Monitoring antibiotic resistance genes in wastewater treatment: current strategies and future challenges

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Abstract

Antimicrobial resistance (AMR) is a growing threat to human and animal health. Progress in molecular biology has revealed new and significant challenges for AMR mitigation given the immense diversity of antibiotic resistance genes (ARGs), the complexity of ARG transfer, and the broad range of omnipresent factors contributing to AMR. Municipal, hospital and abattoir wastewater are collected and treated in wastewater treatment plants (WWTPs), where the presence of diverse selection pressures together with a highly concentrated consortium of pathogenic/commensal microbes create favourable conditions for the transfer of ARGs and proliferation of antibiotic resistant bacteria (ARBs). The recent emergence ARBs and ARGs as well as their potential health effects have re-defined the role of WWTPs as a focal point in the fight against AMR. By reviewing the occurrence of ARGs in wastewater and sludge and the current technologies used to quantify ARGs and identify antibiotic resistant bacteria (ARB), this paper provides a research roadmap to address existing challenges in AMR control via wastewater treatment. Wastewater treatment is a double-edged sword that can act as either a pathway for AMR spread or as a barrier to reduce the environmental release of anthropogenic AMR. State of the art ARB identification technologies, such as metagenomic sequencing and fluorescence-activated cell sorting, have enriched ARG/ARB databases, unveiled keystone species in AMR networks, and improved the resolution of AMR dissemination models. Data and information provided in this review highlight significant knowledge gaps. These include inconsistencies in ARG reporting units, lack of ARG/ARB monitoring surrogates, lack of a standardised protocol for determining ARG removal via wastewater treatments, and the inability to support appropriate risk assessment. This is due to a lack of standard monitoring targets and agreed threshold values, and paucity of information on the ARG-pathogen host relationship and potential risk evolution. These research gaps need to be addressed and research

findings need to be transformed into practical guidance for WWTP operators to enable effective progress towards mitigating the evolution and spread of AMR.

Keywords: Antimicrobial resistance (AMR); Wastewater treatment; Antibiotic resistant genes (ARG) quantification; host identification; horizontal gene transfer (HGT); mobile genetic elements (MGEs).

1. Introduction

Municipal wastewater treatment is essential for the protection of public health and the aquatic environment. A typical wastewater treatment plant (WWTP) integrates multiple engineering processes, including physical, chemical, and biological treatment steps. Biological treatment involves the use of microorganisms to remove wastewater contaminants (i.e. organic carbon, nutrients, and micropollutants). The microorganisms performing this service are extremely diverse, and the microbial community structure of each treatment system is unique and evolves over time.

One of the primary objectives of wastewater treatment is to reduce the transmission of waterborne diseases. Wastewater treatment also plays a critical role in controlling the release of antibiotic resistance genes (ARGs) to the environment. Multiple chemical factors including disinfectants, metals (e.g. copper and zinc), various pharmaceuticals (e.g. antibiotics), and other organic compounds exist within a WWTP. Chemical factors are among diverse selection pressures that influence the transmission, expression and mobilisation of ARGs and drive the emergence, persistence, and proliferation of antibiotic resistant bacteria (ARB) (Guo et al., 2015; Li et al., 2019; Zhang et al., 2017b). Wastewater (sewage) also provides a continuous input of ARGs, ARB, and highly diverse commensal and pathogenic bacteria from human and animal microbiomes into WWTPs. ARGs often assemble in close proximity to one another on mobile genetic elements (MGEs) generating complex resistance regions (CRRs). In such cases, the acquisition of a single plasmid (a type of MGE) can confer a multiple drug resistance

(MDR) phenotype to the host bacterium that acquires it. This is particularly problematic when it occurs on plasmids that carry important virulence gene cargo (Venturini et al., 2010; Venturini et al., 2013; Mangat et al., 2017). There are examples in the literature where *Escherichia coli* with a commensal phylogroup (phylogroup B1) have caused serious human disease (urosepsis), as evidenced by their isolation from multiple body fluids. Subsequent genomic analysis shows the acquisition of a virulence plasmid with a CRR is likely to have precipitated these pathological events (McKinnon et al., 2018). Together, conditions found in WWTPs create an ideal environment for the evolution of new and more complex CRRs as well as their horizontal gene transfer (HGT) to new hosts. Infections caused by ARB, especially those with MDR phenotype, are hard to treat due to reduced antibiotic efficacy, and result in higher medical costs due to prolonged hospital stays and increased morbidity and mortality (World Health Organization, 2020).

Antimicrobial resistance (AMR) has become a cross-cutting, complex, and growing threat to global health. Not surprisingly, dedicated reviews have appeared on this topic, with many focussing exclusively on antibiotic resistance, the fate and distribution of ARGs/ARB during wastewater treatment (Pazda et al., 2019; Rizzo et al., 2013; Sharma et al., 2016), or on the proliferation of ARGs in the environment (Martínez et al., 2015; Partridge et al., 2018; Rice et al., 2020). Unlike these previous reviews, our work aims to provide a new perspective that focuses on the interface between wastewater treatment and microbial genetics. By coupling interdisciplinary perspectives in wastewater treatment with genetic and genomic epidemiology, this review defines a research roadmap to mitigate the evolution and transmission of AMR and to provide new insights to AMR characterization, surveillance and monitoring, and risk modelling and assessment during various stages of wastewater treatment. Discussion and literature data summarised in this review may guide the water industry to play an active role in addressing the threat of AMR to global health.

This review aims to be critical rather than exhaustive and descriptive. It first examines pertinent challenges in quantifying AMR in wastewater and key mechanisms of ARG proliferation. State of the art technologies that demonstrated the capacity to quantify ARGs and identify their hosts (especially pathogenic hosts) are presented, and the risks associated with ARG and ARB in wastewater are discussed. Factors governing ARG removal and transfer are also examined. Data and information compiled for this review are critically analysed to identify key challenges in the monitoring and control of ARGs during wastewater treament and to suggest a roadmap for future research.

2. Antibiotic resistance in wastewater

Numerous ARGs including types and subtypes of almost all common antibiotics have been detected in wastewater influent, effluent and biosolids or sludge. Examples of these ARGs are available in Table 1. The antibiotic classes included in Table 1 cover the most commonly prescribed and consumed antibiotics in the health care, veterinary, and livestock sectors (European Centre for Disease Prevention and Control, 2019; Pazda et al., 2019; Wang et al., 2020). Table 1 provides a snapshot from the recent literature; a more comprehensive list of ARGs in WWTP compartments is available in previous reviews (Pazda et al., 2019; Wang et al., 2020). The occurrence of ARGs in treated effluent and wastewater sludge may pose a risk because these ARGs can potentially be acquired by new bacteria in downstream environments through HGT (Cantón et al., 2012; Perry and Wright, 2013). A major objective of wastewater treatment is to inactivate pathogens prior to effluent discharge. But this remit needs to be reconsidered in the context of AMR because commensal, non-pathogenic bacteria can also be important reservoirs for plasmids and other mobile genetic elements (MGEs) carrying ARGs. WWTPs could provide unparalleled opportunities to control the proliferation of ARGs. The potential role of wastewater treatment as a barrier against AMR is further discussed in Section 4.5.2.

Table 1. Most commonly detected antibiotics and their associated ARGs in WWTPs.

Antibiotic class	**Antibiotic compounds (type)**	**Antibiotic resistant genes (subtype)**	**Sampling location**	**Ref(s)**
Aminoglycoside	Kanamycin, tobramycin, gentamicin	*aad*A, *aac*A4, *aad*B, *aad*E, *str*B	Sewage Sludge	Tang et al. (2017)
β-lactam	Amoxicillin, cloxacillin, penicillin V, ampicillin	$bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{OXA-A}}$, bla_{SHV}, *mecA*	Raw Influent/ Tertiary Effluent/ Activated Sludge	Zhang et al. (2019b); Ziembińska-Buczyńska et al. (2015)
Macrolides	Clarithromycin, erythromycin/erythromycin-H_2O, azithromycin, roxithromycin	*ereA*, *ermB*, *ermC*, *erm43*, *mefC* and *mphG*	Raw Influent/ Secondary Effluent	Sugimoto et al. (2017); Wang et al. (2020)
Quinolone	Ofloxacin, ciprofloxacin, norfloxacin	*qnrS*, *qnrC*, *qnrD*	Raw Influent/ Secondary Effluent/Digested Sludge	Castrignanò et al. (2020a); Castrignanò et al. (2020b)
Sulfonamides	Sulfamethoxazole	*sul1*, *sul2*	Raw Influent/ Secondary Effluent/ Activated Sludge	Chen et al. (2019); Lorenzo et al. (2018); Lye et al. (2019); Rolbiecki et al. (2020)
Tetracyclines	Tetracycline	*tetA*, *tetB*, *tetE*, *tetG*, *tetH*, *tetS*, *tetT*, *tetX*	Raw Influent/ Secondary Effluent/ Anaerobic digested Sludge	Huang et al. (2016); Wang et al. (2020)
Trimethoprim	Trimethoprim	*dhfrA1*, *dhfr14*	Activated Sludge	Ziembińska-Buczyńska et al. (2015)

A major avenue for ARG proliferation is through HGT, which is expected to be prevalent during wastewater treatment. The fate of ARGs and ARB in an environment is

dynamic, and can be affected by changes in bacterial reproduction and decay rate (Fahrenfeld et al., 2014; Gothwal and Thatikonda, 2020). Conjugation frequencies and other mobile genetic events are also impacted by temperature and selection pressures. A standardised approach is thus required for calculating and comparing the removal or generation of ARGs by wastewater treatment. Moreover, although global efforts have been made to curate and regularly update databases of antibiotics and ARGs, such as the Comprehensive Antibiotic Resistance Database (CARD) and Structured Antibiotic Resistance Gene database (SARG) (Boolchandani et al., 2019), more antibiotics and ARGs will continue to evolve and be discovered into the future. Agreed surrogates for antibiotic resistance determinants are needed to effectively track the occurrence and fate of ARGs in WWTPs. These pertinent issues are further elaborated in subsequent sections.

