Environmental antimicrobial resistance: review of biological methods

Chief Scientist’s Group report

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Dr Robert Bradburne
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Executive summary

The importance of the dynamic between the environment and antimicrobial resistance (AMR) has been recognised as part of a multisector 'One Health' approach. While within the clinical and veterinary settings methods used for AMR surveillance are well established, they are driven by clinical cases and so are somewhat reactive. Responding to humans or animals which have already acquired AMR organisms and are presenting with infection or colonisation. On the other hand, environmental AMR surveillance could be proactive since it enables early mitigation of potential risks before they occur. This would be before direct infection of a receptor organism, transfer of resistance genes to a pathogenic organism, by the disruption of natural microbial populations, or even looking for antimicrobial compounds being polluted into the environment which may drive resistance in the environment. The requirements for surveillance of AMR in the environment are in some respects likely to be very different. Nonetheless, a method involving the characterisation of isolated cultures such as species identification, susceptibility testing and whole genome sequencing are likely very similar to clinical uses. In addition, methods that focus on microbial community changes and the impact of pollution of substances known to drive AMR selection are not as well established and will also be required to assess AMR in the environment. So, there is a need to establish a suite of analytical methodologies suitable for environmental AMR surveillance in England.

This review aimed to identify available biological methods to assess AMR organisms and associated genes in the environment and to discuss the benefits and drawbacks of each method which will be evaluated as part of a surveillance pilot.

Methods to be reviewed should include the use of extended spectrum β-lactamase producing *E. coli*, vancomycin-resistant *Enterococci*, and azole-resistant fungi as indicator organisms to assess general levels of AMR in these groups. For the pilot these species should be further characterised using whole genome sequencing and antimicrobial

susceptibility testing. Additionally, the application of broad methods should also be used to determine trends in total AMR, as such both whole metagenomic sequencing and high throughput qPCR should be tested. Additionally, non-biological analyses should be performed to determine how antimicrobial substances may impact and drive AMR in the environment, as well as how the environment acts as a pathway to receptors of importance. A comparison of these biological methods where similar data will be obtained, should be incorporated within the surveillance pilot. This will allow us to assess trends in AMR but also how these methods could be scaled up to a national surveillance programme whilst still being cost effective.

The above methods once tested could allow the Environment Agency to determine changes in the state of the environment that are indicators for an increased hazard to receptors of AMR that are valuable to society and to help wider government agencies determine links between AMR in the environment and that in healthcare and agri-food settings.

Background

Antimicrobials are an essential tool in modern medicine and agriculture. However, the increasing ability of some bacterial, fungal, viral, and eukaryotic pathogens to mitigate the effects of these substances is termed antimicrobial resistance (AMR). While it has been acknowledged that AMR has been part of the natural environment for millennia (D'costa *et al.*, 2011; Waglechner, Culp and Wright, 2021), the increasing use and sometimes misuse of antimicrobials has meant that further resistance has developed rapidly and now threatens our modern healthcare, animal husbandry, and even food security through failure of crop protection (WHO, 2019). The ability of some bacteria to transfer genetic material “horizontally” even between different species, via mobile genetic elements (MGEs), has exacerbated this issue as it means that bacteria do not need to independently evolve resistance mechanisms. In 2019, bacterial AMR alone was directly responsible for over 1.2 million deaths globally (and associated with a further 3.6 million), almost as many as the 1.8 million deaths for COVID-19 reported in 2020 (WHO, 2021b; Murray *et al.*, 2022). Economic reports estimate a global loss of $100 trillion USD, with independent reports suggesting that waterborne AMR could cost the agricultural sector $6 billion USD per annum (O’Neil 2016; World Economic Forum 2021). Whilst most of the focus on AMR to date has been in the clinical and veterinary setting, it has also been acknowledged that the natural environment plays a role as a source, receptor, and vector for transmission of AMR. In addition, the potential impact of AMR on the ecosystems of natural environments remains largely unknown.

The environment is impacted by a range of human, agricultural, and industrial activities. These can result in resistant organisms and associated resistant genes, and antimicrobial substances or other selective agents entering the environment can also lead to the potential development of AMR (Von Wintersdorff *et al.*, 2016). Policy makers acknowledge the role of AMR in the environment, with 103 countries incorporating this into their National Action Plan to tackle AMR. However, at the time of writing, no nation has yet developed an environmental surveillance scheme for AMR to address the environmental aspect. The World Health Organisation (WHO) recently made recommendations for a minimum AMR surveillance programme, which includes the environment and food (WHO, 2021a), but these are modest in scope and may provide only limited information on the sources, development, and spread of AMR or how it impacts on the natural environment as a receptor. Therefore,

questions and considerations remain as to which biological methodologies should be used for a comprehensive environmental AMR surveillance.

Current methodology reviews have limited acknowledgment of antifungal or antiprotozoal resistance and do not fully consider how to build on existing environmental monitoring. AMR is a diverse and complex problem, and no single method can fully capture a representation of AMR in the environment. Therefore, an optimum surveillance programme for environmental AMR is likely to consist of multiple methods, each covering the disadvantages of the other to capture the broad diversity, and extent required.

This report will "identify methods available for the detection of AMR organisms and their associated genes in natural environments" and to discuss their suitability and the practicality of their implementation for surveillance of AMR in the environment. The report considers the costs associated with different methods and that although some methods may be costly now, they may become more cost effective in the near future. Based on the discussion, this report will also make recommendations for methodologies to be trialled in the Environment Agency's pilot surveillance of AMR in the environment.

