**Antimicrobial resistance and bacteriophages: an overlooked intersection in water disinfection**

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**Abstract**

This article focuses on how bacteriophages (phages), antibiotic resistance genes (ARGs) and disinfection practices intersect. Phages are considered the most abundant biological entities on Earth and they have the potential to transfer genes among their bacterial hosts, including ARGs. In the urban water cycle, phages are used as indicators of faecal pollution and surrogates for human viral pathogens but they are also known to withstand common disinfection treatments deployed to produce safe drinking/reclaimed water. Recent studies also suggest that phages have the potential to become an additional footprint to monitor water safety. A precautionary approach should therefore include phages in surveillance programs aimed at monitoring antimicrobial resistance (AMR) in the urban water cycle. This article argues that phages ought to be used to assess the efficiency of disinfection treatments (both classical and novel) on reducing the risk associated with antibiotic resistance. Finally, this article discusses contributions to the advancement of AMR stewardship in aquatic settings and is relevant for researchers and water industry practitioners.

***Keywords:* Antimicrobial resistance; disinfection technologies; horizontal gene transfer; urban water cycle**

**Highlights**

* From a precautionary viewpoint, monitoring of phages and ARGs should be included when designing and developing new disinfection treatments aimed at removing possible AMR risks from treated water.
* Investments in upgrading wastewater treatment plants to decrease AMR risk in treated waters are on the horizon for the water industry.
* Deployment of disinfection to remove phages and the related AMR risk needs further assessment. The method should be cost-effective and should not trigger horizontal gene transfer side-effect. Membrane filtration methods are promising technologies to remove both phages and ARGs, but these still need to decrease in cost.

**Glossary box**

* Antimicrobial resistance (AMR): intrinsic or acquired ability of bacteria to withstand antimicrobial treatment.
* AMR determinants: All genes that encode for mechanisms of AMR. It should be noted that phages or other MGE are not antimicrobial resistance determinants *per se*. AMR determinants are all genes that encode for proteins involved in AMR [1].
* AMR stewardship [2]: coordinated interventions designed to promote, improve, monitor and evaluate the judicious use of antimicrobials to preserve their future effectiveness, and to promote and protect human and animal health.
* Bacteriophages: viruses that infect and replicate in bacterial cells.
* Horizontal gene transfer (HGT): is a process in which an organism (the donor) transfers genetic material to another organism (the recipient) of the same or different species.
* Mobile genetic elements (MGEs): are identified as fragments of DNA that encode a variety of virulence or resistance determinants, as well as the enzymes that mediate their own transfer and integration into new host DNA. Phages, phage-related particles, plasmids, genomic islands, integrons and integrative conjugative elements (ICEs) are MGEs [3,4].
* NDMA (*N*-Nitrosodimethylamine): a well-known DBP (disinfection-by-products) characterized by its toxic and carcinogenic effects.

# Introduction

**Antimicrobial resistance** (AMR) has become a growing global public health concern due to the difficulties and increased costs in treating antibiotic-resistant infections [5,6]. In fact, AMR causes an estimated 700,000 deaths annually worldwide and that has been predicted to exponentially rise to above 10 million deaths annually by 2050 [7]. A better understanding of the mechanisms and pathways underlying AMR is therefore urgently needed to implement effective public health policies, programmes and interventions at all levels. Reclaimed water systems are not exempt from the impact of AMR. Considering that there is increasing evidence that **bacteriophages** may carry antibiotic resistance genes (ARGs) [8,9], their implications for environmental and human health should not be underestimated. Phages – viruses that infect bacterial hosts – are biological entities consisting of single or double stranded DNA or RNA surrounded by a protein coat (capsid), which is able to withstand disinfection treatments [10,11].

Disinfection is an essential step during drinking water production. Most wastewater treatment plants (WWTP) have only up to secondary treatment (focused on the removal of organic matter by activated sludge), and disinfection is mainly limited to when water is intended for reuse [9] or recreational bathing purposes. However, the quest to achieve a circular economy in the water sector [12], driven by a growing global need for reusing water, is expected to increase the application of disinfection methods and tertiary treatment technologies in WWTPs.

This review article puts the spotlight on phages and their contribution to AMR in the context of water treatment. Novel insights on the relationships between water disinfection, antimicrobial resistance, and phages and ARG are presented (**Figure 1**).

**Figure 1.**

# Antimicrobial resistance and phages

Although substantial efforts have been made to understand the mechanisms that promote AMR [13,14], limited information is available about the extent to which phages contribute to the acquisition, maintenance and spread of this phenomenon. Among the main processes responsible for the increasing prevalence of AMR, **horizontal gene transfer** (HGT)plays an important evolutionary role that allows the movement of genetic material between both closely and distantly related organisms. This process is mediated by mobile genetic elements (MGEs), such as phages [3,15,16]. The concentration of phages in the biosphere is estimated at ~1031 phages, thereby increasing the likelihood of phage related HGT events occurring [17,18] (see **Box 1** for more details on HGT).

Phages are mainly involved in HGT by transduction mechanisms. In fact, many studies have provided evidence that phage particles carry genes conferring resistance to different antibiotics and, in some cases, these particles effectively transduce ARGs to recipient bacterial cells [19–21]. By doing this, phages may benefit from host survival under antibiotic selection and thus favour not only their own persistence but also the spread of transferred ARGs [22–24].

