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## Monitoring antibiotic resistance genes in wastewater environments

*The challenges of filling a gap in the One-Health cycle*

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*Published in:*

Journal of Hazardous Materials

*DOI (link to publication from Publisher):*

[10.1016/j.jhazmat.2021.127407](https://doi.org/10.1016/j.jhazmat.2021.127407)

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*Publication date:*

2022

*Document Version*

Publisher's PDF, also known as Version of record

[Link to publication from Aalborg University](#)

*Citation for published version (APA):*

Miłobedzka, A., Ferreira, C., Vaz-Moreira, I., Calderón-Franco, D., Gorecki, A., Purkrtova, S., Jan Bartacek, B., Dziejwit, L., Singleton, C. M., Nielsen, P. H., Weissbrodt, D. G., & Manaia, C. M. (2022). Monitoring antibiotic resistance genes in wastewater environments: The challenges of filling a gap in the One-Health cycle. *Journal of Hazardous Materials*, 424(Part C), Article 127407. <https://doi.org/10.1016/j.jhazmat.2021.127407>

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## Review



# Monitoring antibiotic resistance genes in wastewater environments: The challenges of filling a gap in the One-Health cycle

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## ARTICLE INFO

## Keywords:

Antibiotic resistance  
One-Health  
ARGs  
ARB  
Human health risk  
Wastewater monitoring

## ABSTRACT

Antibiotic resistance (AR) is a global problem requiring international cooperation and coordinated action. Global monitoring must rely on methods available and comparable across nations to quantify AR occurrence and identify sources and reservoirs, as well as paths of AR dissemination. Numerous analytical tools that are gaining relevance in microbiology, have the potential to be applied to AR research. This review summarizes the state of the art of AR monitoring methods, considering distinct needs, objectives and available resources. Based on the overview of distinct approaches that are used or can be adapted to monitor AR, it is discussed the potential to establish reliable and useful monitoring schemes that can be implemented in distinct contexts. This discussion places the environmental monitoring within the One-Health approach, where two types of risk, dissemination across distinct environmental compartments, and transmission to humans, must be considered. The plethora of methodological approaches to monitor AR and the variable features of the monitored sites challenge the capacity of the scientific community and policy makers to reach a common understanding. However, the dialogue between different methods and the production of action-oriented data is a priority. The review aims to warm up this discussion.

## 1. Introduction

Acquired antibiotic resistance (AR) is defined as the ability of a

bacterial clone or lineage to resist the action of an antibiotic agent, to which members of the same species are (or were once) normally susceptible (Grundmann et al., 2006). This type of resistance contrasts with

**Abbreviations:** AR, antibiotic resistance; ARB, antibiotic resistant bacteria; ARDB, Antibiotic Resistance Genes Database; ARGs, antibiotic resistance genes; CARD, Comprehensive Antibiotic Resistance Database; dPCR, digital Polymerase Chain Reaction; epicPCR, Emulsion, Paired Isolation and Concatenation PCR; HGT, horizontal gene transfer; LAMP, loop-mediated isothermal amplification; LCPDb-ARG, literature-based, manually-curated database of PCR primers for the detection of antibiotic resistance genes in various environments; MAGs, metagenome-assembled genomes; MGEs, mobile genetic elements; ReCasp, Resistome Capture; SNP, Single Nucleotide Polymorphism; ssRNA, single stranded ribonucleic acid; qPCR, quantitative PCR; UWTPs, urban wastewater treatment plants; WHO, World Health Organization; WWTPs, wastewater treatment plants.

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<https://doi.org/10.1016/j.jhazmat.2021.127407>

Received 17 March 2021; Received in revised form 22 September 2021; Accepted 29 September 2021

Available online 2 October 2021

0304-3894/© 2021 The Author(s).

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intrinsic AR, typically observed in most or all bacteria of the same species. Intrinsic resistance can be due to mechanisms as diverse as morphological traits (e.g. the absence of cell wall) or the presence of specific genes in all species belonging to a given taxon e.g. *bla*<sub>OXA</sub> or *bla*<sub>SHV</sub> genes in some *Proteobacteria* (Davies and Davies, 2010; Fajardo et al., 2008). While the latter is fairly stable, encoded in the bacterial genome of the host, acquired AR can be efficiently disseminated through both horizontal gene transfer (HGT), facilitated by mobile genetic elements (MGEs), and by clonal proliferation eventually enhanced by selective agents such as antibiotics (Klümper et al., 2019; Larsson et al., 2018; Martínez et al., 2015a). Consequently, acquired AR is of most concern in the field, and is the focus of this review.

AR can jeopardise medical interventions, extend hospitalisation due to infections, and affect the patient's quality of life. These and other reasons led the World Health Organization (WHO) to place antimicrobial resistance (which besides antibiotic resistance includes also virus, fungi, and protozoa) among the biggest threats to human health (Global Antimicrobial Resistance Surveillance System: Manual for early Implementation, 2015). The O'Neil report commissioned by the United Kingdom government (O'Neill, 2014) alerted authorities around the world to the AR threat. The report estimated that if nothing is done to invert the current trends, by 2050 antimicrobial resistant infections will cause 10 million deaths per year, more than cancer or traffic accidents, with serious impacts on the Gross Domestic Product reaching world costs of up to 100 trillion USD. The message is clear - today's expenditure for combating AR must be regarded by nations as priority investments to save human lives, wellbeing and economic impacts.

Opportunistic AR bacterial infections, sometimes associated with different comorbidities, make AR a major threat reported and managed at the clinical level. However, the current knowledge shows the complexity of AR ecology, often led by ubiquitous bacteria and genetic elements adapted to travel through, persist and proliferate in a wide array of hosts and habitats. This comprehensive perspective has led some authors to propose that AR dissemination must be assessed under the One-Health concept, which refers to "the collaborative effort of multiple health science professions, together with their related disciplines and institutions - working locally, nationally, and globally - to attain optimal health for people, domestic animals, wildlife, plants, and our environment" ("The One Health Commission reports and definitions from the meeting," 2018).

When applied to AR, the One-Health concept recommends that surveillance and control measures are implemented across human, animal and natural environments, based on the assumption that bacteria and genes can move freely between those three major compartments (McEwen and Collignon, 2018). Water, which in the One-Health context is classified within the environment compartment, is indeed a major link between humans, animals, and nature. Water is an unconfined path for the transport of nutrients, pollutants, micro- and nanoparticles, and a major bacterial habitat with high potential for AR dissemination (Do et al., 2018; Manaia et al., 2016; Vaz-Moreira et al., 2014). Within the urban water cycle, wastewater represents the liquid emissions resulting from human domestic activities. Urban wastewater treatment plants (UWTPs) are the endpoint of a more or less complex sewer network where sewage is treated, and major contaminants are removed prior to the return to the natural environment, resulting in an effective protection of the environment and human health (Bürgmann et al., 2018). However, antibiotic residues, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), combined with a complex mixture of other contaminants (e.g. pharmaceuticals, hygiene products), are not totally removed during wastewater treatment, even when tertiary treatment is implemented, and are discharged into the receiving environment (Rizzo et al., 2013; Singh et al., 2019).

The widespread occurrence of AR has inspired research in water bodies, wastewater, soils and wildlife, and shown the need to systematically implement monitoring schemes (Barragan et al., 2017; Berendonk et al., 2015; Guerra et al., 2014; WHO/FAQ, 2019). AR monitoring

is considered essential to assess: (i) the overall AR status in a human population or community (Barragan et al., 2017; Berendonk et al., 2015); (ii) the risks that AR evolves and spread across One-Health compartments (Berendonk et al., 2015; Iriti et al., 2020; Larsson et al., 2018); (iii) the risk of AR transmission to humans (Bengtsson-Palme et al., 2017; Manaia, 2017). Although the specific purposes of monitoring will determine the methodology required, some criteria seem to be basis for selecting a method, e.g., sensitivity, reproducibility, operational costs. However, the (not always straightforward) translation of monitoring data into information that can be used to implement control and/or corrective measures is most important for the design of a monitoring scheme.

