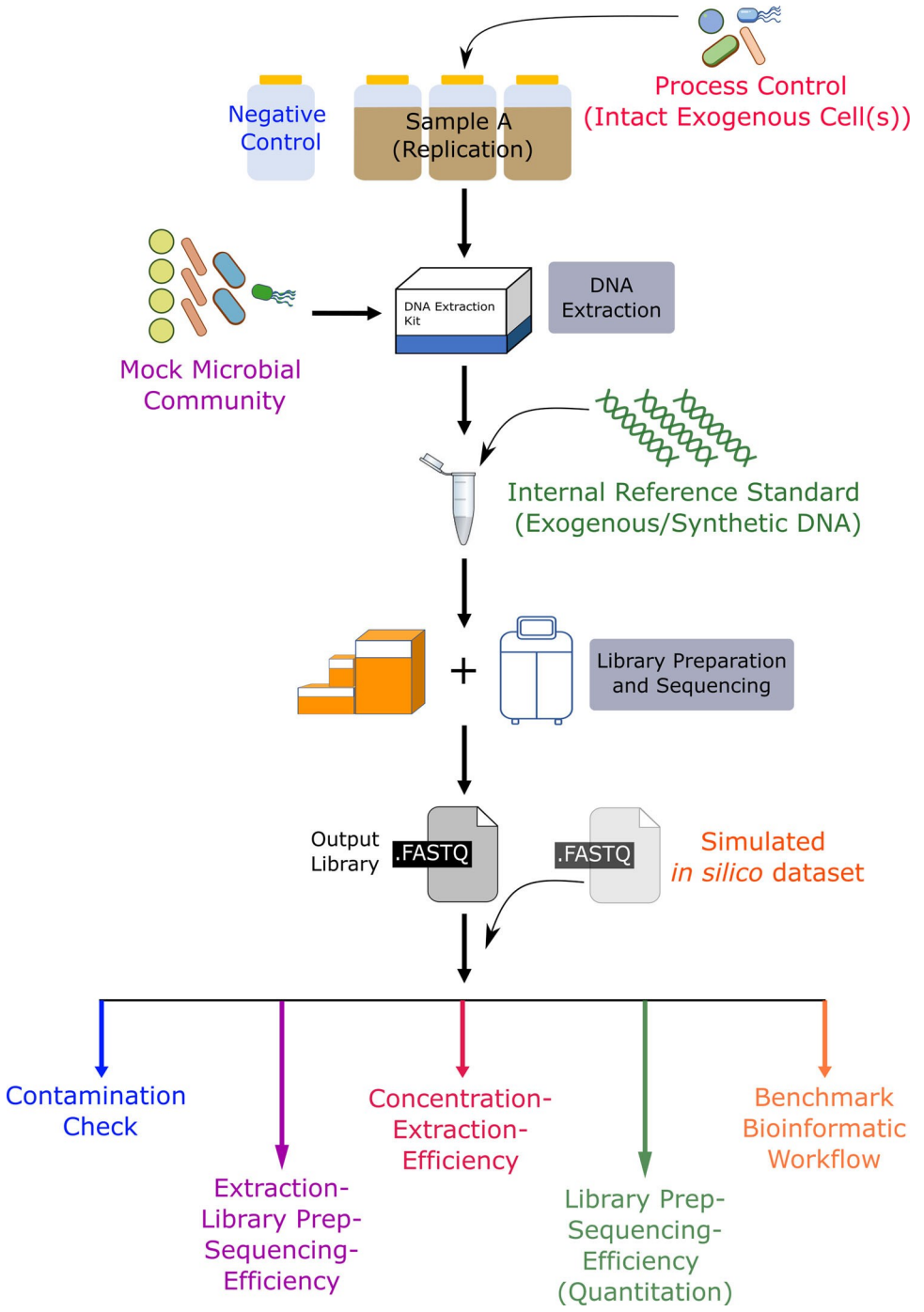

Figure 2. Framework of process controls for metagenomic investigations of environmental AR.

the overall impact of technical variability and batch effects, we recommend adding preservation reagents commensurate with the sample type being stored, and that all samples within a given monitoring campaign be processed and handled uniformly. One example protocol is that of the Global Wastewater Surveillance System, which might provide the additional benefit of being compatible with existing data from this consortium (Munk et al 2022).

Sample concentration techniques

Sample concentration serves to recover an adequate mass of microbial cells for analysis and ideally should be applied in a way that maintains representativeness of the corresponding micro-bial populations. Low mass samples yield low concentrations of DNA, which may preclude library preparation or necessitate amplification, which unfortunately adds a source of bias in the sequencing (see Library Preparation). Among the articles identified in this review, the two most common sample concentration methods were membrane filtration (62 studies) and centrifugation (23 studies). Membrane filtration was more common for less turbid waters (e.g., unimpacted river water, final treated wastewater effluent) and centrifugation was more common for more turbid waters (e.g., raw wastewater, activated sludge).

The most applied membrane pore sizes amongst the identified studies were 0.2 mm (12 stud-ies), 0.22 mm (37 studies), and 0.45 mm (12 studies). Because the smallest prokaryotic cell diameter is approximately 0.2 mm (Staley, 1999), a pore size approaching that threshold will allow for the adequate representation of the bacterial and archaeal composition of a given water sample. The tradeoff of smaller pore sizes is that less water will be able to pass through due to clogging, decreasing the representative volume and increasing the detection limit. Pre-filtration of environmental samples using larger pore size membranes (1.0 mm 1 mm) was common to reduce particulates before passing through subsequent filters, increasing the representative sample volumes. However, pre-filtration effectively eliminates the particle-bound fraction of the microbiome and may significantly alter representativeness of the sample (Xie et al., 2020). For samples with extremely low cell densities and/or turbidity (e.g., advanced water treatment products), ultrafiltration is a means to concentrate volumes up to 100 liters, although this may still be insufficient for recovering enough nucleic acid for sequencing extremely clean samples (Stamps et al., 2018). Centrifugation workflows typically involve pelleting biomass from raw wastewater at 4,000 to 15,000xg. The supernatant is discarded, and the pellet is resuspended in buffered solution that is either then passed through an additional 0.22-mm membrane or directly subject to DNA extraction. Sample concentration methodology can influence the representativeness and comparability of generated metagenomes (e.g., through size exclusion) across studies and it is recommended that researchers utilize a unified sample concentration technique, when possible. The best solution for concentrating various matrices for shotgun metagenomics and routine surveillance remains unclear. Benchmarking experiments and/or controlled comparisons of spiked samples could help to resolve differences introduced due to different concentration methods. However, the most widely used method is filter concentration with pore sizes between 0.2 0.22 mm and has been shown to provide reliable results.