Currently, it is difficult to quantify the exact risk associated with the occurrence of ARGs in wastewater. Detection of ARGs in WWTPs is currently reported in units that cannot be directly used for assessing health consequences and risk. In wastewater treatment, chemical contaminants are commonly expressed in μg/L of wastewater or μg/kg of sludge. Likewise, pathogens are quantified in CFU/g of sludge or CFU/mL (CFU stands for colony forming unit) (World Health Organization, 2020). These units (i.e. μg/L, μg/kg, CFU/g and CFU/mL) can be directly linked to relevant guidelines or standards to evaluate the associated risk via a dose-response relationship. In other words, there are defined threshold concentrations of chemical contaminants or pathogens to trigger regulatory responses. By contrast, ARG in water samples are expressed in ppm (one ARG per million reads), copies/mL or normalized by 16S copies to account for sequencing depth (Al-Jassim et al., 2015; Christgen et al., 2015; Ferro et al., 2016). Unlike the units of chemical contaminants and pathogens, these ARG concentration or abundance units are not comparable and can only be indirectly converted to one another with some uncertainties. Chandrasekaran and Jiang (2019) provided arguably the first example of

such indirect dose-response model by establishing the relationship between stochastic dead rate (indirect) and the occurrence of gentamicin resistant *E. coli*. A direct dose-response model would require a common unit for ARG concentration that can be used consistently across samples to establish a dose-response relationship for risk assessment.

3. ARG development and proliferation

A bacterial host cell can acquire antibiotic resistance through three different routes: vertical gene transfer (VGT), *de novo* mutation, and HGT (Hiller et al., 2019). VGT is the inheritance of ARGs through bacterial reproduction, and there is a difference in VGT of chromosomally-associated and plasmid-associated ARGs. Chromosomally-associated ARGs would undergo stable inheritance by all daughter cells. On the other hand, plasmid-associated ARG inheritance depends on plasmid incompatibility. When two or more incompatible plasmids (they have identical replication systems) are in the mother cell, each daughter cell will have a potentially different plasmid profile (Clark et al., 2019); thus, a different ARG profile. *De novo* mutations are single nucleotide polymorphisms that occur rarely due to low frequency errors arisen during DNA replication and proliferate under a selection pressure (Händel et al., 2014). The evolution and transmission of newly developed ARGs depend upon multiple factors, including the rate of mutation, level of resistance conferred, strength of selection pressure, and the relative fitness of ARB (Melnyk et al., 2015; Yadav and Kapley, 2019).

HGT is the process of transferring ARGs between different bacterial cells (Soucy et al., 2015) and in the context of wastewater treatment, this is thought to play a significant role in the spread of ARGs. There are three HGT mechanisms: transduction, conjugation, and transformation (Figure 1). Transduction involves the transfer of bacterial DNA via bacteriophage or gene transfer agents (Gómez-Gómez et al., 2019; Lang et al., 2012).

Conjugation refers to the transfer of DNA between bacterial cells via physical contact through pili. Transformation is the uptake of naked extracellular DNA by bacteria.

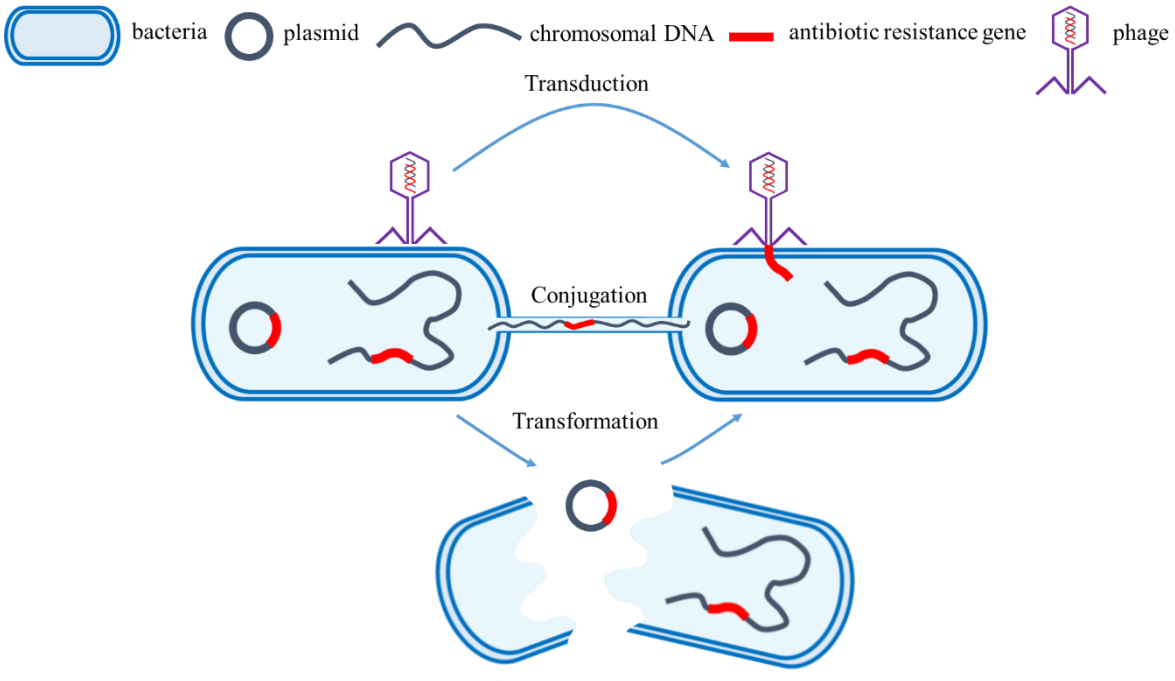

Figure 1. The three mechanisms of horizontal gene transfer: transduction, conjugation and transformation. Adapted from United States Centers for Disease Control and Prevention (2020).

HGT of ARGs is more likely to occur when ARGs are carried by MGEs. Intercellular MGEs are those that can transfer between bacterial cells, including conjugative plasmids, bacteriophages, gene-transfer agents (phage-like particles), and integrative conjugative elements. By contrast, intracellular MGEs, including insertion sequences, transposons, and integrons, can only transfer within the same bacterial cell. Interestingly, intercellular and intracellular MGEs can interact with each other to enhance ARG stability and dissemination. For example, insertion sequences or integrons can be integrated into plasmids and then participate in HGT events (Che et al., 2019). In addition, insertion sequences can potentially influence a plasmid long-term stability in the host cell by mediating deletions of genetic regions within the plasmid's backbone (Porse et al., 2016). Insertion sequence IS26 has the ability to

form pseudo-compound transposons (Harmer et al., 2020), facilitate plasmid fusion events and create hybrid plasmids (Du et al., 2020; Mangat et al., 2017). It is also linked with the mobilisation of many key ARGs of clinical significance (He et al., 2015; Partridge et al., 2018).