This review aims to offer a broad perspective of monitoring approaches commonly used to characterize AR diversity and abundance in wastewater environments, from sampling to results output. The major motivation behind this review is that although AR data are increasingly available in public databases, scientific publications and other open science formats, the scientific community still faces the challenges of monitoring the humans-animals-environment continuum that the One-Health envisages. Also, despite that prolific accumulation of AR data, it is often difficult to ascertain their meaning in terms of risks. These are major limitations for authorities and policymaking entities aiming at the definition of guidelines and recommendations. The current knowledge suggests that such gaps can probably only be properly addressed by the scientific community, although policy-oriented discussions in this field are urgently needed. The main aim of this paper is to promote the discussion around the challenges and limitations of framing wastewater AR monitoring within the One-Health cycle, while it is quarrelled how data can be translated into information that can be used to support actions and to incentivise the scientific community to make significant advances in the field.

## 2. From the environment to the extracted DNA

Historically, culture-dependent methods were used to assess water microbiological quality based on the quantification of indicator bacteria, such as coliforms and intestinal enterococci. However, cultivable bacteria represent a small fraction of the whole microbiota in a given environment. In aquatic systems or even in activated sludge of UWTPs, the cultivability of bacteria can be lower than 0.1% (Amann et al., 1995; Vaz-Moreira et al., 2013). Multiple factors, such as slow growth, specific growth requirements, the occurrence of cell injuries, or dependence on symbiotic interactions with other microorganisms underlie the apparent un-cultivability (Manaia, 2017; Vaz-Moreira et al., 2011; Weissbrodt et al., 2020). Therefore, even when culture-based methods are used, AR monitoring mostly relies on the direct inspection of ARGs and associated MGEs in total DNA extracts. Sample collection and processing are the first steps in AR monitoring, followed by DNA extraction and analysing methods. Table 1 summarizes the steps mentioned above and the methodological options that may influence the results.

### 2.1. Sample collection and processing

After the definition of sites and times, one of the first decisions about sampling refers to the use of grab (single) or composite (multiple/combined; different times or sites). Grab samples represent a single moment or space, while composite samples can be representative of a whole day or bulk. Composite samples may offer an 'overall picture', comprising several sampling times over a day, several sites, or a bulk fraction (e.g. cross sections of a river or lake). While the use of composite samples of sludge or sediments is normally the wisest option, a different logic may be used for liquids (see Section 2 and Table 1). The decision of using time composite samples, may depend on the availability of sampling methods (manual or automatic) and on the transformations that may take place during storage. The use of 24-h composite wastewater samples to assess AR loads is frequent (Cacace et al., 2019; Fernandes

**Table 1**

Examples of methodological options available during the experimental design to measure AR in WWTPs and potential associated biases.

| Monitoring stage            | Methodological options  | Potential associated biases   |
|-----------------------------|---|---|
| <b>Sampling and storage</b> | Number of sample replicates   | Reduced number of samples may not be representative or give an instant image of the resistance load.  |
|                             | Grab vs. composite sample (normally 24 h), and sample storage prior to analysis | Grab samples may give an instant image of the resistance load, but can be used to follow day-long variations. Grab samples are appropriate to analyse specific variations of parameters as temperature, pH and dissolved nitrogen, although may not foresee temporal fluctuations resulting in higher variations between replicates (Human Health Risk Assessment, 2017). Composite samples and storage may lead to the alteration (increase or decrease) of the resistant population in relation to the total. Composite approach enables assessing variations of the microbial community, including resistance, abundance and prevalence. Composite samples are representative of the average wastewater characteristics during the sampling period, frequently 24 h sampling (Human Health Risk Assessment, 2017). |
| <b>Sample processing</b>    | Distance gradients and Seasonality  | Gradients and seasonality are FAQs regarding AR variations but will need a very large number of replicates (e.g. over distinct seasons) to allow any conclusions.   |
|                             | Pre-filtration to remove solids   | Pre-filtration can remove the microbiota fraction adsorbed on particles. The inclusion or not of this step will influence the results since sediments/particles and planktonic communities will hold different microbial communities, and consequently, different fractions may present different loads of ARGs.  |
| <b>DNA extraction</b>       | Pore size and type of filtering membranes                                       | The type of membrane may affect the capacity of retention of bacteria and DNA. Smaller pore size, of 0.1–0.22 $\mu\text{m}$ , can lead to higher recovery of extracellular DNA that may influence the ARGs quantification.  |
|                             | Extraction method   | Different methods may have different efficiencies in the removal of contaminants present in environmental samples that will influence the DNA yield.  |

**Table 1 (continued)**

| Monitoring stage                              | Methodological options                               | Potential associated biases   |
|---|--|---|
| <b>Analysis methods: qPCR or metagenomics</b> | Matrix effect  | The composition of the samples will affect the efficiency of the DNA extraction.  |
|   | DNA storage  | The preservation (at room temperature, cooled or frozen) of DNA for future analyses may influence its quality (mainly degradation).   |
|   | Primers and PCR conditions                           | Selection of universal primers specific for all AR variants of a given ARG may be difficult to achieve. PCR inhibition may be a problem that can be overcome by DNA extract dilution, but this procedure will hamper the quantification of rare ARGs. |
|   | Metagenomics sequencing depth                        | The coverage of the analysis may affect the detection of rare ARGs.   |
| <b>Analysis methods: qPCR or metagenomics</b> | Databases and pipelines used for ARGs identification | The identification of ARGs from raw reads or assembled contigs by comparison with ARGs databases only predicts the presence of known ARGs.  |

et al., 2019; Manaia et al., 2018b; Pärnänen et al., 2019), although grab sampling is widely used too (Cacace et al., 2019; Hendriksen et al., 2019a; Karkman et al., 2016).

The combination of field sample collection with the laboratory processing and analysis is another aspect of the experimental design that may influence the results. The decision is normally between sample storage refrigerated [4 °C up to 24 h; (Novo et al., 2013)] or frozen at –80 °C for longer periods (Hendriksen et al., 2019b). Although frozen samples have been the preferred method for long term (up to some months) sample storage (Hendriksen et al., 2019a; Li et al., 2018), immediate sample processing, in spite of logistics constraints, has been used frequently (Cacace et al., 2019; Pärnänen et al., 2019; Rocha et al., 2020).

For AR analysis, sample processing is required for example, to concentrate the ARGs, to remove impurities and/or promote sample homogeneity. The major processing steps often include filtration and/or centrifugation. In liquid samples filtration is normally used, while in samples with a high percentage of solids, e.g. activated sludge, centrifugation is preferred (Auerbach et al., 2007; Zhang et al., 2019). The options are customized depending on the situation and research goals, and will have implications on the results of the resistome analysis. Different wastewater samples have different bacterial cell loads. The bacterial load from distinct municipal WWTPs can vary between 8 and 9 log-units (of cell equivalents)  $\text{mL}^{-1}$  in the influent to one to two log-units less in the effluent, with variable loads in the intermediary treatment processes (Manti et al., 2008; Pallares-vega et al., 2019). Such differences in bacterial load affect the sampling and analytical design when it comes to obtaining enough biomass for DNA extraction. For membrane filtration, the selection of the membrane material (e.g., cellulose nitrate, polyethylene sulfone, polyvinylidene fluoride, polycarbonate) and porosity is of utmost importance and can influence the flow rate of filtration (Liang and Keeley, 2013). Membrane pore sizes between 0.1  $\mu\text{m}$  and 0.45  $\mu\text{m}$  are reported, although 0.22  $\mu\text{m}$  is the most used. Lower porosity of 0.22  $\mu\text{m}$  or 0.1  $\mu\text{m}$  lead to higher DNA recovery, including extracellular DNA (Liang and Keeley, 2013). One of the problems of sample filtration is the clogging of the pores, which can be overcome by a pre-filtration step capable of removing larger particles and solids.

While the pore size will strongly influence the microbiota recovered for further analysis, the preliminary pre-filtration may remove the microbiota fraction adsorbed onto particles.

The extraction of extracellular or cell-free versus total DNA (mainly intracellular) must be decided according to the aim of the monitoring process. If the goal is to recover only free-floating DNA, methods such as high-speed centrifugation or membrane filtration with recovery of the filtrate may be used together with the removal of polysaccharides or proteins that may be attached to the DNA fragments (Calderón-Franco et al., 2021; Wu and Xi, 2009). Total DNA extracts contain a large fraction of non-viable cells that, as the cell-free DNA, sometimes play an unclear role on AR dissemination. The distinction of viable from non-viable cells can be overcome through the use of ethidium monoazide bromide or propidium monoazide. These dyes bind irreversibly to the DNA from dead cells or free DNA, whereas viable cells with intact membranes remain unaffected, thus enabling PCR amplification of their DNA (Varma et al., 2009; Wagner et al., 2008).