Approach to Method Identification

Using the guidance by Peters et al. (2015), a review of the literature was conducted with the objective to: "identify methods available for the detection of AMR organisms and their associated genes in natural environments". This review focussed on antibacterial and antifungal resistance and excluded the antiviral and antiparasitic aspects of AMR. However, many of the genomic methods identified could be applicable for other types of resistant organisms, such as viruses and protozoa. Relevant peer reviewed articles were identified by searching the Scopus database using the search terms listed within Figure 1. Articles were identified based on the following selection criteria:

1. Source: literature was limited to open access, primary research articles that had been peer-reviewed to ensure the quality of the source.
2. Time: only articles published within the last 10 years have been considered, as methods identified need to be relevant and up to date.
3. Language: only articles written in English were included.

For the initial search the term “enviro*” was used, however this was not effective at limiting the articles to the natural environment. Therefore, the search terms for individual environments were included within the search (figure 1).

.(KEY(((AMR) OR (antifungal) OR (antimicrobial resistan*)) AND ((air) OR (*aerosol*) OR (river) OR (estuar*) OR (stream) OR (pond) OR (lake) OR (catchment) OR (drain*) OR (wastewater) OR (sewage) OR (sewer*) OR (ocean*) OR (sea*) OR (coast*) OR (beach*) OR (bath*) OR (soil) OR (sedim*) OR (silt) OR (compost) OR (shellfish) OR (oyster) OR (mussel) OR (clam) OR (wildlife))) AND (LIMIT-TO (DOCTYPE,"ar")) AND (LIMIT-TO (PUBYEAR,2022) OR LIMIT-TO (PUBYEAR,2021) OR LIMIT-TO (PUBYEAR,2020) OR LIMIT-TO (PUBYEAR,2019) OR LIMIT-TO (PUBYEAR,2018) OR LIMIT-TO (PUBYEAR,2017) OR LIMIT-TO (PUBYEAR,2016) OR LIMIT-TO (PUBYEAR,2015) OR LIMIT-TO (PUBYEAR,2014) OR LIMIT-TO (PUBYEAR,2013) OR LIMIT-TO (PUBYEAR,2012)) AND (LIMIT-TO (LANGUAGE,"English")) AND (LIMIT-TO (OA,"all")) AND (LIMIT-TO (SUBJAREA,"BIOC") OR LIMIT-TO (SUBJAREA,"ENVI") OR LIMIT-TO (SUBJAREA,"AGRI") OR LIMIT-TO (SUBJAREA,"MULT") OR LIMIT-TO (SUBJAREA,"EART")))

Figure 1. Detailing final search keywords used in the scoping stage of the review

The abstracts and metadata for papers identified (*n=717*) using the search terms outlined in Figure 1 were downloaded on 2nd November 2021. Based on the content of the abstract, the identified articles were reviewed to determine whether they were relevant to biological factors of AMR surveillance in bacteria or fungi in natural environments. Articles that did not meet these criteria were removed (*n=481*). In cases where it was uncertain if the inclusion criteria were met based on the abstract, a full text screening was applied. For the remaining articles (*n=236*), the full texts were reviewed to identify the type of methods used and which organisms and genes were analysed. During this stage, articles were excluded if they did not meet any of the previous selection criteria (removal of n=80). For example, in one paper the water analysis mentioned in the abstract was performed on tap water, which was regarded as outside of the scope of the natural environment (Aabed, Moubayed and Alzahrani, 2021). In total, 156 articles were used for this review.

Overview of Available Methods

Phenotypic Selection

A phenotype is a physical characteristic which can be a result of genomic and environmental differences. As such, phenotypic methods test bacteria and fungi based on their phenotypic qualities for example, lactose fermentation in bacteria. Most articles did utilise some phenotypic methods (*n=124*). The use of phenotypic methods for surveillance particularly in a multidisciplinary field can be desirable. It ensures the organisms are viable and therefore can actively spread AMR and cause disease if given the opportunity. Phenotypic methods also ensure that the organisms, when exposed to antimicrobials, do express genes that confer resistance and are still able to survive in such conditions. Additionally, due to the relative simplicity of the methods it is easier to explain to and share the resulted data across a wide range of stakeholders (e.g., from non-experts to experts)/ policy makers.

There is multiple rationale for choosing a specific microorganism, but usually they fall into either being pathogens or indicator organisms. Pathogens are organisms which when exposed to their host cause disease. Indicator organisms are a surrogate for pathogenic organisms. Advantages of these indicator organisms are that they are found in higher abundancies than the pathogenic organisms and so are easier to detect.

Table 1: Summary of the target groups of organisms considered in the reviewed articles.

