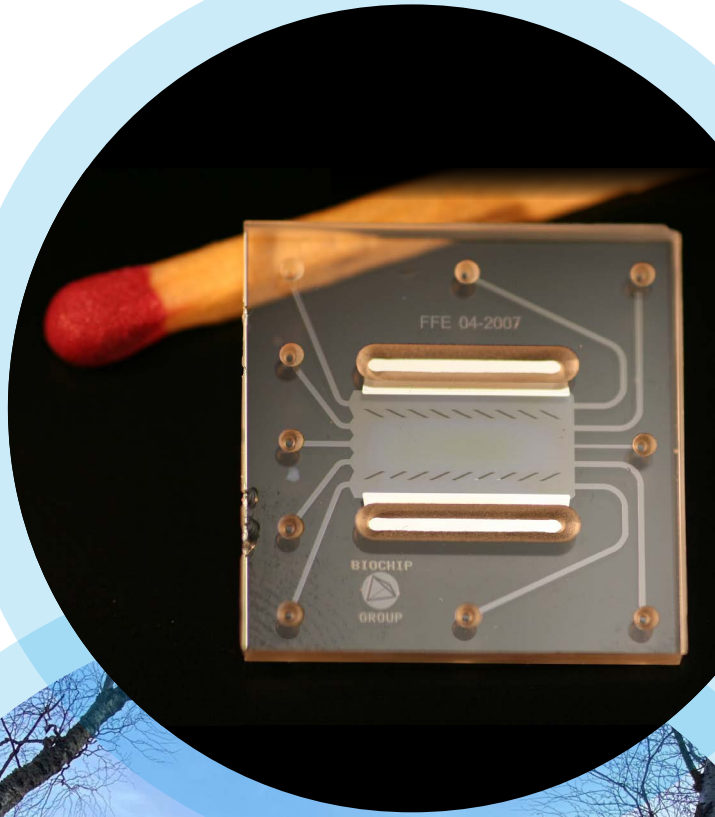
CREW CENTRE OF EXPERTISE FOR WATERS

Technologies for Monitoring and Treatment of Antimicrobial Resistance in Water

CREW Policy Brief

Appendix



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Technologies for Monitoring and Treatment of Antimicrobial Resistance in Water

Appendix - Full literature review

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CREW CENTRE OF EXPERTISE FOR WATERS

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Contents

Appendix A: Review of methods to detect AMR in the environment and the associated biases

This technical appendix is aimed at scientific researchers. Table A1 below provides examples of studies that have used each antimicrobial resistance detection method. Sections 1 to 9 expands on each detection method, including an outline of how they work and associated biases. Section 10 outlines the results of the questionnaires used to garner expert opinion on the best detection method to use from n=24 experts from Scotland and abroad.

1. Cultivation

1.1 Cultivation-dependent methods used to characterise antimicrobial resistance in the environment typically focus on specific species of interest. This requires selective media and then confirmation of the isolates through biochemical tests or genotypic methods such as 16S rRNA sequencing. The antibiotic resistances of the isolates are then tested either phenotypically through growth on media containing different antibiotics, or genotypically through various molecular methods (e.g. whole genome sequencing, multiplex PCR).

1.2 The advantages of this approach are that there are standard methods already established, it is cheap and easy to carry out, and the WHO are already monitoring ESBL (Extended-Spectrum Beta-Lactamase) *E. coli* under the 'Tricycle protocol,' allowing researchers to compare their results to those obtained through the WHO.

1.3 Between 2018-2021, SEPA isolated *E. coli* isolates from bathing waters around Scotland and tested them for resistance to cefotaxime and other antibiotic resistances (ARHAI, 2020). In another study, over 600 enterococci from surface waters were tested for antibiotic resistance (determined by Minimum Inhibitory Concentrations; MIC) and showed that surface waters harboured diverse species of antibiotic resistant enterococci (Cho et al., 2020a). Furthermore, strains of the *Bacteroides fragilis* group were isolated from wastewater and subjected to antibiotic susceptibility testing; this showed that drug-resistant and multi-drug resistant strains were being released from wastewater treatment plants to the environment (Niestepski et al., 2019). See Table A1.

1.4 **Quantitative Culture** is the same as cultivation, but the species of interest are enumerated. McInnes *et al.* used quantitative culture on water and sediment samples to screen for ESBL producing coliforms using Brilliance ESBL agar; this showed that there were significantly more ESBL-producing coliforms in urban compared to rural samples (McInnes et al., 2021). See Table A1.

1.5 **Biases** associated with cultivation-based methods include the following:

1. Non-culturable bacteria (VNCB) will not be detected.
2. Lack of representation of the full microbial community, especially if only one species is analysed (Franklin et al., 2021).
3. They (the methods) only select fast-growing, easily cultivable bacteria (many environmental bacteria require longer incubation times) (Franklin et al., 2021).
4. Selection of the most abundant strains (Anjum et al., 2021).
5. The use of different selective media to isolate specific bacterial species can affect the species populations recovered from the environment (Cho et al., 2020b).
6. Differences in incubation conditions may affect the recovery and selection of certain species (Cho et al., 2020b), e.g. more enterococcal isolates can be recovered at 37°C than at 42°C or 45°C (Jackson et al., 2005).
7. A lack of standard procedures for species identification can lead to an over- or under-estimation (Cho et al., 2020b).
8. Cultivation of naturalised bacteria (e.g. *E. coli*) may confound findings if attempting to measure anthropogenic sources of AMR (Anjum et al., 2021).