Although each type of MGEs play a role in HGT, plasmids and class 1 integrons are recognised as the two most important MGE types involved in ARG proliferation. Plasmids can replicate themselves independently of the bacterial chromosome, cross phylogenetic barriers (i.e. transfer between phylogenetically distant Gram-positive and Gram-negative bacteria), and evolve to increase their stability in the host cell and broaden their host range (De Gelder et al., 2008; Porse et al., 2016; Sota et al., 2010; Yang and Walsh, 2017). A recent analysis of 10,000 reference plasmids showed that 60% of plasmids have host ranges beyond the species barrier and up to 10% can cross order barriers; forming a vast network for HGT in bacteria (Redondo-Salvo et al., 2020). The plasmid transfer rate can increase in heterogeneous bacterial communities, such as those in WWTPs (Svara and Rankin, 2011). Besides plasmids, class 1 integrons also play a major role due to their ability to acquire and disseminate gene cassettes, in a process of site-specific recombination (Partridge et al., 2000). The combination of class 1 integrons with insertion sequences allows recruitment of multiple ARGs and the duplication and transfer of large chromosomal inversions, resulting in the co-localization of ARGs, development of complex ARG cassettes and MDR bacteria (Johnson et al., 2016). Class 1 integrons have been found in the environment (Zhang et al., 2020; Zhu et al., 2019), in the commensal flora of swine (Reid et al., 2017; Zingali et al., 2020) and poultry, and in pathogenic *E. coli* causing colibacillosis (Cummins et al., 2019) indicating that they are globally important environmental pollutants (Gillings, 2018). Class 1 integrons are often components of other MGEs (transposons and plasmids) and CRRs (Zhu et al., 2017). Clinical class 1 integrons appear to have a single origin, indicating HGT as their dissemination mechanism (Gillings et al., 2008).

Plasmids and class 1 integrons linked to the proliferation of ARGs through HGT have been detected in all compartments of a typical WWTP (Figure 2). Of particular note is the significantly higher abundance and diversity of plasmids and Class 1 integrons in activated sludge and anaerobically digested sludge compared to primary (raw) sludge and tertiary effluent. Although it has been challenging to confirm the actual presence of ARGs in plasmid and class 1 integrons in a high throughput manner, previous studies have shown a linear correlation (Pearson's R^2 = 0.78-0.92) between the ARG abundance and diversity of these MGEs (Han and Yoo, 2020; Ma et al., 2014; Tian et al., 2016). However, the number of studies on plasmids and class 1 integrons is far fewer compared to that on ARGs in wastewater, and most of these studies are relatively recent. Due to the difference in reporting units of plasmid and integron abundance and different sequencing depths, it is not possible to normalise all available data for an exhaustive list. However, Figure 2 provides an illustration of relative abundance and diversity of plasmids and class 1 integrons in WWTP compartments using available data reported in ppm. The whisker-plots in Figure 2 are constructed from one data point for raw sewage and effluent, and 2-4 data points for activated and anaerobically digested sludge.

The current lack of data on the occurrence and fate of plasmids and class 1 integrons in wastewater treatment can also be attributed to technical difficulties in detecting and quantifying these MGEs due to their variable nature (e.g., frequent recombination and ancestral versions). Further research is recommended to elucidate the exact role of MGEs in the proliferation of ARGs during wastewater treatment.

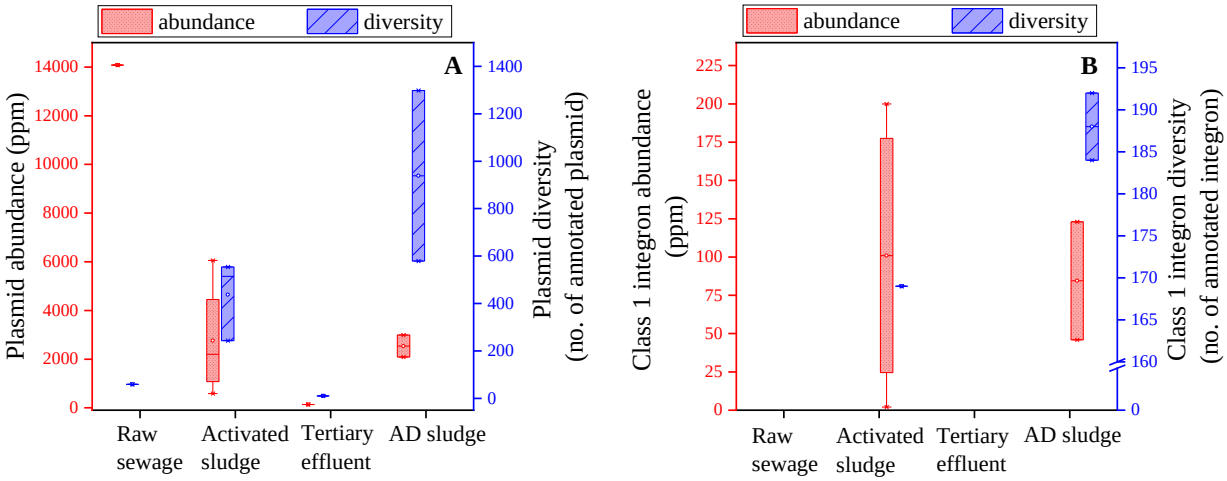

Figure 2. The abundance and diversity of (A) plasmids and (B) Class 1 integrons in WWTP compartments. Data from: Han and Yoo (2020); Lira et al. (2020); Ma et al. (2014); Tao et al. (2016); Tian et al. (2016); Yoo et al. (2020) with reported abundance and diversity. The ppm unit means one read of plasmid or Class 1 sequence in one million reads of metagenomic sequences. The whisker-plots are constructed from one data point for raw sewage and effluent, and 2-4 data points for activated and anaerobically digested sludge. AD sludge: anaerobically digested sludge.

4. ARG-ARB-pathogen relationships

A major challenge in ARG management is the identification of ARG-hosts (ARB), of which human pathogens present the greatest risk. Host-identification is necessary to understand how ARGs might spread to pathogens – both existing and emerging. This section will discuss the context in which ARGs in wastewater microorganisms can become problematic, provide an overview of current technologies that can be used to elucidate ARG and host relationships, and summarise relevant findings revealed using these technologies to date.

4.1. Risks associated with ARGs and ARB in wastewater

4.1.1. ARGs in wastewater impact routes on humans

ARGs in WWTPs can pose human health risks through several routes (Figure 3). WWTPs can act as a reservoir of ARGs and facilitate ARG exchange via HGT. Indeed,

non-ARG hosts in WWTP influents can potentially acquire ARGs while passing through the wastewater treatment process (Hultman et al., 2018). This premise is supported by Jacquiod et al. (2017) who reported that WWTP effluent microbiome had a higher diversity of ARG hosts than the WWTP influent microbiome. During the treatment process, a large proportion of ARB and ARGs are removed from the water phase and partitioned into the sludge phase, resulting in high concentrations of ARGs in sludges and biosolids (up to 10^9 copies/g) (Munir et al., 2011). Many ARGs also remain in the treated effluent (Calero-Cáceres et al., 2014; Hiller et al., 2019), and as biosolids and effluent are eventually returned to the natural environment, wastewater-derived ARB and ARGs can potentially come into contact with environmental bacteria, wildlife, domestic animals, and humans. WWTP effluents contribute significantly to the number of detected ARGs, transposon, and integrons in the receiving river's water and downstream sediments (Berglund et al., 2015; Makowska et al., 2016; Quintela-Baluja et al., 2019).

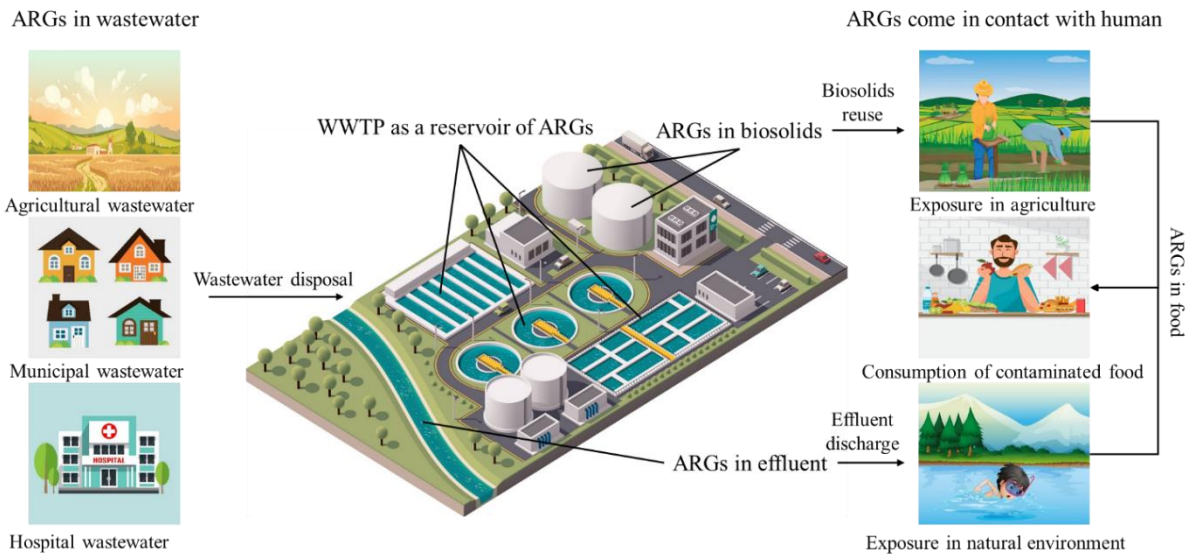

Figure 3. Impact routes of antibiotic resistance genes (ARGs) on humans.