DNA extraction dictates representativeness and comparability of metagenomes

Because no DNA extraction approach is 100% efficient or unbiased, DNA extraction methodologies should be consistent across sample sets intended to be compared by metagenomics. This can be challenging when seeking to compare metagenomic data across published studies, especially as DNA extraction kits and procedures continue to evolve. At a minimum, DNA extraction method and protocol versions need to be reported in associated metadata so that they can be accounted for in any future meta-analyses. Ideally, positive controls such as sample processing controls (mock community processed as separate sample) or internal standards (exogenous whole-cells, DNA or RNA added to a sample matrix) should be included to identify potential biases in the extraction. These can also be used for the identification of biases in sample concentration and bioinformatic analyses. Process controls were almost entirely absent from workflows reported in the identified literature (Fig. 2 and Table S4). Generally, process controls are comprised of known mixtures of organisms with varying susceptibility to common lysis methods (e.g., Gram-positive

bacteria, Gram-negative bacteria) and thus serve to assess the efficiency of the DNA extraction method and give insights into the representativeness and reproducibility of NGS workflows. Process controls are standard practice in many fields of molecular biology, the most recent example being the inclusion of Bovine Coronavirus as a surrogate RNA extraction control in the wastewater monitoring of SARS-CoV-2 (Natarajan et al., 2021).

Recently, mock communities have been used as process controls to characterize DNA extraction and bioinformatic workflow bias in interlaboratory studies, illuminating large deviations in the observed abundance of specific taxa (Han et al., 2020; O'Sullivan et al. 2021). Although it is possible to generate reproducible metagenomes across labs implementing the same DNA extraction workflow (Li et al., 2018a), such reproducibility cannot always be assumed. One study found that batch effects across sequencing runs resulted in loss of detection of key taxa (Yeh et al., 2018). Mock communities and process controls are therefore recommended to help assess reproducibility across space, time, and laboratories. Mock community confirmation, which were utilized only twice in the reviewed articles (Table S4), would also be helpful during submission to public data repositories to support suitability of inclusion of publicly-available data for specific studies. However, it is acknowledged that mock communities are typically much less diverse than the target environment of interest and therefore cannot fully reproduce the sampling environment (Sinha et al., 2015; Yeh et al., 2018). We believe that a single process control used to measure "total recovery efficiency" of the metagenomic workflow would be the most advantageous control to implement for the field moving forward.

Bead beating kits are ideal for short-read metagenomics

Across the studies identified, almost all DNA extractions were performed using commercial kits that employ both chemical lysis and bead beating, along with purification through a spin column (93%). The most popular were the FastDNA Spin Kit for Soil (36 studies), PowerWater or PowerSoil Kits (30), and the QIAamp DNA Stool Mini Kit (6). Previous studies comparing the efficacies of commercial DNA extraction kits for metagenomic sequencing found that the FastDNA Spin Kit for Soil (MP Biomedicals) generated the highest yield and purity of DNA from three commonly sampled WWTP compartments (influent, activated sludge, final effluent), resulting in the detection of the greatest diversity of ARGs when compared using an Illumina sequencing platform (Guo & Zhang, 2013; Li et al., 2018). Two main distinctions of the FastDNA Spin Kit for soil are that it employs a range of bead diameters, and that the DNA is suspended with the binding matrix during isolation as opposed to the binding matrix being confined to the spin column. A modified standard protocol using the QIAamp DNA stool Mini Kit (Qiagen) has also proven to be a popular and unbiased approach for aquatic resistome sampling that uses both mechanical and enzymatic lysis (Knudsen et al., 2016). These approaches aim to evenly lyse both Gram-negative and Gram-positive cells using a combination of high shear forces, enzymatic lysis of cellular membranes, and chemical precipitation of protein debris and are near ideal for large-scale environmental monitoring projects.

High molecular weight DNA extraction optimizes long-read sequencing

The above-cited studies were conducted for optimization of short-read sequencing platforms and therefore DNA damage during extraction is less of a concern. While bead-beating can reduce bias in DNA recovery, it also shears and fragments DNA (Quick & Loman, 2019). Commercial spin column kits with bead-beating generally produce fragment lengths 60 kbp (Quick & Loman, 2019). Short and damaged DNA fragments can be detrimental to optimized long-read sequencing which preferentially sequence shorter sequences at higher molarity and thus high-molecular weight (HMW) DNA extraction methods should be prioritized. For instance, the traditional

phenol-chloroform method can recover DNA with average fragment lengths approaching 150 kbp and maximum fragment lengths > 1 Mbp, although this method is inefficient for large numbers of samples and utilizes carcinogenic reagents. Several commercial HMW kits have been developed but have not been fully benchmarked for complex environmental matrices or resistome analysis. It should further be noted that minimum per-sample DNA inputs of 0.4–2 mg are required for long-read Nanopore sequencing, and this may be difficult to obtain from some aquatic sample types.