Table A1. Summary of example studies using each ARG detection method

Method	Example study	Geographic location	Matrix	What was measured
Cultivation	(ARHAI, 2020)	Scotland	Bathing water	Cefotaxime resistant *E. coli*
	(Cho et al., 2020a)	USA	Surface water	Resistant enterococci
	(Niestepski et al., 2019)	Poland	Wastewater	Resistant *Bacteroides fragilis* strains
Quantitative culture	(McInnes et al., 2021)	Bangladesh	Surface water	ESBL-producing coliforms
WGS	(Falgenhauer et al., 2021)	Germany	Surface water	Whole genomes of ESBL-producing *E. coli*
	(Lepuschitz et al., 2019)	Austria	Surface water	Whole genomes of ESBL-producing and carbapenem resistant *K. pneumoniae*
	(Nuesch-Inderbinen et al., 2021)	Switzerland	Surface water	Whole genome of an *optrA*-carrying *Entercoccus faecalis* ST16 isolate
FACS	(Jacquiod et al., 2017)	Denmark	Wastewater	16S rRNA gene of transconjugants
	(Li et al., 2018)	Denmark	Activated sludge	16S rRNA gene of transconjugants
Multiplex PCR	(Sakkas et al., 2019)	Greece	Hospital raw sewage	Carbapenemases in *K. pneumoniae* isolates
	(Borowiak et al., 2020)	Germany	Environment	*mcr-6* to *mcr-9* in *Salmonella enterica* isolates
	(Knapp et al., 2017)	Australia	Soil	*tet* genes
LAMP	(Stedtfeld et al., 2017)	USA	Surface water and sediment	*intI*1
qPCR	(Fu et al., 2022)	China	Surface water, groundwater and sediment	*ermX, ermF, ermB, ermC, qnrA, sulII, sulI, sulA,* bla_{TEM}, bla_{OXA-10}, bla_{OXA-1}, bla_{CTX-M}, bla_{shv}, *aac, aph,* and *aadD*
	(Zou et al., 2021)	China	Surface water, groundwater, acid mine wastewater	16S rRNA gene, 18 ARGs, Tn916/1545, *intI1* and *intI2*
HT-qPCR	(Zhou et al., 2021)	China	Surface water, fish	319 ARG, 57 MGE, 7 bacterial taxonomic genes
	(Lai et al., 2021)	Sweden	Surface water connected to drinking water reservoir	268 ARG, 8 integrons, 20 other resistances
	(Zheng et al., 2017)	China	Surface water	285 ARG, 8 transposases, 1 integron
dPCR	(Griffin et al., 2019)	USA	Coastal sediments and soils	*tetB, tetG, tetL, tetM, tetO, tetW, ampC, vanA, ermB, mecA,* bla_{SHV}, bla_{PSE}, *floR, aadA2*
	(Stachler et al., 2019)	USA	Surface water	*crAssphage, sul1, sul2, tetO, tetW, ermF, intI1*
epicPCR	(Hultman et al., 2018)	Finland	Wastewater	16S rRNA gene, tetM, intI1, qacEΔ1, bla_{OXA-58}
Genome cross-linking	(Stalder et al., 2019)	USA	Wastewater	Sequences of ARGs cross-linked to MGEs
Funtional metagenomics	(Wang et al., 2017)	China	Soil	Metagenome
	(Marathe et al., 2018)	India	Sediment	Metagenome
Correlation analysis	(Narciso-da-Rocha et al., 2018)	Portugal	Wastewater	16S rRNA gene, *qnrS,* bla_{CTX-M}, bla_{OXA-X}, bla_{TEM}, bla_{SHV}, *sul1, sul2, intI1*
Metagenomics	(Ng et al., 2017)	Singapore	Surafe water, wastewater	Metagenome
	(Corno et al., 2019)	Italy	Surface water, wastewater	Metagenome
	(Chen et al., 2019)	China	Surface water	Metagenome

2. Whole Genome Sequencing (WGS) of isolates

2.1 WGS determines the genomic sequence of isolates cultivated from the environment, and therefore requires a collection of isolates of interest to be established. Since the whole genome is sequenced, several ARG determinants and their subtypes can be determined at once. WGS data are generated from sequencing platforms such as Illumina and Nanopore, producing large amounts of short reads (100-400 bp) that are assembled into contigs mapped onto a reference genome, and later combined to comprise a draft genome. The large number of overlapping reads ensures that sequencing errors are reduced (Anjum et al., 2017).

2.2 The draft genome can then be searched for genetic determinants relating to AMR by comparison to ARG databases such as the Antibiotic Resistance Gene Database (ARDB). Such databases tend to contain well defined genes or specific single point mutations where there are documented correlations between genotype and phenotype (Anjum et al., 2017).

2.3 There are several freely available bioinformatic tools for detecting ARG in WGS data using the ARG databases. The commonly used bioinformatic tools include ResFinder, CARD, ARG-ANNOT, KmerResistance and SRST2 (Anjum et al., 2017).

2.4 WGS of ESBL-producing *E. coli* isolated from surface water revealed a clonal relationship amongst a sub-set of the isolates, and that they were closely related to human clinical isolates (Falgenhauer et al., 2021). WGS of ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from surface water also showed resemblance to clinical isolates suggesting their release from wastewater into the environment (Lepuschitz et al., 2019). In a further study, the genome of an *optrA*-carrying *Entercoccus faecalis* isolate cultivated from river water was determined. This showed that *optrA* was located on a Tn*6674*-like transposable element, and that 14 other resistance genes and 16 virulence genes were detected (Nuesch-Inderbinen et al., 2021). See Table A1.

2.5 The **biases** associated with WGS include the following:

1. Biases associated with cultivation methods (see Section 1.5).
2. False positive results from traces of contaminant DNA (Anjum et al., 2017). It can be difficult to apportion genes to contaminant DNA, so good laboratory practices are encouraged.
3. This method depends on the reliability of the ARG databases. These only contain well characterised ARG determinants, so new gene families, novel genes or new point mutations will not be detected (Anjum et al., 2017).
4. ARG determinants vary among bacterial species and the sensitivity of the databases may be too low to pick this up resulting in false positives (Anjum et al., 2017).
5. Parts of the genome that have a low quantity of sequence reads or the presence of several resistance genes with almost identical sequences will affect DNA assemblers and may lead to false negative results (Anjum et al., 2017).
6. The bioinformatic tools used to detect ARG may contain biases e.g. ResFinder only detects acquired resistance genes; it does not detect chromosomal mutations, multidrug transporters or intrinsic genes (Anjum et al., 2017).

3. Fluorescence-activated cell sorting (FACS) and sequencing

3.1 FACS combines flow cytometry with cell sorting to separate cells of interest. ARG hosts can be tagged with fluorescent labels using bioreporter genes, or detected using fluorescence in situ hybridisation (FISH) techniques. FACS is then able to select ARG hosts, which are subjected to 16S rRNA gene sequencing to identify the host (Nguyen et al., 2021).