Interestingly, a recent study has shown that environmental phage fractions contain genes conferring resistance to *β*-lactamase and carbapenems (7.3% to 64.9%, respectively) at a greater proportion than in bacterial fractions (5 to 36.8%, respectively) [19]. Some authors, however, argued that ARGs are more abundant in bacteria than in phages [20,21]. Also, phages in the human microbiome rarely encode ARGs [25]. In clear contrast, phages from non-human sources (e.g., pig faeces, raw sewage, and freshwater and marine environments) contain a large reservoir of ARGs [26]. Despite the controversy, a recent study has demonstrated that phages isolated from wastewater successfully transduced β-lactamase genes into *E. coli* [27]. Further efforts are needed to elucidate the rate at which phages actively contribute to the transfer of ARGs among environmental bacteria in aquatic settings.

**Box 1. Horizontal gene transfer and phages**

Mobilization of genes (including ARGs) among bacterial cells occurs through three main mechanisms: (i) conjugation (mediated by plasmids or conjugative transposons); (ii) transformation(the uptake of free DNA from the surrounding milieu); and (iii) transduction (mediated by phages). Three transduction mechanisms have been described, namely generalized, specialized and lateral [10,23,28]. The latter has been recently described in temperate phages of *Staphylococcus aureus* and its characteristic feature is that prophages excise later in their life cycle, allowing for an exacerbated (up to 1,000 greater that previously observed) random packaging of host genome fragments. This process will generate both true or competent phages and transducing particles containing bacterial DNA, and it is considered key to bacterial evolution [28].

**Phage life cycles: lytic and lysogenic pathways**

Depending on the phage, the infection of the bacterial host may follow either a lytic or a lysogenic pathway. In the lytic cycle, the infecting (or infectious) phage uses the cell machinery to replicate itself, to assemble new viral particles and to lyse the host cell, thereby resulting in the release of its progeny. The lysogenic (or temperate) cycle usually involves the integration of the phage genome into the host chromosome and the maintenance of a latent state – the prophage – that perpetuates until environmental cues (nutrient imbalance, UV light, chemicals) trigger the lytic pathway (induction).

**Phages and transducing particles**

Errors in the packaging of phage genomes during assembly of new virions may result in the formation of viral particles containing hybrid genomes (in specialized transduction this correspond to a defective phage genome + bacterial genes) or particles containing only bacterial genome fragments (transducing particles in generalized transduction) [19–22]. Both hybrid genomes and transducing particles can infect the host, but they cannot multiply inside the host cell. Only “true” phages(those which contain the complete viral genome) are able to carry out the viral cycle, multiply inside the host and release progeny.

# Disinfection of phages and ARGs

Phages are usually considered surrogates of human viral pathogens and thus it is important that their removal be monitored to ensure water safety. Phages have recently been suggested as more reliable indicators of the occurrence of viral pathogens than traditional indicator bacteria (*E. coli*, coliforms, etc.) [29]. New commercially available tests that utilize phage kits (BluePhage®) [30] are thus gaining market traction. Therefore, we foresee the surveillance of phages being implemented at larger scale in WWTPs and water reuse scenarios.

Most disinfection studies to date, both in the lab and in real scale, have focused on the removal of faecal bacterial indicators (FBIs). In this context, data on phage and ARG removal are still scarce. A precautionary approach to deal with the possible AMR risk is therefore necessary. Advanced tertiary treatments (which may include certain disinfection or membrane methods) have a better potential to remove phages and **AMR determinants**. In this article, we argue that phages ought to be used to assess new disinfection treatments, so that the potential removal of phages carrying ARGs and the possible associated AMR risks are more fully comprehended.

Representative data on the responses of phages and ARGs to various disinfection methods are compiled in **Table 1**. Filtration methods have been included for comparison purposes. For the evaluation of the disinfection efficiency, it is necessary to count phage plaques or halos (lytic zones caused by infection of a sensitive bacterial host by a phage particle) on double agar overlay plaque technique [31]. In this way, the information available from the disinfection literature regarding phage disinfection originates mostly from studies targeting true phages and not transducing/defective particles. As regards disinfection of ARGs, the data shown in **Table 1** were resourced from studies targeting disinfection of, in most cases, extracellular ARGs. We have only encountered one study that targeted disinfection of ARGs in the phage fraction of wastewater samples [32]. Each disinfection method is commented on in more detail below.

**Table 1.**

From Table 1, we observe an overall trend: the disinfection dose to achieve a 1–Log reduction (90%) of ARG concentration is commonly greater than the dose required to achieve a similar reduction of phage counts. The specific reasons for these differences need to be analysed by taking into consideration the environmental conditions under which the disinfection assays were performed. Factors such as aqueous media composition, competing COD (chemical oxygen demand), and specific biochemical features of the ARG and phages involved may play a role in the response to a disinfectant [33,34]. Also, from the reviewed data, it is possible to conclude that disinfection of ARGs and phages is not yet cost-effective. High doses of disinfectant would be required to (i) achieve the disassembly of the viral capsid, and (ii) ensure enough contact time to inactivate the ARG. If the total elimination/disinfection of ARGs or phages is still not a feasible target, the alternative goal should be to monitor traditional indicators of AMR such as antibiotic-resistant bacteria (ARB).

## 3.1. Ultraviolet Radiation

In wastewater treatments, generally the type of UV deployed for microbial activation is the germicidal wavelength of monochromatic lamps emitting UV light at 253.7 nm (or UV-C). Other wavelengths and lamps may be utilised, although UV-C is the one that is most commonly used. Doses of UV are calculated as a function of the lamp or reactor emission in mW per cm-2 versus exposure time in seconds, which in turn is equated to a value in mJ. UV-C doses range between 5 and 400 mJ/cm2, which corresponds to a reduction of gene copies in the range between 0.2–6 Log [32,33,35–37]. UV-C doses to achieve reduction of phage particles between 4–7 Log were relatively lower, that is between 5–250 mJ/cm2. From these values, described in detail in **Table 1**, it seems that Log reductions of phages are more easily achieved by UV than Log reductions of ARG copies. However, it is important to highlight that, in some cases, deployment of high UV doses has been shown to increase the abundance of ARGs [38].

