Environmental Sustainability
https://doi.org/10.1007/s42398-024-00322-y

REVIEW

Potential use of bacteriophages as biocontrol agents against multidrug-resistant pathogens in wastewater treatment: a review

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Received: 17 February 2024 / Revised: 27 August 2024 / Accepted: 28 August 2024

Abstract

The conventional methods of wastewater treatment are essentially used to remove contaminants and pathogens from wastewater before it is released into the environment or used for other purposes. With the increasing number of Multidrug-Resistant (MDR) organisms in wastewater, the potential usefulness of conventional treatment methods has been re-evaluated. The conventional biological treatment and disinfection have been proven in many studies to increase the prevalence of Antibiotic Resistance Genes (ARG) in bacteria. More than 25 genes responsible for antibiotic resistance were found to be increased from influent to effluent in different Wastewater Treatment Plants (WWTPs). Additionally, many studies have discussed the high abundance of several Mobile Genetic Elements (MGEs) after disinfection by chlorination and ozonation. Bacteriophage-based therapy has emerged as an innovative method for effectively managing microorganisms in wastewater treatment and in various other applications. Bacteriophages can be utilized to kill pathogenic bacteria and eradicate the biofilms formed by the bacteria in wastewater treatment plants with low intrinsic toxicity. However, the use of bacteriophages has been associated with some limitations, including the narrow host range spectrum. This review provides a critical overview of the recent knowledge on the effect of biological treatment and disinfection on spreading antibiotic resistance. In addition, we highlight the interactions among bacteria and phages to sustain the water treatment process. We also emphasize the proposed improvement in wastewater treatment using bacteriophage-based therapy. Our focus is identifying gaps, opportunities, and critical concerns that should be addressed in further research.

Keywords Wastewater · Biological treatment · Antibiotic resistance · Phage therapy

Introduction

Water covers 70% of the Earth's surface, yet only 3% of this water is classified as fresh. Of that portion, two-third exists as frozen glaciers or is unsuitable for human consumption (Choi et al. 2011). In 2018, the World Economic Forum (WEF) reported that water crises were among the top five global risks with the most significant potential impact over the next decade. The situation will worsen if consumption continues at its current rate without immediate solutions. By 2025, approximately two-third of the global population will face the consequences of water scarcity, while ecosystems worldwide will experience notable deterioration (World Wildlife Fund 2022). To tackle this probable water scarcity crisis, several countries have dedicated their research and efforts to using treated wastewater as an alternative water source to meet the growing demand. This approach addresses the need for water reuse by utilizing treated

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Published online: 19 September 2024

wastewater in agriculture or replenishing groundwater aquifers for future use (AbuZeid and Elrawady 2014).

Several new techniques have been used to enhance treatment process, such as chemical precipitation, reverse osmosis, ion exchange, nanofiltration, and biosorption methods (Gadirova 2021). Nevertheless, the excessive use of antibiotics in the industrial, medical, and agricultural fields has increased the presence of pathogenic and antibiotic-resistant bacteria (ARB) in wastewater that have become more resistant to damage by conventional biological and chemical treatment methods. Some hospitals discharge residual antibiotics into wastewater without filtering, putting selection pressure on the microorganisms and increasing the possibility of spreading antibiotic resistance genes (ARGs) (Rodriguez-Mozaz et al. 2015). A previous study reported the dominant presence of tetracyclines, macrolides, sulfonamides, and quinolones antibiotics in domestic sewage (Booth et al. 2020). The prolonged contact between microorganisms in the treatment plants facilitates the horizontal gene transfer between bacteria in the plants; antibiotic-sensitive bacteria acquire the ARGs by the horizontal gene transfer to form ARB, while resistant bacteria obtain the ARGs to generate multidrug-resistant bacteria (MDRB) (Lerminiaux and Cameron 2019).

ARB can survive sewage treatment and transfer to humans through food supply, direct contact with animals, or indirect contact through various environmental pathways (Angulo et al. 2004; Weber et al. 2013). Therefore, drug-resistant pathogens can develop in locations far from the initial drug prescription, and this emergence may occur long after the application of initial selection pressure (Tello et al. 2012). Human pathogens did not initially harbor many of these resistance-associated genes, suggesting that the sole viable origin for these genes was the environment's microbiota (Aminov 2011). Around 700,000 deaths of antibiotic resistance were recorded annually before 2016, and it is suspected that by the year 2050, antibiotic resistance will lead to 10 million deaths annually (O'Neill 2014). Therefore, there is an urgent need to solve the problem of antibiotic resistance emergence.

Bacteriophages (commonly referred to as phages) are viruses that have been thoroughly studied for their structure, size, and genetic content. They have a protein core with a nucleic acid called capsid and a thin tail with fibers; nucleic acid can be DNA or RNA. The phage's proteins attach to specific receptors on the surface of the targeted bacteria, giving them high specificity. Some phages have a broad host range and can recognize multiple receptors on multiple bacterial species and genera. Bacteriophages have two life cycles: lysogenic and lytic. In the lysogenic cycle, phages insert their nucleic acid into the host's genetic material, replicating with the bacterial cell. During the lytic cycle, phages inject their genetic material into the bacterial host, eventually lysing the host cell wall. This characteristic makes them suitable for biocontrol applications. Those lytic cycle phages, or virulent phages, rapidly lyse their host, using enzymatic machinery to replicate and synthesize new particles (Silpe and Bassler 2019).

Recently, there has been an increasing interest in bacteriophages as targeted biological control agents in water and wastewater treatment. This interest comes from phages' ability to specifically eliminate antibiotic-resistant bacteria and disrupt bacterial biofilms in wastewater environments, all while possessing minimal inherent toxicities (Mukherjee et al. 2021; Pazda et al. 2019). The notable specificity of bacteriophages towards their hosts is due to the existence of proteins located at the tip of phage tail fibers. These proteins specifically attach bacterial receptors, initiating the injection of phage genetic material into the host membrane (Mathieu et al. 2019). Many studies have reported the high efficacy of phage against ARB and MDRB in wastewater (Kwiatek et al. 2017; Lin et al. 2010; Mathieu et al. 2019; Turki et al. 2012). However, some limiting factors may affect the efficacy of the treatment process in practical applications. Therefore, this review summarizes the conventional wastewater treatment technology and evidently discusses the effect of traditional disinfection technologies on ARG transfer and ARB prevalence in the environment, including chlorination and ozonation. The main aim is to give insight into the new perspectives on the efficacy of using bacteriophage to control the emergence of antibiotic resistance in Wastewater Treatment Plants (WWTPs) and enhance treatment technologies. Furthermore, the review provides an overview of the limitations and obstacles of phage-based treatment, based on the knowledge collected from recently published literature.