## 2.2. DNA extraction

Ideally, the DNA extraction procedure should have maximum efficiency, remove contaminants capable of interfering with the analytical methods (e.g. PCR or shotgun sequencing), and minimize DNA fragmentation and degradation (Z. Li et al., 2018; Thomas et al., 2012; Volkmann et al., 2007). Most monitoring studies have used commercial kits for DNA extraction e.g. DNeasy PowerWater kit (QIAGEN, Hilden, Germany), FastDNA Spin Kit for Soil (MP Biomedicals, USA), or PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., USA) adequate for environmental samples. Such kits are designed to efficiently lyse the cells (using glass beads, ceramic and silica beads, or enzymatic lysis) and remove environmental contaminants such as detergents, phenolic compounds, humic acids or heavy metals that may affect the DNA stability or inhibit the DNA amplification (Bessetti, 2007; Djurhuus et al., 2017; Hinlo et al., 2017; Z. Li et al., 2018). Popular DNA extraction kits are the Power Water DNeasy kit (Calderón-Franco et al., 2021; Pärnänen et al., 2019) or FastDNA Spin Kit for Soil (A.-D. Li et al., 2018), although the associated procedure may have influence on the DNA yield and quality. For instance, sludge homogenization and disruption by bead-beating has been shown to be an essential step in commercial kits for DNA extraction. Guo and Zhang (2013) showed that kits without bead-beating steps may yield lowered amounts of DNA which can result in the significant underestimation of bacteria hard to lyse, such as Gram-positive *Actinobacteria*.

Cheaper DNA extraction kits, such as NZY Tissue gDNA Isolation kit (Nzytech, Portugal) or ZR Fecal DNA MiniPrep (Zymo Research Corporation, USA) (Li et al., 2018; Rocha and Manaia, 2020) can also be used with good DNA yields. Despite the convenience of using commercial kits, the fact that users do not fully know their composition sometimes limits full understanding of the drawbacks that may bias the DNA extraction procedure. Also, the amount and diversity of impurities can hamper DNA extraction in different ways. The sample matrix effect can have important implications in the DNA extraction yield, mainly due to DNA adsorption onto matrix particles after cell lysis. Sample impurities can cause significant DNA loss during extraction, resulting in unpredictable or unavoidable effects that vary with the sample type. One of the ways to overcome these biases is the use of internal standards (Skooog et al., 2017). Although internal standards are frequently used in chemical analyses, they are not widely practised in AR studies. Approaches to assess the matrix effect include the use of standard DNA added to the sample before total DNA extraction (internal control) or spiking the DNA extract with a DNA fragment (external control) (Burggraf and Olgemöller, 2004; Volkmann et al., 2007). A cell-based approach in which samples are spiked with a known amount of a reference bacteria before sample processing and DNA extraction has been proposed as an approach to normalize samples in different laboratories aiming at comparing final results (Rocha and Manaia, 2020).

## 3. Exploring the environmental resistome

Due to the limited cultivability of environmental bacteria, the screening of ARGs and MGEs directly on DNA extracts is now the common practise. The detection, quantification and characterization of specific ARB and ARGs in different environmental compartments have been supported by the molecular biology-based method, qPCR and metagenomic analysis in numerous studies (Gandra et al., 2020; George, 2019; Hendriksen et al., 2019a; Hendriksen et al., 2019a; Munk et al., 2018; Waseem et al., 2019). Quantitative PCR is advantageous for epidemiological studies as it can track a specific ARG through different compartments and establishes a proxy for the risk of transmission from the environment to humans (Anjum, 2015; Kralik and Ricchi, 2017; Manaia et al., 2018a; Rocha et al., 2019; Smith and Osborn, 2009; Wang et al., 2016; Yang et al., 2013). This paradigm-change has been strongly influenced by recent advances such as the wider accessibility to molecular biology techniques, the lowering of sequencing prices, the establishment of public databases on microbiology (e.g. MiDAS: Field Guide to the Microbes of Activated Sludge and Anaerobic Digesters <https://www.midasfieldguide.org/guide>) and resistance data [Comprehensive Antibiotic Resistance Database (CARD) <https://card.mcmaster.ca/>; (Alcock et al., 2020)], or the refinement and user-friendliness of bioinformatics pipelines. Indeed, the qualitative and quantitative characterization of the environmental resistome, meaning the array of ARGs present in a given place, has been boosted to unprecedented levels (Hendriksen et al., 2019a; Karkman et al., 2019; Pärnänen et al., 2019; Zhang et al., 2020). However, although the selection of methods must be in accordance with the monitoring objectives, the proliferation of data from different research groups using different techniques has made comparisons between datasets and studies questionable. This impedes our ability to develop control measures (Berendonk et al., 2015). PCR- and DNA sequencing-based methods are preferred by the scientific community for quantifying ARGs, although other methods are also used (Table 2). In the absence of guidelines to implement standardized methods, each study can only compare its own data, which seriously hampers the potential applications of the immense amount of information generated over the last decade. The results obtained by each method are impacted by user skills, repeatability, reproducibility, and high-throughput vs. fast-throughput constraints and costs.

### 3.1. Amplification-based methods

In general, the major aims of characterizing the environmental resistome include: (i) quantify ARGs, (ii) assess the diversity of ARGs and/or (iii) assess the range of hosts of ARGs. The latter has been traditionally supported by cultivation methods, but is nowadays moving to culture-independent approaches.

One of the most important characteristics of amplification-based methods is the possibility of developing a targeted search for specific genetic determinants, such as those that are reported in human infections. Quantitative PCR (qPCR) provides the opportunity to determine the abundance of a given genetic element per volume or mass of sample or per total bacteria in a sample, giving an estimate of the density or prevalence.

The qPCR approaches are supposed to be more sensitive than metagenomics (Manaia et al., 2018b), although require higher efforts to cover a wide array of genes or taxonomic markers. These limitations can be overcome by multiplexing qPCR with the simultaneous quantification of multiple ARGs and housekeeping genes. However, novel technical PCR solutions, such as the digital PCR (dPCR) offer high throughput gene monitoring. While qPCR relies on a calibration curve and calculation of the number of cycles needed to achieve gene amplification in a sample, in dPCR gene amplification occurs in thousands of independent compartments (microwell, capillary, oil emulsion or array) being the quantification based on the estimation of the number of positive and

Table 2

Strengths and weaknesses of methods that can be used for detecting and quantifying ARGs.

| Methods                 | Strengths   | Weaknesses  | References   |
|-------------------------|---|---|--|
| qPCR                    | Fast, effective, enabling quantification of gene and/or transcript numbersHigh sensitivity and specificity towards targeted sequences in mixed communitiesWide dynamic range for quantification (7–8 log <sub>10</sub> )Well developed technologyObjective and simple interpretation of resultsAllows analysis of a large number of samplesAllows expression analysis (RNA)No post-PCR manipulations minimize the chances for cross-contamination in the laboratory | Biases in the template to product ratios of target sequenceBiases increase with increasing numbers of PCR cyclesNeed of normalization improvementsqPCR inhibitors present in environmental matricesExpensive reagentsAvailable only for known ARGsRNA liability compared to DNA requires careful isolationDesign of primers and standards can be a constraint | (Anjum, 2015; Girones et al., 2010; Kralik and Ricchi, 2017; Manaia et al., 2018b; Smith and Osborn, 2009; Valasek and Repa, 2005; Wang et al., 2016; Yang et al., 2013) |
| LAMP                    | Rapid (provide results in less than half an hour)Simple and low-cost effective equipment, no need of thermo-cyclerGood and effective detection method for developing countriesBoth amplification and detection of the target gene are done in a single stepIt can be visually detected through fluorescenceHigh sensitivity and specificity when one gene is targetedMultiple commercial detection oligonucleotides   | More sensitive than qPCR to inhibitorsLess versatile than qPCRDesign of primers and standards can be a constraintNo single amplicon obtaining: banding pattern on a gelPoor sensitivity and specificity for multi-LAMP assaysHigh initial design effortsLarge amount of DNA requiredCross-hybridizations may happen if not optimized                          | (Notomi et al., 2000; Sahoo et al., 2016; Zhong et al., 2019)  |
| Whole Genome Sequencing | Allow individual isolates studies with high precisionHigh sensitivity and specificityHigh level of detail at gene level (specific allele profiles and SNP)  | Need of bioinformatics skillsTime and labour consuming  | Hendriksen et al. (2019a); Oniciuc et al. (2018); Shelburne et al. (2017)  |
| Metagenomics Sequencing | Information on the whole microbial community, at the phylogenetic and functional levelsIdentifies uncultivable, unknown or emergent microorganismsAllows detection of ARGs in complex samplesProvides information on AR prevalence, distribution and routes of transmissionDetects simultaneously phage and bacterial DNAIdentification of  | High bioinformatics skillsNeed of continuously updated databases and toolsHigh "noise" due to multitude of databases and non-relevant ARGsHigh costsTime and labour consumingWet-lab and dry-lab standardization and optimization   | (De, 2019; Hendriksen et al., 2019a; Oniciuc et al., 2018; Pollock et al., 2020)   |