Phage genomes are enclosed by a protein shell (i.e., the capsid), which provides protection against environmental challenges including UV radiation. In fact, the deactivation of ARGs in phage fractions of wastewater are delayed in comparison to the deactivation of ARG in bacterial fractions [32]. Other influential factors in UV disinfection are aqueous media composition, such as suspended particles, which may shield ARGs and phages from UV radiation, and aggregation of viruses to particles.

## Chlorination

In WWTPs of USA and Canada, disinfection is often required prior to wastewater effluent discharge into the environment. The first and most widely used method of water disinfection results, unfortunately, in the generation of disinfection-by-products (DBPs). Although required in these North American countries, at global scale, disinfection of wastewater is generally not a standard practice in WWTPs [39]. In WWTPs, standard doses of chlorination are 5 to 20 mg/L versus a contact time which depends on physicochemical features of the wastewater [40]. Impairment of ARGs and phages are likely to occur by chlorine but largely depend on aqueous media composition. The dose that has been reported to reduce phages by 1–Log is 1 mg /L × 30 min. On the other hand, doses that were reported to achieve up to 6–Log units of ARG reduction ranged between 1–1000 mg/L (time and aqueous media varied) [34,36,37]. More detailed metrics on disinfection of ARGs and phages can be found in **Table 1**.

## Advanced Oxidative Processes (AOPs)

AOPs present a promising technology for microbial reduction of viruses; however, they are not yet scalable for large applications [41]. Available both as a homogeneous (only aqueous phase reagents with or without a light source) and a heterogeneous phase (solid catalyst or semiconductor involved plus a light source) [42], the main downsides to AOPs include the likelihood of microbial or ARG repair and hydroxyl (or other) radical scavenging. General comments about AOPs are listed next (with detailed appraisals in **Table 1)**. Both homogeneous and heterogeneous catalysts have been shown to be effective at removing phages, but less effective in removing ARGs. The ranges of disinfection reported of phage and ARGs, in various types of waters matrices (such as buffers or distilled water, or artificial wastewater) and in lab scale, were up to 10–Log reductions of PFU/ml (plaque forming units per mL) for phages and to 4–Log reduction for ARGs. Also, in the case of heterogeneous photocatalysis, immobilised catalysts provide lower quantum yield because of the reduced surface area. Although more efficient, suspended catalysts have been proved to not be feasible, thus far, for deployment at large-scale because of post treatment separations. Finally, various efforts to change the characteristic of catalysts [41], such as doping, to increase absorption of visible wavelengths and result in improved quantum yield have been shown to contribute to improved disinfection [41–47]. Homogeneous photocatalysis, such as Fenton reaction, have gained traction in lab scale testing; however, ARG and phage inactivation by this method are still low or subject to recovery after post-treatment incubation (**Table 1**). More studies in the area of photo-Fenton disinfection are thus necessary [48].

## Ozonation

Less frequently employed than chlorination, ozonation has a lower risk of DBPs generation during disinfection in WWTPs. However, there are significant downsides to implementing this method in large-scale applications. These include high cost, technical difficulties with dosing, and no lasting disinfectant residual concentration [48]. Ozonation doses reported to achieve inactivation of ARGs (1–6 Log) ranged between 0.20–0.9 mg O3/mg DOC. On the other hand, inactivation of phages (4 –9 Log) required ozone doses between 0.25–0.6 mg O3/mg DOC [37,42,49–51]. From Table 1, it seems the method is highly efficient for disinfecting both phages and ARGs. However, while considering ozonation in the context of water reuse, one must monitor DBPs such bromates and *N*-Nitrosodimethylamine (**NDMA**), as well as be aware of the need for downstream toxicity tests of treated water to avoid adverse health effects [42].

## Peracetic acid and performic acid

In the search to find alternatives that are more sustainable and possess a lower risk of DBP generation than chlorine disinfection, various alternative disinfectants are currently being investigated. Peracetic acid (PAA) (CH3CO3H) is a new sterilizing agent, which has been gaining attention in the water treatment sector. Efficient at inactivating both bacteria and viruses, PAA possesses a lower risk of generating DBPs [48]. In fact, this method has been shown to inactivate ARB in wastewater aquatic settings [52]; however, regrowth of bacteria was observed, and might be related to the formation of the easily assimilable acetic acid [53]. Rizzo et al. [42] advised that to target ARB, PAA is not efficient enough, and needs to be used with a coadjutant disinfection method. This approach may also be necessary to disinfect phages and ARGs, which are more problematic targets for disinfection [54]. Another disadvantage of PAA is its high cost.

Alternatively, Performic Acid (PFA) (CH2O3) is up to 20 x faster and more efficient as a disinfectant than PAA, as evidenced by tests done on coliforms and murine norovirus in wastewater [55]. It has also been recently used for treating municipal wastewater and combined sewage overflows [54]. PFA is the strongest oxidising (oxidizing potential of 2.70 V) disinfectant currently available and it has been shown to rapidly decompose into CO2 and water. It has been shown that this method will work more effectively at a pH of 7 and its efficiency decreases with lower temperatures [53,54]. To the best of our knowledge, PFA has not been yet explored for the disinfection of phages and ARGs and this remain to be explored; thus, the method is not covered in Table 1. Also, a major concern with PFA is the feasibility of ensuring the safety of operators during its deployment in WWTPs.