3.2 FACS combined with 16S rRNA sequencing was used to identify the diversity of bacterial phyla that could host ARG-carrying plasmids by selecting for transconjugants. Furthermore, this study showed keystone bacteria (e.g. *Arcobacter*) that were involved in plasmid transmission between distant Gram-positive and Gram-negative phyla (Jacquiod et al., 2017). In a similar study, FACS was used to select for transconjugants in activated sludge, which revealed that ARG carrying-plasmid dissemination was widespread (Li et al., 2018). See Table A1.

4. Polymerase Chain Reaction (PCR)

4.1 PCR involves the exponential amplification

of target genes from extracted genomic or environmental DNA using forward and reverse PCR primers and DNA polymerase in the presence of deoxyribonucleotides. Conventional PCR involves repeated cycles of denaturation of the double-stranded DNA at 95°C, annealing of the PCR primers at 50-60°C and then extension of the DNA at 72°C. The PCR-amplified product is visualised on an agarose gel containing a DNA stain such as ethidium bromide, and its size is determined by running the product alongside a marker containing fragments of DNA of known sizes.

4.2 In **Multiplex PCR**, several target genes are amplified simultaneously using different primer pairs. The target genes must be of different sizes so that they can be resolved on an agarose gel. Multiplex PCR was used to screen *Klebsiella pneumoniae* isolates from hospital wastewater for carbapenamse genes. This showed that the isolates contained class A (bla_{KPC}) and class B (bla_{NDM} and bla_{VIM}) genes (Sakkas et al., 2019). Additionally, a specific multiplex PCR for identifying *mcr* gene variants (*mcr-6* to *mcr-9*) was developed and tested on *Salmonella enterica* isolates from the environment, feed, animals and food (Borowiak et al., 2020). Furthermore, a multiplex PCR for tetracycline resistance was used to show that these genes were linked to concentrations of zinc, copper and manganese cations in soil (Knapp et al., 2017). See Table A1.

4.3 **Loop Mediated Isothermal Amplification (LAMP)** differs from conventional PCR by the process occurring at a constant temperature, typically 60-65°C. It uses 4-6 primers recognising 6-8 regions of target DNA. Two specifically designed primers form loop structures, which facilitates subsequent rounds of amplification using the strand-displacing DNA polymerase. The resulting amplicons are long, consisting of concatemers of the target genes connected by single-stranded loop regions. Agarose gel detection is compatible with this method, but since large quantities of DNA are produced, the amplicons can be detected through photometry methods when a DNA dye is used. A LAMP assay was developed for the detection of *intI*1 as a surrogate to measuring multiple ARG. It was tested on DNA extracted from river and lake samples, and results showed a high correlation between the *intI*1 LAMP assay and ARG relative abundance measured through quantitative PCR (qPCR) (Stedtfeld et al., 2017). See Table A1.

4.4 **Quantitative PCR (qPCR)** differs from conventional PCR by allowing the PCR to be tracked in real time, either using a DNA dye such as SYBR Green or by using a fluorescently labelled probe as in the TaqMan assays. Fu *et al.* used qPCR to track 16 ARG in the environment (surface water, groundwater and sediment) and correlated their relative abundance with concentrations of antibiotics. They found that *sulA* was significantly correlated with spiramycin and lincomycin, while bla_{OXA-1} was significantly correlated with roxithromycin, ciprofloxacin, ofloxacin and sulfapyridine (Fu et al., 2022). Zou *et al.* used SYBR Green qPCR to show that the ARGs confering resistance to sulfonamides, chloramphenicols and tetracyclines were enriched around areas of the river affected by mining activities that released heavy metals into the environment (Zou et al., 2021). See Table A1.

4.5 **High Throughout qPCR (HT-qPCR)** involves the simulatanous real time amplification of numerous ARG in nanoliter reactions run on high throughput analysis platforms such as the Wafergen SmartChip Real Time PCR System. HT-qPCR was used to identify 80 and 220 unique ARG subtypes in fish and surface water, respectively, demonstrating shared ARG profiles between the fish and water (Zhou et al., 2021). In addition, HT-qPCR was used to profile the resistome of urban recipient water systems connected to Sweden's major drinking water reservoir (Lai et al., 2021). Furthermore, ARG profiling of surface water using HT-qPCR revealed that mobile genetic elements (MGEs) were positively correlated to nutrients, while ARG was influenced by the bacterial community (Zheng et al., 2017). See Table A1.

4.6 **Digital PCR (dPCR) or droplet digital PCR (ddPCR)** partitions the PCR solution into nanolitre-sized droplets so that each droplet conducts a single PCR reaction (using a fluorescent probe similar to TaqMan) on a single DNA molecule. If the gene of interest is present, the droplet will fluoresce and the fraction of fluorescing droplets is recorded. dPCR was used to assess the presence of 15 ARG in coastal sediments and soils along the eastern seaboard of the USA (Griffin et al., 2019). dPCR was also used to show a high correlation between a fecal pollution indicator (crAssphage) and ARG in and urban stream receiving combined sewer overflows (Stachler et al., 2019). See Table A1.

4.7 **Single-cell fusion PCR or Emulsion Paired Isolation and Concatenation PCR (epicPCR)** involves encapsulating single cells followed by PCR amplifcation of the 16S rRNA gene fused with ARGs. The resulting PCR products are then sequenced to identify the bacterial host of the ARGs. EpicPCR was used to show that the majority of ARG hosts in wastewater belonged to the *Proteobacteria* and *Firmicutes*, while a few were associted with *Fusobacteria*, *Gracilibacteria* and *Tenericutes* (Hultman et al., 2018). See Table A1.

4.8 The PCR-based methods are more sensitive than the sequencing, but they only target well known ARGs. Targets must be selected a priori and therefore must be "known" targets.