Once in contact with humans, ARG-hosts can transfer ARGs to commensal bacteria and pathogens via HGT. For example, the widespread ARG *bla*$_{\text{CTX-M}}$ (encoding extended-spectrum-*β*-lactamases - ESBLs), which is mobilised globally on plasmids, is

suggested to originate from the chromosomal *bla* gene of soil *Kluyvera* species (Cantón et al., 2012). Similarly, globally disseminated quinolone resistance genes probably had their origins in the chromosome of *Shewanella* spp. (Melvold et al., 2017). There is also evidence of ARG exchange between environmental bacteria from soil and swine farms with clinical pathogens, including two high-risk species *Klebsiella pneumoniae* and *Acinetobacter baumannii* (Forsberg et al., 2012; Johnson et al., 2016; Perry and Wright, 2013). These putative HGT events were proposed based on 100% gene sequence similarity between species, encompassing ARGs, MGEs (integrons and insertion sequences) as well as non-coding regions (Forsberg et al., 2012). Of particular concern, ARG and virulence genes can co-localize on the same MGE, hence allowing bacteria to acquire both resistance and virulence in a single conjugation event and develop the ability to infect the human body (Beceiro et al., 2013). An example of this phenomenon is the co-localization of ARGs and virulence genes on the same plasmids (McKinnon et al., 2018; Venturini et al., 2010; Venturini et al., 2013). The global spread of pathogens carrying such plasmids (e.g. *E. coli* B2 025:H4-ST131 strains) in recent years may indicate the simultaneous selection of resistance and virulence (Beceiro et al., 2013; Bevan et al., 2017).

4.1.2. **Risks associated with ARGs**

It is noteworthy that not all ARGs pose the same risk level to human health. A conceptual framework was proposed to classify ARG candidates into different risk levels (Martínez et al., 2015). The classification criteria include sequence similarity between the ARG candidate and known ARGs, co-localization with MGEs (mobility), types of resistance mechanism (e.g. efflux pump, target modification, or novel mechanism), type of antibiotics the ARG confers resistance to, and presence in human pathogens. A publicly available tool for ranking ARG risk using metagenomic data – MetaCompare – was also introduced (Oh et al., 2018). This tool evaluates ARG candidates based on similar criteria with

the abovementioned conceptual framework and assigns a resistome risk score. The ARGs with the highest risk scores are those that confer resistance to antibiotics currently in use, are associated with MGEs, and present in human pathogens. In contrast, the detection of an ARG is of lesser significance if the ARG presents in environmental bacteria, with a low likelihood of transferring into human pathogens (e.g. not associated with MGEs). The classification of ARGs into health risk levels within an environmental context, could assist the development of high resolution risk models and specific recommendations for AMR mitigation (Pruden et al., 2018).

4.1.3. **Risks associated with ARB**

The likelihood of ARG introduction into human pathogens should be assessed based on their hosts (ARB), rather than the ARGs themselves. ARB can be differentiated as reservoirs, carriers, and vectors (Vaz-Moreira et al., 2014), with different risk levels to human health, and they are linked together in a transmission chain (Figure 4). Reservoir bacteria consists of ARB with intrinsic resistance (develop/acquire antibiotic resistance naturally), most of which are probably strictly environmental (Costa et al., 2006). Carrier bacteria and vector bacteria refer to bacteria that are abundant in the environment, have high genome plasticity, and acquire ARGs from reservoirs under selective conditions of anthropogenic activities. Carriers and vectors are the key players in ARG spread among different bacterial populations. Carriers cannot colonize or infect the human body; however, their proliferation can increase the abundance and diversity of ARGs in vectors. Vectors can colonize and proliferate in the human body. Pathogens are vectors that can infect the human body. Non-pathogenic vectors can transfer ARGs to commensal bacteria and opportunistic pathogens, which might subsequently cause an infection (Manaia, 2017). DNA sequences encoding ESBLs have been detected in vectors of vegetable origin including *Rahnella aquatilis* and *Pseudomonas teessidea* (Raphael et al., 2011; Ruimy et al., 2010). ARG transfer from a vector of fish origin (*Aeromonas salmonicida* subsp.

salmonicida) to human pathogens (e.g. *Aeromonas hydrophila*, *E. coli*, and *Salmonella*) has also been widely documented (Heuer et al., 2009; Rolain, 2013). Vectors can also become opportunistic pathogens, especially in immunocompromised patients (Raphael and Riley, 2017). Humans and animals can also act as carriers and vectors for ARB spread through migration (Cummins et al., 2020; Nesporova et al., 2020); however, this topic is beyond the scope of this review.

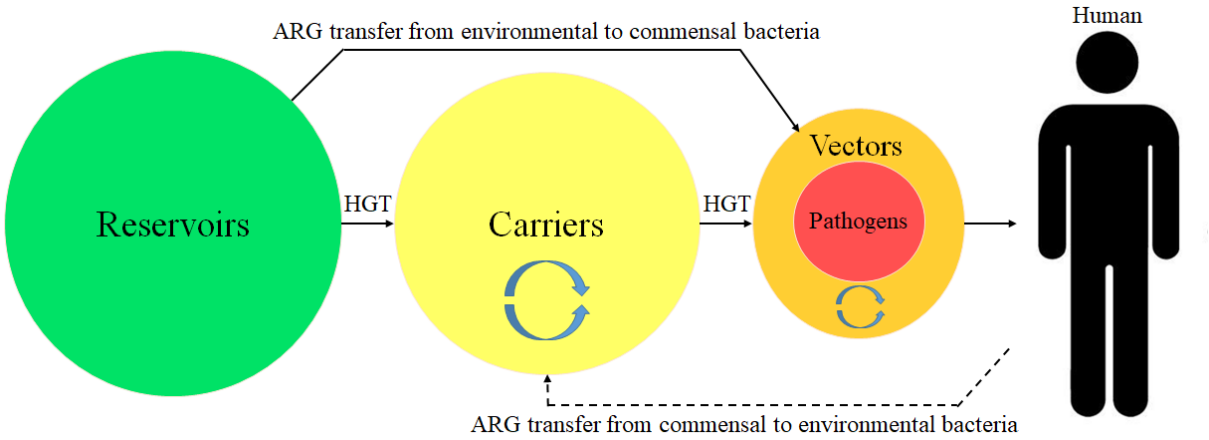

Figure 4. Chain of antibiotic resistance genes (ARGs) transfer between reservoir, carriers, vectors and pathogens. The colour of each circle represents the associated risk, with green representing the lowest risk level and red representing the highest risk level. The size of each circle represents the number of bacteria belonging to each category. HGT: horizontal gene transfer.

The risk associated with a particular ARB depends on multiple factors in addition to its classification as reservoir, carrier, vector, or pathogen. These factors include the frequency of exposure to a human body (Johnson et al., 2016), modes of transmission and portal of entry, the infectious dose (the number of cells required to colonize or infect humans), the capacity to acquire and disseminate ARGs to the host microbiome, and the types and diversity of ARGs it harbors (Manaia, 2017). A pathogen with a low infectious dose, residing in an environmental compartment with high exposure to humans, conferring resistance to

last-resort antibiotics or multiple antibiotics, will be classified at the top level of risk. It is important to note that bacteria can occur in high-density aggregates (i.e. biofilm) where they can reach clinically relevant infectious doses, even if their average abundance in the environmental is below the infectious dose (Manaia, 2017).

4.2. Technologies to identify, quantify and track ARGs in wastewater and hosts

Advances in culture-independent molecular biology techniques have facilitated the study of ARGs both qualitatively and quantitatively. Several analytical tools can be deployed for detecting and/or quantifying ARGs in wastewater (Table 2). Many of these tools are based on polymerase chain reaction (PCR) and have been developed further to incorporate recent advancements in microbial genetics. Details on the advantages and disadvantages of each tool are available elsewhere (Ishii, 2020; Rice et al., 2020). For ARG quantification, amplification-dependent methods such as quantitative PCR (qPCR), high-throughput qPCR (HT-qPCR) and digital PCR (dPCR) are particularly useful, in part due to their ease of execution, robustness, specificity and sensitivity. qPCR can provide information on the abundance of the targeted ARGs in different genetic contexts including viable bacteria, mobile DNA fragments like MGEs and "free" environmental DNA (extracellular DNA), depending on the DNA extraction technique (Dong et al., 2019; Eramo et al., 2019). For example, propidium monoazide can be used to remove DNA from dead cells and extracellular DNA in the sample during the extraction process, thus allowing for the obtainment of DNA from live cells only (Wagner et al., 2008). Similarly, it may also be possible to selectively target plasmids and separate them from chromosomal DNA. qPCR has also been used to estimate plasmid transfer frequency in bacterial communities since 2010 (Bonot and Merlin, 2010). Several tools in Table 2 can be used to identify ARG-hosts. Further discussion of these tools for ARG-host identification and findings from their recent applications are discussed below.