Conventional wastewater treatment process

WWTPs primarily focus on removing contaminants from wastewater through two main processes: primary treatment, and secondary treatment (Nathanson and Ambulkar 2023). Preliminary treatment, which is sometimes used before the primary treatment, involves screening, grit removal, and skimming to remove large objects (Nathanson and Ambulkar 2023). Primary sedimentation aims to eliminate remaining organic matter and suspended materials, forming primary sludge (Demirbas et al. 2017; Oakley 2019). Secondary treatment follows, eliminating biodegradable contaminants, residual organics, and solids that weren't removed in the primary treatment phase. Secondary treatment involves suspended and attached growth systems. In suspended systems, microbes metabolize organic materials in wastewater, while

attached growth systems use solid media to grow microorganisms. The effluent flows through the media, forming a biofilm that detaches or falls off through the sloughing process (Parkin and Speece 1983; Tatoulis et al. 2015). Tertiary treatment, an optional process after secondary treatment, removes remaining contaminants and eliminates microorganisms using chlorine disinfection (Serra et al. 2014). This involves adding bleaching powder or purging chlorine gas to ensure the removal of pathogenic microorganisms from the water (Mazumder 2011).

Evaluating antibiotic resistance occurrence and spreading in the treatment plants

Conventional WWTPs have played a role in eliminating bacteria within the treatment plants. This can be attributed to the substantial volume of bacteria and other microorganisms that flow through these facilities on a daily basis (Mukherjee et al. 2021; Pazda et al. 2019). Furthermore, various forms of resistance genes can be present through conjugation-capable resistant bacteria, free plasmids/DNA, and phage particles. This increases the likelihood of horizontal transfer of ARGs among microorganisms (Parsley et al. 2010).

Bacterial horizontal gene transfer includes three mechanisms: transformation, transduction, and conjugation. In conjugation, two bacteria connect through pili to transfer the moveable genetic materials, such as plasmids or transposons, from a donor cell to a recipient cell. The frequency of conjugation was observed to be significantly increased in *Escherichia coli* under Minimum Inhibitory Concentrations (MIC) of gentamicin (0.1 mg/L), triclosan (0.1 mg/L), sulfamethoxazole (1 mg/ L), and chlorhexidine (24.4 µg/L) (Jutkina et al. 2018). Transformation is the process by which the extracellular DNA is absorbed by a competent bacterial cell and recombined into its genome. In a previous study, the environmentally detected concentrations of triclosan (0.2–20 µg/L) significantly enhanced the transformation of plasmid-borne ARGs into *E. coli* DH5α by up to 1.4-fold (Lu et al. 2020). The environmental pressure on bacteria in the treatment plants is known to induce transduction and increase the probability of gene transfer (Bouki et al. 2013). The transduction process occurs when bacteriophage infects a donor cell, and environmental stress factors induce SOS reactions in bacteria, initiating prophage excision and replication. The phage particles will contain both phage genetic material and random DNA, including ARGs, and will support ARGs transfer with other recipient antibiotic-susceptible bacteria (Fig. 1).

Gene transfer is more likely to occur when heavy metal ions, antibiotics, hazardous chemicals, and UV radiation are present at subclinical levels in wastewater (Motlagh et al. 2015). The role of WWTP as a source of increased resistance and virulence gene acquisition was studied by Ragab et al. (2023) They examined six different genes (traT, FimH, blaCTX, tetA, blaTEM, and blaSHV) in a total of 28 *E. coli* strains collected from Egyptian wastewater plants. The results showed that all isolates carried traT, FimH, and tetA genes. Additionally, the occurrence of ESBL genes blaCTX, blaTEM, and blaSHV was found to be 96.4%, 89.3%, and 57.14%, respectively (Ragab et al. 2023).

Various studies have discussed that the high presence of antibiotics in different wastewater samples can increase resistance rates (Sutradhar et al. 2023; Maghsodian et al. 2022; Mutuku et al. 2022). Currently, 70 out of 100 types of antibiotics used by humans and other organisms have been found in surface waters and sediments (Maghsodian et al. 2022). Tetracycline, a broad-spectrum antibiotic widely used to treat infections in humans and animals, is found in wastewater, surface water, and drinking water samples. Over 70% of tetracycline antibiotics are released into the environment in an active form by humans and animals (Daghrir and Drogui 2013). Due to their highly stable nature in water and tendency to bind to suspended matter during wastewater treatment, tetracyclines are frequently detected in wastewater treatment plants. In Sweden, Hong Kong, Norway, and Germany, five tetracycline antibiotics were identified in hospital samples, and wastewater treatment plant influent and effluent (Minh et al. 2009; Watkinson et al. 2009; Rossmann et al. 2014; Mutuku et al. 2022). Additionally, antibiotics such as sulfonamides, tetracyclines, macrolides, and quinolones were predominantly identified in the domestic sewage water (Lyu et al. 2020).

The relation between biological treatment and antibiotic resistance development

Bacteria resistant to antibiotics and their acquired genes are collected from a broad range of sources, especially in WWTPs, such as municipal, medical, and slaughter wastewater. Sludge was shown to have higher concentrations of resistant bacteria than wastewater, with levels reaching up to 10^8 CFU/mL (Withey et al. 2005). Consequently, raising the question about the safety of using activated sludge systems in biological treatment methods is crucial. It was previously discussed that WWTPs with activated sludge systems showed higher levels of ARGs in the effluents than other treatment technologies (Table 1). Ebomah and Okoh (2020) conducted a study to detect the percentage of carbapenem-resistant genes occurrence in *Klebsiella* species recovered from nine different environmental niches in the eastern Cape province- South Africa. The results declared the high occurrence of the resistant genes in *Klebsiella*