Target organisms/ phenotypic work performed	Number of articles	Percentage of articles with phenotypic analysis
Gram-negative bacteria	86	69
Gram-positive bacteria	27	22
Non-specific bacteria	25	20
Fungi	11	9

Table 1 summarises the target groups of organisms identified in this review. Most articles identified in this review are targeted gram-negative bacteria. Of the gram-negative bacteria, *Escherichia coli* (*E. coli*) was the most observed species (*n=63*), likely because it is associated with faecal pollution and can be easily cultured. Additionally, the WHO has released a tricycle method for extended spectrum β–lactamase (ESBL) producing *E. coli* to enable comparisons across different sectors, and internationally (WHO, 2021a). Detection of *E. coli* could easily be applied to other environmental matrices to make resulting data standardised and therefore more comparable across different studies and sectors and thus allows for greater spatial comparisons.
The other commonly used gram-negative species discussed within the literature were *Pseudomonas* spp. (*n*=9) and *Salmonella* spp. (*n*=10). *Salmonella* spp. carry a variety of AMR genes, however, require multi-step confirmation methods for species and strain identification. Additionally, studies which used *Salmonella* spp. were predominantly focused on wildlife swabs (among others gulls, wild boar, reptiles, shellfish, marine mammals, etc.) and thus required enrichment or onsite filtration to detect them, meaning that bacterial concentrations were not quantifiable (Palhares *et al.*, 2014).
Pseudomonas spp. are known to be present in environmental samples (although there is little contemporary literature on concentrations in environmental samples), and environmental *Pseudomonas* spp. are known to be multi-drug resistant. Therefore, the use of this species will hinder a surveillance if the question involves movement of mobile genetic elements (Luczkiewicz *et al.*, 2015).

Of the gram-positive bacteria both *Enterococcus* spp. (n=16) and *Staphylococcus* spp. (n=8) were the most widely used, and both are listed on the WHO priority pathogens list (WHO, 2017). Similarly, to *E. coli*, *Enterococcus* spp. would be a good choice for environmental AMR surveillance as it is already known to be a suitable faecal indicator organism. Whereas there is limited knowledge of the survivability of *Staphylococcus* spp. within the environment, with some research showing that survival is negatively affected in freshwater environments (Levin-Edens *et al.*, 2011), but may be suitable for other environmental matrices like aerosols, which are less well characterised.

Within this review few articles were identified that looked at AMR in fungal species (*n=11*). Of the eleven articles, five used *Aspergillus spp.* two used all yeast species, and two explicitly for *Candida* spp., and two articles considered total fungi. However, there is a knowledge gap surrounding antifungal resistance in the environment, and a high likelihood that these species were chosen as indicators of human associated antifungal resistance.

Because of this, antifungal resistance surveillance should not discriminate using taxa until more information can be gathered about the abundance and diversity of antifungal resistance in the environment. Instead, selection should be done using an azole antifungal agent as they are a group of antifungals used in both agriculture and healthcare. Azole-resistant fungi are therefore more likely to pose a risk to and develop within the environment, as well as impacting on human health from the environment. Detection of Tebuconazole could be a good candidate for a suitable azole within an environmental surveillance, due to being the most widely used agricultural antifungal agent in the UK (Ridley *et al.*, 2021), and that it cross selects for the common clinical antifungal fluconazole (Bastos *et al.*, 2018).

Enumeration and Isolation

Three methods to enumerate and/ or isolate microorganisms will be discussed in this review, namely membrane filtration, most probable number (MPN), and spread plating.

Membrane filtration is the use of a filtration manifold and vacuum to pass a liquid sample through a membrane, capturing the bacterial or fungal organism of interest. Membranes are then added onto an agar or to a broth that selects for the organism of interest. The main benefit of this method is the ability to process larger volumes of sample. However, it is limited by sample turbidity, with turbid samples causing the filter to clog, and thus impacting the readability of the sample. This method also allows for enumeration and isolation in one step with a lower limit of detection compared to the other isolation methods discussed below. Therefore, this method lends itself to the detection of low numbers of organisms in water samples. However, it is not appropriate for more turbid sample types, such as soils with large particles or shellfish flesh, which would clog the membrane too quickly.

MPN methods are the enumeration of microorganisms which involve inoculating broths with various dilutions of a sample, which when the target organism is grown changes colour. It is typically used when samples are too lumpy or turbid for membrane filtration or spread plating, although it is comparatively labour intensive as each sample must be setup with multiple dilutions. Additionally, to do further tests the culture must be streaked on a plate to obtain a pure isolate.

Spread plating is spreading the sample directly onto the plate. It lends itself to samples containing a high number of organisms of interest, which do not need to be concentrated.

Phenotypic Characterisation

Characterisation of bacterial and fungal isolates can be undertaken using a suite of different methods, and often depends on the organism being targeted and the type of information required. In the context of AMR, phenotypic characterisation can include confirmation of antimicrobial and metal resistances, species, or strain identification, and for some species the host of the isolates, which can be used to fully explore the data gathered as a whole.

Characterisation at Enumeration and Isolation

One way of characterising resistance is during the enumeration and isolation stage directly, for example with the addition of antimicrobials in an agar plate. This method has the benefit of being very cheap, and screens large numbers of organisms for AMR. This benefit is synergistic with methods, such as membrane filtration that allow for very low limits of detection, and when used in combination with testing for non-resistance organisms still shows AMR prevalence. However, this is only testing for resistance in one species (or group) and only to one or a mixture of antimicrobial(s) at a time and only at one concentration. The selection of which antimicrobial at what concentration to test becomes an important choice, and whichever antimicrobial is chosen will likely bias results of any further isolate characterisation.

Antimicrobial Susceptibility Testing

The characterisation of the antimicrobial properties of an isolate can be done by antimicrobial susceptibility testing (AST) which is advantageous over genomic methods in some respects as it looks for phenotypic AMR. Of the 124 articles reviewed that utilised phenotypic data, 79% of them performed AST to an array of antimicrobials. The two most widely accepted methods are micro-broth dilution and disc diffusion (ISO, 2019; EUCAST, 2022). AST by disc diffusion involves the spreading of a lawn of an isolate onto a non-selective agar plate, placing a disc infused with antimicrobial substance(s) on top and measuring the zone of inhibition (Figure 2).