373 **Table 2.** Summary of current technologies for ARG quantification and host identification.
374 qPCR – quantitative polymerase chain reaction, HT-qPCR – high-throughput qPCR, dPCR –
375 digital PCR, FACS - fluorescence-activated cell sorting.

Method	**Quantitative**	**Host identified**	**Ref.**
qPCR	Yes	No	Cheng and Hong (2017); Du et al. (2015); Kappell et al. (2018); Munir et al. (2011)
HT-qPCR	Yes	No	Bueno et al. (2020); Karkman et al. (2016); Sandberg et al. (2018)
dPCR	Yes	No	Gao et al. (2018); Griffin et al. (2019); Stachler et al. (2019
Single-cell fusion PCR	No	Yes	Hultman et al. (2018)
16S rRNA sequencing + correlation analysis	Yes	Yes*	Li et al. (2015); Luo et al. (2017); Narciso-da-Rocha et al. (2018); Quintela-Baluja et al. (2019); Su et al. (2017); Tian et al. (2016)
Metagenomic sequencing	Yes	Yes*	Arango-Argoty et al. (2019); Che et al. (2019); Jia et al. (2017); Liu et al. (2019); Ma et al. (2016)
FACS + sequencing	Yes	Yes	Gallego et al. (2020); Jacquiod et al. (2017); Li et al. (2018b); Qiu et al. (2018)
Genomic cross-linking	Yes	Yes	Stalder et al. (2019)

376 * ARG hosts are inferred (potential hosts) but not directly identified**.**

377 4.2.1. **Single-cell, fusion PCR**

378 Single-cell fusion PCR or emulsion paired isolation and concatenation PCR (epicPCR)
379 involves single-cell encapsulation, followed by fusion of a bacterial phylogenetic marker gene
380 (e.g. 16S rRNA) with ARGs using PCR, and subsequent sequencing of the PCR products for
381 taxonomical identification. Although the epicPCR technology has similar primer bias and off-

target amplification drawbacks to the conventional PCR method (Rice et al., 2020), it can directly identify ARG hosts. The majority of identified hosts in wastewater belong to phyla *Proteobacteria* and *Firmicutes*, and a few were associated with phyla *Fusobacteria*, *Gracilibacteria*, and *Tenericutes* (Hultman et al., 2018). *Arcobacter* was found to harbor all the investigated ARGs (*tetM, bla, intl, qac*) and are considered important carriers in wastewater (Hultman et al., 2018). These results were in agreement with findings from other studies using the combination of 16S rRNA sequencing and correlation analysis as well as fluorescence-activated cell sorting (FACS) (Jacquiod et al., 2017; Narciso-da-Rocha et al., 2018). In addition, the wastewater treatment process appears to decrease the ARG-host range, despite the HGT of ARGs to bacterial species (taxa) that were previously not ARG-hosts.

4.2.2. **16S rRNA sequencing and correlation analysis**

Potential ARG-hosts can be inferred from the correlation between ARG abundance (obtained using qPCR) and bacterial species abundance (obtained using 16S rRNA sequencing). This method assumes that a positive correlation indicates co-occurrence between an ARG and a taxon, and a stronger correlation means a higher likelihood of the taxon to be the ARG-host. Using a combination of qPCR/16S rRNA sequencing and correlation analysis between ARG abundance and bacterial taxa abundance, previous studies have identified multiple potential ARB at the species level such as *Bacteroides*, *Clostridium*, and *Escherichia* (Supplementary Information). Most potential ARB belong to *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* phyla, which are dominant bacterial phyla in wastewater and sludge (Quintela-Baluja et al., 2019; Su et al., 2017). The application of the correlation method has also yielded novel findings on the relationship between ARGs (i.e. co-localization of multiple ARGs), and the impact of environmental factors (i.e. temperature, seasonal changes) and MGEs on ARGs (Li et al., 2015; Luo et al., 2017; Narciso-da-Rocha et al., 2018; Su et al., 2017; Tao et al., 2016; Tian et al., 2016). It is noteworthy that spurious correlations can emerge through the data

normalization process (i.e. relative abundance data), and further research using whole-genome sequencing or genomic cross-linking methods (Section 4.2.5) are needed to confirm the actual link between ARG and the correlated hosts (Rice et al., 2020).

4.2.3. **Metagenomic sequencing**

Metagenomic sequencing relies on the assembly of contigs or reconstructed microbial genomes from sequencing reads to link ARGs to a specific taxonomy. ARG-hosts can be identified from the assembly of ARGs with host phylogenetic biomarkers (16S rRNA gene), or the annotation of genes co-located with the ARG of interest (Rice et al., 2020). Nevertheless, caution needs to be taken in interpreting results derived from the assembly of short sequence reads due to the possibility of assembly errors (Arango-Argoty et al., 2019; Suzuki et al., 2019). Metagenomic sequencing yielding long reads (e.g. Nanopore sequencing) can reveal more information about the ARG genetic context and potential for mobility, whether it is plasmid- or chromosomal-associated, and if it is co-located with MGEs and metal resistance genes (MRGs). Besides *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* members, taxa within the *Actinobacteria* and *Spirochaetes* have been identified as ARG-hosts using this method (Jia et al., 2017; Liu et al., 2019; Luo et al., 2017). ARGs in WWTPs were found to be frequently associated with MGEs (i.e. plasmid and class 1 integron) (Che et al., 2019), and the genetic context exerts a substantial impact on ARGs persistence and expression, with plasmid-associate ARGs more likely to be expressed than exclusively chromosomal ARGs (Liu et al., 2019). In addition, it has been observed that the microbial community composition determines ARG composition (Jia et al., 2017; Liu et al., 2019; Luo et al., 2017), and ARGs frequently co-occur with MGEs due to co-localization (Che et al., 2019; Luo et al., 2017; Ma et al., 2016).

4.2.4. **Fluorescence-activated cell sorting and sequencing**

FACS combines flow cytometry with cell sorting based on fluorescence emission. ARG-hosts can be tagged with fluorescent labels using bioreporter genes (enabled by HGT

events) (Pinilla-Redondo et al., 2018), or detected using fluorescence *in situ* hybridization (FISH) techniques such as rolling circle amplification FISH (RCA-FISH), tyramide signal amplification FISH (TSA-FISH) (Gallego et al., 2020) and catalyzed reporter deposition FISH (CARD-FISH) (Rice et al., 2020). The FACS-sorted bacterial cells are subjected to sequencing for taxonomical identification. Studies applying this method to track ARG-hosts have revealed multiple ARG-host taxa belonging to the *Gammaproteobacteria* class, as well as some novel ARG-hosts from *Chloroflexi*, *Ignavibacteriae*, *Nitrospirae*, *Planctomycetes*, *and Gemmatimonadetes* phyla (Jacquiod et al., 2017; Li et al., 2018b; Qiu et al., 2018). Among them, *Arcobacter* showed a high plasmid transfer potential and was suggested as a keystone taxon involved in HGT between distant Gram-positive and Gram-negative phyla.

4.2.5. **Genomic cross-linking method**

Similar to epicPCR, the genomic cross-linking method also relies on the fusion of ARGs and 16S rRNA genes to create hybrid products for sequencing (Lieberman-Aiden et al., 2009). However, the hybrid product was created using proximity ligation cross-linking and restriction enzymes rather than PCR (Schmitt et al., 2016). Stalder et al. (2019) reported using this method to identify 12 taxa as ARG-hosts, among which *Aeromonadaceae* was considered a keystone taxon in wastewater. This taxon is linked to at least 18 ARGs in two WWTPs, conferring resistance to eight antibiotic classes. The broadest host range in wastewater was observed for IncQ plasmids and class 1 integrons, while several narrow-host-range plasmids were almost exclusively linked to *Enterobacteriaceae*.

4.3. ARGs detected in pathogenic hosts

Many studies investigating ARG-hosts have not identified whether these hosts are reservoirs, carriers, vectors, or pathogens. Thus, there is still a gap in the literature regarding the relationship between ARGs and pathogenic species. For example, pathogens were detected in activated sludge, swine wastewater, and the receiving water, but there was no information

on whether they are ARB or not (Jia et al., 2017; Yadav and Kapley, 2019). This is due to the existence of multiple strains within a species (with different ARG carriage) as well as the limitations of ARG-host identification technologies (discussed in Section 4.2) in resolving the host down to strain, and sometimes species level in complex microbial communities found in WWTPs. Nevertheless, a few studies have successfully identified ARG-hosts with high sequence identity to MDR pathogenic species (Arango-Argoty et al., 2019; Che et al., 2019), including those in the ESKAPEEc panel (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *E. coli*) (De Angelis et al., 2018). These pathogens are the major culprits responsible for severe infection in the clinical context, and their acquisition of resistance to last-resort antibiotics has significantly contributed to morbidity and mortality (Göttig et al., 2014; Rice, 2008).