## Monochloramine (NH2Cl)

Monochloramine (NH2Cl) is a less efficient disinfectant than chlorine but also less prone to generate DBPs such as trihalomethanes. Although NH2Cl has an overall low reactivity towards carbohydrates, proteins, and nucleic acids [34] disinfection was still feasible. In fact, this method of disinfection has been applied to avoid microbial regrowth in membrane bioreactors that treat secondary wastewater effluent prior to reverse osmosis (see discussion on membrane methods below) [56]. Results were more promising in buffers than in wastewater, with doses ranging from 1228 mg × min/L for 1–Log removal of phages [57] to 1.5–3.0 ×105 mg × min/L for 4 to 6–Log removal of ARGs [33]. However, it should be noted that this method is not yet scalable for disinfection of phages and ARGs and further investigations are warranted.

## 3.7. Filtration methods

Our rationale for including filtration methods in the current discussion is that they have competitive removal rates when compared to chemical, UV and AOPs-mediated disinfection. The aim of filtration treatments is not inactivation of ARGs, phages or bacteria, but rather their physical removal from drinking and wastewater. Membrane-based processes present a wide array of removal efficiencies, membrane setups, applications and materials, and costs. They are generally applied to complement other disinfection methods in the water treatment process chain.

Filtration methods are typically classified according to their size-exclusion cutoffs, as follows: membrane filtration (MF) allows separation of particles greater than ~100 nm; ultrafiltration (UF) is the separation of macromolecules with molecular weight between ~1 kDa to 1000 kDa; nanofiltration (NF) can remove both macromolecules and ions (~1 kDa or less), while reverse osmosis (RO) can remove ions (~100 Da or less) [58]. As a matter of comparison, most phages range in size from ~20 to 200 nm in length [59], which is a relatively low variability and might be unlikely to cause major effects on the exclusion response of phages to disinfection (although experimental data are lacking). On the other hand, phage genomes can vary from ~3.0 kb to over 500 kb [60], whereas ARGs range from ~200 bp to over 2000 bp [61]. As can be seen from **Table 1**, UF, NF and RO can achieve the highest removals for both phages and ARGs (4.4–7 Log for phages, and 5.9–9.5 Log for ARGs) [62–64] when compared to all other methods. To be effective, these membranes however require pre-treatment of water to prevent clogging. Also, NF and RO treatments require post-treatment of membrane concentrate and high energy input, which means that careful feasibility assessments are necessary to remove phages and ARGs prior to implementing these solutions at a larger scale [42].

# Knowledge gaps and outstanding questions

From a precautionary point-of-view, stakeholders acting on global **AMR stewardship** should be informed about where to devote their efforts [65]. To date, the risk that phages pose to ARG spread in aquatic settings has not been established. Questions about the relationship between phages and ARGs in the context of AMR and disinfection are discussed in the Outstanding questions box. A few clues to address these questions are also presented as follows:

1. In a disinfection system, it is not currently possible to specifically target phages containing ARGs. Methods of disinfection applied to reduce phage particles, if cost-effective, could meet the criteria of the precautionary approach to mitigating AMR risks relating to phage particles in aquatic settings.
2. It is not yet possible to distinguish between true phages and transducing particles. Advanced microscopy techniques such as Transmission Electron Microscope (TEM) could help in assessing alterations in the morphology of phage particles caused by disinfection treatments. Investigations on developing more accessible methodologies to assess the different ways in which disinfection methods affect various phages particles are needed.
3. A clearer correlation between the decrease of AMR risk in aquatic settings and the disinfection of both phages and ARGs needs to be established so that AMR efforts can be best applied.
4. As faecal indicator bacteria (FIB) play a role in assessing the microbiological risks of water sources, future studies should examine the relationships between indicator phages, ARGs, and AMR risk. Our group is currently working to assess the efficacy of novel disinfection methods on the reduction of phages, ARGs and the overall HGT risk. We encourage other research groups to also pursue this effort, and to focus on removal or reduction of other MGEs from aquatic settings.
5. The cost-effectiveness and feasibility of disinfection technologies to remove phages and ARGs should be carefully considered. Two case-studies in large-scale are briefly presented next in the treatment of hospital wastewaters [66] and toilet-to-tap reuse scenarios (<https://www.ocsd.com/>). While these studies resulted in a measurable reduction in ARB and ARGs, the deployment of such treatments requires high financial investment. The Grundfos BioBooster system [66] claimed reduction of pharmaceuticals and ARB using a combined point-of-use tertiary treatment to treat hospital wastewater (Herlev hospital, Denmark). Treatment included a membrane bioreactor/filters, ozone above 4 mg O3/ mg DOC-1, followed by granular activated carbon and UV, thus resulting in complete removal of ARB. In the BioBooster system, phages were not monitored; however, a 4–5 Log reduction in waterborne virus was achieved. Investment necessary for the BioBooster system ranged between 3.3–4.7 million euros. Another example comes from California Orange County Sanitation District (<https://www.ocsd.com/>), which used an advanced water treatment facility to treat wastewater for both aquifer refill and potable reuse. In their case, treatment methods included chlorination, micro-filtrations, reverse-osmosis, ultraviolet disinfection and advanced oxidation systems. Although ARGs were reduced to levels under the detection limit (<50 copies per L) after treatment, they did increase back in the aquifer and in the distribution systems [67].
6. It should be noted that the water sector does not assess the potential risk associated to phages carrying ARGs. Nanofiltration and reverse osmosis methods have been shown to reduce the amount of phages + transducing particles + ARGs and other MGEs. Subject to further feasibility studies, they might be the only current solution to target these various types of AMR contaminants.

# Concluding remarks

The role of phages in the acquisition and spread of ARGs in aquatic settings is now undisputable. Our opinion is that, from a precautionary viewpoint, the monitoring of phages and ARGs should be included when designing and developing new disinfection treatments aimed at removing possible AMR risks. Currently, such studies have proved more feasible with infectious phages, although transducing phage particles and other MGEs should also be considered. Our conclusion from the review is that in water disinfection and antimicrobial resistance research, bacteriophages really matter.