Table 2 (continued)

| Methods         | Strengths   | Weaknesses   | References   |
|-----------------|---|--|--|
| Hi-C Sequencing | HGT phenomena and novel antibiotic synthesis pathwaysInformation on the whole microbial community and how they interactAllows quantification of interactions between all possible pairs of DNA fragments simultaneouslyAllows linkage of specific plasmids harbouring integrons, transposons and ARGs to bacterial hostsAllows HGT phenomena detection across urban water systems | High bioinformatics skillsHigh costsTime and labour consumingHigh abundant genomes may produce clustering artefactsContig resolution between closely related bacterial species may produce defective links | (Burton et al., 2014; Fraser et al., 2015; Press et al., 2017; Stalder et al., 2019) |

negative reactions (Kuypers and Jerome, 2017). Hence, dPCR spares a calibration curve and may have a considerably lower limit of quantification. The broader use of dPCR may contribute to leverage the capacity to target specific and minor genes in the environmental resistome, although at the moment it is not the state of the art (Dungan and Bjorneberg, 2020; Liu et al., 2021). Depending on laboratory resources and study aims, a range of PCR based methods can be used, including multiplex PCR, qPCR, some forms of shotgun sequencing and microarrays (Anjum et al., 2017; Fluit et al., 2001; Waseem et al., 2019), often decided according to the means available in the laboratory and aims of the study.

Gene-targeted quantifications in environmental samples using qPCR have been employed for almost two decades (Fernandes et al., 2019; Narciso-da-rocha et al., 2018; Rodriguez-Mozaz et al., 2015; Schwartz et al., 2003). However, it is uncertain if these data is comparable, as qPCR is susceptible to factors such as the reaction components, master mixes, primers design and choice, the analytical equipment, and the operator, as well as the type of sample (e.g. wastewater, treated wastewater and river water). Although further studies are needed to assess qPCR data comparability, some inter-laboratory qPCR assays have suggested that the differences may be acceptable to assess patterns or variations (Rocha et al., 2020; Rocha and Manaia, 2020).

For PCR applications, primers are designed based on a specific target ARGs. However, given the expansion on the number of ARG sequences available, there are new opportunities to reassess the range of targets for the primers recommended in the literature. Single nucleotide primer changes can affect the pool of amplicons produced by PCR from environmental samples, resulting in either too narrow determinations or in inaccurate findings (Thomas et al., 2011; Wei et al., 2018). For this reason, it is recommended to test primers with tools such as ResFinder or AMRfinder. In addition, validation against a high-quality curated AMR gene reference database is also recommended (Hendriksen et al., 2019a). Based on these principles, a literature-based, manually-curated database of PCR primers for the detection of antibiotic resistance genes in various environments was recently proposed [LCPDb-ARG; (Gorecki et al., 2019)]. Quality criteria in qPCR measurements used are also important to improve data reliability and for sake of inter-laboratory comparisons. Rocha et al. (2019) summarized a set of criteria recommended for qPCR analyses for the quantification of ARGs in wastewater samples. Mainly they referred to the melting temperature of the amplicon, the quality and amplitude of the calibration curve in relation to the analyte, as these were observed to be possible sources of errors, neglected in some studies. The DNA dilution and concentration in the PCR reaction mix is also critical, as excess DNA or the presence of PCR inhibitors may cause a reduction of the amplification efficiency and

consequently inaccurate quantification (Manai et al., 2018b). To determine the qPCR inhibition due to excess of DNA and/or presence of inhibitors in a sample, serial dilutions of the DNA extract or a DNA spike-in with a known amount of a target gene may be used (Fortunato et al., 2018; Rocha et al., 2019). The dilution of the DNA extract will promote the dilution of the inhibitors, but may also dilute the target ARGs to a level below the limit of quantification (Rocha et al., 2020). Finally, it is important to note that the limit of quantification of traditional qPCR is generally high (Fortunato et al., 2018). However, mainly in risk assessment contexts, it must be noted that the lack of detection or quantification does not mean gene absence.

Loop-mediated isothermal amplification (LAMP) that can be performed in a single tube has been described as highly specific and rapid (less than 1 h) (Notomi et al., 2000). It may be a suitable alternative when other equipment is not available. Here, the amplification reaction occurs under isothermal conditions (60–65 °C), eventually minimizing the biases due to temperature settings that may occur in classical PCR. Generally, products can be visualized with simple instrumentation over time, based on nucleic acid binding dyes such as SYTO-9 or SYBR green fluorophores, e.g. measuring OD at 440 nm. The result can be monitored by naked eye by comparing colours of amplification test reactions. A positive result is indicated by a green colour after SYBR addition, contrasting with the brownish of negative reaction. If electrophoresis is available, the products can be also separated on agarose gels and stained. LAMP has been used to detect multiple bacteria (Nzulu et al., 2019; Sagcan and Turgut Kara, 2019), and was lately used to quantify and monitor ARGs such as mobilized colistin resistance (*mcr*) gene (Zhong et al., 2019) or a combination of ARGs (with multi LAMP) (Kostić et al., 2015). LAMP is suggested as fast and specific detection method with potential to be used for ARGs cross-field monitoring, due to its capacity to generate results with using minimal laboratory equipment (Rivoarilala et al., 2018).

### 3.2. Metagenomics based methods

Metagenomics is a non-targeted method used for exploring the taxonomic and functional genetic diversity in complex environments. Metagenomic analysis permits the determination of the relative abundance of given gene/sequence categories allowing inferences about occurrence and proportions of distinct groups in complex microbial communities (Committee on Metagenomics: Challenges and Functional Applications, 2007). The large amount of information provided makes metagenomics one of the most attractive tools to explore natural environments (Che et al., 2019; Hiraoka et al., 2016; Huson et al., 2018). The metagenomics analysis of the environmental resistome has been largely encouraged by the availability of public databases of ARGs and MGEs. Major examples of these are the ResFinder web server that uses whole genome sequencing data for identifying acquired antimicrobial resistance genes in bacteria (Bortolaia et al., 2020), CARD, the reference centre for bacterial insertion sequences ISfinder (Siguier et al., 2006), or the Integron Database INTEGRALL [(Moura et al., 2009); <http://integrall.bio.ua.pt/>]. Metagenomics offers identification of hundreds of ARGs and MGEs (Hendriksen et al., 2019a), and if adequate statistical analyses are used, it is possible to explore inferences about the relationship between the resistome and mobilome profiles and the microbiome composition or the overlapping of resistomes in distinct environments (Che et al., 2019; Hendriksen et al., 2019a; Karkman et al., 2019; Lira et al., 2020; Munck et al., 2015). The importance of metagenomics approaches on the description of general AR patterns, with the definition of critical geographical or activity-related areas or temporal AR trends (e.g. sewage, pharmaceutical industry) has been highlighted in different publications (Aarestrup and Woolhouse, 2020; Hendriksen et al., 2019a; Id et al., 2019; Sukhum et al., 2019).