4.9 The general **biases** associated with PCR-based detection methods include the following:

1. Differences in sample storage prior to DNA extraction will influence microbial community composition and the ARG contained therein. Freezing can damage cells and diminish efficacy of capture of DNA (Keenum et al., 2021).
2. Biases associated with DNA extraction methods from environmental samples; some methods fail to extract DNA from certain species.
3. Reactions may be compromised by inhibitors that were not removed during DNA extraction from environmental samples, particularly soil, resulting in false negative results or under-estimation of ARG abundance.
4. Primers and probes are designed against well characterised ARG, so novel ARG subtypes will not be detected. As WGS increases the number of available genomes from resistant isolates, more primers and probes will become available.
5. Certain environments may be prone to non-specific amplification, resulting in over-estimation of ARG (Keenum et al., 2021).
6. Contaminant DNA may produce false-positive results or an over-estimation of ARG adundance, so clean lab practices and inclusion of negative controls are encouraged.
7. Inability to distinguish between live and dead cells or intracellular and extracellular DNA will result in over-estimation of ARG (Franklin et al., 2021).
8. Preferential amplification of ARG in high abundant microbes; conversely ARG in low abundance taxa may not be detected (Franklin et al., 2021).
9. Only a selection of ARG tend to be analysed at a time.

4.10 Specific biases associated with HT-qPCR include:

1. The inability of HT-qPCR to optimise individual assays due to all the assays experiencing the same qPCR cycling conditions will bias results towards targets whose cycling conditions match those used (Waseem et al., 2019).

4.11 Specific biases associated with multiplex PCR include:

1. Biases associated with cultivation methods (see Section 1.5) if using this method to characterise specific isolates.

5. Genomic Cross-Linking

5.1 This method fuses different sections of DNA present in a single cell together (i.e. chromosal, plasmid or integron DNA). Formaldehyde is used to induce covalent bonds between DNAs that are in the same cell. Restriction enzymes are then used to cut the portions of covalently bound DNAs, which are then selected for using ligation followed by junction enrichment. These fragments are then sequenced to give non-contiguous DNA sequences from the same cell. Graph clustering is used to deconvolute the contigs into their original cellular groupings, including both chromosomes and plasmids (Stalder et al., 2019).

5.2 This methods was used to determine the reservoir of ARG and the plasmids and integrons that carry them in a wastewater community. The study showed that *Moraxellaceae*, *Bacteroides*, *Prevotella* and *Aeromonadaceae* were the most likely reservoirs of ARGs in wastewater (Stalder et al., 2019). See Table A1.

5.3 The **biases** associated with this method include:

1. Biases associated with sample storage (see Section 4.9).
2. Highly abundant genomes may produce spurious links leading to erroneous clustering (Stalder et al., 2019).
3. Contigs that are shared between closely related bacterial species or plasmids may produce spurious links, making it difficult

to pinpoint the linkage of the marker to a specific bacterial strain (Stalder et al., 2019).

6. Functional Metagenomics

6.1 Functional metagenomics requires contruction of metagenomic libraries prepared from environmental DNA. The clones of interest contain recombinant plasmids carrying a resistance gene and are selected by growing them on agar plates containing antibiotics. The plasmid inserts are analysed to determine the gene sequence, which can be compared to sequence databases. Functional metagenomics is useful for identifying the true diversity of ARGs, new resistance mechanisms and their ecological roles (dos Santos et al., 2017).

6.2 Functional metagenomics was used to show that efflux pumps were the main resistance mechanisms in soils around China. In the same study, functional metagemonics identified novel ARGs belonging to the family of tetracycline-inactivating enzymes (Wang et al., 2017). In a further study, functional metagenomics was used to identify a novel mobile beta-lactamase (bla_{RSA2}) in river sediments contaminated with antibiotic production waste (Marathe et al., 2018). See Table A1.

6.3 The **biases** associated with functional metagenomics include:

1. Biases associated with sample storage prior to DNA extraction (see Section 4.9).
2. Biases associated with DNA extraction methods from environmental samples (see Section 4.9).
3. ARG that are expressed at low levels or not expressed at all (i.e. cryptic resistance genes) will not be detected (dos Santos et al., 2017).
4. Codon usage bias varies across host species, so each host will provide expression of a different set of ARG. The use of a single host will therefore bias results (dos Santos et al., 2017).
5. The method relies on sequence databases, which likely contains well characterised ARGs; novel ARGs may not be identified as being responsible for a resistance phenotype (dos Santos et al., 2017).
6. The metagenomic library tends to contain inserts no larger than 10 kb. Therefore, libraries containing small insert sizes will only allow for idenitification of single genes able to confer resistance by themselves (as opposed to resistances that are only conferred as part of an operon or gene cluster) (dos Santos et al., 2017).
7. Inability to distinguish between live and dead cells or intracellular and extracellular DNA will result in over-estimation of ARG (Franklin et al., 2021).

7. Meta-taxomonic sequencing and Correlations Analysis

7.1 This approach combines bacterial species abundance obtained from meta-taxomonic analysis (16S rRNA gene sequencing) with ARG abundance obtained through qPCR, and correlates the two to infer potential ARG hosts. It assumes that there is a positive correlation between an ARG and a taxon, with stronger correlations increasing the likelihood of the taxon to be the host (Nguyen et al., 2021).

7.2 Correlation analysis between Illumina sequencing data (16S rRNA gene) and qPCR of ARGs showed that ARGs were strongly correlated with taxa more abundant in raw wastewater than other types of wastewater, and were associated with *Campylobacteraceae*, *Comamonadaceae*, *Aeromonadaceae*, *Moraxellaceae* and *Bacteroidaceae* (Narciso-da-Rocha et al., 2018). See Table A1.

7.3 The **biases** associted with this approach include:

1. Spurious correlations can emerge through the data normalisation process (i.e. relative abundance data) (Nguyen et al., 2021).
2. Biases associated with sample storage prior to DNA extraction (see Section 4.9).
3. Biases associated with DNA extraction methods from environmental samples (see Section 4.9).

8. Metagenomic Sequencing

8.1 Metagenomics involves sequencing all the genomes in a given environmental sample through high throuput sequencing methods such as Illumina and Nanopore. The resulting reads must go through various quality control processes using approporiate bioinformatics pipelines. The metagenome is then searched for ARG by comparison to sequencing in ARG databases.