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**References**

1 Nesme, J. and Simonet, P. (2015) The soil resistome: A critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. *Environ. Microbiol.* 17, 913–930

2 National Collaborting Centre for Infectious Disease (2016) Glossary of Terms: Antimicrobial Resistance. [Online]. Available: https://nccid.ca/publications/glossary-terms-antimicrobial-resistance/

3 Frost, L.S. *et al.* (2005) Mobile genetic elements: The agents of open source evolution. *Nat. Rev. Microbiol*, 722–732

4 Sui, Q. *et al.* (2018) Does the biological treatment or membrane separation reduce the antibiotic resistance genes from swine wastewater through a sequencing-batch membrane bioreactor treatment process. *Environ. Int.* 118, 274–281

5 Jit, M. *et al.* (2020) Quantifying the economic cost of antibiotic resistance and the impact of related interventions: Rapid methodological review, conceptual framework and recommendations for future studies. *BMC Med.* 18, 1–14

6 Roope, L.S.J. *et al.* (2019) The challenge of antimicrobial resistance: What economics can contribute. *Science,* 80*,* 364 (6435)

7 Government, H. (2016) *Book review: Tackling drug-resistant infections globally*.

8 Calero-Cáceres, W. *et al.* (2017) Bacteriophages as Environmental Reservoirs of Antibiotic Resistance. *Trends in Microbiol*. 27, 570–577

9 Debroas, D. and Siguret, C. (2019) Viruses as key reservoirs of antibiotic resistance genes in the environment. *ISME J.* 13, 2856–2867

10 Clokie, M.R.J. *et al.* (2011) Phages in nature. *Bacteriophage* 1, 31–45

11 Muniesa, M. *et al.* (2011) Bacteriophages and genetic mobilization in sewage and faecally polluted environments. *Microb. Biotechnol.* 4, 725–734

12 Neczaj, E. and Grosser, A. (2018) Circular Economy in Wastewater Treatment Plant–Challenges and Barriers. *Proceedings* 2, 614

13 Karkman, A. *et al.* (2018) Antibiotic-Resistance Genes in Waste Water. *Trends Microbiol.* 26, 220–228

14 M Pärnänen, K.M. *et al.* (2019) Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Sci. Adv.* 5, 1–10

15 Leplae, R. *et al.* (2004) ACLAME: A classification of mobile genetic elements. *Nucleic Acids Res.* 32, D45–D49

16 Soucy, S.M. *et al.* (2015) Horizontal gene transfer: Building the web of life. *Nature Reviews Genetics*, 16, 472–482

17 Subirats, J. *et al.* (2016) Metagenomic analysis reveals that bacteriophages are reservoirs of antibiotic resistance genes. *Int. J. Antimicrob. Agents* 48, 163–167

18 Brown-Jaque, M. *et al.* (2015) Transfer of antibiotic-resistance genes via phage-related mobile elements. *Plasmid* 79, 1–7

19 Zhang, A. *et al.* (2019) β-lactam resistance genes in bacteriophage and bacterial DNA from wastewater, river water, and irrigation water in Washington State. *Water Res.* 161, 335–340

20 Wang, M. *et al.* (2018) Metagenomic insights into the contribution of phages to antibiotic resistance in water samples related to swine feedlot wastewater treatment. *Front. Microbiol.* 9, Article 2474

21 Yang, Y. *et al.* (2020) Profiles of bacteria/phage-comediated ARGs in pig farm wastewater treatment plants in China: association with mobile genetic elements, bacterial communities and environmental factors. *J. Hazard. Mater.* DOI: 10.1016/j.jhazmat.2020.124149

22 Modi, S.R. *et al.* (2013) Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature* 499, 219–222

23 Taylor, V.L. *et al.* (2019) The Diverse Impacts of Phage Morons on Bacterial Fitness and Virulence. In *Advances in Virus Research* 103pp. 1–31, Academic Press Inc.

24 Wettstadt, S. (2020) Protect thy host: Pf4 phages shield Pseudomonas aeruginosa from antibiotics. *Environ. Microbiol.* 22, 2461–2462

25 Enault, F. *et al.* Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses. DOI: 10.1101/053025

26 Lekunberri, I. *et al.* (2017) Exploring the contribution of bacteriophages to antibiotic resistance. *Environ. Pollut.* 220, 981–984

27 Gunathilaka, G.U. *et al.* (2017) Phages in urban wastewater have the potential to disseminate antibiotic resistance. *Int. J. Antimicrob. Agents* 50, 678–683

28 Chen, J. *et al.* (2018) *Genome hypermobility by lateral transduction, Science*. 362, 207–212

29 Dias, E. *et al.* (2018) The application of bacteriophages as novel indicators of viral pathogens in wastewater treatment systems. *Water Res.* 129, 172–179

30 Muniesa, M. *et al.* (2018) Bluephage: A rapid method for the detection of somatic coliphages used as indicators of fecal pollution in water. *Water Res.* 128, 10–19

31 International Organization for Standardization (2000) *ISO 10705-2:2000(en) - Detection and enumeration of bacteriophages — Part 2: Enumeration of somatic coliphages*

32 Calero-Cáceres, W. and Muniesa, M. (2016) Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater. *Water Res.* 95, 11–18

33 He, H. *et al.* (2019) Degradation and Deactivation of Bacterial Antibiotic Resistance Genes during Exposure to Free Chlorine, Monochloramine, Chlorine Dioxide, Ozone, Ultraviolet Light, and Hydroxyl Radical. *Environ. Sci. Technol.* 53, 2013–2026

34 Dodd, M.C. (2012) Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. *J. Environ. Monit.*, 14, 1754–1771

35 Collivignarelli, M.C. *et al.* (2018) Overview of the main disinfection processes for wastewater and drinking water treatment plants. *Sustain.* 10, 1–21

36 Hiller, C. X. *et al.* (2019) Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review. *Sci. Total Environ.* 685, 596–608

37 Olivieri, A. *et al.* (2016) *Expert Panel on the Feasibility of Developing Uniform Water Recycling Criteria for Direct Potable Reuse*, 17NLM (Medline).