Che et al. (2019) have detected 10 ARB species that are potential pathogenic bacteria across the treatment process in three WWTPs, and five of them are members of the ESKAPEEc panel. These pathogens, including *E. coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *A. baumannii*, and *P. aeruginosa*, possess high ARG diversity (at least four ARG types). Four of them were found at all treatment stages, indicating their risk of passing the wastewater treatment process and entering the receiving environments. Arango-Argoty et al. (2019) also identified *A. baumannii*, *Enterobacteriaceae*, *Neisseria gonorrhoeae*, and *P. aeruginosa* as ARG-hosts in WWTP samples, with *P. aeruginosa* carrying up to 74 ARGs. The presence of ARG-carrying pathogens in WWTP effluent is a water quality concern and a major risk factor associated with water recycling.

4.4. ARB and ARGs in wastewater

4.4.1. ARB in wastewater

Previous studies have revealed some common ARB detected in WWTPs (Section 4.2, Supplementary Information). At the phylum level, *Proteobacteria* harbour the highest number

of identified ARB, followed by *Firmicutes* and *Bacteroidetes* (Liu et al., 2019). At the order level, *Bacteroidales, Clostridiales*, *Burkholderiales* and *Enterobacterales* are notable ARG-hosts. These are also the most common taxa in wastewater. Liu et al. (2019) reported that activated sludge samples of Taiwanese WWTPs contained *Proteobacteria* harbouring a diverse range of ARGs (88 were identified), while *Burkholderiaceae* were hosts to 50 different ARGs. The order *Burkholderiales* contains environmental saprophytic organisms, human and animal pathogens, which therefore pose a risk of spreading AMR. Several genera including *Acinetobacter* and *Pseudomonas* have been frequently detected in wastewater as active ARG carriers and vectors (Jia et al., 2017; Manaia, 2017).

It is possible that the frequently detected taxa above act as ARG transfer hubs and form a "core permissive fraction" (Jacquiod et al., 2017; Li et al., 2018b). This core fraction of keystone taxa possesses high plasmid permissiveness (i.e. the capability to transfer an exogenous plasmid within a microbial community) (Musovic et al., 2010). Plasmid permissiveness may be influenced by factors such as the type of plasmid donor and recipient, and exposure to metal and antibiotic stressors (Jacquiod et al., 2017). Li et al. (2018b) reported different plasmid transfer frequencies across different types of ARG-carrying plasmids and plasmid donor bacteria (i.e. *E. coli* and *P. putida*) within an activated sludge microbial community. The plasmid recipient community (i.e. the transconjugant pools) was dominated by *Acinetobacter* genera, *Enterobacteriaceae* and *Pseudomonadaceae* families.

To further support the "core permissive fraction" theory, it has been proposed that specific microbial taxa carry specific ARGs. Liu et al. (2019) reported that among the 159 ARGs detected in activated sludge samples, only seven ARGs were shared by the primary ARG-carrying phyla. A significant number of ARGs (62.3%) were carried by unique host phyla (Liu et al., 2019). This phenomenon can be linked to the capability of specific species to host specific plasmids (Qiu et al., 2018; Redondo-Salvo et al., 2020). Thus, developing a database

of ARGs and their associated hosts is crucial in managing and mitigating ARG dissemination in general, and in identifying suitable ARB and ARG surrogates in particular.

The lack of agreement on ARB and ARG surrogates to serve as environmental monitoring targets is a major challenge for antibiotic resistance mitigation (Pruden et al., 2018). Faecal coliforms, *P. aeruginosa*, *Enterococci*, and *Enterobacteria* have been considered as ARB surrogates (Hiller et al., 2019). They are omnipresent in the wastewater ecosystem and frequently detected as active ARG carriers and vectors. In addition, their abundances are highly quantifiable, as they have already been used as faecal contamination indicators. Thus, these bacteria appear to be ideal ARB surrogates, and in fact, ESBL-producing *E. coli* has been chosen as the target for a pilot surveillance program initiated by WHO, the EU, and several Asia and Africa countries (Jorge Matheu, 2017). Other representative Gram-positive and Gram-negative indicator bacteria are also worthy of consideration.

4.4.2. **ARGs in wastewater**

An ARG surrogate should allow for direct confirmation of the existence of antibiotic resistance. Similar to the requirements for suitable ARB surrogates, ARG surrogates should ideally be ubiquitous in wastewater, and easily and accurately quantified using current technology. Frequently detected ARGs conferring resistance to broad-spectrum antibiotics such as sulphonamide (*sul1* and *sul2*) and tetracycline (*tetA, tetB, tetO* and *tetW*) are likely to be useful surrogates for the evaluation of treatment efficiencies (Hiller et al., 2019). As noted in Section 4.2.2, multiple ARGs show strong co-occurrence due to their co-localization on the same MGE (e.g. plasmids and conjugative transposons) (Jia et al., 2017; Soge et al., 2009). Frequently detected ARGs (Table 1) exhibited higher non-random co-occurrence events in wastewater samples than random events (Jia et al., 2017). Their co-occurrence expands the possibility of identifying suitable ARG surrogates. For example, Li et al. (2015) revealed that *tetM* and aminoglycoside resistance protein were the main hubs of an ARG co-occurrence

network built from 50 environmental samples using metagenomics and network analysis. These ARGs could be useful surrogates to quantitatively estimate the abundance of 23 other co-occurring ARG subtypes by power functions. Besides correlation and network analysis, modelling and machine learning approaches can be applied to identify ARG surrogates and develop ARG-predictive models for routine monitoring (Ishii, 2020; Li et al., 2018a). MGEs should also be taken into consideration; for example, the class 1 integron integrase gene can serve as an excellent indicator of MDR bacteria and anthropogenic pollution (Gillings et al., 2015; Leverstein-van Hall et al., 2003). It is also suggested that data on associated environmental variables (e.g. temperature, water turbidity, faecal indicator, and pathogen levels) should be collated for the determination of potential ARG indicators.

4.5. Factors governing ARGs removal or transfer

An important aspect of AMR dissemination is the interplay among the various factors that can affect ARG removal and transfer (Figure 5). This section will discuss previously identified factors and their mechanisms of promoting/reducing ARG in WWTPs.

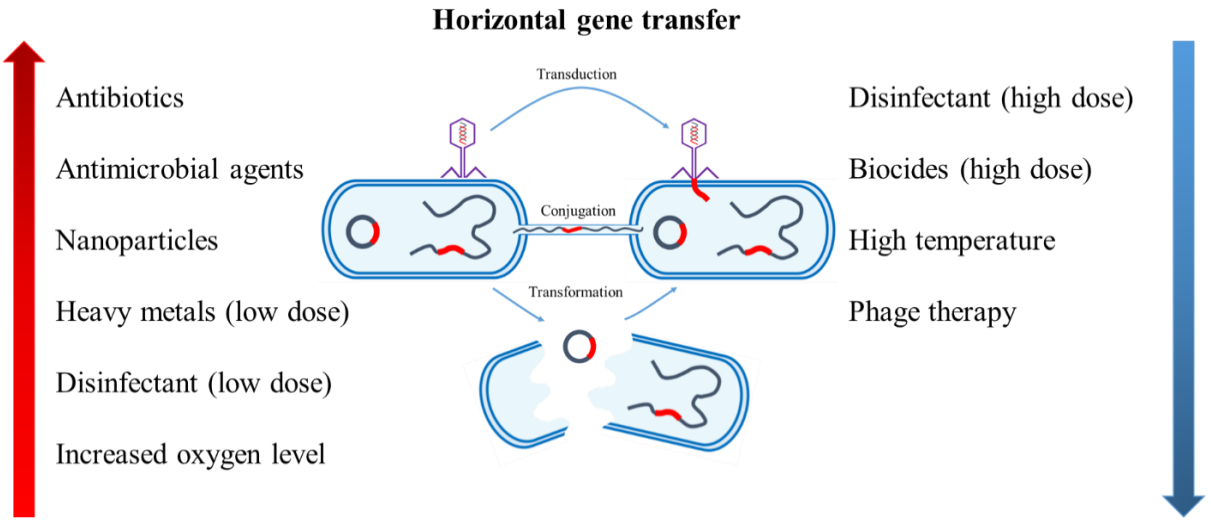

Figure 5. Conditions that promote/reduce antibiotic resistance genes transfer in wastewater treatment.