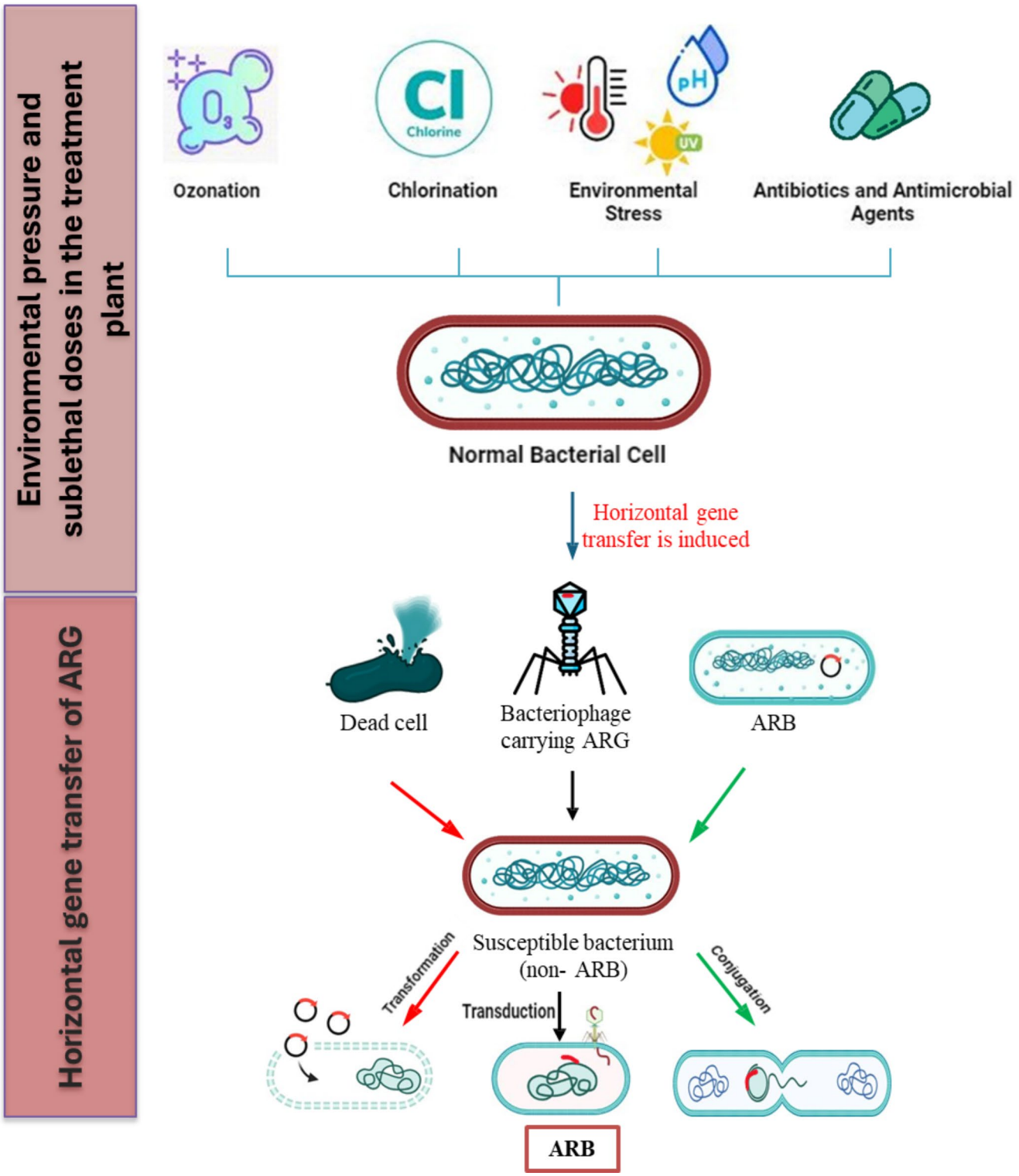

Fig. 1 Effect of subclinical doses of chlorine, ozone, or antibiotics, combined with environmental pressures, on horizontal gene transfer (HGT) and the emergence of antibiotic resistance in water treatment plants. Sublethal concentrations of these agents can stimulate HGT mechanisms (transformation, transduction, and conjugation) in bacterial populations, facilitating the acquisition of antibiotic-resistance genes (red color line) and the conversion of non-antibiotic-resistant bacteria (non-ARB) into antibiotic-resistant bacteria (ARB)

Table 1 Overview of the genes responsible for antibiotic resistance in various wastewater treatment plants (WWTPs) from influent to effluent

Antibiotic Class	Antibiotic-resistant genes (ARGs)	Wastewater sample source	Treatment technology	Treated wastewater discharge	Country	Reference
β–Lactams	bla_{AmpC}	Municipal wastewater	Activated sludge system	-	Germany	(Alexander et al. 2015)
	bla_{CMY-13}	A mix of municipal, hospital and industrial wastewater	Sand filtration, mesophilic anaerobic digestion and activated sludge system	-	Sweden	(Bengtsson-Palme et al. 2016)
	$bla_{CTX-M-1}$	Domestic Sewage and Industrial wastewater	Trickling filtration	Mondego river	Portugal	(Amador et al. 2015)
	$bla_{st-M-12}$	Municipal and hospital wastewater	Activated sludge system	-	Canada	(Biswal et al. 2014)
	bla_{FOX}	Domestic Sewage and Industrial wastewater	Trickling filtration	Mondego river	Portugal	(Amador et al. 2015)
	bla_{OXA}	Domestic Sewage and Industrial wastewater	Trickling filtration	Mondego river	Portugal	(Amador et al. 2015)
	bla_{TEM}	Domestic Sewage and Industrial wastewater	Trickling filtration	Mondego river	Portugal	(Amador et al. 2015)
	bla_{TEM}	Municipal and hospital wastewater	Activated sludge system	-	Canada	(Biswal et al. 2014)
	bla_{TEM}	Municipal and hospital wastewater	-	Ter River	Spain	(Rodriguez-Mozaz et al. 2015)
	bla_{VIM-1}	Municipal wastewater	Activated sludge system	-	Germany	(Alexander et al. 2015)
Quinolone	gnrC	Sewage wastewater	Activated sludge system	Rivers in China	China	(Xu et al. 2015)
	gnrS	Municipal and hospital wastewater	Waste stabilization pond system and mechanical filtration	Clyde River	Canada	(Neudorf et al. 2017)
	gnrS	Municipal and hospital wastewater	-	Ter River	Spain	(Rodriguez-Mozaz et al. 2015)
Macrolide	ereA	Municipal and hospital wastewater	Activated sludge system	-	Canada	(Biswal et al. 2014)
	ereB	Municipal and hospital wastewater	Activated sludge system	-	Canada	(Biswal et al. 2014)
	ereB	Municipal and hospital wastewater	-	Ter River	Spain	(Rodriguez-Mozaz et al. 2015)
	mph(A)	Municipal and hospital wastewater	Activated sludge system	-	Canada	(Biswal et al. 2014)
Tetracycline	tetB(P)	A mix of municipal, hospital and industrial wastewater	Sand filtration, mesophilic anaerobic digestion and activated sludge system	-	Sweden	(Bengtsson-Palme et al. 2016)
	tetE	Sewage wastewater	Activated sludge system	Rivers in China	China	(Xu et al. 2015)
	tetV	Sewage wastewater	Activated sludge system and anaerobic digestion sludge	-	China	(Yang et al. 2014)
	tetZ	Sewage wastewater	Activated sludge system	Rivers in China	China	(Xu et al. 2015)
	dfrA3	A mix of municipal, hospital and industrial wastewater	Sand filtration, mesophilic anaerobic digestion and activated sludge system	-	Sweden	(Bengtsson-Palme et al. 2016)
	sulI	Municipal and hospital wastewater	Activated sludge system	-	Canada	(Biswal et al. 2014)
	sulI	Sewage wastewater	Activated sludge system and anaerobic digestion sludge	-	China	(Yang et al. 2014)
	sulII	Municipal and hospital wastewater	Activated sludge system	-	Canada	(Biswal et al. 2014)