8.2 Metagenomic analysis was conducted on surface waters receiving WWTP (wastewater treatment plant) effluent, which showed that 21 ARG were shared between the wastewater, treated effluent and surface water (Ng et al., 2017). In a similar study, taxonomic information was also gathered from the metagenome, which showed that increased WWTP effluent stablised the resistome of the receiving surface waters, but this was irrespective of the microbiome (Corno et al., 2019). In a further study, metagenomics conducted on lake samples identified novel ARG subtypes not identified when using culture-dependent or qPCR approaches (Chen et al., 2019). See Table A1.

8.3 The **biases** associated with metagenomics include:

1. Biases associated with sample storage prior to DNA extraction (see Section 4.9).
2. Biases associated with DNA extraction methods from environmental samples (see Section 4.9).
3. ARGs harboured by microbes that are in low abundance may not be detected if sequencing depths are not sufficiently high. Conversely ARG harboured in microbes that are in high abundance will skew the data (Anjum et al., 2017).
4. Inability to distinguish between live and dead cells or intracellular and extracellular DNA will result in over-estimation of ARG (Franklin et al., 2021).
5. Results are dependent on library preparation and bioinformatic workflows (Franklin et al., 2021).

9. Other methods for screening ARG not included in the review

9.1 Other methods to monitor ARG were identified but were not included in this review because they either have not been applied to environmental samples (but have been applied to e.g. clinical or food samples) or they have not been used by researchers within the last 5 years. These include:

1. DNA microarray
2. MALDI-TOF
3. Recombinase Polymerase Amplification

10. Results from Questionnaires

Questionnaires used to garner expert opinion on the best detection method to use was completed by 25 participants from the academic, industry, water industry and regulator sectors. The results of 24 questionnaires showed that there was no consensus regarding the best detection technique to use. The most popular method mentioned in the questionnaires were PCR-based techniques (40%), followed by cultivation and susceptibility testing (30%). Other methods mentioned were metagenomic sequencing (15%), whole genome sequencing (5%) and Raman spectroscopy, chromatography, lateral flow test and flow cytometry (2.5% each). Cultivation-based techniques are the easiest and cheapest methods, so are more accessible to researchers. The more complex and expensive methods (e.g., metagenomics) are limited to those with access to bioinformatic expertise and larger sources of funding.

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Appendix B: Wastewater based epidemiology

Wastewater based epidemiology (WBE) for AMR could incorporate several levels of evaluation, e.g.

1. the use of culture based methods to exploit existing faecal indicator bacteria infrastructure and provide a measure of viable ARBs of concern;
2. qPCR based analysis to enable highly sensitive identification of specific ARGs of concern; and
3. metagenomics to offer rich contextual data on microbial communities without defining targets and allowing for the identification of new and emerging ARGs (Pruden, 2021).

Choi et al predict expansion of WBE surveillance for AMR in the future once methods and targets are established (Choi, 2018), while Pruden and colleagues call for the immediate implementation of a global surveillance based on WBE for AMR (Pruden, 2021), citing several main reasons:

1. To identify hotspots of AMR evolution and spread, to target regulation and action
2. To support doctors and veterinarians in antibiotic selection based on knowledge of community level AMR dissemination
3. To generate data that can inform risk assessments and therefore regulations, as well as support modelling of key drivers of AMR, and additionally assessments of treatment and mitigation approaches

Chau reviewed WBE AMR surveillance finding 29 studies covering 72 countries from 2007-2020 with majority from last couple of years. Overall, they concluded that AMR characterisation in wastewater reflected AMR in human populations, irrespective of target species, target resistance and study location, although there was variation in the strength of the association depending on these, and other, factors (Chau, 2020). Nyugen et al consider that existing approaches to WBE AMR surveillance lack the ability to support appropriate risk assessment due to a lack of standardised targets (surrogates) and agreed threshold levels along with standard reporting methods. Consideration of the ARG-ARB pathogen relationship is also critical in accurate assessment of the risk (Nyugen, 2021).

The fate and survival of different microorganisms in wastewater will depend upon the wastewater composition, the wastewater treatment processes, pH, temperature, transit times and presence of biofilms. As such concentrations of microorganisms in wastewater can vary seasonally and daily, and the impact of this variation will need to be better understood to enable the predictive value of WBE for AMR. Utilisation of WBE for AMR determination has been reported though the existing studies have concentrated on common ARGs, leaving a knowledge gap in relation to other ARGs, and also little is known about the impact on seasonality on WBE for AMR (Sims, 2020).

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Appendix C: Emerging Monitoring Technologies

Lab-on-a-chip

Lab-on-a-chip refers to the miniaturisation of lab protocols towards portable cartridges integrating and automating multiple process steps. The term microfluidics is also used to describe the flow of samples through such devices. Integrated systems coupling sample handling and detection protocols offer advantages in testing. A variety of microfluidic tools have been developed, usually focused on AST and MIC testing for medical applications, with research publications reporting simple, robust and cost-effective AMR testing though this has yet to be fully exploited in the clinic, much less in the environment (Hassan, 2020). Measures such as surface-enhanced Raman spectroscopy (Kaprou, 2021), flow cytometry (Toku blog) or impedance cytometry (Spencer, 2020) have also been demonstrated on chip. Mass spectral techniques combined with electrochemical immunoassays and enhanced with nanoparticles have also been applied (Pugia, 2021).

Genotypic testing on microfluidics is also emerging, coupled with isothermal amplification techniques (avoiding the need for thermocycling on-chip) (Kaprou, 2021). Sample complexity remains a major challenge for microfluidics, though advances such as analysis of single trapped cells or smartphone imaging offer potential solution (Needs, 2020).

Paper microfluidics is a sub-category where the device itself is made of paper, which results in a very low-cost and easily portable system. Such devices have been utilised for AMR, translating a growth inhibition assay into a paper microfluidics format with performance in environmental water samples similar to traditional lab techniques at a fraction (1/100th) of the cost (Boehle, 2017). Multiplexed analysis of the resistance against a range of different antibiotics and concentrations on paper microfluidics has been demonstrated for clinical analysis (Peijun, 2020).