DNA quality control necessary for successful library preparations

It is good practice to preserve DNA extracts in buffer, rather than water, for long-term storage. This helps to avoid DNA hydrolysis during long-term storage and freeze-thaw cycles. Additional purification steps for the removal of PCR inhibitors from DNA extracts was uncommon among the reviewed workflows but is generally recommended. Common PCR inhibitors; such as humic/-fulvic acids, tannins, melanin, and lingering reagents from DNA extraction, have been shown to interfere with NGS library preparation (Sidstedt et al., 2020). Inhibitors such as EDTA and other salts can also cause library preparation failure. DNA sequencing cores commonly determine the quantity and purity of submitted DNA extracts as a prerequisite for sequencing. A minimum of 1 ng of DNA per sample is generally acceptable for PCR-based library preparation (Illumina, 2017). Among the reviewed articles, DNA was quantified using three different platforms: Qubit™ dsDNA HS Assay Kit (Life Technologies, 27 articles), Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, 5 articles), and the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific, 24 articles). Qubit and PicoGreen assays use fluorescing dyes that are highly specific to double-stranded DNA and accurately quantify 10 pg/mL to 100 ng/mL. NanoDrop uses spectrophotometry to assess the absorbance profiles of nucleic acids, proteins, and other contaminants. A 260 nm/280 nm (DNA/protein) optical density ratio (OD) of 1.8 to 2.0 is considered high quality DNA suitable for library preparation. Gel electrophoresis is also commonly used to assess DNA integrity and the presence of RNA contamination. Samples can be run before and after shearing and after adaptor ligation to ensure the correct insert sizes of the final library. We recommend using either PicoGreen or Qubit assays for DNA quantification and the NanoDrop or DeNovix spectrophotometers with an Agilent TapeStation (gel electrophoresis) for quality control of extracts. Twenty-six of the ninety-five identified articles did not report quantification or QA/QC of their DNA extracts and is considered good practice to assess yields and purities from extrac-tion kits and matrices to assess overall data quality.

Different sequencing platforms achieve different monitoring objectives

Roche 454 Pyrosequencing was the first highly parallelized platform (released in 2005) applied for shotgun metagenomics in environmental research (Barba et al., 2014), but has since been discontinued, with the Ion Torrent (Thermo Fisher) (released in 2010) and Illumina sequencing platforms (MiSeq released in 2011) still in use today. These technologies all yield relatively short reads (75–300 bp for Ion Torrent and Illumina and 800 bp for 454) (Metzker, 2005). Long-read sequencing, including PacBio (Pacific Biosciences) and Nanopore (Oxford Nanopore Technologies) platforms, entered the market more recently and are advantageous when the objective is to examine the genetic context of ARGs with greater accuracy (i.e., their association with MGEs and host organisms). Short-reads are limited in this regard because they must be assembled into longer contigs in order to examine neighboring genes, which introduces substantial uncertainty and bias (Bengtsson-Palme et al., 2017) (See below section on Metagenomic Assembly for ARG Contextualization). The tradeoff is that long-read sequencing tends to be relatively shallow (5.4 Gb maximum identified in this review), while deep Illumina sequencing was reported to reach 77.5 Gb (Liu et al., 2019) for wastewater samples and thus can more comprehensively profile ARGs

(Fig. 3). The average base error rates for Nanopore platforms are also higher (1–20%) (Sahlin et al., 2021) compared to Illumina (0.1%) (Stoler & Nekrutenko, 2021). Studies surveyed indicated recovery of 1–500 million reads per sample for Illumina sequencing, while reports of Nanopore sequencing of aquatic matrices to date were in the tens to hundreds of thousands, limiting the absolute number of genomic inquiries per sample. These issues of sequencing depth and read lengths ultimately factor into the degree of sample coverage achievable by each platform (i.e., the fraction of the total genomic information from the microbial community that was sequenced) (Fig. 3). However, with the advent of newer Nanopore (PromethION) and PacBio platforms (Sequel II with HiFi reads), this gap in depth, error rate, and ultimately sample coverage will continue to shrink between long-read and short-read platforms.

Among the studies identified by the search criteria, 90% utilized Illumina sequencing, 4% Oxford Nanopore sequencing, 3% Ion Torrent sequencing, and 3% Roche 454 Pyrosequencing. Thus, current understanding of optimal conditions for metagenomic monitoring of AR in aquatic environments is largely based on what has been learned from Illumina sequencing. However, it is important to also look to the future as long-read DNA sequencing is rapidly gaining ground and presents many advantages for certain monitoring objectives, specifically assessing the mobilization and host-context of ARGs (Che et al., 2019; Dai et al., 2022). Hereafter, we primarily focus on what is known based on Illumina sequencing but point out distinctions and opportunities related to long-read sequencing where relevant.

Library preparation techniques exhibit inherent biases

Library preparation generally comprises three steps: DNA fragmentation to a uniform insert size (enzymatic or mechanical), repairing and end polishing of fragmented DNA, and ligation of platform-specific adaptors (Sato et al., 2019). Illumina library preparation was almost exclusively performed by the core facility performing the sequencing analysis. Consequently, available options are often restricted to the research facility providing the service.

There are two main categories of library preparation, PCR-free and PCR-based, with the latter introducing biases associated with PCR amplification. The choice between the two is typically a function of available sample DNA, where a threshold mass is required (25 ng) for PCR-free preparations. PCR-based library preps, like the Nextera XT DNA Library Preparation Kit, use a transposome complex to simultaneously shear and ligate adaptor sequences to fragments (tagmentation) (Sato et al., 2019). Research by (Bowers et al., 2015) documented the effect of input DNA quantities and library preparation methods on the ability to reconstruct a mock community consisting of pre-extracted genomic DNA. Input DNA quantities reaching as low as 1 picogram could successfully pass library preparation using PCR-based kits, but bias toward GC rich sequen-ces was apparent as DNA inputs fell below 1 ng, as compared to a control generated with the PCR-free TruSeq kit and 200 ng of DNA. (Sato et al., 2019) carried out a similar study and found that PCR-based kits were unable to accurately reflect extremes in genomic GC content. The most variable reconstructions of mock communities were derived from the Nextera XT and TruSeq nano kits, presumptively due to nonrandom DNA fragmentation during sonication and PCR amplification. Other kits, including the newer Nextera DNA Flex (now simply Illumina DNA Prep) and the TruSeq DNA and KAPA HyperPlus PCR-free workflows, reconstructed statistically identical mock communities, even at a shallow sequencing depth (1 Gb) (Sato et al., 2019). These studies indicate that PCR-free library prep is the best option, but that newer PCR-based methods can help to reduce bias observed in previous generation kits. Regardless, metagenomes will be most comparable when generated from the same library prep method.