However, as any other method, metagenomics resistome analysis is not exempt from biases. For example, sequencing depth can influence the abundance and diversity of ARGs detected in the sample

(Bengtsson-Palme et al., 2017). In addition, the reads generated for the same sample can vary between runs, requiring a balance between the number of replicates and the sequencing depth to detect statistically significant differences (Bengtsson-Palme et al., 2017; Knight et al., 2012). As opposed to qPCR, which permits the estimation of absolute quantification from gene copy number per volume or weight of sample and relative abundance from gene copy number per 16S rRNA copy number, metagenomics sequence data is expressed in relative abundance, as the number of ARGs reads per total number of sequenced reads or per 16S rRNA gene or other housekeeping genes reads such as *rpoB* (Bengtsson-Palme et al., 2017). Dissonances in reporting ARGs load in UWTPs, like abundance per volume or mass vs. normalization per 16S rRNA gene or cells number need to be clearly considered in any discussion concerning AR in UWTPs (Ju et al., 2019). One of the limitations of metagenomics for ARGs identification may be the failure to detect rare genetic elements, which is heavily dependent on the DNA sequencing depth (Yang et al., 2014).

This limitation can be curbed through the use of targeted metagenomics that may increase the sensitivity and therefore detect some specific genetic elements, based on the preparation of libraries for specific genes further analysed based on high-throughput sequencing (D'Costa et al., 2011; Gatica et al., 2016). Targeted approaches can also be used at the data analysis step, as is the case of ResCap, a bioinformatic platform, developed by Lanza et al. (2018). This platform enables the targeted sequence capture in metagenomics datasets, with beneficial implications on the detection of minority populations. Including thousands of probes for antibiotic, biocide and metal resistance genes, mobile genetic elements, as well as antibiotic target genes. ResCap was described as a specific core reference database rooted in SeqCapEZ (NimbleGene) technology. The ResCap outperformed the metagenomic shotgun sequencing for 17 fecal samples by improving “gene abundance” from 2.0% to 83.2% and “gene diversity” with up to 300 times more mapped reads. The workflow includes whole-metagenome shotgun library construction followed by hybridization and capture, finally sequencing of the captured DNA (Hendriksen et al., 2019a; Lanza et al., 2018).

### 3.3. Who is who in the environmental resistome?

The dissemination of ARGs and associated risks are strongly influenced by the ecology and physiology of the host bacteria. The assessment of the diversity and abundance of ARGs seems insufficient to uncover the ecology of AR, how it can be controlled and if there are direct or indirect risks for human health. Also, the investigation of the association between ARGs and MGEs and the range of specific microbial hosts can contribute to determining the rate of DNA exchange or uptake. The identification of the ARGs' hosts in different environments is, however, an ambitious goal. Different approaches have been developed, for instance, Emulsion, Paired Isolation and Concatenation PCR (epicPCR) aims at linking functional genes with phylogenetic markers that permit the taxon identification (Spencer et al., 2016). It was described as a promising single-cell analysis approach that contours cultivation needs, but probably due to the technical complexity versus the obtained outputs it is less common in the literature than it could be expected (Hultman et al., 2018).

The growing availability of metagenomes and consolidation of bioinformatics capabilities and resources has encouraged the recovery of metagenome-assembled genomes (MAGs) from distinct environmental compartments greatly contributing to expanding the tree of life and improving the current perspective of key genes or functions held by specific taxa (Parks et al., 2017). One of the interesting applications refers to analyses of ARGs in MAGs. As an example, recently, 1083 high-quality MAGs incorporating full-length 16S rRNA genes were recovered from Danish activated sludge plants (Singleton et al., 2020). ARG screening of these MAGs using Abricate (<https://github.com/t-seemann/abricate>) and the ResFinder database (Bortolaia et al., 2020)

revealed 21 MAGs encoding ARGs, representing well-known and abundant wastewater microbes in the *Candidatus* Accumulibacter and the genus *Rhodoferrax* (Fig. 1 - heatmap/ARGs; Supplementary Information). All MAGs, except three identified as *Candidatus* Accumulibacter phosphatis, were novel at the species level, and their abundance could be tracked across Danish WWTPs using the MiDAS3 database [(Nierychlo et al., 2020); https://www.midasfieldguide.org/guide; Fig. 1 - heatmap/ARGs]. The most common ARGs identified were for beta-lactam (*bla*), sulphonamide (*sul1*) and macrolide resistance (*mef(C)* and *mph(G)*; Table S1). Combining this information with the functional and eco-physiological characteristics available in the MiDAS3 database and Field Guide will contribute to improve the understanding of ARB's functioning, diversity and abundance in the activated sludge system.

Shotgun metagenomics enables deep insight into overall genetic richness in an analysed environment, including ARGs. However, the assembly of large genomic contigs is still difficult, which especially hampers analyses of ARGs integrated or linked to repetitive regions, e.g. transposable elements, whose reconstruction from the metagenomic data is challenging (Bengtsson-Palme et al., 2014; Ellington et al., 2017). The existing bioinformatic pipelines are still not capable of dealing with these limitations and sometimes may fail to identify ARGs in metagenomic datasets, for instance due to limited sequence coverage or low sequence identity values, both factors contributing to the possible artefactual ARG identification. The most common method is alignment-based homology search against ARGs reference databases, e.g. ARDB (Liu and Pop, 2009), CARD (McArthur et al., 2013) and ResFinder (Bortolaia et al., 2020). Limitations related with the databases and pipelines include (i) lack of full representation of ARGs diversity in the reference databases, (ii) lack of optimization of pipelines to search for ARGs with low similarity to a given database resources, (iii) inability of using gene fragments for reliable searches (Berglund et al., 2019). An alternative is direct aligning of the sequencing reads with the reference database using, e.g. ARGs-OAP (Yin et al., 2018), AmrPlusPlus (Lakin et al., 2017) and ARIBA (Hunt et al., 2017). Currently, the continuous progress in bioinformatics resulted in development of several novel and more sensitive tools for ARGs identification, including: (i) hidden Markov models-based system – Resfams (Gibson et al., 2015), (ii) a machine learning system utilizing information about the protein structure – PCM

(Ruppé et al., 2019), (iii) artificial neural networks-based tool for identification of novel ARGs directly from shotgun metagenomic data – deepARG (Arango-Argoty et al., 2018a, 2018b) and (iv) a tool using probabilistic gene models optimized to accurately identify previously uncharacterized resistance genes, even when showing only limited similarity with known ARGs – fARGene (Berglund et al., 2019).

The identification of chromosomal ARGs through MAG analysis is an important tool used to explore the environmental resistome. However, other complementary approaches are needed as the AR ecology is shuffled by highly dynamic ARGs vectors, mostly plasmids. The high inter-strain and inter-species mobility, as well as the extensive repetitive regions, impair straightforward ARGs-plasmid analyses. Different strategies have been designed to tackle that with long-read sequencing, capable of providing a plasmid nucleotide sequence in a single contig, being the Oxford Nanopore and Pacific Biosciences (PacBio) the most promising. Combined methods, e.g. Illumina producing short reads with low error rate and Nanopore producing long-reads with comparatively lower read coverage and accuracy, have been successfully used to unveil the diversity of ARGs, the respective bacterial hosts and the associated MGEs (Arango-Argoty et al., 2018a, 2018b; Ashton et al., 2015; Białasek and Mitobedzka, 2020; Che et al., 2019). The approach worked for various types of DNA samples. Namely, the chromosomal DNA extracted with the Wizard genomic DNA purification kit (Promega); the metagenomic DNA for the water samples extracted with the PowerWater® DNA Isolation kit (MO BIO Laboratories Inc.), DNA extracts for the influent, activated sludge, and effluent obtained with FastDNA® Spin Kit for Soil (MP Biomedicals, USA) as well as the total genomic DNA of the mixed isolates extracted with DNeasy PowerSoil Kit (Qiagen, Germany) (Ashton et al., 2015; Białasek and Mitobedzka, 2020; Che et al., 2019).

Long-read sequencing will bring important insight into AR ecology. The fact that Nanopore-based MinION technology does not require sophisticated equipment, and can be adapted for AR monitoring in real time with the possibility of feeding a global and centralized database, may place this method in the strategic plans of authorities aiming to launch global monitoring programmes.