isolates from the environmental samples, with percentages reaching 45% for isolates from the soil, 33% from hospital effluents, and 22% from vegetables. Additionally, 69% of all isolates were resistant to meropenem antibiotics when tested by disc diffusion. Their results demonstrated the high efficacy of environmental pathways in functioning as reservoirs for resistance genes (Ebomah and Okoh 2020).

The traditional biological treatment technologies used in the plants mentioned in Table 1 facilited gene transfer; higher levels of ARGs and ARB were observed from influent to effluent, which has been reported to be discharged into rivers for reuse in various applications. Therefore, various pathogenic bacteria, including *E. coli*, *Mycobacterium tuberculosis*, *Salmonella*, *Staphylococci*, and *Vibrio cholerae*, have acquired or increased their resistance to antibiotics. Consequently, this has resulted in a decline in the effectiveness of commonly employed antibiotics (Berryhill et al. 2021; Lilic et al. 2020; Morita et al. 2020; Singh et al. 2021; Zhi et al. 2020). Therefore, infection with these pathogenic bacteria reduces the cure probability, resulting in severe illness and even death (Zhi et al. 2020).

The role of conventional disinfectants in elevating antibiotic resistance

Chlorination

The inactivation mechanism of bacteria by chlorination depends on the oxidation of the chlorinating agent, which causes hydrolysis, leading to mechanical destruction of the bacterial cell wall and changing its permeability (Bommer et al. 2018). After chlorine gains access to the cytoplasm, it begins to act on target molecules, and intracellular molecules such as DNA, RNA, and protein, leak out of the cell; these leaked intracellular molecules are damaged by the chlorinating agent outside the cell (Ofori et al. 2018). A study reported that the cell membrane was damaged at low doses of chlorine (< 5 mg/L NaOCl), while DNA was slightly damaged at high doses (> 5 mg/L NaOCl). Furthermore, the bacterial DNA repair function (RecA and mRNA) was entirely inhibited after 30 min of exposure to 5 mg/L chlorine. Any increase or decrease in these doses was proven to develop into what are called chlorine-resistant strains (Luo et al. 2021; Xu et al. 2018). The question that should be highlighted here is "Does chlorination disinfection increase the abundance of antibiotic resistance in the environment?" Significant upregulation was noticed in the pertinent defense systems against oxidative stress and antibiotics following the comprehensive analysis of the metagenome; microorganisms present in the chlorinated drinking water distribution system were sequenced. These defense systems included Efflux pump-related genes, β-lactamase, several genes regulated by RpoS, the OxyR system, and the SoxRS system, which are among the elements that exhibit regulation in this context (Douterelo et al. 2018). Additionally, according to some studies, the number of Mobile Genetic Elements (MGEs) became more abundant after chlorination, leading to the transfer of ARGs to other bacteria (Shi et al. 2021; Zhang and Hu 2013). Another study has shown that the chlorination process aided in transforming opportunistic pathogens from non-ARB to ARB by gene transfer (Jin et al. 2020).

A previous study used high-throughput sequencing and metagenomic techniques to examine changes in the bacterial population and ARGs over time in a Drinking Water Distribution and treatment System (DWDS). It was found that chlorination had mixed effects; it could successfully eliminate *Ethylotenera*, *Limnobacter*, *Methylophilus*, and *Polynucleobacter* while causing an increase in the incidence of *Acidovorax*, *Plemons*, *Pseudomonas*, *Sphingomonas*, and *Undibacterium* in the water sources. Drinking water contained 151 ARGs among 15 types, and chlorination clearly raised their overall prevalence while decreasing their variability in opportunistic bacteria ($p < 0.05$) (Jia et al. 2015). Chlorine-resistant *Acidovorax* and *Pseudomonas* were the main carriers of the bacitracin resistance gene (bacA), primarily responsible for the increased abundance of ARGs (Jia et al. 2015).

Several studies have examined how disinfectants affect ARGs in biofilms; one of these studies examined the development of ARB and the role of biofilms in a Water Distribution System (WDS). Researchers monitored various ARB resistant to clindamycin, norfloxacin, sulfamethoxazole, and tetracycline for an entire year in a WDS. The findings showed that the concentration of ARB in tap water increased, most probably as a result of biofilm detachment (Giacometti et al. 2021). According to high-throughput sequencing, the relative levels of the antibiotic-resistant *Acinetobacter*, *Bradyrhizobium*, and *Sphingomonas* were higher in outflow than in intake water. These findings imply that disinfectants induced biofilm dissociation and had an impact on the overall development of bacterial antibiotic resistance in the microorganisms found in tap water (Giacometti et al. 2021).

Ozonation

Gaseous disinfectant technologies represent an effective method of manual disinfection and a suitable alternative to chlorine in water disinfection processes. Ozone (O^3), the second-strongest oxidant for water treatment after the hydroxyl radical, has proven to be the most successful among the gaseous sanitizers studied recently, along with cold plasma and chlorine dioxide (ClO_2) (Nguyen et al. 2021). Ozonation inactivates bacteria through a mechanism

that mainly depends on the cleavage of the cell wall, which causes the denaturation of nucleic acids and depolymerization of proteins through the breakage of carbon and nitrogen bonds (Jäger et al. 2018).