Sensors

Sensor technologies can detect changes in bacterial growth or presence or amount of ARGs to report on AMR (Reali, 2019). Sensor technologies can be divided into different categories based on the method of signal transduction, e.g. magnetic, mechanical, mass-based, electrochemical or optical. All types of sensors have been investigated for phenotypic susceptibility assays and performance Tables can be found below. Genotypic methods have also been integrated into sensor platforms, using short sequences of nucleic acids to recognise and capture target genes. The most common types of sensor approach here is electrochemical and SPR, an optical method, and some approaches enabled detection of ARGs without prior amplification of the targets. However, the variation in reporting units makes it challenging to compare sensor performances. Additionally, there are still many challenges to be overcome to enable more widespread application of sensor technologies, which could be met by advances in materials, coupling with microfluidics and moving towards standardisation (Reynoso, 2021). Tables C1-C4 (below) are from Reynoso et al, 2021 and are reproduced under Open Access permissions

Table C1. Different magnetic, mass, and mechanical sensor for AST.

Technic/Signal/Type	Target	Antibiotic	MIC (µg/L)	Limit of Detection	Time	Reference
Asynchronous magnetic bead rotation	*E. coli*	Gentamicin	1	Single bacterium binding events	15 min	[87]
Atomic force microscope cantilevers	*E. coli*	Ampicillin	12.5–50	NM, 1×10^5 CFU/mL **	45 min	[100]
Asynchronous magnetic bead rotation	*E. coli*	Ampicillin	8	Single bacterium binding events	1.5 h	[103]
Asynchronous magnetic bead rotation	*E. coli*	Gentamicin	1	50 cells per drop	100 min	[104]
Asynchronous magnetic bead rotation	*E. coli*	Gentamicin	2	Single bacterium binding events	91 min	[105]
Brownian motion	*P. aeruginosa*	Gentamicin	2	One bacterium	2 h	[106]
Multi-channel series piezoelectric quartz crystal	*E. coli*	Ampicillin	32	5×10^5 CFU/mL **	5–8 h	[107]
Spectral amplitude modulation MZO- QCM	*S. epidermidis*	Ciprofloxacin Oxacillin Ciprofloxacin	0.5 1 1	1×10^5 CFU/mL	1.5 h	[108]
Orthogonal quartz crystal microbalance	*E. coli*	Ciprofloxacin Ceftriaxone Tetracycline	12.5 15 150	5×10^8 CFU/mL**	1 h	[109]
Indirect series piezoelectric	*M. tuberculosis*	Isoniazid Rifampin Ethambutol Streptomycin Capreomycin p-Aminosalicylic acid Ethionamide Rifabutin	0.1 1.0 2.5 2.0 10 2.0 5.0 0.5	1×10^3 CFU/mL **	>1 day	[110]
QCM under an external magnetic field	*D. desulfotomaculum*	Vancomycin	NR	1.8×10^4 CFU/mL **	30 min	[111]
Cantiliver NMS under an external magnetic field	*M. bovis*, *M. abscessus*	Amikacin Rifampin Isoniazid	1.7 0.15 0.17	100 bacterial cells	30 min	[112]
Atomic force microscope cantilevers	*E. coli*, *S. aureus*	Ampicillin Kanamycin	2.0 70.0	4.6 ± 0.5 bacteria/100 µm^2	30–40 min	[113]
biomaterial microcantilever with an embedded microfluidic channel	*E. coli*	Ampicillin Kanamycin	NM	1×10^5 CFU/mL **	30 min	[114]
Atomic force microscope cantilevers	*B. Pertussis*	Erythromycin Clarithromycin	0.06 0.12	NM	20–40 min	[115]

** Initial concentration; CFU, colony-forming unit; MZO, magnesium zinc oxide; NM, not measured; NMS, nanomotion sensor; NR, not reported; QCM, quartz crystal microbalance.

Table C2. Optical (bio)sensors for determination of susceptibility to antimicrobials.

Technique	Recognition Probe	Target	Antibiotic	MIC (µg/L)	Limit of Detection	Time	Reference
Colorimetric	Tetrazolium salts-8	*E. coli*	Ampicillin	128	10 CFU/mL	2 h	[122]
Fluorescence	TDN-aptamer/SYTO 9 Green	*E. coli*	Kanamycin Streptomycin Ofloxacin Norfloxacin Chloramphenicol	4.0 8.0 0.5 1.0 2.0	10 CFU/mL	5 h	[130]
SPR	2PAC—Au nanosphere/block copolymer templates (PS-*b*-PMMA)	*E. coli*, *P. aeruginosa*	Carbenicillin Gentamicin Rifampicin	100 1 Resistant	NR	30 min	[131]
laser-patterned paper-based	Chromogenic agar CHROMagar/photopolymer DeSolite®	*E. coli*	Amoxicillin	30	2.5×10^9 CFU/mL	18 h	[132]
SPR	Poly-L-lysine/glass slide coated with gold sensor chip	*MRSAMSSA*	Cefoxitin	32 to >128 1 to 4	5×10^5 CFU/mL *	3 h	[133]
Colorimetric	Endogenous H2S/AgNRs	*E. coli*	Ampicillin	100 (MBC)	10^2 cell/mL *	4–6 h	[134]
Fluorescence	Resazurin	*E. coli*	Gentamicin	4	Single cell	1 h	[135]
Fluorescence imaging	anti-E.coli antibody/streptavidin-coated polystyrene microsphere	*E. coli*	Ceftazidime Levofloxacin	4 32	Single cell	30 min	[136]
SERS	Gold nanoparticles	*L. lactis*	Ampicillin Ciprofloxacin	NR	NR	1.5 h	[137]
Fluorescence	PDMS/TLFM	*E. coli*	Ampicillin Cefalexin Chloramphenicol Tetracycline	8 12 8 2	Single cell	2–4 h	[138]
SPR	Poly-L-lysine/Au thin film	*E. coli* *S. epidermidis*	Ampicillin Tetracycline	3 10	NR	2 h	[139]
SERS	Bacteria-aptamer/AgNPs	*E. coli* *S aureus*	Tigecycline Vancomycin	0.02 0.2	5×10^3 CFU/mL *	2 h	[140]
Raman tweezers	fused-silica microfluidic chip	*S. aureus*	Oxacillin	2000	10^{12} cells/L	4 h	[141]

* Initial concentration; 2PAC, two-dimensional physically activated chemical assembly method; AgNPs, silver nanoparticles; AgNRs, silver nanorods; MBC, minimal bactericidal concentration; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; NR, Not reported; PDMS, polidimetilsiloxano; PS-*b*-PMMA, poly(styrene-*block*-methyl methacrylate); SERS, surface-enhanced Raman spectroscopy; SPR, surface plasmon resonance; TDN, tetrahedral DNA nanostructure; TLFM, time-lapse fluorescence microscopy.