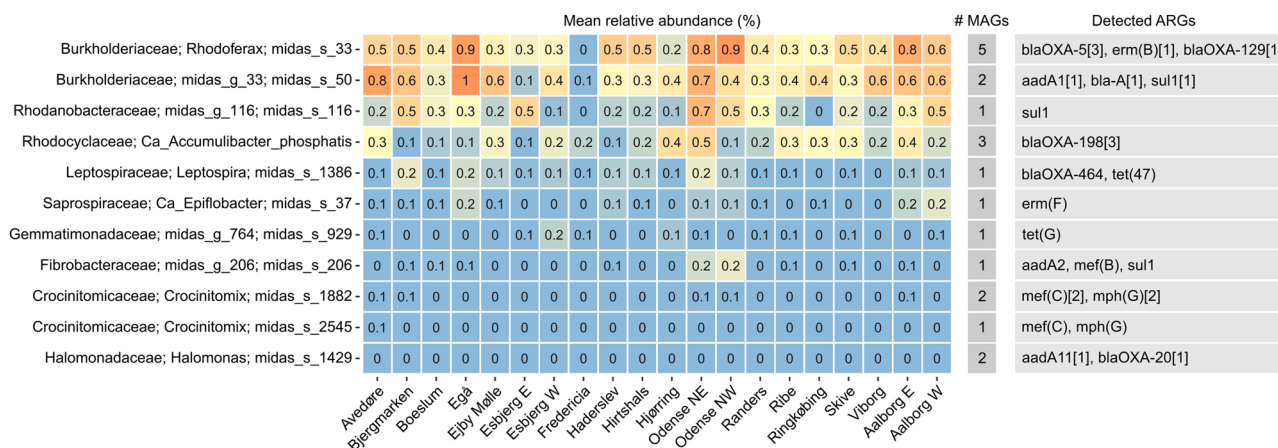


Fig. 1. Relative abundance of species encoding ARGs across Danish WWTPs using the MiDAS3 database. The MiDAS3 family, genus and species name is indicated per row. The relative abundance represents the average abundance of taxa in all samples collected from given WWTP in years 2006–2018 and is given as a percentage of the total community based on 16S rRNA gene ASV abundance. Number of MAGs with ARGs belonging to the species is indicated in the column #MAGs. The ARGs detected are shown, and if there is more than one MAG with ARGs, the number of MAGs in the species encoding the ARG type is indicated by the square brackets. The identified ARGs: *bla*<sub>OXA-5</sub> - oxacillin-hydrolyzing class D beta-lactamase OXA-5, *erm*(B) – 23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(B), *bla*<sub>OXA-129</sub> - OXA-5 family class D beta-lactamase OXA-129, *aadA1* - ANT(3'')-Ia family aminoglycoside nucleotidyltransferase AadA1, *bla*A - class A beta-lactamase, *sul1* - sulfonamide-resistant dihydropteroate synthase, *bla*<sub>OXA-198</sub> - class D beta-lactamase OXA-198, *bla*<sub>OXA-464</sub> - class D beta-lactamase OXA-464, *erm*(F) – 23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(F), *tet*(G) - tetracycline efflux MFS transporter Tet(G), *aadA2* - ANT(3'')-Ia family aminoglycoside nucleotidyltransferase AadA2, *mef*(B) - macrolide efflux MFS transporter Mef(B), *mef*(C) - macrolide efflux MFS transporter Mef(C), *mph*(G) - Mph(G) family macrolide 2'-phosphotransferase, *aadA11* - ANT(3'')-Ia family aminoglycoside nucleotidyltransferase AadA11, *tet*(47) - tetracycline destructase Tet(47), *bla*<sub>OXA-20</sub> - oxacillin-hydrolyzing class D beta-lactamase OXA-20.



### 3.4. Novel approaches for renewed AR insights

The study of the environmental resistome has motivated the development of innovative diagnostic tools worldwide. The feasibility, quality of output and costs of these methods will determine their use in the future. FLASH (Finding Low Abundance Sequences by Hybridisation) provides an example of an under-development hybridisation method (Quan et al., 2019). FLASH is a new-generation CRISPR diagnostic tool using the genomic DNA or cDNA for multiplexed detection of ARG sequences. The FLASH method combines the use of a set of Cas9 guide RNAs designed to target and cleave sequences of interest, in this case ARGs, into fragments with adequate size for Illumina sequencing. Depending on the guide RNAs, the method offers high efficiency, specificity and flexibility to target different ARGs and its allelic variants, and it can be adapted for multiplexing thousands of targets. It results in up to five orders of magnitude of enrichment and sub-attomolar ( $<10^{-18}$  moles  $L^{-1}$ ) gene detection with minimal background. The accuracy can be improved together with internal confirmation of sequence identity as in traditional next generation sequencing readout by FLASH (Quan et al., 2019). Currently only tested in isolates, FLASH has the potential to unveil a variety of ARGs, even those occurring in low abundance or in rare bacteria.

Another type of targeted analysis uses CRISPR-based tools and promises the simplification of the workflow and reduction of cost and time of analysis. The SHERLOCK (Specific High Sensitivity Enzymatic Reporter UNLOCKING) system aims to characterize resistant pathogens or human associated bacteria (Gootenberg et al., 2017). SHERLOCK makes use of a quenched fluorescent ssRNA reporter added to the reaction. The generated quantifiable signal of the targeted nucleic acid can be detected with Cas13 protein. The upgrade of this system (SHERLOCKv2) makes it easy to transport and provides results within an hour, enabling the possibility of field use. The presence of DNA/RNA of interest within a given sample using the SHERLOCK inspires its adaptation for AR detection (Gootenberg et al., 2018; Myhrvold et al., 2018; Niewoehner and Jinek, 2016; Zetsche et al., 2015). Initial equipment or human resources investments are not required, which makes it a possible tool for global monitoring.

There is no universal monitoring method available: either a set of specific genes is selected in order to get an incomplete picture of ARGs composition in a sample, or non-targeted shotgun analysis is performed, likely underestimating the role of genes and microorganisms present at low abundance. The method to be chosen will mainly depend on researcher preferences and budget, but above all on the type of information aimed. Table 3 presents examples of international comparisons of ARGs occurrence, based on metagenomics or qPCR. On one hand, a combination of methods may be suitable to unveil the AR ecology, as it will offer a more comprehensive overview of the resistome, which is a major goal of numerous research efforts. Novel and easy to use methods and technologies, and portable approaches, should be prioritised for AR diagnosis projects and use in global AR monitoring programmes.

### 4. AR monitoring: lessons and future perspectives

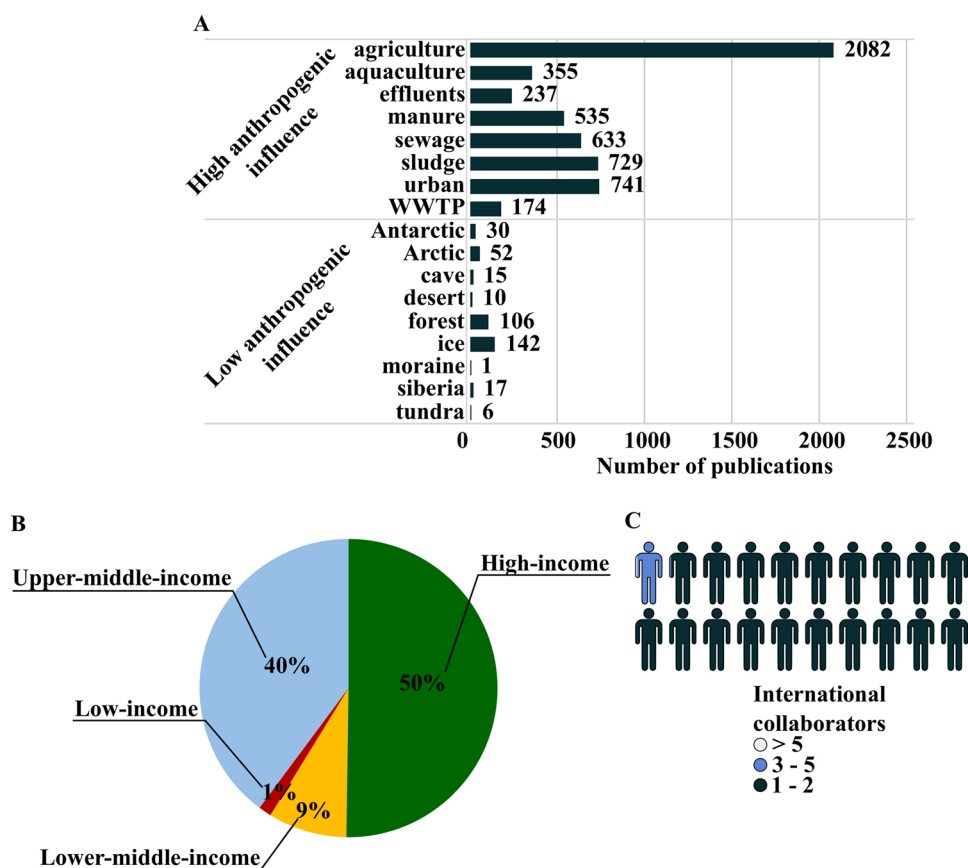
The occurrence of ARGs in environments with different levels of anthropogenic influence has attracted the attention of the scientific community (Fig. 2A). Understandably, remote and/or pristine areas with minimum anthropogenic pressure, e.g. Arctic, Antarctica, deserts, have received less attention than sites under intense human pressure. Yet, microbial communities originating from remote and pristine environments may provide a valid genetic resource for exploring the evolutionary origin of natural AR from the pre-antibiotic era. Such pristine niches constitute the reservoirs of poorly analysed pools of novel ARGs (Bhullar et al., 2012; D'Costa et al., 2011; Segawa et al., 2013; van Goethem et al., 2002). Considering various environmental compartments and ARGs circulation, sewage and WWTPs have been the object of diverse international monitoring studies (Table 3). These have been