Library preparations for long-read sequencing, specifically on Nanopore platforms, were done in-house and are less flexible. All four articles identified in this review used the SQK-LSK108 1D ligation genomic DNA kit (Oxford Nanopore Technologies) in-house, with 1—2 mg of input

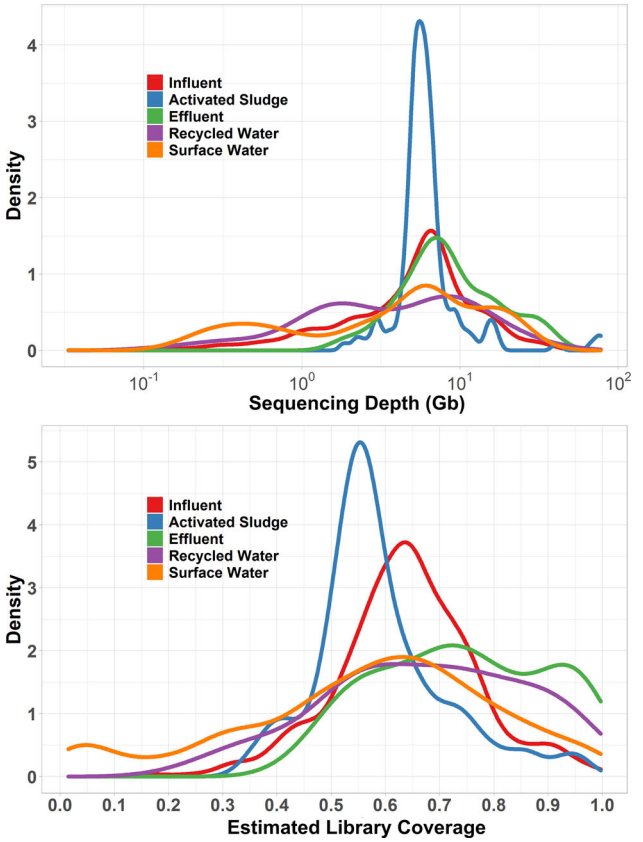

Figure. 3. Sequencing depth and coverage by water matrix. Sequencing depths were determined from all publicly-available paired-end Illumina metagenomes from the 95 studied articles downloaded from the Sequence Read Archive and European Nucleotide Archive (1474 metagenomes). Metagenomic library coverage was estimated using Nonpareil (Rodriguez-R et al., 2018) with option "-T kmer" on all cleaned and trimmed forward reads. Y-axis represents the density of individual metagenomes occurring at that depth or coverage factor.

DNA per sample for sequencing on the MinION platform (Białasek & Miłobędzka, 2020; Che et al., 2019; Hamner et al., 2019; Yadav et al., 2020). This library preparation involves four steps: end-repair of extracted DNA, Nanopore-specific adaptor ligation, barcoding, and purification. As noted above, the DNA extraction strategy employed will determine the suitability of DNA frag-ment size distributions for long-read sequencing. A study conducted by (Che et al., 2019) used a bead-beating and spin column DNA extraction approach for wastewater samples and then selected DNA fragment sizes > 8 kb by manually excising them from an agarose gel for library preparation. They then compared long-reads with sequenced and assembled Illumina data and found that the average N50 from Nanopore was 8.1 kbp (average depth 3.4 Gb), compared to 1.7 kbp from Illumina (14.5 Gb). All four articles used a bead-beating and spin column DNA extrac-tion approach for long-read sequencing, but as HMW extraction techniques continue to emerge (Maghini et al., 2021), reconstruction of complex microbial communities and optimization of long-read sequencing from environmental samples will continue to improve.

Sequencing parameters dictate depth and coverage of metagenomes

When selecting a sequencing technology and associated parameters, the platform, target read length, and depth per sample must be considered. The primary unit of "currency" for sequencing

platforms is the flow cell. Each flow cell contains one to multiple lanes, i.e., physically partitioned regions of the solid surface that enable multiple experiments to be run in parallel, but independ-ent of each other (i.e., without cross contamination). For example, the NovaSeq 6000 platform (Illumina) has SP (2 lanes, 800 M reads per lane), S1 (2 lanes, 1.5B reads per lane), S2 (2 lanes, 1.8B reads per lane), and S4 flow cells (4 lanes, 2.5B reads per lane). These flow cells can then be run with varying numbers of paired-end sequencing cycles (50–250 bp reads), which dictates the number of base pairs generated for each experiment. The number of reads generated per flow cell (and therefore per lane) fall within narrowly defined ranges, meaning the number of reads gener-ated per sample will be a function of the number of samples multiplexed on that flow cell.

There is typically a need to strike a balance between the depth of sequencing and level of replication needed to achieve monitoring objectives, while also bearing in mind cost. The level of microbial diversity anticipated in the sample and the need to detect rare sequences and taxa will both drive the need for deeper sequencing. Careful consideration is needed when choosing sequencing depths. Comparing two environmental samples with significant differences in cover-age interferes with accurate and ecologically-relevant insights into microbiome and resistome dynamics (Gweon et al., 2019; Rodriguez-R & Konstantinidis, 2014; Zaheer et al., 2018). NGS platforms preferentially sequences the most abundant features, thus, shallow sequenced datasets are severely disadvantaged in their ability to detect differentially abundant features at low abundances (Rodriguez-R & Konstantinidis, 2014). To provide guidance with respect to sequencing depths, metagenomic library coverage was empirically estimated as a function of dataset size using Nonpareil (Rodriguez-R et al., 2018) across all publicly-available paired-end Illumina data from the studied articles ($n = 1474$) (Fig. 3; Tables S5 and S6). The generated models were then used to predict library coverage at a range of depths (1 Gb, 5 Gb, 10 Gb, 25 Gb, 100 Gb) and found that 10 Gb was an optimum metagenome size that will reliably achieve 0.80 coverage across diverse environments (Table S6). The authors of Nonpareil observed that metagenomes with coverages 0.60 performed better in terms of assembly and detection of differentially abun-dant genes and can be regarded as a universal minimum. Comparing samples with greater than two-fold differences in coverage should be avoided (Rodriguez-R & Konstantinidis, 2014). Effort is needed to determine whether these general guidelines are also suitable for resistome analysis, especially considering that ARG diversity does not correspond 1:1 with phylogenetic diversity. Across these studies, the mean library size of Illumina datasets was 7.0 Gb, which would corres-pond to 0.74 theoretical coverage as estimated by Nonpareil (Fig. 3; Tables S5 and S6).