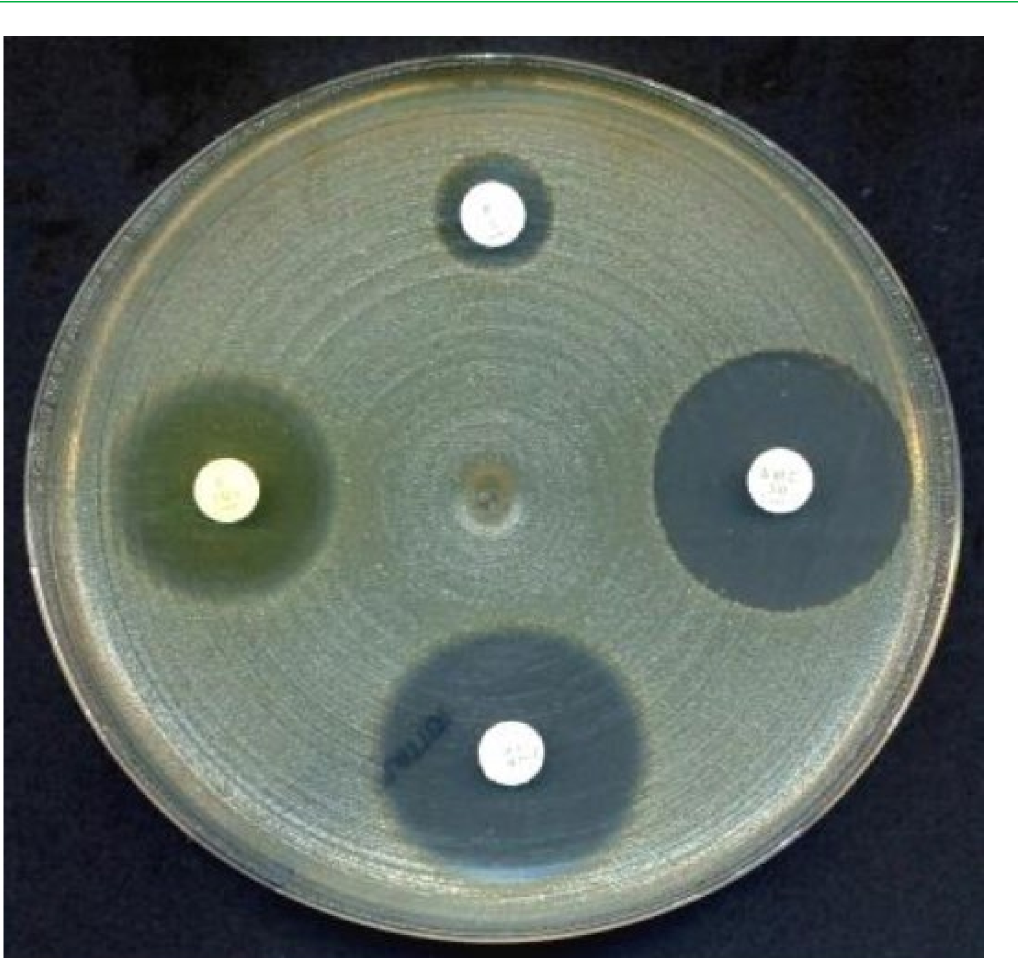

Figure 2. Showing cultured lawn on agar with zones of inhibition caused by antimicrobials. Source: EUCAST disk diffusion method for antimicrobial susceptibility testing, 2022

AST by broth dilution involves inoculating the microorganisms to be tested into broths containing a dilution range of antimicrobial substances and determining at what concentration there is no growth. Micro-broth dilution is the development of broth dilution, adapted to a microplate, which can then be read automatically using a microplate reader. Both micro-broth dilution and disc diffusion have their benefits and drawbacks, with micro-broth dilution being a more automated system allowing for a higher throughput. However, its drawbacks include being subjective to read, especially without a plate reader. Disc diffusion is simpler, having a lower start-up cost but it is limited to organisms that form a lawn and hence would not be appropriate to use for the investigation of some fungal species e.g., those that form hyphae.

Once set up both are affordable when compared to genomics methods, although the cost does depend on how many antimicrobials are tested. Despite the limitation of AST not being able to explain the underlying mechanism of how the organism acquired resistance, it is

important to test environmental samples for AMR phenotypically. Due to the clinical bias of existing genomic databases, the sole use of these database may lead to mischaracterisation if the gene was novel and thus not present in the database

Matrix-assisted laser desorption/ ionization time of flight mass spectrometry methods

Matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF MS) is a method that utilises mass spectroscopy for biological samples, mainly species identification. The proteins of an isolate are ionised and measured using mass spectroscopy and time of flight, generating a fingerprint of the cell proteome which can be compared to known databases and samples. Despite 16% of the 156 studies identified using MALDI-TOF MS, only one study used it for direct detection of AMR whereas all the other studies used it for species identification only. This is likely because detection of AMR using MALDI-TOF MS is limited by the molecular weight (size) of the proteins that confer resistance being too large for the currently available technology (Florio *et al.*, 2020).

MALDI-TOF MS is a useful cost-effective tool for rapid identification of species, and which can distinguish a limited range of resistances to antimicrobials. However, for its environmental AMR applications, this method is limited as the target organism must be isolated and cultured, to have sufficient material for its identification. Additionally, the ability to detect differences in AMR and non-resistance proteins in practice have been primarily limited to the detection of β–lactams and carbapenems (Feucherolles, Cauchie and Penny, 2019). Furthermore, data analysis, whilst not as complex as sequencing bioinformatics, still requires matching to databases of known proteomes and mass to charge ratios.