O_3 is immensely reactive to the proteins, peptidoglycans, and lipids in the cell wall and cell membrane, especially amino acids and unsaturated carbon-carbon bonds. Consequently, when the cell wall and the cell membrane are damaged, O_3 could additionally attack the intracellular ARG (iARG) (Dodd 2012). Extracellular ARG (eARG) is free DNA independent of bacteria; it results from the release of the intracellular ARG (iARG) and is more vulnerable to O_3 oxidation attacks compared to iARG. Different bacterial sensitivity to ozonation was observed, particularly associated with the guanine-cytosine content of the target organism's genome (Alexander et al. 2016). ARGs with lower guanine-cytosine content may be readily damaged by O_3. However, the presence of hydrogen bonds between the DNA's double strands could have resulted in lower-than-expected O_3 reactivity to double-stranded DNA (Oncu and Balcioglu 2013). Another study outlined the current advancements in ozone application for enhanced chemical and biological contaminants treatment in wastewater; these included DBP issues and the effectiveness of elimination/disinfection at various ozone doses, or DOC-normalized ozone doses. The study noticed that specific ozone doses feasible for full-scale application resulted in the disruption of iARGs; however, they may be hindered by floc. Nonetheless, iARG in the wastewater community is not eliminated by ozone doses relevant for micropollutant abatement (Czekalski et al. 2016).

While O_3 exhibits a satisfactory ability to eliminate ARB, its ability to eliminate ARG is less effective. Therefore, a higher ozone dosage may be necessary to completely remove antibiotic resistance in wastewater. A metagenomic assembly analysis performed by another study illustrated that utilizing an O_3/chlorine-coupled disinfection procedure significantly increased the relative abundance of ARB-carrying ARGs and mobile genetic elements; this delivered a contrasting perspective (Zhang and Hu 2013). Despite the wide use of ozonation to remove ARB, it could increase the abundance of ARGs under specific circumstances that create favorable conditions for the development of antibiotic resistance.

The ecology of bacteriophage

Bacteriophages, also known as phages, are viruses that specifically infect bacteria. They can potentially be utilized to control bacterial populations and facilitate gene transfer processes (Clokie et al. 2011). They are extremely abundant, with an estimated titer of ~ 10^{30} to 10^{32} viral particles in the biosphere. At the beginning of the 20th century, phages were used as a selective bio-control agents for various important agricultural and human pathogenic bacteria. Nonetheless, the discovery of antibiotics overshadowed their utilization as they proved to be simpler and more convenient for achieving broad-spectrum antibacterial effects. There has been a focused interest recently in using phages as biocontrol agents because of the high abundance of MDRB in the environment and recognizing the deleterious effect of broad-spectrum antimicrobials on beneficial microbial communities (Nobrega et al. 2015).

The application of phage-based technology in wastewater treatment

Sustainable ecology and public health depend on the treatment of wastewater. Phage treatment is a potential option for targeted bacterial removal in WWTP. Recently, using phages as antibacterial agents has been implemented in water systems to selectively target the pathogenic and ARB without harming the beneficial microorganisms or affecting the biological treatment process (Shivaram et al. 2023). Pathogenic bacteria are controlled by phages through: (1) selectively lysing the strains of target; (2) reducing population fitness of the target by making the more sensitive bacteria more susceptible to competitive exclusion or biocides; (3) removing biofilms that shelter the bacteria of target; (4) removing activated sludge foaming and bulking formed by the targeted bacteria; and (5) the replacement of antibiotics and biocides to reduce their intended or unintended discharge to the environment (Fernandes et al. 2014). These points will be discussed in the following sections.

Phage therapy in controlling pathogenic and multidrug-resistant bacteria in wastewater

Bacteriophages are used as antibacterial agents and can be used to treat pathogenic microorganisms in wastewater. The new phage-based technology helps in reducing the amount of harmful chemical reagents, which are hazardous to beneficial bacteria and the environment (Mathieu et al. 2019). The utilization of phages has the potential to enhance traditional wastewater treatment methods, enabling the safe reuse or discharge of treated wastewater into the environment, including its return to the source waters. Phages could target pathogenic bacteria and ARB in the treatment plants. Recent research has revealed that some WWTPs act as hotspots for the environmental dissemination of MDR genes (superbugs) and ARGs (Luo et al. 2014). This problem could be alleviated using phages by targeting the species that carry

MDR genes, such as *Klebsiella*, *Pneumoniae*, *Staphylococcus aureus*, and certain strains of *E. coli* (Levy and Marshall 2004). Polyvalent phages, for example, thrived and successfully reduced MDR *E. coli* NDM-1 without disrupting overall heterotrophic activity when administered at 10^7 PFU/mL to activated sludge microcosms (Levy and Marshall 2004).

Moreover, implementing lytic phages that specifically bind to receptors on antibiotic efflux pumps or other proteins associated with antibiotic resistance could be a promising strategy for targeting ARB (Yu et al. 2017). For instance, the pathogenic bacteria *Acinetobacter baumannii*, causing nosocomial infections, was treated by phage AB2 isolated from hospital sewage, and it showed strong lytic activity against most of the *A. baumannii* strains (Lin et al. 2010). *Vibrio cholerae* infection is among the most common water-borne diseases caused by bacteria (Yen et al. 2017). A mathematical model was developed to explore the population dynamics of the bacterium and its phages; this model was based on culturing *V. cholerae* with its specific phages (Wei et al. 2011). The results revealed that phages efficiently combat *V. cholerae* and that the synergy between the two was more efficient than using a single phage. Another study conducted by Jun et al. could effectively treat dysentery, a major water-borne disease caused by *Shigella*, by combining two phages (pSf-1, pSb-1, and pSs-1) (Jun et al. 2016).

Phage therapy in biofilm removal

The formation of biofilms is a survival mechanism adopted by some bacteria during infections. Biofilm is a complex of numerous types of bacteria that act collectively to help the bacteria escape the antibacterial effects of antibiotics. This resistance arises from the synergistic action of the bacteria present in the biofilm community (Pires et al. 2017). Biofilms present an ideal growth environment for bacteria, primarily pathogens and ARB, because they protect the bacteria against disinfection and the effects of other antimicrobial agents. Furthermore, biofilms increase the frequencies of mutation and gene transfer within the bacterial population, which accelerates the emergence of antibiotic resistance (Molin and Tolker-Nielsen 2003). As a result, the Minimal Inhibitory Concentrations (MICs) of antibiotics required to inhibit biofilm-associated bacteria can increase up to 1000-fold more than those required for the inhibition of planktonic bacteria, making bacteria forming biofilms significantly more challenging to treat (Høiby et al. 2010). Moreover, in the wastewater treatment process, the presence of biofilms is a primary concern. The flow rate of wastewater passing through the membrane surface is significantly less than the regular flowrate without the biofilms, affecting the device's normal operation (Wu et al. 2017). Thus, finding new approaches to eliminate biofilms while avoiding their undesired consequences is necessary.