Table C3. Electrochemical (bio)sensor for determination of susceptibility to antimicrobials.

Electrode—Recognizing Element	Respond	Target	Antibiotic	MIC (µg/L)	Limit of Detection	Time	Reference
Au SPE—antibody alkaline phosphatase	DPV	*S. aureus*	MRSA strain	Nm	845 CFU/mL	4.5 h	[143]
SPE—Thiolated oligonucleotide capture probes	Amperometric	*E. coli*	Ciprofloxacin	2	10^3 CFU/mL	5 h	[150]
G-FET—peptide probes	Dirac voltage	*S. aureus*, *A. baumannii*	colistin resistant strain	NM	10^4 cells/mL	5 min	[165]
SPCE/MWCNTs/AuNPs—reduction of resazurin	DPV	*S. gallinarum*	Ofloxacin Penicillin	32 16	10^2 CFU/mL	1 h	[166]
Au—aptamers	Capacitance	*E. coli* *, *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *E. faecalis*	Amikacin Ampicillin Aztreonam Cefepime Cefotaxime Ceftazidime Gentamicin	≤2 ≥32 ≤1 ≤1 ≤4 ≤1 ≤1	10^5 CFU/mL **	6 h	[167]
Au SPE—agarose-based hydrogel deposit	EIS	*S. aureus*	Amoxicillin Oxacillin	8 in both	10^7 CFU/mL (50,000 CFUs) **	45 min	[168]
Pt deposited over a glass substrate—reduction of resazurin	DPV	*E. coli*, *K. pneumoniae*	Ampicillin Kanamycin Tetracycline	NM	10^4 cells/mL	4 h	[169]
Two working electrodes (Au and Pt)—POA detection	CV	*M. tuberculosis*	Pyrazinamide	NM	40 µM of POA	NR	[170]
Array of interdigital electrodes—FD of the impedance of living and dead microorganisms	Impedance	*E. coli*	Ceftazidime Ceftriaxone Benzylpenicillin	NR	NR	2 h	[171]
Silicon nanowire FETs	Current caused by varying pH values	*E. coli*	Kanamycin Cefotaxime Ofloxacin	1–4 0.1–6 5	Single cells	6 h	[172]
3-APBA modified electrode bind with cis-diol groups on the cell wall	Capacitance	*E. coli*, *S. thyphi*, *P. aeruginosa*, *S. epidermidis*, *S. aureus*, *B. subtilis*	Ceftriaxone Q. infectoria Ampicillin Vancomycin Rhodomyrtone	0.03 20 0.5 1.25 0.5	10^8 CFU/mL	2.5 h	[173]
SPE plastic-based microchips—antibodies	Impedance	*E. coli*, *S. aureus*	Erythromycin	0.1	10^3 CFU/mL **	1.5 h	[174]
Interdigital electrodes—antibodies	Impedance	*S. aureus*	Flucloxacillin	100	10^4 cells/mL **	2 h	[175]

* Antibiotic and MIC correspond to *E. coli*; ** Initial concentration; 3-APBA, 3-aminophenylboronic acid; AuNPs—gold nanoparticles; CV, cyclic voltammetry; DPV, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; FD, frequency dependence; G-FET, graphene field effect transistors; MRSA, methicillin-resistant Staphylococcus aureus; MWCNTs, multiwalled carbon nanotubes; Not measured; Not reported; POA, pyrazinoic acid; SPCE, screen-printed carbon electrodes; SPE, screen-printed electrodes.

Table C4. Different genosensor for determination of antimicrobial resistance genes.						
Technic	**Recognition Element**	**Target**	**Type of Resistance**	**Limit of Detection**	**Previous Amplification**	**Reference**
Electrochemical—EIS and CV	DNA probe	rpoB	Rifampicin resistance	0.08 fmol/L	Yes	[179]
Optic—fluorescence	DNA probe	CTX-MNDM-1	Cephalosporins resistanceCarbapenems resistance	<10 copies of the gene	Yes	[182]
Optic—SERS	Hairpin-structured	tetA	Tetracycline Resistance	25 copies/μL	No	[189]
Optic—fluorescence	Fluorescent nucleic acid probe	VIM NDM IMP KPC	carbapenem antibiotic resistance genes	$(1.8 \pm 0.7) \times 10^6$ beads/mL per target	No	[190]
Electrochemical—EIS and CV	DNA probe	rpoB	Rifampicin resistance	0.2 fM	Yes	[191]
Optic—fluorescence	Binary deoxyribozyme	rpoB katG inhA	Rifampin resistanceIsoniazid resistanceFluoroquinolone resistance	5 fg–15.6 pg	Yes	[192]
Electrochemical—EIS and CV	DNA probe	rpoB	Rifampicin resistance	0.1 fM–1 pM	No	[193]
Electrochemical—capacitance	DNA probe	ampR	Ampicillin resistance	1–4 pM	No	[194]
Electrochemical—DPV	DNA probe	MDR1	Multidrug resistance	2.95×10^{-12} M	No	[195]
Optic—fluorescence	DNA probe	rpoB	Rifampicin resistance	1 nM ssDNA in 1 mL sample volume	Yes	[196]
Optic—fluorescence	DNA probe	rpoB	Rifampicin resistance	100 nM	Yes	[197]
Optic—SPR	DNA probe	rpoB	Rifampicin resistance	NR	No	[198]
Electrochemical—DPV	PNA probe	rpoB	Rifampicin resistance	1 CFU/ml	No	[199]
Optic—fluorescence	Fluorescence DNA hairpin	mecR	Methicillin resistance	1 nM	Yes	[200]
Optic—fluorescence	Ab-DNA probe	lamB	Increases resistance to chlortetracycline, ciprofloxacin, balofloxacin and nalidixic acid	4–250 pM amplicon concentrations	Yes	[201]
Electrochemical—DPV	DNA probe	rpoB	Rifampicin resistance	20 fM	Yes	[202]
Mechanic—piezoelectric	DNA probe	mecR	Methicillin resistance	0.125 μM	Yes	[203]

Ab, antibody; CV, cyclic voltammetry; DPV, differential pulse voltammetry; EIS, electrochemical impedance spectroscopy; IMP, Imipenemase; KPC, Klebsiella pneumoniae carbapenemase; NDM, New Delhi metallo-β-lactamase; NR, not reported; SERS, surface-enhanced Raman spectroscopy; VIM, Verona integron metallo-β-lactamase.