Sharing of comprehensive metadata is needed to reap the value of metagenomic data

Collection and sharing of raw data and relevant metadata is a challenge across the microbiome field and should be a critical feature of any standardized framework for AR monitoring. This is critical as resistomes and microbiomes are inextricably linked to the broader environment of ori-gin as well as the microbial processing that yields the NGS sequences. Efforts have been made by the Genomics Standards Consortia through the MiXS checklist and the STORMS reporting guide-lines to generate recommended meta-data templates and reporting guidelines for human and environmental microbiome samples these should be followed for water and wastewater AR monitoring (Mirzayi et al. 2021). Specifically, such metadata includes not only physicochemical parameters (temperature, pH, turbidity, VSS, BOD, etc.), but also water volumes collected, sample preservation (if any), DNA extraction methods/kits, and library preparation methods/kits (Liang et al., 2021). These metadata should be shared by researchers in all available instances, especially when uploading raw data to public repositories. Sparsely collected or vague reporting of metadata and effects on interpretation of results were common problems across the articles examined in this study. Notably, inspection of metadata reported across 1474 publicly-available metagenomes housed by SRA and ENA revealed several instances of sample types labeled "wastewater metagenome"

without specifying the stage of biological wastewater treatment. Given that each stage of wastewater treatment is a distinct microbial ecosystem, lack of reporting of this nature renders the data difficult to contextualize in any subsequent meta-analyses.

Online platforms for resistome analysis

Depending on level of expertise, online data processing tools may be the most feasible option for resistome analysis (Table S7). These tools also tend to be publicly-available data analysis pipelines, such as those hosted by Galaxy web portals (Giardine et al., 2005), can be beneficial where computational resources are minimal (no access to a computational server), for labs early along the adoption curve, or eventually, for when metagenomic methods for resistome monitoring become more standardized in common practice. The most commonly used online platform for environmental resistome analysis was ARGs-OAP executed in Galaxy with its latest version utilizing DIAMOND (Buchfink et al., 2015) and minimap2 (Li et al., 2018) against a custom, dereplicated database of ARGs, the Structured ARG Reference Database (SARG) (Yang et al., 2016; Yin et al., 2018). MetaStorm is another online platform with dedicated computational servers that enable the user to upload custom databases (Arango-Argoty et al., 2016). Although implemented in the command line, MEGARes and its pipeline AmrPlusPlus, is another excellent standalone analysis resource for environmental AMR analysis with a convenient acyclic hierarchical ARG ontology to simplify count-based (short-read) analysis (Doster et al., 2020).

Familiarity with command line data handling and processing for large datasets is advantageous for more advanced metagenomic analysis. This allows exploration and optimization of new analytical tools as they become available. As metagenomic profiling of ARGs is still largely implemented in the research domain, it is critical to be aware that there are numerous analytical parameters to choose from and each have implications for the research/monitoring objectives. As progress is made toward standardizing metagenomics for monitoring of resistomes in water and wastewater, agreement will be needed on default parameters (e.g., % identity, query coverage, amino acid length), depending on specific monitoring objectives, databases, and ideally, individual reference sequences. New workflows used to analyze metagenomic data should be made publicly available to aid in reproducibility of data analysis. Workflow tools such as Snakemake (Köster & Rahmann, 2012) and Nextflow (e.g., nf-core) (Ewels et al., 2020) make workflows more sharable across researchers and scalable.

Read QA/QC and merging essential to accurate resistome analysis

Following the generation and backup of sequencing reads, a critical first step is QA/QC assessment of the generated sequences to distinguish between correct and incorrect base calls and remove technical artifacts (i.e., adaptors and primer fragments). Because each sequencing run is unique in the quality of generated data, exploratory analysis of library quality is useful in determining the degree of cleanup needed. FastQC (Andrews, 2010) with MultiQC (Ewels et al., 2016) was found to be the most commonly employed software for this purpose, providing visualizations of key summary statistics of raw data, including read length, GC content, quality score distribu-tions, number of duplicated reads, adaptor contamination, and number of Ns (unknown bases). These summary statistics can then inform appropriate read preprocessing, which involves trim-ming adaptors and low-quality ends, removing low quality and truncated reads, and choosing an acceptable number of Ns that define a valid sequence.

Across the studies identified in this critical review, the most frequently implemented trimming and filtering tools were Trimmomatic (31 articles) (Bolger et al., 2014), Sickle (7) (Joshi & Fass, 2011), Fastx-toolkit (5) (Hannon, 2009), BBduk (5) (Bushnell, 2017), Trim Galore! (4) (Babraham Bioinformatics, 2012), and Cutadapt (3) (Marcel, 2011), although many others exist and perform

similar functions. The parameters used with each software were study specific, as the degree of quality filtering is dependent on the outcome of each sequencing run and the researcher's discretion. Reporting of trimming and filtering parameters, though, is essential for the reproducibility of metagenomic studies, as improperly cleaned data can result in artifacts that distort interpretation of the data due to the presence of erroneous sequences in unfiltered reads (Bharti & Grimm, 2021; Del Fabbro et al., 2013). The removal of reads originating from host organisms (i.e., host filtering) as a preprocessing tool was uncommon, although some chose to fil-ter out reads aligning to Homo sapiens when analyzing municipal wastewater. This step is used by the Joint Genome Institute Metagenomics pipeline and may boost the reliability of assembly-based analyses by removing contaminant reads that might contain spurious overlaps with the microbiome (Clum et al., 2021).

After reads have been filtered and trimmed, merging of the paired-end sequences via their overlapping regions was performed by a minority of studies (15 articles) using FLASH (Magoc & Salzberg, 2011), Vsearch (Rognes et al., 2016), SeqPrep (St. John, 2011), or PEAR (Zhang et al., 2014). When insert sizes in paired-end Illumina libraries are shorter than twice the read length, read pairs can be merged via overlapping regions to generate longer reads (Magoc & Salzberg, 2011), which can improve genome assembly, binning, and read mapping algorithms. Merging of read pairs should be included in workflows whenever possible; however, it should be noted that having too small insert sizes can negatively affect genome assembly (Bushnell et al., 2017).