Phages help eradicate biofilm and biofouling in drinking water and wastewater treatment and distribution systems. They are very effective in biofouling control because they can control the structural bacteria that retain biofilm integrity (i.e., "keystone" species) (Bhattacharjee et al. 2015). In addition, phages were used as antibacterial agents to increase the net flow rate of wastewater, as biofilms can reduce the water flow rate through the devices' membranes. A study showed that using the lytic phage DTP1 isolated from a wastewater treatment system was highly effective in targeting *Delftia tsuruhatensis* biofilms. The results showed a 70% increase in the membrane flux (Bhattacharjee et al. 2015). In another study, *E. coli* phage P2 was administered to clean an ARB-contaminated nanocomposite membrane. The synergetic effect of combining a modified nanocomposite membrane with a phage showed promising results that could simultaneously bypass the problem of bacteria and their proteins and consequently improve the membrane flux (Ayyaru et al. 2018).

Using phage cocktails is essential in promoting the host range of phages, consequently improving biofilm removal (Mathieu et al. 2019). Applying nanoparticles to formed biofilms can enhance the efficiency of phages in removing biofilms and assist phages in reaching inaccessible locations in biofilms (Li et al. 2017). Generally, combining new technologies with phages to create a synergistic effect is a promising approach to control biofilm pollution in the future.

The role of phage lytic enzymes in eradicating biofilm

Bacteria can develop various mechanisms to evade phages' lytic and lysogenic effects. Therefore, phage lytic enzymes are used to combat bacteria and reduce biofilm formation (Knecht et al. 2020). Phage-derived enzymes, also known as enzybiotics, are divided into two major classes. The first class is the Peptidoglycan Hydrolases (PGHs), responsible for the peptidoglycan breakdown in the bacterial cell wall. The second class is the Polysaccharide Depolymerases (PSDs), which are responsible for the degradation of extracellular polysaccharides as slime layers, capsules, and biofilm (Knecht et al. 2020).

Phage-derived enzymes are characterized by their specificity and efficiency in targeting pathogens with a minimal risk of developing bacterial resistance. They facilitate phage entry into cells and can break down the chains of peptidoglycan, capsular polysaccharides, and exopolysaccharides, as shown in Fig. 2. While certain bacteria may include all these components in their capsules, many of them are essential components of biofilms (Knecht et al. 2020). Phages that encode these depolymerases may have better access

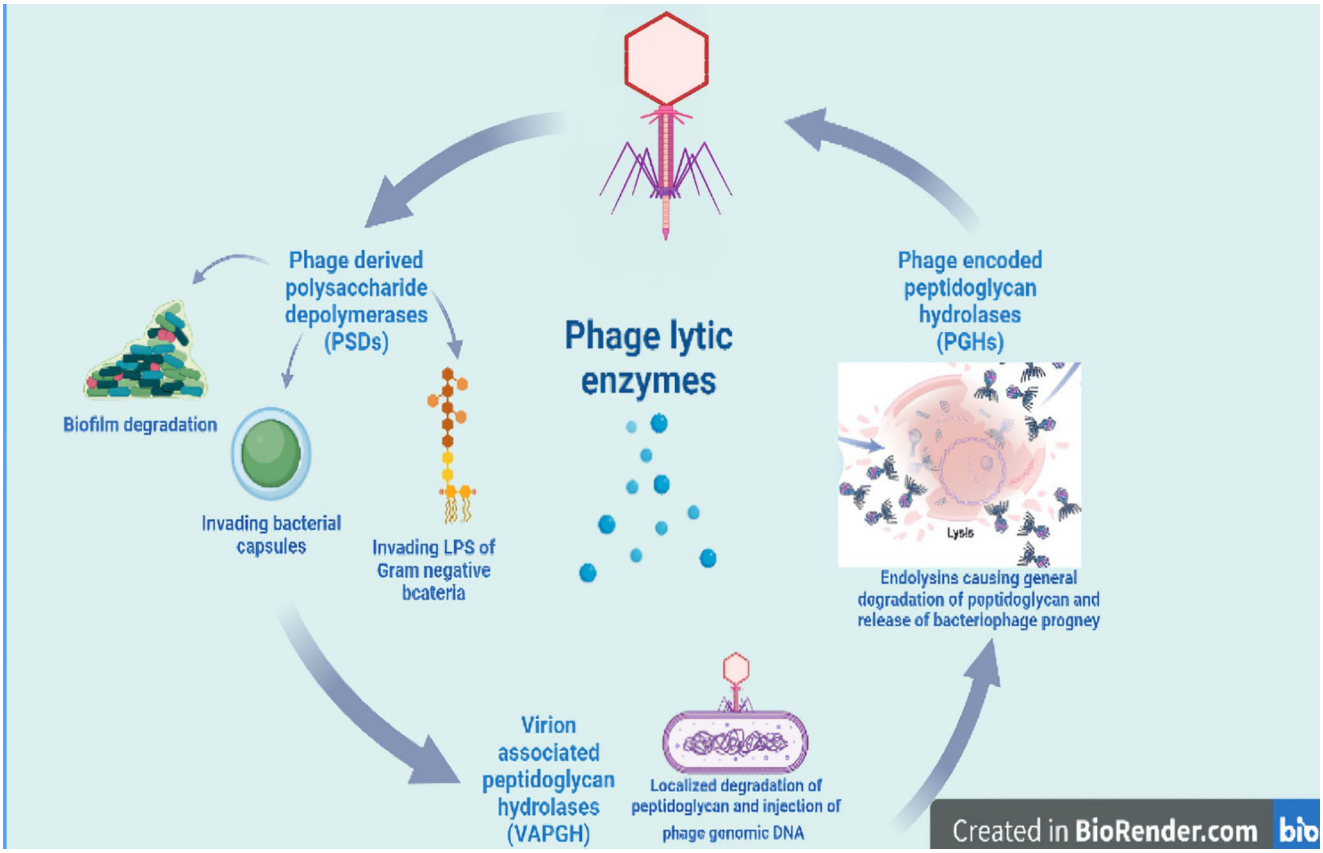

Fig. 2 Types of phage-lytic enzymes. Phage-derived polysaccharide depolymerase, virion-associated peptidoglycan hydrolases, and phage-encoded peptidoglycan hydrolases represent the three primary classes of enzymes involved in phage-mediated bacterial cell lysis