Long read sequencing technologies

Oxford minION, and other tools like PacBio, offer long read AMR sequences which are advantageous as the presence of ARGs can be understood in the context of neighbouring genes, giving valuable insight into mobility, co-selection and pathogenicity (Che, 2019). Computational technologies and databases to analyse the resulting data are also emerging. The minION is a portable system to sequence DNA, based on passing the DNA through a nano-scale pore and utilising electrical measurements to read the DNA code (Arango-Argoty, 2019). The system has also been successfully implemented in clinical blood samples (Taxt, 2020).

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Appendix D: Treatment technologies

An overview of the various techniques that can be applied for AMR removal is given below along with performance data from a review of the literature. The key missing understanding is in relation to the determination of the relationships between the effectiveness of AMR removal and factors like process design, operational conditions, water quality and catchment area. The review focuses on wastewater treatment plants (WWTP) though the technologies could be adapted for removal of AMR at source.

WWTP have been identified as hotspots for proliferation of AMR. However, the traditional role of WWTP has been to remove organic components from waste and reduce the risk of pathogen spread, and not specifically to tackle challenges like AMR removal. Despite that, many scientific studies have confirmed that WWTP can effectively reduce total number of bacteria and absolute abundance of ARGs although the relative ARG abundance depends on the different genes.

Methods for the removal of AMR from water can be divided into biological, physical and chemical approaches (Li, 2021), with techniques including:

International case study: Dutch WWTP

Pallergas-Vega investigated 62 Dutch WWTP, distributed across the country, demonstrating an average 2.3 log ARG removal. However, that still resulted in an effuluent load of 1 million ARG copies per litre of water. Relationships between WWTP AMR removal performance and a range of factors identified rainfall as the most significant factor reducing WWTP AMR.

Table D1: Types of AMR removal technologies

Biological	Physical	Chemical
• Conventional activated sludge (use of microorganisms to remove wastewater contaminants) • Constructed wetlands • Anaerobic membrane bioreactors (including developments to try to overcome fouling like fluidised systems or those incorporating electrochemistry)	• Filtration methods (from traditional sand based filters to membrane filters) • Adsorption, e.g. by biochar	• Disinfection • Oxidation processes

A review article by Hiller et al from 2019 summarised the results of studies looking at a range of the above processes and their performance at removing ARBs and ARGs. However, the data is often built on individual or a small number of studies (Hiller, 2019). Additionally, effective combination of the above methods is not considered, whereas there are literature reports of combined disinfection processes delivering effective results. While overall research has demonstrated the potential for WWTPs to reduce AMR by several orders of magnitude there is a high degree of variability in reported removal efficiencies, attributable to operational and site-specific parameters. Additionally, lots of work considers the overall removal performance of a WWTP without investigating the efficiency of individual components. There is still more research required to understand the working mechanisms within AMR removal methods, and therefore optimise operating parameters (if possible since for example, temperature effects appear to be ARG specific), and to consider whether methods could also contribute to AMR (e.g. low doses of chlorination has been shown to trigger horizontal gene transfer) (Nyugen, 2021).

Other factors which contribute to AMR dissemination and transfer within a WWTP are stress-inducing conditions (e.g. heavy metals, antimicrobials, low levels of disinfectants), which are ubiquitous within WWTP and hard to control for. Even low levels of antibiotics increase the transfer rate of ARGs highlighting the importance of antibiotic stewardship. However, the role of hospital effluents is unclear with Pallergas-Vega (2019) finding little correlation between ARG levels in WWTP with hospitals in the catchment whereas Nguyen (2021) suggest that treatment of hospital effluent before discharge would

be effective in reducing AMR in the environment. The importance of source control of other contaminants (heavy metals, nanoparticles, textile dyes etc) was also highlighted by Nyugen (2021).

Methods not included in Table D1 are some newer technologies and approaches, including:

- constructed wetlands (CW), where up to 99% removal of ARGs has been reported, although like WWTP, CW can also act as a reservoir for ARGs and a site of dissemination and transfer
- use of algae-based treatment, which at the lab scale has been shown to deactivate particular plasmids
- employing new materials, either natural like fruit waste or engineered like designed nanoparticles, e.g. zinc oxide or titanium dioxide in disinfection processes
- phage based therapy or graphene based materials in adsorption processes

Li et al (2021) considered that WWTPs urgently needed to be updated to deal with the threat of AMR, to enhance removal efficiency and to consider possible secondary environmental impacts, e.g. if ARBs and ARGs are removed from water does that result in a concentration within sludges and how is this disposed of safely. Cui et al (2022) reviewed methods for ARG removal in sludge concluding that traditional anaerobic and aerobic digestion methods could be effective, especially if combined with pre-treatment approaches, like thermal hydrolysis, microwave-based or free ammonia, which typically offer a higher ARG removal efficiency. However, like methods from removal from water the lack of standardised methods, and units, for measurement made comparison of different technologies challenging. Additionally, the issue was raised that many of the enhanced processes are as yet unproven on a larger commercial scale, despite successful bench and batch scale demonstration.

Further systematic studies are required and Hiller (2019) and Li (2021) highlighted key areas for future research to focus upon:

- Consideration of the individual stages of WWTPs, including treatment conditions and hydraulic residence times
- Investigation of the performance of physical methods of ARB and ARG removal, which have received less attention so far
- Elucidation of the underlying mechanisms behind the observed performances, along with the impact of different operating conditions on behaviour
- Explore combinations of different processes to maximise performance
- Standardised analytical approaches to verify and compare removal efficiencies of different processes

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