Database selection and curation for ARG annotation

Metagenomic sequence data must be aligned to a database to identify genes of interest. Across the included studies, the most frequently used databases for ARG annotation were the Comprehensive Antibiotic Resistance Database (CARD; 42%) (Alcock et al., 2020; Jia et al. 2017), the Antibiotic Resistance Genes Database (ARDB; 20%) (Liu & Pop, 2009), Structured Antibiotic Resistance Genes (SARG; 11%) (Yin et al., 2018), ResFinder (10%) (Bortolaia et al. 2020), ARG-ANNOT (4%) (Gupta et al., 2014), and MEGARes (2%) (Doster et al., 2020) (Table S8). ARDB and ARG-ANNOT, it should be noted, are no longer maintained and all sequences have been incorporated into several other databases. ResFinder, SARG, MEGARes, and CARD remain actively curated. In many cases, a collection of these databases are manually combined and dereplicated on a per-study basis to increase the breadth of ARG detection (Ju et al., 2019; Liu et al., 2019; Subirats et al., 2016). To overcome the difficulties in curation and discrepancies in ARG nomenclature, ARGminer (Arango-Argoty et al., 2020) is a platform and database that seeks to maintain active curation through crowd-sourcing, and is useful for exploratory research, bearing in mind that not all ARGs have been functionally validated in the laboratory.

When choosing an ARG database, it is important to consider that each one is curated for specific purposes and has strengths and weaknesses. In terms of routine ARG monitoring, a common objective may be to conservatively identify all known and functionally-validated ARGs with associated peer-reviewed literature references, such as those in the CARD database. ResFinder focuses specifically on acquired resistance genes and contains only nucleotide references. On the other hand, if the objective is to identify potentially new ARG variants that could be of concern in a community, then the deep-learning enabled DeepARG or the probabilistic gene model based fARGene (Berglund et al., 2019) pipelines might be advantageous.

Another concern is that many housekeeping genes confer resistance via single nucleotide polymorphisms (SNPs), for example, the rpoB2 gene variant in Nocardia spp. found in the protein homolog database of CARD. Although a match may be found in the metagenomic dataset, even at 80–100% sequence homology, it cannot be guaranteed that the variant conferring resistance was detected without significant lateral coverage. This is an intrinsic limitation of short-read shot-gun metagenomics, where the length of the query is only a fraction of the reference sequence,

and the sequencing error rate precludes confidence in detecting a SNP without sufficient query depth. ARGs that are known to be caused by SNPs such as parE, rpoB, phoP, phoQ, evgS, evgA, crp, evgA, envR, marA, cpxA, cpxR, ompF, gyrA, gyrB, parC, and blaR should be checked for 100% peptide homology over a significant portion of the reference to prevent the overrepresentation of wild types (Doster et al., 2018). ARGs that are known as global regulators of efflux pump complexes are also commonly manually excised from databases before annotation (Lee et al., 2020). Past efforts were made to manually remove such ARGs from the CARD database (e.g., through the development of SARG), but recent updates to CARD have continually improved this issue by demarcating homolog versus SNP database (Alcock et al., 2020).

Read alignment tools and parameters dictate detection stringency

When performing read alignment to identify ARGs and other relevant genes, it is critical to assess what level of stringency is needed for the monitoring objective. Among the studies examined here, BLAST and its variants (Johnson et al., 2008), such as DIAMOND (Buchfink et al., 2015) and UBLAST/USEARCH (Edgar, 2010), are the dominant family of read annotation tools. BLAST is known for its alignment accuracy (Buchfink et al., 2021), but DIAMOND and USEARCH provide much more reasonable turnaround time for metagenomic alignment. Given the goal of monitoring would be to identify best ARG hits with high sequence similarity, the accuracy is comparable across methods.

It is critical to report any cutoff parameters applied, such as the e-value, amino acid identity, query coverage, and bit score, as these will dictate the stringency of database hits. Some articles identified in this review did not report these cutoffs, particularly when using online platforms. Across studies utilizing short-read sequences, the e-values used ranged from 1E-10 to 1E-4, while amino acid identity ranged from 50–95 percent, depending on the research question (Table S9). When objectives are to conservatively identify known and functionally verified ARGs of clinical concern, parameters are stricter (e.g., query coverage 80%, amino acid identity 90%, e-value 1e-10). The most applied alignment tool was BLASTx implemented in DIAMOND with an amino acid length of 25 at 80% identity. These parameters were first introduced by (Kristiansson et al., 2011) and have since been propagated throughout the field. Although data-bases such as CARD provide recommended bit score cutoffs for specific protein models, which can help to reduce guesswork in homology-based cutoffs, these parameters are benchmarked for full-length gene queries (e.g., long-reads and contigs) and will preclude short-read alignments.

Additionally, traditional sequence-based homology frameworks are not ideal for new gene discovery, where expanded databases and deep learning models (e.g., DeepARG and HMD-ARG (Li et al., 2021b)), Hidden Markov Model-based approaches (e.g., ARGsOAP v2 (Yin et al., 2018) and ResFams (Gibson et al., 2015)), and probabilistic gene models (e.g., fARGenes (Berglund et al., 2019)) have been developed; although the need for further validation has been duly noted (Bengtsson-Palme, 2018). Permissive parameters are sometimes applied to more broadly capture putative ARGs. In any case, an agreed upon classification of allowable stringent to permissive alignment parameters would greatly enhance the comparability of resistome monitoring studies.

Normalization and comparison of ARGs across environmental samples

Metagenomic data are affected by several sources of systematic technical (e.g., inconsistent DNA extraction, differential sequencing depths) and biological (e.g., differences in average genome sizes and GC content) variability. Normalization of gene abundances serves to account for such systematic variability, while also maintaining statistical power and reducing false positives (Pereira et al., 2018). Unfortunately, consistency in normalization is notably lacking and detracts from comparability across studies.