Next Generation Sequencing

Whilst the ability to sequence genetic material has been possible for some time, recent advances termed next generation sequencing (NGS) technologies allow for high throughput gene sequencing and will be the focus of this review. Currently NGS technologies provide two types of data dependent on the technology used to determine the sequences: short, accurate sequence “reads” using Illumina based technology (Bentley *et al.*, 2008), and longer but less accurate sequence reads using either Oxford Nanopore (Mikheyev and Tin, 2014) or PacBio SMRT technologies (Eid *et al.*, 2009). Each have their own application depending on the question asked, although it is likely in the future that long accurate reads

will be possible. The high-throughput nature of NGS technologies has allowed the analysis of whole genomes of multiple species (whole genome sequencing) to be investigated, and, in the context of AMR, the “resistome” of these communities (metagenomic sequencing).

52% of the 156 articles identified used sequencing methods (all sequencing technologies), 14% used whole metagenomic sequencing, and 8% looked at whole genome sequencing (WGS). Other studies reviewed sequenced specific genes, such as 16S metabarcoding or multi-locus sequence typing for species identification, as well as sequencing specific AMR-related gene alleles. In this review, only WGS and metagenomic sequencing are being discussed as they capture a broader range of information on environmental AMR. Due to the large number of genes that may confer resistance the genes themselves will not be directly discussed. Although their use to answer specific questions around relative abundance and sequence type is valid, it is beyond the scope of this review to compare which are most significant. “Metabarcoding” such as 16S or ITS (internal transcribed spacer) assays, do not target resistance genes and so would not be appropriate to look at AMR directly but could be used in conjunction with methods described here to determine the antimicrobials effect on the ecosystem.

All NGS based sequencing methods discussed below are expensive methods, although costs have improved recently due to improvements in sequencing devices such as the Illumina NovaSeq6000 and are likely to fall further as technologies develop. As a result, the numbers of samples that can realistically be analysed at present is very limited, but as the technology improves and costs fall may be more cost effective than other methods in the future. Additionally, these methods require bioinformatic pipelines: the statistical analysis of DNA sequence data, turning the data into usable outputs. These typically involve discarding reads of poor quality, contig/ genome assembly (Bankevich *et al.*, 2012), and matching to databases (Altschul *et al.*, 1990). Databases can include data for taxonomic assignment using core-genome multilocus sequence typing (cgMLST), virulence genes, AMR genes, mobile genetic elements (MGEs). These pipelines can have large IT requirements (computing power and storage space), and additional technical staff which need to be accounted for.

Whole Genome Sequencing (WGS)

WGS is the sequencing of an organism’s whole genome and can characterise a microbial isolate in much greater detail than traditional characterisation methods. WGS methods are applicable to both bacterial and fungal isolates. Briefly, organisms of interest are isolated

and cultured, the DNA is extracted, then sequencing libraries prepared and run on the sequencing instrument. After sequencing, *in silico* bioinformatics analyses as discussed above are required to take the data from raw sequences to a readable format and allow a comprehensive characterisation of the isolates.

There is much potential for WGS for AMR in environmental surveillance, as isolates can be stored and analysed later, when an area or catchment of interest is identified. For example, to determine the effects on environmental microorganisms after an unusually high use of antimicrobials in an area of interest or after the release of untreated sewage. Detailed information about the isolate can be determined from even partially assembled genomes and at a higher resolution compared to phenotypic methods as specific alleles can be detected. Furthermore, this method allows resistance genes to be linked to one another, as well as genes that indicate the species host adaptations, pathogenicity, and virulence. In combination with an isolate database, the identification of clonal or related strains can provide insights into sources and pathways for transmission which could be shared easily across multiple sectors. Additionally, analyses can be repeated with updated, more comprehensive databases as understanding improves, which allows for historic analyses of recently discovered AMR genes. Additionally, WGS data analysis can allow for the application of a novel approach to AMR gene discovery by mining the data using artificial intelligence and machine learning (Liu *et al.*, 2020). However, it must be acknowledged that even if all isolates could be analysed, it is unlikely to be able to represent the full genetic diversity of the sample. Because there is limited availability and comprehensiveness of fungal AMR genes databases, so initially antifungal resistance would be underrepresented until a database can be curated.

After the bioinformatics pipeline a typical output would be a spreadsheet that can be easily imported into databases or statistical software, such as R. The practicality and approach of WGS in relation to an environmental surveillance will depend on the aim of the investigation. It is often unknown how many isolates would be needed to be representative of a given sample population and depends on the variability of the population, and its stability over time. Nevertheless, WGS is likely to be a key tool in any environmental AMR surveillance programme due to the benefits outlined above.

Non-Targeted Whole Metagenomic Sequencing

Whole metagenomic sequencing (WMS) uses the same sequencing technologies as described above, but instead of sequencing individual organisms, random environmental

DNA within a sample is captured and sequenced. The result is the sequencing of a broad range of genes and species present in the environment. This includes organisms and genes that are not of primary interest. The benefit of this method is the ability to detect the most abundant AMR genes, and MGEs that confer AMR resistance in the environment across a broad range of species (Hendriksen *et al.*, 2019). A further advantage of this method is that potential pollution sources can be determined using microbial source tracking (Li, Yin and Zhang, 2018). However, the disadvantage of this non-targeted WMS is the loss of power to link together organism species, host, and AMR genes.