549 4.5.1. **ARG transfer during wastewater treatment**

550 Stress-inducing conditions such as exposure to antimicrobials, heavy metals, and
551 disinfectants at low doses, can stimulate ARG development and dissemination. These stressors
552 share a common stimulating mechanism through multiple alterations in bacterial gene
553 expression. Stressors increase the expression of the SOS response system, which in turn
554 increases genetic instability, promoting DNA mutations (Händel et al., 2014). The reactive
555 oxygen species generated in response to stress can also damage bacterial membranes, resulting
556 in enhanced cell permeability and facilitating HGT events. In addition, stressors can alter the
557 expression of conjugation-relevant genes, e.g. inducing more sex pili on cell surfaces. These
558 act as pathways for ARG transfer (Guo et al., 2015), and reduce the activity of regulatory genes.
559 Despite the understanding of their stimulating mechanisms, controlling stress-inducing
560 conditions in WWTPs is highly challenging, since these stressors are ubiquitous in wastewater
561 at trace levels.

562 Exposure to antibiotics accelerates the transfer rate of ARGs in environmental samples.
563 This arises from an antibiotic's ability to exert pressure on exposed microorganisms/bacteria
564 thus inducing resistance to itself, and/or stimulate the transfer of MGEs responsible for the
565 dissemination of resistance determinants (Depardieu et al., 2007). Exposure to the antibiotic
566 trimethoprim significantly increased the rate of HGT in an activated sludge bacterial
567 community (Li et al., 2019). Triclosan exposure at concentrations frequently detected in
568 wastewater (0.02–20 μg/L) could stimulate HGT of plasmid-encoded MDR genes within and
569 across genera (Lu et al., 2018). Even at a low concentration of tetracycline (10 μg/L which is
570 150 times below the minimal inhibitory concentration (MIC) of the ARG recipient), HGT of
571 ARG determinants in WWTP activated sludge and effluent could still be stimulated (Jutkina et
572 al., 2016; Kim et al., 2014). This may explain the higher ARG abundance and diversity in
573 sludge from pharmaceutical wastewater treatment compared to municipal WWTP sludge (Tao

et al., 2016). Efforts in antimicrobial stewardship (i.e. strategies to improve appropriate use and minimise adverse effects of antibiotics) within hospitals and communities could contribute significantly to the mitigation of ARG occurrence in wastewater. These stewardship programs have proven to effectively reduce antibiotic dosages and resistance (Nathwani et al., 2019; Zhang et al., 2017c), and provide greater opportunities for engineers to monitor and mitigate AMR in hospital effluent before discharging it to local sewer systems.

The presence of antimicrobial organic compounds, nanoparticles, and heavy metals in wastewater can significantly influence the ARG transfer rate. The frequency of HGT of ARG-carrying plasmids in textile dyeing wastewater increased up to 200-fold under low doses of quaternary ammonium compounds (e.g. malachite green, ethylbenzene, trioxymethylene and o-xylene) (Jiao et al., 2017). Qiu et al. (2012) revealed a similar increase in HGT frequency under the presence of nanomaterials (e.g. nanoalumina). Copper nanoparticles and copper ions have also been reported to stimulate the HGT of MDR genes at environmentally-relevant and sub-inhibitory concentrations (i.e. 1–100 μmol/L) (Zhang et al., 2019c). Notably, metal stress can increase the plasmid permissiveness of the microbial community by more than 1000-fold (Klümper et al., 2017). These findings highlight the vital importance of source control to decrease the release of metals, nanoparticles and organic contaminants into wastewater and the wider environment.

Besides the aforementioned stimulators, other conditions such as wastewater disinfection, oxygen level, and the spatial distribution of bacteria can also accelerate ARG dissemination. Several studies have demonstrated that sub-inhibitory (0.1–1 mg Cl_2/L) or low doses of chlorine (< 40 mg Cl_2 min/L) led to the increases in intra-genera and inter-genera HGT of ARGs by 2 to 7.5-fold (Guo et al., 2015; Zhang et al., 2017b). Meanwhile, oxygen level can affect bacterial community composition and in turn affect ARG profileration. For example, aerobic sludge reportedly has a higher proportion of *Proteobacteria* (27%) than anaerobic

599 sludge (21%), thus allowing for two times higher plasmid abundance (Tao et al., 2016).
600 Furthermore, bacterial biofilms, particularly at the air-liquid interface (i.e. higher oxygen
601 level), are potential hotspots for plasmid-mediated ARG transfer due to the high densities of
602 plasmid donor and recipient cells (Król et al., 2011).

603 4.5.2. **ARG reduction during wastewater treatment**

604 The type of wastewater treatment processes can impact the removal of ARG and ARB.
605 Membrane-based technologies such as membrane bioreactors (MBR) are regarded as the most
606 effective technologies among primary and secondary treatment processes (Hiller et al., 2019;
607 Lu et al., 2020; Wang and Chen, 2020). Le et al. (2018) observed that MBR outperformed
608 conventional activated sludge (CAS) in the elimination of ARB (5.0–7.1 vs. 1.0–5.3 log
609 removal) and 13/16 selected ARGs (1.3–6.5 vs. -0.3–6.1 log removal). Munir et al. (2011) also
610 reported significantly higher removal of ARG (*tetW* and *tetO*) and ARB in MBR facility (2.57–
611 7.06 log removal) compared to conventional treatment plants that employ CAS, oxidative ditch
612 and rotatory biological contactors (2.37–4.56 log removal). Better performance of MBRs can
613 be attributed to the ability to effectively separate sludge from effluent and retain ARB inside
614 the reactor. Nevertheless, the fact that some ARB and ARGs can persist through the MBR
615 process (Ng et al., 2019) highlights the need for further research on this topic.

616 The retardation of ARG transfer and the removal of antibiotic resistant determinants in
617 WWTPs can also be facilitated through chemical treatment processes, including advanced
618 oxidation (AOP) and disinfection (e.g. chlorination, ultraviolet irradiation and ozonation).
619 Fenton oxidation offers complete reduction (5-log decrease) of ARB to below the detection
620 limit with relatively short treatment time (20 minutes) and lower energy (0.98 kJ/L) compared
621 to other solar driven AOPs (i.e. H_2O_2/sunlight, TiO_2/sunlight, H_2O_2/TiO_2/sunlight) (Ferro et
622 al., 2015). A sufficiently high dose of chlorine (>80 mg Cl_2 min/L) applied within a short
623 contact time (~30 min) can inactivate ARB and mitigate their regrowth or reactivation (Guo et

al., 2015), thus decreasing ARG abundance (Guo et al., 2015; Pei et al., 2019). Zhang et al. (2017b) also observed that exposure to chlorine, chloramine and hydrogen peroxide concentrations higher than MICs significantly suppressed conjugative transfer within *E. coli* strains and across genera from *E. coli* to *Salmonella enterica* serovar Typhimurium. Similarly, high UV doses (>10 mJ/cm^2) can exhibit lethal effects on bacterial communities, thus reducing the number of ARB to below 10^4 CFU/mL, and suppressing the conjugative transfer of ARG-carrying plasmids (i.e. HGT) (Guo et al., 2015).

Although disinfection demonstrates high efficacy in ARG removal, it is also necessary to recognise their limitations and disadvantages. For example, UV treatment processes at WWTPs are less efficient than in simulated laboratory experiments partly due to the high doses required (Chen and Zhang, 2013; Zhang et al., 2017a). High doses of chlorine (>80 mg Cl_2 min/L) needed for efficient ARB inactivation are also not practical due to high corrosion risk, toxicity and harmful chemical byproducts, thus requires increased dechlorination and safety regulations. Ozonation process offers greater reduction of ARB, pB10 plasmids, and pB10 plasmid transfer rate compared to chlorination (Pak et al., 2016). However, an excessive ozone dose (>0.55 g O_3 g DOC^{-1}) can result in harmful by-products (e.g. nitrosamines or bromate) (Czekalski et al., 2016). Disinfectants might also result in the selection of more resistant strains that can regrow during and subsequent treatment (Huang et al., 2011; Xi et al., 2009).

Similar to disinfectants, exposure to metal stressors at high doses (mainly at the influent e.g. from animal manure discharge) can decrease HGT. Klümper et al. (2017) demonstrated that the presence of heavy metals (e.g.: Cu, Cd, Ni, and Zn) at inhibitory concentrations (i.e. causing 20 and 50% bacterial growth inhibition) reduced the plasmid conjugative transfer events by 30 to 100%. It is noteworthy that the current knowledge regarding the mechanism behind the influence of metal stressors to plasmid transfer inhibition is still insufficient. It is likely that high metal doses inhibit bacterial growth and reduce ARB abundance, thus

649 limiting ARG transfer rate. However, metal stressors can select for MRG which co-localize
650 with ARGs as discussed in Section 4.2.3.