38 Guo, M. T. *et al.* (2013) Ultraviolet reduction of erythromycin and tetracycline resistant heterotrophic bacteria and their resistance genes in municipal wastewater. *Chemosphere* 93, 2864–2868

39 Simhon, A. *et al.* (2019) Enteric viruses in municipal wastewater effluent before and after disinfection with chlorine and ultraviolet light. *J. Water Health* 17, 670–682

40 US Environmental Protection Agency (1999) Wastewater Fact Sheet, *Chlorine Disinfection*.

41 Habibi-Yangjeh, A. *et al.* (2020) Review on heterogeneous photocatalytic disinfection of waterborne, airborne, and foodborne viruses: Can we win against pathogenic viruses? *J. of Colloidal Int. Sci.* 580, 503–514

42 Rizzo, L. *et al.* (2020) Best available technologies and treatment trains to address current challenges in urban wastewater reuse for irrigation of crops in EU countries. *Sci. Total Environ.* 710, 1–17

43 Mamane, H. *et al.* (2007) Inactivation of E. coli, B. subtilis spores, and MS2, T4, and T7 phage using UV/H2O2 advanced oxidation. *J. Hazard. Mater.* 146, 479–486

44 Yoon, Y. *et al.* (2017) Inactivation efficiency of plasmid-encoded antibiotic resistance genes during water treatment with chlorine, UV, and UV/H2O2. *Water Res.* 123, 783–793

45 Zhang, Y. *et al.* (2016) Reduction of antibiotic resistance genes in municipal wastewater effluent by advanced oxidation processes. *Sci. Total Environ.* 550, 184–191

46 Karaolia, P. *et al.* (2017) Investigation of the potential of a Membrane BioReactor followed by solar Fenton oxidation to remove antibiotic-related microcontaminants. *Chem. Eng. J.* 310, 491–502

47 Cacace, D. *et al.* (2019) Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. *Water Res.* 162, 320–330

48 Zhang, G. *et al.* (2020) Problems of conventional disinfection and new sterilization methods for antibiotic resistance control. *Chemosphere* 254, 126831

49 Wolf, C. *et al.* (2018) Kinetics of Inactivation of Waterborne Enteric Viruses by Ozone. *Environ. Sci. Technol.* 52, 2170–2177

50 Hembach, N. *et al.* (2019) Dissemination prevention of antibiotic resistant and facultative pathogenic bacteria by ultrafiltration and ozone treatment at an urban wastewater treatment plant. *Sci. Rep.* 9, 1–12

51 Iakovides, I.C. *et al.* (2019) Continuous ozonation of urban wastewater: Removal of antibiotics, antibiotic-resistant Escherichia coli and antibiotic resistance genes and phytotoxicity. *Water Res.* 159, 333–347

52 Huang, J.J. *et al.* (2013) Inactivation and regrowth of antibiotic-resistant bacteria by PAA disinfection in the secondary effluent of a municipal wastewater treatment plant. *Biomed. Environ. Sci.* 26, 865–868

53 Hawrlylik, E. (2020) Methods using in disinfection of wastewater and sewage sludge – short review. *Archit. Civ. Eng. Environ.* 2, 57–63

54 Campo, N. *et al.* (2020) Inactivation kinetics of antibiotic resistant Escherichia coli in secondary wastewater effluents by peracetic and performic acids. *Water Res.* 169, 115227

55 Maffettone, R. *et al.* (2020) Performic Acid Disinfection of Municipal Secondary Effluent Wastewater: Inactivation of Murine Norovirus, Fecal Coliforms, and Enterococci. *Environ. Sci. Technol.* DOI: 10.1021/acs.est.0c05144

56 Farhat, N.M. *et al.* (2018) Application of monochloramine for wastewater reuse: Effect on biostability during transport and biofouling in RO membranes. *J. Memb. Sci.* 551, 243–253

57 Dunkin, N. *et al.* (2017) Reduction of Human Norovirus GI, GII, and Surrogates by Peracetic Acid and Monochloramine in Municipal Secondary Wastewater Effluent. *Environ. Sci. Technol.* 51, 11918–11927

58 Madaeni, S.S. (1998) The application of membrane technology for water disinfection. *Water Res.* 33, 301–308

59 Brock, T*. et al.* (2015) Biology of Microorganisms, 14th Edition, Pearson.

60 Hatfull, G.F. and Hendrix, R.W. (2011) Bacteriophages and their Genomes Graham. *Curr. Opin. Microbiol.* 1, 298–303

61 Van Hoek, A.H.A.M. *et al.* (2011) Acquired antibiotic resistance genes: An overview. *Front. Microbiol.* 2, 1–27

62 Lan, L. *et al.* (2019) High removal efficiency of antibiotic resistance genes in swine wastewater via nanofiltration and reverse osmosis processes. *J. Environ. Manage.* 231, 439–445

63 Slipko, K. *et al.* (2019) Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. *Water Res.* 164, 114916

64 Lood, R. *et al.* (2017) Revisiting antibiotic resistance spreading in wastewater treatment plants – Bacteriophages as a much neglected potential transmission vehicle. *Front. Microbiol.* 8, 1–7

65 Berendonk, T.U. *et al.* (2015) Tackling antibiotic resistance: The environmental framework. *Nat. Rev. Microbiol.* 13, 310–317

66 Nielsen, U. (2016) *Full scale advanced wastewater treatment at Herlev Hospital Treatment performance and evaluation Grundfos BioBooster A/S Report*.