IT requirements for WMS approaches are higher than other NGS methods due to the larger datasets. Additionally, as there is no initial selection this results in sequencing of non-AMR organisms, which adds to the noise of the final data. WMS methods are also more costly than other NGS analyses, compounded by questions regarding appropriate sequencing depths, which is a compromise between cost, capturing sample diversity, and the scientific question being investigated (Gweon *et al.*, 2019).

After analysis data can be represented in various ways: detection of key genes in a sample, with relative abundance of sequence; detection of species present; and dominant sources of pollution. Additionally, in a similar way as WGS, further analysis can involve identification of novel alleles or genes as potential AMR related genes through algorithmic artificial intelligence or machine learning (Liu *et al.*, 2020). Non-targeted WMS is likely a useful tool to be used to provide broad overviews of AMR in the environment. This could include for example, to initially probe the natural environment to determine which AMR genes should be targeted, as well as to ensure that the methods characterise AMR in that area (spatially) are still relevant.

Targeted Metagenomic Sequencing

Instead of sequencing random DNA within a sample, DNA can be targeted to capture the genetic sequences of multiple organisms of interest. Targeting can be done by a few different methods, which each have their own benefits and drawbacks.

One approach of selection is the targeting of microorganisms using phenotypic methods, as described above. The key benefit here is the reduced cost compared to WGS, as multiple organisms from the same sample are pooled together to determine resistances of a population of a species of interest. An example of this approach has been described for *E. coli* in bathing waters (Leonard *et al.*, 2018). The downside of this method is the reduced

power to link genes together. Although larger sequence assemblies (contigs) can be assembled in some cases, the similarity of genomes introduced by selection means more similar regions and thus less confidence in these assemblies.

Other broader selection methods exist such as utilising differences in cell lysis in different organisms (MolYsis Basic 5 kit), or by selection based on methylation of the DNA (NEBNext Microbiome DNA Enrichment kit). Both have the advantage of a reduction in sequencing of non-target background DNA. This results in data like non-targeted metagenomics sequencing, with the added benefit of more target sequences, however with an additional cost. It is also unknown if these selection methods introduce a bias to the representation of the microbial communities as there are no independent comparisons available yet. Although it may be justified where it is expected to find large amounts of non-target DNA, such as in shellfish flesh.

One of the articles identified within this review (Macedo *et al.*, 2021) used a set of hybridisation and capture probes, a technology that allows targeting sequencing of multiple genes of interest. SeqCap EZ ResCap Design is a library of customised hybridisation and capture probes that allows more specific sequencing of AMR and related genes by 279 fold (Lanza *et al.*, 2018). Currently this technology looks promising allowing selection and sequencing of multiple AMR related targets. However, as the probe-library was generated using existing genomic databases, it has the potential to have a clinical bias, although data is comparable in human, pig, and cattle faeces. Additionally, the probes are proprietary, which could be a risk if being used in routine surveillance should they cease to be available. Additionally, the probes only target bacterial AMR genes and so would not capture antifungal resistance data at all.

Data from targeted metagenomic sequencing will be in similar formats to the non-targeted method described above. And whilst all will increase the relative abundance of AMR genes in the dataset, there will be limitations for each method and how the data can be used based on which targeting method used.

Quantitative PCR

This review will focus on conventional quantitative polymerase chain reaction (qPCR, used within 24% of the identified articles) and high-throughput chip array qPCR (HT-qPCR) (used in 4% of articles). Related, but not discussed methods within this review are droplet digital PCR (ddPCR) and conventional PCR. ddPCR was omitted herein as it is not suited for the

high sample throughput that would be required within a surveillance programme (Zhao *et al.*, 2016). Conventional PCR was omitted, as whilst it is a valid method used in 39% of all articles, it is outdated and is labour intensive which increases costs. It also lacks quantification in comparison to qPCR based methods.

Conventional qPCR

There are two main approaches to qPCR, either using intercalating dyes or fluorescent probes (Smith and Osborn, 2009). qPCR can be highly specific, can be multiplexed (probe based approach only), can be scalable, and allows for accurate quantification. However, qPCR isn't without its limitations, specifically the validation of multiplex assays is difficult and often involves compromising on the assay specificity or accuracy of quantification. Published qPCR assays aren't always fully validated, and often need additional work to be used for routine surveillance (Thalinger *et al.*, 2021). Furthermore, this method can have limited detection in samples that are difficult to concentrate or contain PCR inhibitors, such as soils and sediments. The cost of qPCR is proportional with the amount of markers (targeted genes) the surveillance question needs to answer, although as previously mentioned multiple markers can be multiplexed.

Data from qPCR is often expressed in target copies (or log10 copies) per amount of sample, for example copies/L of water. In terms of practicality, the use of qPCR for an AMR in the environment investigation would be most suitable for the detection of key marker genes. These key marker genes will first need to be identified as a gene of interest by broader screening which may need to be on a site by site basis. Other uses of this method could include the testing for dominant sources of bacterial and fungal pollution and thus to determine likely causes of AMR pollution.