**Table 3**

Examples of centralized and inter-lab comparative studies on the quantification of ARGs.

| Comparison of  | Potential bias factors  | Analysis method   | Key conclusion  | Reference                 |
|--|---|---|---|---------------------------|
| Untreated sewage from 79 sites in 60 countries   | Sampling; sample storage and shipping                                 | Metagenomics  | Gene diversity and abundance vary by region and strongly correlate with socioeconomic, health and environmental factors.  | Hendriksen et al. (2019a) |
| Raw and treated wastewater between different European WWTPs  | Sampling, DNA extraction and shipping                                 | High-throughput qPCR array  | Antibiotic use, environmental temperature, and treatment plant size are important factors related to resistance persistence.  | Pärnänen et al. (2019)    |
| Samples of treated wastewater collected in three different countries were analysed by different laboratories based on common DNA extract pools | Sampling, DNA extraction, shipping, protocols and equipment, operator | qPCR  | Inter-laboratory variation in genes quantification was below 28%. qPCR equipment and the nature of environmental DNA extracts are the factors adding higher bias to ARGs quantification by qPCR.                | Rocha et al. (2019)       |
| Impact of 16 WWTP effluents, from ten different European countries, in the receiving environment (river)                                       | Sampling, DNA extraction and shipping                                 | qPCR  | The river catchment, the local hydrological situation or the distance to the discharging point together with the effluent load, are major factors that affect the AR quantification in the receiving river.     | Cacace et al. (2019)      |
| Effluent samples from 12 European WWTPs and sludge, soil and effluent samples from a saturated aquifer treatment system                        | Sampling, DNA extraction and shipping                                 | Illumina MiSeq sequencing of integron gene cassette-associated amplicons (by PCR) | Differences in DNA extraction efficiencies in aqueous and sludge matrices is suggested as one factor explaining the differences in the distribution of integrons found between the effluent and sludge samples. | Gatica et al. (2016)      |

conducted to assess the occurrence of ARGs in sewage, the efficacy of removal of AR, mainly ARGs, by treatment systems in different geographic regions, to assess the risks of possible dissemination to the environment or to infer geographic contrasts (Cacace et al., 2019; Gatica et al., 2016; Hendriksen et al., 2019a; Marano et al., 2019; Pärnänen et al., 2019). These studies were mostly designed with the intention of avoiding inter-laboratory variations. However, this may not always be



**Fig. 2.** Summarized global representation of the search of scientific literature concerning antibiotic resistance genes (based on PubMed database screened with methodology described in [Supplementary Material](#)). (A) Number of released (as of 26th January 2021) publications describing the issue of AR in various environments under high and low anthropogenic influence. (B) Contribution of scientific groups researching ARGs affiliated to countries of various economic income. (C) Co-authorship affiliated in LMCIs of publications on ARGs within international collaborations.

feasible. Since the number of studies monitoring AR worldwide is increasing, a major question is if studies performed in different laboratories are indeed comparable. [Rocha et al. \(2020\)](#) addressed these aspects, observing, for instance, that the operator may have a low impact on the quantification of ARGs, with the qPCR equipment being the most likely cause of inter-laboratory variations and representing a difference in the quantification of up to 28% regarding gene copy number. On the contrary, using specific qPCR protocols and specific equipment, results produced in Portugal and USA for the same DNA extracts were statistically non-significantly different ([Rocha et al., 2019](#)). These are promising results for the scientific community which may encourage comparison of AR quantifications in different world regions. However, these efforts need to be continued in order to give an added-value to the large dataset that has been generated and that can be shared through open science options.

The migration of AR from regions with high population density to less populated areas is of major concern for the authorities. Some of these regions coincide with low and middle-income countries (LMICs), where antibiotic use and environmental policies may facilitate AR dissemination. LMICs are still poorly studied ([Harbarth et al., 2015](#); [Seale et al., 2017](#)). The literature search (methodology described in the [Supplementary Material](#)) showed that only 13% of articles describing ARGs in anthropogenically-shaped environments were conducted by researchers affiliated to low-income countries and collaborations with LMICs were also rare ([Fig. 2B and C](#)). Meanwhile, some LMICs exhibit an increasing trend of antibiotic consumption as shown by analyses of defined daily doses of antibiotics per 1.000 inhabitants per day over a 15-year period (2000–2015) ([Klein et al., 2018](#)). Low national funding for science in LMICs limits capabilities for AR research. Increasing the involvement of these countries in international projects would help fill the knowledge gap. Conducting joint projects (involving socioeconomically diverse countries) is critical for drawing conclusions about the

global resistome, hot spots of AR outbreak, spread and phylogeography ([Hendriksen et al., 2019a](#)). Partnerships between LMICs and high-income countries will permit the development of advanced AR monitoring and will support the global AR. LMICs should be supported regarding bioinformatics and supporting management systems, personal training and financing ([Vegyari et al., 2020](#)).

## 5. The challenges of transforming numbers into One-Health useful information

Environmental monitoring has the potential to target two types of risk assessment, the one associated with the dissemination and amplification of ARB and ARGs across environmental compartments, and the probability of transmission to humans. The research methods used to reach these two goals can be the same, although the data may need to be explored and interpreted in different ways. A major gap in understanding the human health risks posed by the acquired environmental resistome is due to the fact that ARGs that critically threaten human lives (e.g. encoding carbapenemase or ESBL) hardly can be traced across the distinct One-Health compartments, being almost impossible to track their path from the environment to humans. This is due to the extremely low abundance that these ARGs reach in the environmental compartments on the One-Health cycle, although hardly can be understood as risk absence ([Manaia, 2017](#)). Therefore, the risk of transmission cannot be calculated based on the current state of the art, but it can be inferred from multiple concepts and measurable variables ([Fig. 3](#)). This type of inference seems to be the most straightforward way for filling the gap due to wastewater within the One-Health cycle. As [Martínez et al. \(2015b\)](#) discussed, the potential harmful effects of ARGs are proportional to the likelihood that it can be horizontally transferred to human pathogens. Hence, the highest risk is posed by AR associated with MGEs, although even these may represent different risks of propagation. In