to the recipient bacterium, facilitating infection because of their capacity to break down the polysaccharides in biofilms and bacterial capsules. Depolymerase activity is especially interesting for removing biofilms since it modifies the EPS structure and reduces bacterial pathogenicity (Gutiérrez et al. 2015). A study has shown that Dpo7 (depolymerase) lowered biofilm density in *Staphylococcus* sp. by 53–85%. Another study showed that Dpo42 (depolymerase) was studied on *E. coli* and demonstrated biofilm inhibition (Pires et al. 2017). Furthermore, phage K1F produced endosialidases to break down *E. coli* polysaccharide capsules by cleaving α2,8 in sialic acid polymers (Scholl et al. 2005), and phage H4489A produced hyaluron lyases to degrade the hyaluronan capsule of *Streptococcus pyogenes*.

Phage therapy in activated sludge bulking treatment

In the process of water treatment, foam flocs can form while using an activated sludge system due to the overgrowth of filamentous microorganisms. The formation of foam can hinder the sedimentation of solid particles. Filamentous microorganisms can often cause the formation of viscous foams that are not easily removed (Aracic et al. 2015). The persistent existence of these foams affects the activated sludge system machinery and must be removed regularly. The removal method must ensure the presence of the microorganisms is sustained. Hence, using physical and chemical methods that might eliminate beneficial microorganisms must be excluded. Due to these reasons, the use of phage technology is proposed to deal with the problems of bulking and foaming (Mathieu et al. 2019).

In laboratory-scale investigations, foam stabilization was achieved using polyvalent bacteriophage GTE7, which lysed both *Gordonia* and *Nocardia* species (Petrovski et al. 2022). In a previous study, the filamentous bacterium *Haliscomenobacter hydrossis*, which causes sludge bulking problems, was treated by phage HHY. The study showed that batch reactors infected with a lytic phage identified from wastewater mixed liquor (phage HHY from the *Myoviridae* family, MOI = 0.001) removed 54% of the bulking bacterium *H. hydrossis*, leading to a faster sludge settling rate and a 33% lower sludge volume index. Moreover, the addition of the phage decreased neither the efficiency of chemical demand nor nutrition. There was also no harm to the other microorganisms existing in the system. This demonstrates

the good effect of using phages in sludge systems (Li et al. 2015). Additionally, *Gordonia* phages were isolated for use in a stimulated aeration tank system test in a study that showed that *Gordonia* phages could survive, along with reducing the volume of sludge sedimentation (Liu et al. 2015). This study proposed that phage efficiency is not limited to lab work but expands to aeration tank systems.

Phage therapy in wastewater quality assessment

Treated wastewater quality assessment is essential to indicate the suitability of reusing treated water according to global standards. Some standardized parameters are tested for quality assessment, including faecal coliform presence, pH, temperature, dissolved oxygen ratio, total solids, phosphates, turbidity, biochemical oxygen demands, and total nitrates (Şener et al. 2017). Faecal indicator bacteria (FIB) are used as contamination indicators, particularly *E. coli*. Regardless of its efficiency, performing basic tests to measure the concentration of FIB is time-consuming and does not provide scientists with accurate information on pathogenic bacteria (Khan and Gupta 2020).

Due to some deficiencies in the conventional monitoring methods, phage-based devices have been developed and shown significant potential for rapid assessment and real-time monitoring of pathogenic microorganisms in water. These devices have been introduced due to the phages' stability under environmental conditions and their high selectivity in recognizing viable cells from nonviable cells. One of the devices is a nanostructured electrochemical biosensor, through which phages are used as biorecognition elements as they are immobilized on the surface of the electrode to detect bacteria. Therefore, phage characteristics qualify this method for biorecognition (Zhou et al. 2017). Another currently used method is Electrochemical Impedance Spectroscopy (EIS), which could successfully detect *E. coli* and *S. aureus* bacteria using phage-based electrochemical bacterial detection (Richter et al. 2018; Zhou et al. 2017). In addition to this method, a phage-based electro-chemiluminescent biosensor has been developed and successfully detected *Pseudomonas aeruginosa* (Yue et al. 2017). Furthermore, using phages as capturing elements for pathogenic bacteria is a promising approach, as it combines them with specific synthesized nanoparticles to form bacteria-capturing conjugates. The superiority of this method stems from its fast performance as the detection assay can be done in only a few minutes (Richter et al. 2018).

Microfluidics has great potential as a field; one approach is using microfluidic chips engineered with phages. Those chips are advantageous as they are rapid and highly selective in detecting pathological bacteria (Dow et al. 2018). A recommendation was presented in another study to combine the use of microfluidics with phage-based electrochemical sensors to create a synergistic effect (Ji et al. 2021). Another rapid detection method that can improve the detection limit is magnetic separation. One of its major advantages is detecting bacteria in complex samples and separating them with a magnet to reduce matrix effects on signal detection (Richter et al. 2018). The application of this concept involved using T7 phage, which was conjugated to Magnetic Beads (MBs) to capture and isolate *E. coli* BL21 from water samples (Chen et al. 2015). Phage-MBs capture bacterial cells before bacterial cell lysis, and then bacteria are suspended in a buffer. Following the cell lysis process, the release of β-galactosidase (β-gal) occurred, and its presence was subsequently detected using a colorimetric substrate (Chen et al. 2015). Additionally, phage-based magnetic separation was coupled with q-PCR to capture, quantify, and separate *E. coli* cells from water with a concentration of up to 100 CFU/mL in 2 h without the need for a pre-enrichment step (Wang et al. 2016).

Challenges and possible solutions to phage-based microbial control

Efficient bacterial control is achieved by delivering the appropriate phages to the site of pathogenic bacterial infections. The target pathogenic and MDR bacteria could exist in hard-to-reach locations, including biofilms, sludge flocs, and microcolonies that affect the application of phage therapy in water systems. The current primary challenges facing phage application in water systems are further discussed below.

Identifying the target bacteria

The host range of phages is considered narrow, so phylogenetic identification and discernment of the target bacteria should be well-established to identify the appropriate phages. Recently, gene sequencing and culture-independent techniques were improved to analyze almost the entire 16 S rRNA gene and assign the bacteria taxonomy down to the species level (Paul 2023). Bacterial species found mixed with large communities could be identified using further advanced techniques such as metagenomics and chromosome conformation capture. These advanced techniques identify the pathogenic bacteria without the knowledge or isolation required of the community composition (Emerson et al. 2010).