High-throughput qPCR

Whilst the fundamental principle is the same as for conventional qPCR, differences in application mean that high-throughput chip array qPCR (HT-qPCR) has very different uses. The benefit of HT-qPCR is the ability to run large amounts of reactions per sample simultaneously, leading to large primer sets such as those available for AMR (Wang *et al.*, 2014; Stedtfeld *et al.*, 2018). The main drawback of this method is the high cost due to the large number of reactions required. However, the number of markers could be reduced once sample areas are initially well characterised. The method has the drawback of a higher limit

of detection due to the small reaction volumes used. In addition, there are no primer sets for antifungal genes, however this could be rectified with additional research.

Resulting data is displayed as a spreadsheet showing gene quantification against samples. HT-qPCR has been used successfully in freshwater and sediment samples for environmental AMR (Borsetto *et al.*, 2021; Lai *et al.*, 2021) and may represent a compromise between highly broad sequencing assays and narrower probe-based qPCR assays.

Additional Methodologies

While the above sections focussed on biological based methodologies to investigate AMR in the environment, it is acknowledged that the following methods and analytes (shown in table 2) would be complementary as part of an environmental AMR surveillance programme. Whilst these methods do not directly detect AMR organisms or genes, they do provide an insight in discerning the source and catchment modelling of AMR organisms or genes in the environment.

Release of antimicrobial substances into the environment can play a role in the development of resistance (Gullberg *et al.*, 2011; Murray *et al.*, 2018). Although the gap in our understanding regarding the exact relationship between the occurrence of antimicrobial substances and the development of AMR remains.
For the determination of antimicrobial substances in environmental samples some sample preparation steps, such as extraction methods, are commonly required. For liquid and solid samples these can include for example, liquid-liquid extraction, solid-phase extraction, ultrasonic extraction, and others (Díaz-Cruz and Barceló, 2006; Martínez-Carballo *et al.*, 2007; Holton *et al.*, 2021). After sample preparation, liquid chromatography combined with mass spectrometry, tandem mass spectrometry, or ultra-performance liquid chromatography tandem mass spectrometry (Angeles and Aga, 2018; Holton and Kasprzyk-Hordern, 2021) can provide quantitative data of substances of interest. While these methods allow a specific and sensitive determination of selected substances at very low detection limits, the main disadvantages encompass the limited range of targeted substances and relative high cost of these methods. In recent years screening, either semi-quantitative screening or non-target screening (NTS), for a broader range of substances in environmental samples have been proposed to overcome these limitations (Hollender *et al.*, 2019). While screening has been shown to detect and identify a comprehensive range of emerging contaminants, their disadvantages include large error margins for quantification.

Similarly, metal ions are known to co-select for AMR genes, due to linked chromosomal genes or via MGEs (Wales and Davies, 2015), and in order to account for this effect would also need to be monitored.

Other environmental factors have been highlighted to impact the stability of microorganisms (including resistant organisms) in the environment and thus their viability and bacterial ability to exchange MGEs. For example, pH and temperature have been noted to affect the rate of conjugation (Alderliesten *et al.*, 2020). Other factors such as ammonia and phosphate can indicate what other catchment processes are happening, e.g., sewage overflows and agricultural runoff. Additionally, climate and hydrology data can help provide valuable insights into how AMR is moving through the environment. For example, certain microorganisms or genes could change in their abundance in response to weather conditions. With the potential for some forms of ultraviolet radiation (UV) to increase the rate of mutations in microbial DNA which could lead to cell death or viable microbes with novel mutations (Rastogi *et al.*, 2010). Some of these analytes are already part of existing environmental surveillance programmes and so data could be repurposed for AMR surveillance.

Table 2. Co-analyses for different sample types

Freshwaters	Metal ions, Ultraviolet radiation (UV) (100-400nm), rainfall, pH, salinity, water temperature, flow, ammonia, phosphate, turbidity oxygen levels, antimicrobial agents
Coastal Waters	Metal ions, wind speed, wind direction, tidal rate, UV, rainfall, salinity, water temperature, antimicrobial agents
Soils/ sediments	Metal ions, pH, soil temperature, antimicrobial agents
Bioaerosols	Wind speed, wind direction, UV, rainfall, air temperature, relative humidity

Discussion

A wide range of methodologies for the detection of AMR in the environment have been described in the literature. This review was performed to 'identify methods available for the detection of AMR organisms and their associated genes in natural environments', so that they could be discussed for their suitability for an environmental AMR surveillance pilot. Throughout this review, we applied a forward-thinking approach as technology will develop and may become more cost effective in the future. Owing to the diversity and complexity of environmental AMR, no single method can fully capture a representation of all AMR in the environment. However, what is scientifically most conclusive may not be financially viable and so there needs to be a balance between the cost and benefit of the methods used. For this reason, a wider range of methods will be trailed for the surveillance pilot so that they can be evaluated with actual data.

Phenotypic screening assays are one of the methods that should be included in an environmental AMR surveillance programme. As outlined above phenotypic methods are cheap, quantitative and isolates can be either stored or analysed immediately using the methods discussed. No single microorganism will be representative of all AMR in an environment but may provide an indication of particular risk linkages. Due to the expected low number of AMR pathogens in the environment, phenotypic analysis will likely utilise indicator species instead of direct detection of pathogens. As such "indicator" organisms could be used to assess the general risk and estimate prevalence of AMR, such as the screening of AMR gram-negative, gram-positive bacteria, and fungi. For gram-negative bacteria ESBL-*E. coli* is a logical choice. It is part of the WHO tricycle recommendations, making inter-agency and international comparisons possible (Anjum *et al.*, 2021; WHO, 2021a). Additionally, AMR *E. coli* is the leading cause of AMR related deaths globally according to recent reports (Murray *et al.*, 2022). Furthermore, *E. coli* is already a widely used faecal indicator organism within the Environment Agency for existing programmes, such as bathing water and shellfish water monitoring (*The Bathing Water Regulations* 2013; *The Shellfish Water Protected Areas (England and Wales) Directions* 2016). Although other gram-negatives may be of interest, they face further difficulties such as whether they are at high enough concentrations in environmental samples and require more complex confirmations.