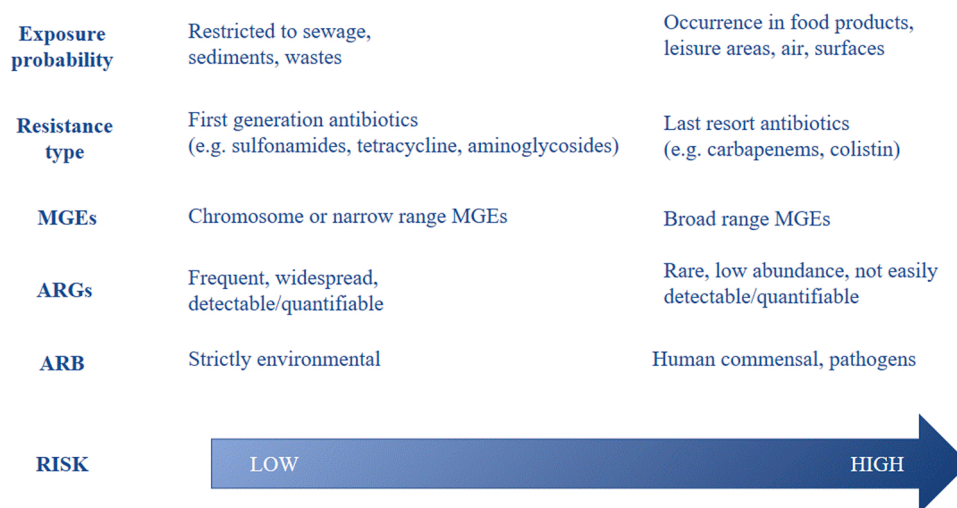


Fig. 3. Conceptual model of variables that may affect the risks due to the occurrence of antibiotic resistance in the environment.

addition, for assessing the risks of transmission to humans, it is important to infer if the ARG host belongs to a bacterial group that may reach, colonise and eventually infect humans, or transfer ARGs to the commensal microbiota (Manaia, 2017). Also, it is important to know the absolute abundance of the bacterial host and if it can occur at relevant infective dose (Ashbolt et al., 2013; Huijbers et al., 2019; Mughini-Gras et al., 2019). These considerations show that the evaluation of risks associated with environmental antibiotic resistance is necessarily multifactorial (Fig. 3). Another aspect that makes this analysis more complex is the fact that the detection of ARGs does not indicate that the resistance function is being expressed, conferring effective resistance, or that the ARG is somehow in a biologically active form, for instance to be acquired. In part, these shortcomings could be overcome by the analysis of the expressed mRNA of key ARGs or MGEs, or of the whole community (metatranscriptomics). However, these approaches still present some methodological challenges to examine environmental samples (Luby et al., 2016). The low yield of mRNA in environmental samples, where metabolism and generation times are delayed, combined with the fast degradation are major limitations still to overcome to reach the reliable measurement of minor genes as is the case of ARGs (Jiang et al., 2015).

Regarding the risks of ARGs dissemination in the environment, a determinant factor refers to the distinct core communities that may exist in each niche, and that may be more or less permissive to the survival and spread of exogenous resistance types. ARGs monitoring based on direct DNA analysis provides data corresponding to read number or gene copy number that can be shown through total bacterial abundance, normally using the 16S rRNA gene (relative abundance), or per sample volume or mass (Auerbach et al., 2007; Bhullar et al., 2012; Cacace et al., 2019; Dumas et al., 2006; Hendriksen et al., 2019a; Pärnänen et al., 2019). These analyses cannot assess if the ARGs were hosted by living or dead bacteria, bacteriophages particles or by free DNA, and therefore do not provide direct evidence of the self-replicative and/or infective potential. However, these data support the comparison of contamination levels, assessment of treatment efficiencies or rough estimates of the risks of dissemination or transmission to humans. For assessing the impacts and risks of dissemination in the environment, it may be important to distinguish between intrinsic and acquired AR, including ARGs that are associated with MGEs (Dumas et al., 2006; Lehtinen et al., 2020; Lira et al., 2020; Luby et al., 2016). For example, in activated sludge samples studied by Yang et al. (2014) some of the most abundant ARGs were assigned to the plasmid metagenome, associated with resistance to tetracycline (27%), macrolide (25%), multidrug (25%), bacitracin (9.9%), and sulfonamide (5%). Also, the assessment of

the relative or absolute abundance may be important to infer the likelihood of horizontal gene transfer or of successful clonal selection under favourable conditions (Almakki et al., 2019; Marti et al., 2013). Despite the importance of HGT for ARGs dissemination, reliable assignment of MGEs carrying ARGs to their microbial hosts in natural communities is still difficult. Even the high throughput metagenomic sequencing is not an appropriate approach because it is difficult to reveal the origin of the particular gene and during sequencing, plasmids (key players in HGT) are assembled as separate contigs that cannot be linked with their bacterial hosts. Methods relying on linking the gene/ARG/MGE with bacterial hosts will be determinant to advance the knowledge in this field. Examples of these are (i) *in vivo* proximity-ligation method Hi-C (Stalder et al., 2019), (ii) Emulsion, Paired Isolation and Concatenation PCR (epicPCR) (Hultman et al., 2018; Spencer et al., 2016) (iii) non-targeted sequencing of single bacterial genomes (Gupta et al., 2020), and long-read sequencing (Van Der Helm et al., 2017).

Despite the apparent intermittence of the occurrence of some ARGs across the One-Health cycle there are reasons to believe that at least some establish a continuum between distinct niches and environmental compartments. This reality may require the use of different methodological approaches may be required, it is necessary to establish integrated monitoring programmes, capable of informing the path of dissemination of some ARB and ARGs across One-Health. Besides the collaboration among institutions and researchers responsible for various One-Health compartments, it is necessary to develop algorithms and other approaches that permit the translation of the outputs provided by different methods and obtained in distinct sites into a cohesive dataset that supports. This vision, that meet the One-Health concept, faces, however, some challenges mainly in what refers to the alignment required between methods and sites. Specifically, (i) the biological entities to pursue, which may be bacteria, MGEs, or ARGs; and (ii) the sensitivity and accuracy of the methods, as the abundance of the transmissible agent can be at trace amounts in some paths of the One-Health cycle, and the existence of numerous genetic variants for the same genetic element can blur a straightforward analysis and reliable risk assessment (George, 2019; Huijbers et al., 2019). The main goal of AR monitoring should be the supply of information that can be used by authorities and policy making entities for the definition of guidelines and recommendations for the prevention and remediation of resistance dissemination. To achieve this goal, the data that the scientific community is producing must be translated into practical information. This is still the major challenge in this field.

## 6. Outlook

The integrated system of AR monitoring conducted at the multinational scale allowing global studies of AR distribution and future trends is still a long way off. The knowledge gaps in environmental AR studies are being slowly tackled. The methodology targeting single genes or a set of ARGs and ARB is delivered by researchers around the world. However, to reach the ambitious goal of combating AR, science and society need to work together.

Scientific research and innovation have a pivotal role in combating AR, at two distinct levels. The first is the study of the ecology and genetics of AR shedding light on the mechanisms of acquisition and transmission, paths of dissemination and major drivers for AR evolution. Control of these factors is essential to halt the increasing AR threat. The second level, more focused on innovation, refers to the development of new tools and portable methods that simplify and promote the feasibility, while ensuring comparability and reproducibility, necessary to implement global AR monitoring schemes. Society, represented by authorities and guided by recommendations and policy, needs to act locally on promoting public awareness and stimulating actions, among which AR monitoring contributes to recognizing critical AR sources and also to building local and global AR databases. The return of this information to the policy making authorities and the scientific community has the potential to leverage AR control in a true One-Health circuit.

### CRedit authorship contribution statement

**Aleksandra Miłobędzka:** Conceptualization, Writing – original draft, Writing – review & editing. **Catarina Ferreira, Ivone Vaz-Moreira, David Calderón-Franco:** Writing – original draft, Writing – review & editing. **Lukasz Dziewit, Sabina Purkrtova, Jan Bartacek, Per Halkjær Nielsen:** Writing – review & editing. **Adrian Gorecki:** Investigation, Writing – review & editing, Visualisation. **Célia M. Manaia:** Data on MAGs, Writing – review & editing. **David Gregory Weissbrodt:** Writing – outlining, review & editing. **Célia M. Manaia:** Conceptualization, Supervision, Writing – review & editing.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Acknowledgements

This work has received funding from the European Research Council under European Union's Horizon 2020 Research and Innovation through the project "Research platform on antibiotic resistance spread through wastewater treatment plants, REPARES" Programme Grant Agreement 857552; H2020-WIDESPREAD-2018-03; NWO TTW Project TARGETBIO (grant no. 15812); VILLUM FONDEN Research grants (16578, 15510); and European Structural and Investment 671 Funds, OP RDE395 funded project 'ChemJets' (No. CZ.02.2.69/0.0/0.0/16\_027/0008351) who supported AM. We thank Dr. Marta Nierychło for her help with proofreading and fruitful discussion on the manuscript.

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2021.127407](https://doi.org/10.1016/j.jhazmat.2021.127407).

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