The most common approaches for normalizing ARG abundances are either biological or technical in nature. Normalizing to the 16S rRNA gene (Li et al., 2015a) as a housekeeping gene pre-sent in all bacteria has been the most common approach and provides a biologically-relevant denominator, e.g., a proxy for ARGs/total bacteria. However, 16S rRNA gene copy numbers vary across species and therefore cannot be interpreted directly as an "ARG/cell" metric, which would be more meaningful from a biological standpoint. More accurate ARG/cell equivalent estimates can be derived through flow cytometry (Liang et al., 2020), by dividing by single copy genes (e.g., the b subunit of bacterial RNA polymerase, rpoB) (Thornton et al., 2020; Zhang et al., 2019), or by dividing the number of ARGs by the average of a set of single-copy housekeeping genes (Dang et al., 2020; Lee et al., 2020; Yin et al., 2018). Because longer genomic fragments will innately generate more reads, gene length is an important component of normalizations. Reads per kilobase million (RPKM) and fragments per kilobase million (FPKM) are metrics derived from RNA-Seq and are common normalization approaches when the aim is to compare samples with significant differences in sequencing depths (Hendriksen et al. 2019; Munk et al. 2022). It is important to be aware that RPKM is derived for single-end or merged reads whereas FPKM is designed for paired-end reads, by restricting the double-counting of pairs of sequences aligning to the same reference. Thus, these two normalizations are not interchangeable. A parts-per-million (PPM) normalization was also common in the literature, which simply divides the number of ARGs found by the number of million-reads queried. A similar metric and interpretation as ARGs/cell, ARG density divides the RPKM of ARGs by the RPKM of a set of 40 single-copy genes (Lee et al., 2020) (Table S10). Rarefaction, i.e., randomly subsampling to a consistent number of reads per sample, should be avoided in almost all cases except estimating diversity indices as it results in the loss of very costly sequencing data and statistical power.

The five most common normalization approaches were RPKM, PPM, ARGs/16S rRNA, ARGs/cell (rpoB), and ARG density (Table S10). Using the 1474 downloaded metagenomes, we assessed the effectiveness of these normalizations in negating the influence of sequencing depth (i.e., $\frac{\text{coverage of community}}{\text{true biological diversity}}$), a prominent source of technical variability. In unnormalized data, we found strong correlations between sequencing depth and total ARG counts across all samples (R^2¼0.410, p¼2.19e-25; Fig. 4A). Comparing R^2 and p-values across other normalization strategies, however, we found that all normalizations except PPM (R^2¼0.21, p¼4.49e-7; Fig. 4C) produced non-significant and near-null slopes when plotted against sequencing depth (Fig. 4B–F). This discrepancy is likely due to the lack of reference gene length normalization in the PPM calculation (Table S10).

The lack of overarching bias across common normalization approaches is encouraging and indicates that ARG abundance data that use these normalizations are comparable across studies, even with disparate sequencing depths. This analysis cannot account for heterogenous workflows in data preparation, which would require controlled experiments, but can serve as an initial validation check. The analysis conducted here is supportive of the ARGs/cell calculation, either using the rpoB gene or the average of a set of single copy genes, as it accounts for both reference gene length as well as sequencing depth and provides the most straightforward biological interpretation. For "sterile" normalizations, the RPKM metric performed the best in terms of highest p-values and can also be appropriate, depending on the aim of the study. Normalizations, however, should be done on a case-by-case basis depending on the nature of the data set and aims of the study, but this ana-lysis, the equations, and data ranges provided can serve as the reference for future studies.

Quantitative metagenomics

Relative abundance metrics are not always ideal for downstream analysis, especially for microbial risk assessment (Garner et al., 2021b; Haas, 2020; Li et al., 2021a). A few studies to date have sought to derive absolute ARG abundances (i.e., ARGs per volume or mass of sample) from

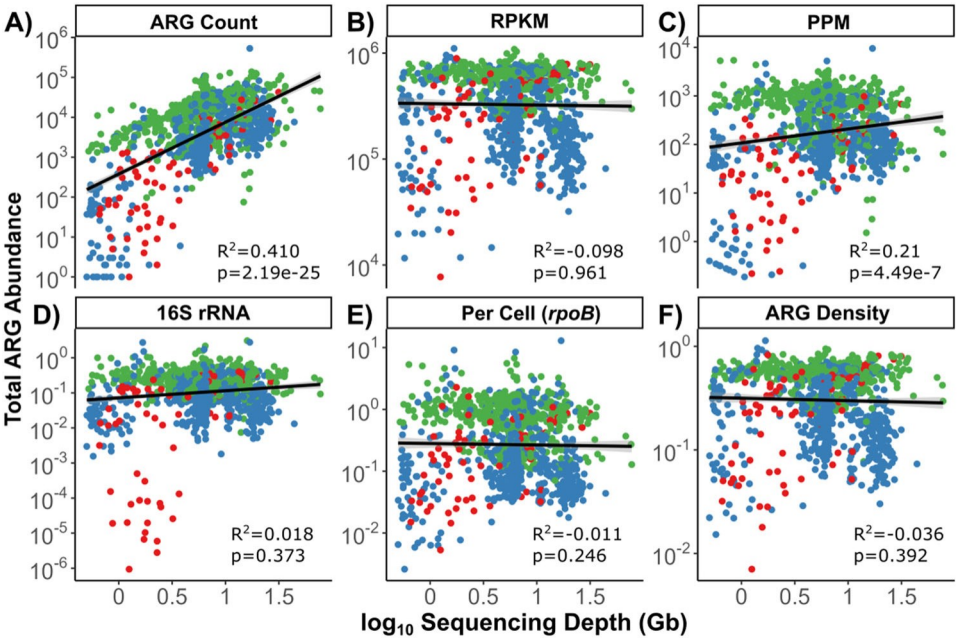

Figure 4. The effect of the 5 most common normalization approaches on negating sequencing depth bias in ARG abundance data across aquatic matrices. Unnormalized data is represented by the "ARG Count" data in panel A. Green dots ¼ wastewater; blue ¼ surface water; red ¼ recycled water. R^2's and p-values are the result of pearson correlations.

environmental metagenomic data, i.e., quantitative metagenomics (qMeta) (Crossette et al., 2021; Li et al., 2021a). Hybrid spike-independent approaches convert relative ARG abundances into absolute abundances by relying on supplementary quantitative analyses. For instance, (Garner et al., 2016), (Garner et al., 2018), and (Davis et al., 2020) determined the relative abundance of ARGs per 16S rRNA copies within the metagenomic dataset and correspondingly quantified the 16S rRNA copies per sample using qPCR. Applying the assumption that the target gene/16S rRNA quotient is equivalent between metagenomics and qPCR, a gene copy per unit volume met-ric is derived. Correlations between absolute ARG abundances derived from qPCR and hybrid spike-independent methods have shown strong correlations across several gene targets (Davis et al., 2020; Majeed et al., 2021). However, the reliability was shown to diminish for low abun-dance ARGs where the limit of detection (LOD) for metagenomics exceeded that of the qPCR assay targets (Davis et al., 2020; Majeed et al., 2021), or where primers fail to capture the full diversity of target ARGs (Crossette et al., 2021).