Gram-positive bacteria in the context of environmental AMR are not as well studied, such as organisms like *E. coli*. It is proposed that vancomycin-resistant *Enterococcus* spp. are

trialled in the surveillance pilot due to the existing use of *Enterococci* as a faecal indicator organism in England as well as its clinical relevance as highlighted by the WHOs pathogen priority list (*The Bathing Water Regulations* 2013; WHO 2017). Due to the large unknowns around antifungal resistance in the environment it makes sense to not select for a particular species or group. However antifungal analysis should focus on azole-resistant fungi, particularly with environmentally relevant azoles.
For the surveillance pilot culture isolates from all three phenotypic screening assays, even if not possible to immediately analyse, isolates can be stored, allowing a future analysis for samples of interest. However phenotypic screening alone cannot capture the diversity of AMR, and so there needs to be either further characterisation of the isolates or of the microbiome.

Further characterisation of AMR isolates is an important part of understanding the complexity of AMR and its potential impact on microbial communities in the environment. Therefore, AST of isolates should be used to identify isolates which are phenotypically resistant, and which can aid in the identification of unknown genotypes. Although this may not be possible for all isolates as part of an actual environmental AMR surveillance programme, for the pilot this could be used to identify gaps in both antibacterial and antifungal AMR genotypes. Characterisation by WGS is a more comprehensive method, with the ability to link AMR genes to strains, virulence and sometimes mobile genetic elements. In the pilot WGS will be used to determine resistance more widely, but in the future, it could also be used for epidemiological purposes to determine how AMR is moving within and between different sectors. Additionally, the use of both AST and WGS together become more powerful as it would allow the expansion of existing AMR gene databases with environmental AMR genes. And whilst it could be argued this could be done by *in silico* mining of clinical datasets, this would miss instances where environmental AMR genes are mobilised. Such instances whilst rare, have the potential to be highly impactful such as the mobilisation of $bla_{CTX\text{-}M}$ from chromosomal bla_{KLU} rapidly spreading in the 1990s with each area in the world having endemic genotypes (Cantón and Coque, 2006). Other examples of this include $bla_{OXA\text{-}48}$ and *qnrB* genes which are thought to have been mobilised from environmental isolates and are now present in clinical pathogens (Ribeiro *et al.*, 2015; Tacão *et al.*, 2018). As part of the surveillance pilot both AST and WGS should be used to characterise isolates to allow assessment for any future surveillance programme.

Total AMR cannot be assessed using phenotypic methods alone and for this, broader molecular methods should also be utilised for the pilot. Two such methods that could be

used are non-targeted WMS and HT-qPCR. Whilst currently these are both costly and only identify genes of high prevalence, intermittent use could ensure that the most abundant resistance genes are accounted for in the other analysis and to provide a broad overview of the diversity of AMR in an area. As future costs come down and the methods become more accessible, these could be further implemented in an environmental AMR surveillance. For the surveillance pilot both methods should be trialled and compared to assess their ability to detect total AMR genes. For a full scale surveillance programme, it is envisioned that these analyses would be used periodically to ensure focus of surveillance on relevant targets as AMR develops in that catchment.

In any environmental AMR surveillance, it is important to consider the factors that drive both the development and dissemination of AMR in a catchment. Factors that can drive AMR selection, such as antimicrobials, metal ions, pH, and temperature should be considered to assess their impact. Such data should be utilised from other environmental surveillance programmes where available and relevant. Initial use of semi-quantitative or NTS to target antimicrobial compounds of interest within a catchment area would be the best use of resources, with the potential to use more targeted analysis if required to confirm any suspected associations with AMR more strongly. Hydrological and meteorological data should also be captured as these factors likely play an important role in the movement and dissemination of AMR in the environment.

Conclusion

This review was performed to identify methods to be used as part of an AMR surveillance pilot. Which together could allow the Environment Agency to measure changes in the state of the environment that indicate for an increased hazard to AMR, were they to be developed into an environmental AMR surveillance programme.

For the surveillance pilot it is recommended that ESBL-*E. coli*, vancomycin-resistant *Enterococci*, and azole resistant fungi are used as indicator organisms to assess general levels of AMR in these groups. For the pilot these species should be further characterised using WGS and AST. However, the use of broad methods should also be used to determine trends in total AMR, as such both WMS and HT-qPCR should be used for the pilot. These methods should also begin to tell us whether this limited set of indicator organisms are sufficient to adequately represent AMR in our waters. Additionally, non-biological analyses should be performed to determine how AMR drivers may impact AMR in the environment,

as well as how the environment acts as a pathway to receptors of importance. Methods which provide similar data should be compared in terms of ability to assess trends in AMR but also how they could scale to a national surveillance programme.

The methods outlined above can together capture the broad diversity and extent of AMR in the environment which is needed for the surveillance of the dynamic between AMR and the environment in the future. However, if or when these methods are put in place for routine surveillance, it is important to re-evaluate these methodologies based on the exact aim and objective being investigated and how the data is used and be open to the use of new methodologies.

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