651 Several studies have highlighted how operating temperature influences ARG transfer
652 and removal rate in sewage sludge (Ghosh et al., 2009; Ma et al., 2011; Zhang et al., 2015).
653 Thermophilic anaerobic digestion (50 – 60 °C) can effectively remove 50 – 99% of
654 tetracycline ARGs and class 1 integrons in both lab-scale digesters and full-scale WWTPs
655 (Diehl and LaPara, 2010; Ghosh et al., 2009). ARG abundance was also decreased by 50%
656 after thermophilic anaerobic digestion (Tian et al., 2016), suggesting that increased
657 temperature can potentially reduce ARG abundance by inhibiting both HGT (e.g. plasmids,
658 insertion sequences, and integrons) and VGT (i.e. regeneration of potential bacterial hosts)
659 pathways. However, several studies have pointed out that the removal efficiency of various
660 sludge digestion conditions (i.e. different temperatures) may be ARG-specific. For example,
661 Ma et al. (2011) revealed that mesophilic anaerobic digestion was effective at removing *sulI*,
662 *tetC*, *tetG*, and *tetX* but enriched *tetW*, *ermB* and *ermF* abundance. In the same study,
663 thermophilic process significantly reduced *ermB*, *ermF*, *tetO*, and *tetW* but poorly removed
664 other ARGs. Zhang et al. (2015) also reported > 90% removal of quinolone resistance gene
665 after thermophilic anaerobic digestion, but a simultaneous enrichment of chloramphenicol
666 resistant gene was observed. These results imply that further research is necessary to have a
667 complete understanding of the impact of operating temperature on ARG removal in sewage
668 sludge.

669 A targeted treatment method using phage therapy or engineered phage lysin to control
670 high-risk ARB was suggested by Rice et al. (2020). Phage/phage lysin can kill specific bacteria
671 with high effectiveness and specificity with minimal disruption of the normal microbial
672 community (Jassim et al., 2016; Yang et al., 2014). Phages are self-replicating and self-
673 limiting, and phage therapy has shown promising results in controlling foaming bacteria in

CAS process. Keystone taxa in the ARG-transfer network (Section 4.4.1) could be regarded as "Achilles heels" to be targeted using phage therapy (Pinilla-Redondo et al., 2018). However, bacteria can develop mechanisms to prevent phage infection such as the restriction-modification system (i.e. CRISPR/Cas system) and modification to their cell wall receptors (Jore et al., 2012). Phage can also contribute to bacterial virulence and HGT of ARG through transduction (lysogenic cycle) or release of ARG during cell lysis (lytic cycle) that can be uptaken by other bacteria via transformation (Section 3). Besides, successful phage therapy requires a comprehensive understanding of the target bacteria in the microbial population, phage–host interactions, dose optimization, and other chemical and physical factors (Jassim et al., 2016). Additional research is thus necessary to evaluate the feasibility of phage therapy for ARG control.

5. Current challenges to monitor and control ARGs

5.1. ARG referencing conditions

The presence of ARGs in any environment is a natural phenomenon. ARGs have been detected in pristine environments not affected by anthropogenic activities (e.g. Antarctic marine water) (Brown and Balkwill, 2008; De Souza et al., 2006; Van Goethem et al., 2018). ARGs should not be merely quantified and reported but need to be interpreted based on the significance of their presence and how it is related to rapid evolution and spread of MDR bacteria (Zhang et al., 2019a). The identification of critical risk thresholds for ARB and ARG exposures that influence human health is also important in developing mitigation strategies (Pruden et al., 2018). However, ARG quality thresholds or standards have not been established even in wastewater and sludge. Several studies have attempted to use ARG concentration in pristine environments (i.e. river's source water) as the natural "resistance background" level or reference to determine the magnitude of the ARG problem in urban stream, hospital effluent, and animal husbandry wastewater (Ouyang et al., 2015; Rowe et al., 2017). This approach

699 appears impractical since the “background” concentration would be different for each
700 geographical location and require re-assessment. A potential solution is to establish a set of
701 threshold values and standardised conditions to serve as the ARG reference, such as those
702 developed by the European Commission for endocrine-disrupting compounds (European
703 Commission, 2011).

704 **5.2. Standardization of ARG unit**

705 ARG quantity in a sample is usually reported in terms of relative or absolute abundance
706 using various units such as one ARG read per one million reads (denoted as ppm), copies of
707 ARG per copies of 16S rRNA gene, or copies of ARG per mL. The difference in methods used
708 for ARG quantification is the underlying reason for these different units. Standardization of an
709 ARG unit is necessary to allow for comparisons between results and effective management of
710 the ARG issue. Ideally, ARGs should be reported in terms of concentration (copies per mL) or
711 relative abundance (copies per bacteria cell) for consistency with the WWTP context and
712 removal calculation. The method for unit conversion was introduced recently, through
713 normalization of ARG abundance by the absolute copy number of 16S rRNA (which can be
714 obtained reliably using qPCR), or by the number of bacterial cells per litre (which can be
715 estimated from 16S rRNA copy number) (Ouyang et al., 2015; Su et al., 2017). However, the
716 current unit conversion method has limitations when applied to microbial communities with
717 very different copy numbers of 16S rRNA. Further details of the conversion method are
718 available in the Supplementary Information.

719 **5.3. Assessment of WWTP performance in removing ARG**

720 The removal efficiency of ARGs by WWTPs is often considered by the difference
721 between the total abundance of ARGs in the influent (i.e input) and effluent (i.e. output)
722 (Section 4.5.2). In some studies, the performance of individual treatment stages (e.g. primary,
723 secondary, and tertiary treatment) on ARG removal within the overall WWTP workflow has

been reported. This process of reporting neglects the behaviour of ARGs and ARB within each treatment processes (Cheng and Hong, 2017; Du et al., 2015; Kappell et al., 2018; Liang et al., 2021; Lin et al., 2021). In the context of ARGs in WWTP, apart from the ARG input in the influent, other contributing factors such as bacterial growth and decay, ARG transfer due to VGT and HGT, and ARG loss from a host (segregation) must all be assessed. Many of these factors have not been mathematically described and fully understood to assess the performance of WWTP in the literature. Thus, an insignificant difference between the ARG abundance/richness in the influent and the effluent does not necessarily imply a poor performance of WWTP process.

6. Future roadmap

AMR will continue to be a priority global issue for the foreseeable future. Previous studies have revealed part of the AMR picture, such as the stimulators contributing to ARG dissemination. Future research will need to address current challenges such as the inconsistent ARG reporting units or the lack of standard ARG threshold for monitoring. Three key areas need to be prioritised in the future: AMR characterization, surveillance and monitoring, and risk modelling and assessment.

More studies are needed to clarify the mechanism of ARG selection, transfer, propagation, and the impact of environmental and operational, socioeconomic, and legal/regulatory factors (Pruden et al., 2018). Methods to enhance the removal efficiency of ARG as part of the current effective treatment technologies (e.g., MBR, advanced oxidation/disinfection) needs to be identified (Wang and Chen, 2020), especially for waste streams with high AMR potential, such as those from pharmaceuticals and hospitals. Research findings on AMR characterization needed to be translated into practical, meaningful, and actionable guidance for WWTP designers and operators. Mitigation strategies must be

harmonized with the need for water sustainability and reuse. For example, developing countries would require cost-effective ARG treatment technologies.

AMR surveillance and monitoring can provide an overall picture and help identify effective actionable points to place AMR barriers. It is essential to agree upon monitoring targets (surrogates), monitoring thresholds, and standard reporting methods as soon as possible. Modelling and machine learning approaches can pinpoint ARG surrogate, the most influential factors, and the most promising targets to control. Predictive models can also be used for routine monitoring of ARGs (Ishii, 2020; Li et al., 2018a). Li et al. (2019) successfully described HGT kinetics using an epidemic infection model combined with quantitative measures of HGT and VGT using microfluidics. This microfluidic system provides a promising tool to study and predict ARG dynamics spread in real-world microbial communities. Advanced digital tools such as machine learning, data mining, and predictive analytics have the potential to improve ARG identification (Arango-Argoty et al., 2018), more accurately predict resistance phenotypes from whole genome sequencing data (Kim et al., 2020; Liu et al., 2020; Mahé and Tournoud, 2018), and track ARG pollution from different sources (Li et al., 2018a). Last but not least, epidemiological studies that examine the extent of ARB/ARG exposures (e.g. on livestock farmers/ WWTP operators) in the environment and correlate such exposures to associated health risks would be of value.

7. Conclusions

Recent progress in metagenomics and molecular microbiology has generated database of ARGs and ARG-hosts (ARB). These databases are essential to the understanding of ARG dissemination, especially in the wastewater system. Solutions for AMR control, such as ARB-targeted therapy, must be developed from this expanding knowledge of ARGs and the associated context (e.g. environmental conditions and genetic elements that influence their abundance). This review highlights the role of WWTP in AMR mitigation and reveals a dearth

773 of data on the risk associated with ARGs and ARB, the relationship between ARGs and
774 pathogenic species, and standardized approaches to assess ARG removal efficiency in
775 WWTPs. More research is also necessary to shed light on how WWTPs can evolve into
776 effective gatekeepers guarding us against AMR.

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