Narrow host range

Depending on the number of infected bacteria, the phage host range could be narrow or broad (Yu et al. 2017). Most phages exhibit narrow specificity, which protects the beneficial and non-pathogenic bacteria from being killed during the treatment. On the other hand, phage specificity could be disadvantageous when it limits its utility to a small number of possible pathogens, including many taxonomically distant species, and requires more attacking phages. Additionally, the narrow host range makes it difficult and hazardous to scale up the isolation and production of phages targeting the pathogenic bacteria (Reyneke et al. 2024; Davis et al. 2005). However, new approaches were established to isolate polyvalent phages that are isolated using non-pathogenic and fast-growing bacteria. Such polyvalent phages are more resistant to environmental stresses and can infect bacteria from different genera or species at the same time (Zhao et al. 2019; Yu et al. 2017).

Bacterial resistance to bacteriophage

Bacteria may develop resistance to the attacking phages via various methods, such as preventing phage attachment and nucleic acid penetration, breaking phage genetic material, and inhibiting the phage life cycle (Reyneke et al. 2024). Phage cocktails are made up of different individual phages that target multiple receptors on the bacterial host and counteract the resistance mechanisms that bacteria develop against phages (Labrie et al. 2010). To prevent total phage resistance, it is crucial to comprehend the bacterial resistance mechanisms while trying to construct phage cocktails for application purposes. Restriction-modification systems defend bacteria against incoming foreign DNA, including phage DNA. However, some phages can modify their DNA through methylation to evade endonuclease digestion (Caflisch et al. 2019; Samson et al. 2013).

The Defense Island System Associated with Restriction Modification (DISARM) is a bacterial phage defense system that consists of five gene cassettes (defense islands), each of which contains the genes for a DNA methylase, a protein containing the helicase domain, a protein containing the Phospholipase D (PLD) domain, a protein containing the DUF1998 domain, as well as a gene with an unidentified function (Azam and Tanji 2019; Ofori et al. 2018). The double-stranded DNA phages commonly found in activated sludge appear to be the most susceptible to this DISARM defense mechanism. There have been reports of additional defense mechanisms that defend certain bacteria from phage invasion. For instance, the defense mechanism known as Bacteriophage Exclusion (BREX) consists of six gene cassettes that inhibit phage replication; the genome of *Gordonia pseudamarae* is known to have a BREX system (Petrovski et al. 2022). Therefore, studies have shown that microbial biocontrol can be further enhanced by using polyvalent phages, and the phage genomes of virulent genes should be well-screened to decrease the frequency of bacterial phage resistance development (Nale et al. 2016).

Genes transduction

The ability of therapeutic phages to transduce bacterial genes, such as virulence factors, toxins, and ARGs, should be avoided. The phage's genome packing method appears to be essential for generalized transduction (Reuter and Kruger 2020). It is believed that phages that initiate packing from non-specific or low-specificity DNA sequences will also incorporate foreign genetic material during maturity. Some phages, such as classical T3 and T7, need specific sequences in their genomes to start DNA packaging; as a result, they shouldn't be able to bind to and incorporate foreign genes. Furthermore, phages that break down the infected cell's DNA and use it as building blocks for their genome's replication should be regarded as non-transducers (Lobocka 2014). Microbiological approaches, PCR-based techniques, sequencing, and bioinformatic prediction tools can all be used to identify generalized transducing phages (Lobocka 2014). When a prophage is induced during specialized transduction, extra packaging of nearby bacterial genes into the phage particles may result. As a result, only temperate phages can perform specialized transduction; these phages should not be used therapeutically (Zalewska-Piątek 2023; Abdelsattar et al. 2022; Reuter and Kruger 2020).

Phage inactivity in the harsh environmental conditions

Environmental stresses may destroy or inactivate phages before they attach to the target bacteria. Phages are mainly inactivated due to structural protein degradation, phage genome damage, or envelop lysis. Phages could be protected in liposomes or encapsulated with materials, such as alginate-chitosan, to overcome inactivation in harsh environmental conditions (Chaudhry et al. 2015). In addition, bacterial spores can be used as protective shells to shield phage nucleic acid against extreme conditions, including desiccation, pH, UV radiation, and inappropriate temperatures. Polyvalent phages are highly recommended in these harsh conditions (Reyneke et al. 2024; Malik et al. 2017).

Conclusion

Conventional methods for wastewater treatment are becoming ineffective and can increase antibiotic resistance in the environment. Bacteriophage-based therapy is a promising alternative for managing microorganisms in water treatment, specifically targeting MDR bacteria in WWTPs. However, harsh environmental conditions and unpredictable situations may affect phage titers and cause bacterial resistance to phages. Therefore, the conditions for phage application should be optimized, and the titer of phages released into the environment should be well-monitored to determine the best time and delivery dose. Additionally, phage cocktails can be used to overcome the resistance of host bacteria and broaden the host range by combining multiple phages with complementary characteristics. Integrating phage therapy into wastewater treatment could be an important solution for enhancing microbial management, combating antibiotic resistance in the ecosystem, and promoting environmental sustainability. This proactive approach reduces reliance on chemical treatments and is crucial for a sustainable future.

Acknowledgements Thanks are to the Egyptian Sciences and Technology Development Fund (STDF), which funded this research under a grant number #41909.
Geolocation information This research study was conducted at Center for Microbiology and Phage Therapy, Zewail city for science and technology, October Gardens, First 6th of October, Giza Governorate-12578 (location on Google map: W3V7 + XH First 6th of October).

Author contributions Conceptualization: Ayman El-Shibiny and Samar Ragab. Writing - original draft: Samar Ragab, Mohamed Kamal Mustafa, Yara Y. Hassan, Alaa Nasr, and Bassant H. Abd El Hady. Writing - review & editing: All authors. Supervision: Ayman El-Shibiny. Project administration: Ayman El-Shibiny. Funding acquisition: Ayman El-Shibiny. All authors read and approved the final version of the manuscript.

Funding Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). This research was funded by the Egyptian Sciences and Technology Development Fund (STDF), under Grant #41909.

Data availability No data was used for the research described in the article.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate The authors agree to participate.

Consent for publication The authors agree to submit the article.

Generative AI in scientific writing The authors declare no generative AI in scientific writing.

Conflict of interest The authors declare no competing interests.

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