67 Harb, M. *et al.* (2019) Background Antibiotic Resistance and Microbial Communities Dominate Effects of Advanced Purified Water Recharge to an Urban Aquifer. *Environ. Sci. Technol. Lett.* 6, 578–584

68 Weng, S.C. *et al.* (2018) Infectivity reduction efficacy of UV irradiation and peracetic acid-UV combined treatment on MS2 bacteriophage and murine norovirus in secondary wastewater effluent. *J. Environ. Manage.* 221, 1–9

69 Zhang, C. *et al.* (2019) Progress and challenges in photocatalytic disinfection of waterborne Viruses: A review to fill current knowledge gaps. *Chem. Eng. J.* 355, 399–415

70 Ferro, G. *et al.* (2016) Antibiotic resistance spread potential in urban wastewater effluents disinfected by UV/H2O2 process. *Sci. Total Environ.* 560–561, 29–35

71 Karaolia, P. *et al.* (2018) Removal of antibiotics, antibiotic-resistant bacteria and their associated genes by graphene-based TiO2 composite photocatalysts under solar radiation in urban wastewaters. *Appl. Catal. B Environ.* 224, 810–824

72 Chen, S. Z. M. *et al*. (2019) Degradation of extracellular genomic, plasmid DNA and specific antibiotic resistance genes by chlorination. *Front. Environ. Sci. Eng.* 13, 38

73 Zhuang, Y. *et al.* (2015) Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection. *Environ. Sci. Pollut. Res.* 22, 7037–7044

74 Zhang, C. *et al.* (2019) Higher functionality of bacterial plasmid DNA in water after peracetic acid disinfection compared with chlorination. *Sci. Total Environ.* 685, 419–427

75 Hu, J.Y. *et al.* (2003) Removal of MS2 bacteriophage using membrane technologies. *Water Sci. Technol.* 47, 163–168

76 Gómez, M. *et al.* (2006) Urban wastewater disinfection by filtration technologies. *Desalination* 190, 16–28

77 Singh, R. *et al.* (2020) *Nanofiltration technology for removal of pathogens present in drinking water*, Elsevier.

78 Breazeal, M. V.R. *et al.* (2013) Effect of wastewater colloids on membrane removal of antibiotic resistance genes. *Water Res.* 47, 130–140

**Table and Figure captions**

**Table 1.  Responses of phages and ARGs to various disinfection treatments.**

**Figure 1. A potential intersection between phages, antimicrobial resistance and disinfection practices.** Aquatic settings (circle 1): these include urban water cycle wastewater treatment and drinking water systems. Phage–mediated HGT risks (circle 2): there are several unassessed AMR risks in aquatic settings. These include ARB, MGEs, ARGs (in the form of free DNA), true phages and transducing particles. Disinfection treatments (circle 3): the need and the feasibility of disinfection methods to remove phage–mediated HGT risks needs to be assessed further. Arrows indicate that, from a precautionary viewpoint, monitoring phages and ARGs should be included when designing and developing new disinfection treatments aimed at removing possible AMR risks from aquatic settings. All icons were obtained from The Noun Project (<https://thenounproject.com>).