Spike-dependent methods use internal nucleic acid reference standards that are incorporated directly into samples after DNA extraction (Fig. 2). The reference standards are selected to be highly unlikely to be present in the sample, allowing them to be distinguished from the native microbial community. Recently, (Crossette et al., 2021) spiked genomic DNA from an exogenous marine organism (Marninobacter hydrocarbonoclasticus) into DNA extracted from digested and undigested cow manure to quantify tetracycline ARGs. Reads were mapped to all 4,272 genes comprising the genome and the average ratio of known spiked-in gene copies to reads mapped were used to calculate absolute abundances on a per-mass basis. The authors found that qPCR and qMeta were in strong agreement, but qPCR displayed a lower LOD than qMeta (2 to 8 copies/mg versus $3x10^4$ copies/mg). The LOD for qMeta is directly proportional to the sequenc-ing depth. Synthetic DNA reference standards (Li et al., 2021a) and quantitative ladders (Hardwick et al., 2018) have recently been developed and are worthy of exploration to support quantitative environmental monitoring of ARGs.

Metagenomic assembly for ARG contextualization

Environmental metagenomes are especially difficult to assemble due to the intermingled genomes of thousands of species at unknown abundance distributions, many of which are closely related or are not represented in databases (Liao et al., 2019). There are numerous options to assembling short-read data, each with their own assumptions, computational requirements, and overall limitations (Ayling et al., 2020). Across the identified studies, MEGAHIT (Li et al., 2015b) (8 articles), IDBA-UD (Peng et al., 2012) (7 articles), SOAPdenovo2 (Luo et al., 2012) (7 articles), and metaSPAdes (Nurk et al., 2017) (2 articles) were the most commonly used assemblers. Universally, assembly algorithms dimin-ish the absolute quantitative value of the data and direct comparisons to short-read "count" abundan-ces should be avoided. One workaround is to derive the relative abundance of contigs within assembled metagenomes by mapping short-reads back to the assembled contigs (Ng et al., 2017; Zhao et al., 2020; Zhou et al., 2019). Still, the larger challenge is uncertainty in the accuracy of short-read assembly and lack of means to formally assess the accuracy. Long-read sequencing is a promising way to circumvent this and has recently been demonstrated for ARG monitoring (Che et al., 2019; Dai et al., 2022), but comes with the tradeoff of shallower sequencing depth and lower coverage. A recent systematic evaluation of various assembly approaches for contextualizing ARGs found that a hybrid assembly approach resulted in the least number of erroneous contigs, suggesting a 10 minimum depth to minimize chimeric contigs that may skew resistome analysis (Brown et al., 2021).

Resistome risk assessment models

Looming large over efforts to monitor aquatic resistomes is the need to take steps toward translating the measurements to human and ecological health risks. The original framework proposed by (Martınez et al., 2015) ranks the "risk" posed by individual ARGs as a function of their documented ability to cause treatment failure, their association with MGEs, their carriage by human and animal pathogens, and their propensity for being transferred into pathogens. This framework was translated into a comparative resistome risk metric by (Oh et al., 2018), where the metagenomic reads are de novo assembled and annotated to identify ARGs, MGEs, and pathogen markers and their co-occurrence patterns. MetaCompare calculates a resistome score and ranking for each sample in accordance with these co-occurrences to identify potential "hot spots" for AMR evolution and transmission. A key limitation to this approach is the algorithm's inability to rank the relative importance of individ-ual ARGs and taxonomic sub-groups of bacteria. For instance, differentiating the relative importance of MGE-borne carbapenamases in Enterobacterales over ubiquitous efflux pumps in environmental strains of human pathogenic taxa is a critical distinction (CDC, 2019). More recently, a similar omics-based framework and software package "arg_ranker" was developed to categorize individual gene targets by their enrichment in anthropogenically-impacted environments, their history of mobility, and their presence in ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.) pathogens (Zhang et al., 2021). The framework made marked progress in sorting high- from low-risk ARGs from an environmental perspective and determined human-associated, functionally verified, and mobilized ARGs to constitute only 3.6% of the known resistome. ARGs found by sequenced-based homology are ubiquitous in the environment, but only a small fraction pose a direct threat to human health (Fitzpatrick & Walsh, 2016), while others may serve better as indicators of conditions that are conducive to the evolution and selection of resistant strains.

Conclusion

Metagenomics has emerged as a powerful tool for the routine monitoring of environmental resistomes. The sequencing of all genomic fragments in a sample without a priori identification of

gene targets allows for comprehensive assessments of microbial dynamics and risk factors for the development and proliferation of AR. However, several aspects of the workflow, from sample collection to NGS data generation and analysis, require careful consideration to ensure that monitor-ing objectives can be met, and that data generated are comparable across space and time. Experimental controls were noticeably absent from identified studies applying NGS for AR monitoring of aquatic environments and should be included in future studies. Sequencing depths should be appropriately targeted based on the monitoring objective and internal and external standards should be included to verify the accuracy and improve the quantitative capacity of resulting metagenomic data. The recommendations here can aid in the generation of comparable sequencing datasets needed to support broader ecological studies and environmental surveys. Sharing of metadata can also support larger-scale computational modeling. Given that a major advantage of NGS is the ability to store and analyze data retrospectively, the sooner the field can move toward improved quality and consistency in application of NGS for environmental AR monitoring, the better off we will be in our ability to accurately harvest the information needed to effectively combat the spread of AR.

Acknowledgements

We thank Jennifer Jenrette at Virginia Tech's Genomic Sequencing Center for providing comments on Illumina library preparation.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Water Research Foundation (WRF) Project 5052: Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water. Support was also provided by National Science Foundation Awards OAC 2004751, OISE 1545756, NRT 2125798, and ECCS 2025151.

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