**Table 1.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Process | Target | Dose/Treatment | Log Reductiona | Aquatic Environmental  Settingsc | Ref. |
| UV-C (253.7 nm) germicidal) | Phages | 5.94ꟷ178.2 mJ/cm2 | 7 | wastewater | [32] |
| 5ꟷ250 mJ/cm2 | 4.5–5.5 | buffer/wastewater | [68] |
| ARGs | 5ꟷ178 mJ/cm2  (ARG in phage genomes) | 0.2b–1 | mesocosm | [32] |
| 10ꟷ400 mJ/cm2 | <1–4 | wastewater | [37] |
| 50ꟷ250 mJ/cm2 | 3–6 | buffers | [33] |
| 10ꟷ150 mJ/cm2 | <1 | wastewater/ drinking water | [34] |
| Advanced Oxidative Processes (AOPs) | Phages | UV > 295 nmplus 0ꟷ25 mg/L H2O2  (15 min) | 1–2.5 | buffers, surface water | [43] |
| UVA/B/C/or sunlight plus TiO2 photocatalysis in solution or immobilised  (2ꟷ2280min) | 1–10  (not scalable) | lab matrices, distilled water, wastewater | [69] |
| ARGs | UVA-B/H2O2 UV @ 320ꟷ450nm plus  20 mg/L to 340 mg/L H2O2 (up to 240 min) | 0b–4  (not scalable) | wastewater | [70] |
| 33–72 mg × min /L chlorine and 50–130 mJ/cm2 and 10 mg/L for UV/H2O2. | 4 | buffers, wastewater | [44] |
| Fe2+/H2O2 molar ratio 0.1 and a H2O2 [0.01mol/L] pH=3.0 120 min  Fenton > UV/H2O2 | 2.5–3.8 | wastewater | [45] |
| UV/Fe/H2O2 [Fe2+]0= 5 mg/L plus[H2O2]0= 50 mg/150 min ARGs persisted | 97% total DNA | wastewater | [46] |
| TiO2-graphene based composite,  Xenon lap=63 W/m2 | Some removal | wastewater | [71] |
| Chlorination | Phages | 30 mg x min/L | 1 | mesocosms | [32] |
| ARGs | 15ꟷ450 mg x min/L | <1–2 | drinking water, wastewater | [37] |
| 1ꟷ20 mg Cl2/L,  2 mg x 30 min (initial [ ] 105 copies/µl),  DNA fragmentation and reduction, genomic DNA more sensitive than plasmid borne DNA/ARG | 70% reduction DNA signal | ultrapure  water | [72] |
| 1ꟷ20 mg x min/L  ARG in phages | 0.1b–0.6b | mesocosms | [32] |
| 50 ꟷ150 mg x min/L | 4–6 | buffers | [33] |
| 180ꟷ1000 mg x min/L – extracellular fragmented plasmid and 16S rDNA depended on aqueous media composition.  10ꟷ100 mg x min/L intracellular DNA | NR (various)  Likely to occur | buffers | [34] |
| Ozonation | Phages | 0.25ꟷ0.6 mg O3 x mg DOC, MS2 tipically inactivated in WWTP doses (0.25ꟷ 1 mg O3 x mg DOC) | 4–9 | buffers | [49] |
| ARGs | 0.1ꟷ200 mg x min/L | 1–3 | wastewater | [37] |
| 0.8ꟷ0.12 mg x min/L | 4–6 | buffers | [33] |
| 0.1ꟷ 1 mg x min/L  15 mg x L (15 min) plasmid DNA | NR  (DNA fragmented) | buffers | [34] |
| (27ꟷ178 mg/L) 177.6 mg /L O3 (corona discharge, time not mentioned | 1.7–2.5 | wastewater | [73] |
| 0.25ꟷ0.75 mg O3/g DOC x 10 ꟷ40 min, various ARGs | 2–6 | wastewater | [51] |
| 0.2ꟷ0.9 mg O3 /g DOC various HRT, depends on wastewater features, reduces ARGs, selects for bacterial resistance, recovery upon few days storage | various | wastewater | [42] |
| Peracetic acid | Phages | 0ꟷ10 mg/L x 30ꟷ120 min plus UV-C @ 20 mJ/cm2 (low reduction alone or in wastewater) | 1–5 | buffer, wastewater | [68] |
| 1254 mg x min/L greater removal in buffers | 1 | buffers, wastewater | [57] |
| ARGs | 25 mg/L x 15 min  (plasmids reduced transforming activity) | 0.3b | buffer | [74] |
| Monochloramine | Phages | 1228 mg x min/L greater removal in buffers | 1 | buffers, wastewater | [57] |
| ARGs | 1.5ꟷ3.0 x105 mg x min/L (not scalable) | 4–6 | buffers | [33] |
| Ultrafiltration (~ 1kDa to 1000 kDa) | Phages | Polyamide polysulfone membrane 10ꟷ40 psi | 0.3b–1.8 | tryptic soy broth | [75] |
| Membrane of polyvinylidene- fluoride 0.05 µm (0.2 to 0.6 Bar) | ~0.1b–1 | wastewater | [76] |
| Various types of membranes and membrane sizes | 2–7 | wastewater, drinking water | [37,58] |
| Various sizes (review chapter) 0.01ꟷ0.5 µm membranes | 6 | drinking water | [77] |
| ARGs | Various sizes (reviews) (increase reported in treated water) | 1–6 | drinking water | [37,50] |
| 1.2 µmꟷ1kDa PVDF, and cellulose membranes Millipore | 0.9b–5.9 | wastewater | [78] |
| polysulfone polyamide membrane 0.15MPa, 80-100 KDa | iARGd removed | swine wastewater | [62] |
| 2.5ꟷ300KDa, 2ꟷ24bar polyether sulfone and polyamide thin | 0.1b–3.1 | filtered secondary wastewater, distilled water | [63] |
| Nanofiltration (~1kDa or less) | Phages | polysulphone, cellulose acetate (60ꟷ100 psi) | 1.9–3 | tryptic soy broth | [75] |
| Various configurations <100 nm, including carbon nanotubes | 0.5b–9 | drinking water | [77] |
| ARGs | polyamide (2.0 MPa)  <500 Da | 4.9–8.1 | swine wastewater | [62] |
| 15ꟷ300 or 400 Da,  38-40 bar, polyamide | 3–3.6 | filtered secondary wastewater, distilled water | [63] |
| Reverse Osmosis (~100 Da or less) | Phages | Polyamide, cellulose acetate membranes, pore: 3–4 nm up to 23 nm (100ꟷ160psi) | 3.5–4.4 | tryptic soya broth | [75] |
| ARGs | polyamide (3.6MPa)  (ARG increase after treatment in wetlands) | 5.2–9.5 | wastewater, wetlands | [62] |
| 200 Da, 40 bars, polyamide | 4 | filtered secondary wastewater, distilled water | [63] |

**Table 1** shows the overall efficiency of removal of “true phages” (or “infectious phages”) and ARGs (primarily in extracellular form) through classic and novel disinfection treatments, in a range of aquatic settings.

aLog reduction: ARG=Log gene copies, Phage=Log.

bLog disinfection values lesser than 1 and greater than 0 Log are possible when the count of gene copies (in the case or ARGs) or PFU/ml (in the case of phage plaques) are between 1 and 10 gene copies or PFU/ml, respectively. Note that while phage cultivation requires a cultivation method on agar through bacterial infection to quantify plaques, generally gene copies will be determined by a suitable molecular method, such as qPCR. Accessibility of working with molecular methods, however, is not straight-forward for most water monitoring microbiology labs.

c Data were collected from studies in WWTPs, drinking water treatment, and lab-scale and buffered water matrices, with the latter being the most frequent source.

d=iARG= intracellular ARGs

NR=Not reported.

Observation: Detailed reviews on the disinfection and removal treatment of ARB have been covered extensively elsewhere [22–24].