RESEARCH ARTICLE

Bacteriophages in water pollution control: Advantages and limitations

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GRAPHIC ABSTRACT

ABSTRACT

Wastewater is a breeding ground for many pathogens, which may pose a threat to human health through various water transmission pathways. Therefore, a simple and effective method is urgently required to monitor and treat wastewater. As bacterial viruses, bacteriophages (phages) are the most widely distributed and abundant organisms in the biosphere. Owing to their capacity to specifically infect bacterial hosts, they have recently been used as novel tools in water pollution control. The purpose of this review is to summarize and evaluate the roles of phages in monitoring pathogens, tracking pollution sources, treating pathogenic bacteria, infecting bloom-forming cyanobacteria, and controlling bulking sludge and biofilm pollution in wastewater treatment systems. We also discuss the limitations of phage usage in water pollution control, including phage-mediated horizontal gene transfer, the evolution of bacterial resistance, and phage concentration decrease. This review provides an integrated outlook on the use of phages in water pollution control.

1 Introduction

Human demand for safe drinking water is rising, but water resources are limited worldwide. To alleviate this disparity, effective treatment and reuse of wastewater are becoming increasingly important. The existence of pathogenic bacteria and viral pathogens in wastewater poses a threat to human health and has become a major public health issue (Mathieu et al., 2019). Residual antibiotics may be discharged into wastewater from hospitals without treatment, creating selection pressure on the microorganisms in the water and accelerating the development of antibiotic-resistance genes (ARGs) (Rodriguez-Mozaz et al., 2015).

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Hence, monitoring water quality and wastewater treatment is particularly important. At present, antibacterial agents are still widely used in many areas to eliminate some microorganisms in wastewater. These agents not only harm some benign bacteria but also cause antibiotic resistance in some pathogenic bacteria (Deng et al., 2018). Therefore, antibacterial agents are becoming increasingly ineffective, and the wastewater treatment process employing them has an unpredictable impact (Mathieu et al., 2019). More precise and efficient methods need to be developed and used in water pollution control.

Phages are some of the most abundant organisms on Earth. It is estimated that the total number of phages in the biosphere is $\geqslant 10^{31}$ (Batinovic et al., 2019). Phages usually accompany their host bacteria in a variety of environments. They can interact with bacterial surface receptors to identify unique regions and thus infect specific hosts. When a lytic phage invades its host, its genome is first injected; then, progeny phages are produced through bacterial metabolic systems (Batinovic et al., 2019). These progeny phages can infect and kill new hosts. As a biological control method, phage therapy employing lytic phages has made breakthrough progress in agronomy, pharmacy, medicine, and other fields (Doss et al., 2017). Inspired by these studies, more researchers are opting to use phages to solve the problem of water pollution (Mathieu et al., 2019). Compared with other wastewater treatment methods, phage usage has the following advantages: (1) Specificity: Unlike antibacterial agents, phages are highly specific for their host and do not damage beneficial bacteria. (2) Sustainability: Many progeny phages can be released after phages infection. (3) High efficiency: Phages can rapidly lyse host bacteria.

In this Review, we explore the potential applications and limitations of phage usage in water pollution control. We examine the role of phages in monitoring pathogens, tracking pollution sources, treating pathogenic bacteria, infecting bloom-forming cyanobacteria, and controlling bulking sludge and biofilm pollution in wastewater treatment systems (Fig. 1). We then point out some limitations in the practical application of phage-based technology and provide corresponding solutions.

2 Applications of phage-based technologies in wastewater

2.1 Monitoring water pollution using phage-based technology

2.1.1 Monitoring pathogenic bacteria in wastewater

Water quality is gradually becoming a focus of attention in research. For water quality, the assessment of some parameters, such as pH, temperature, turbidity, fecal coliforms, dissolved oxygen, total phosphates, biochemical oxygen demand, total solids, and nitrates is considered a standardized method for comparing water sources in different regions (Şener et al., 2017). Reproduction and
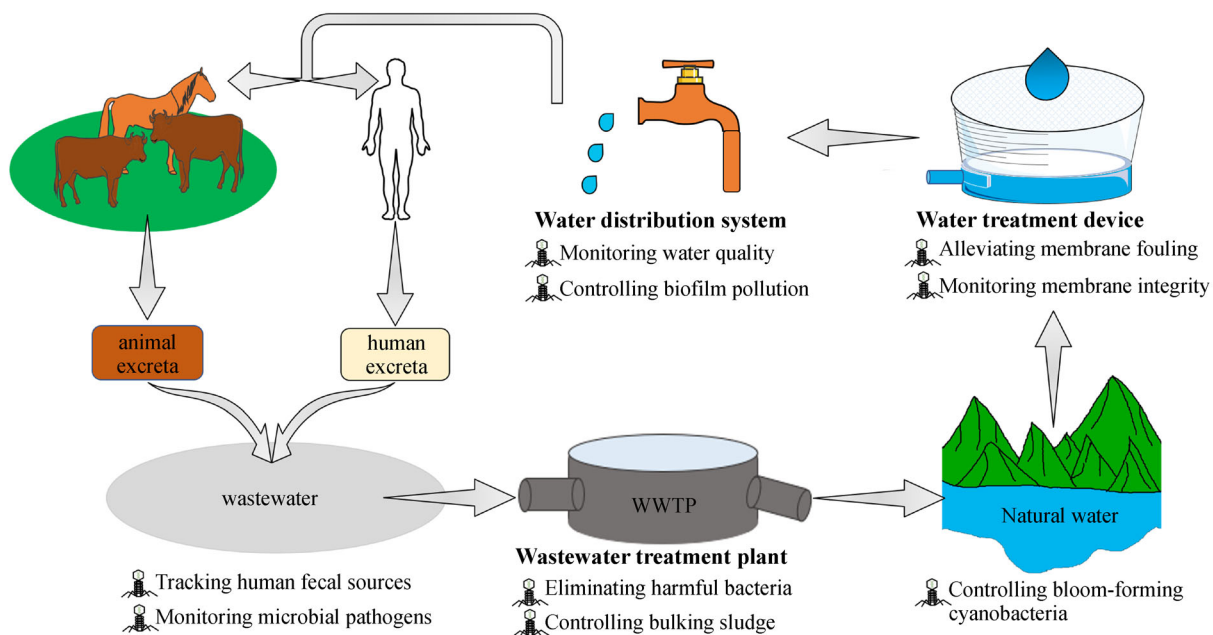

Fig. 1 The advantages of phages in controlling wastewater contamination.

dispersion of pathogens in water caused by fecal contamination is a major infection risk (Khan and Gupta, 2020). Because millions of people die from waterborne diseases every year, a safe, simple, and effective method is needed to evaluate water quality and prevent the spread of diseases. Indicator properties mainly include the following: (1) the indicator organism is more tolerant to disinfectants as well as other treatment conditions than pathogenic bacteria or enteric viruses; (2) the indicator organism is able to grow on a relatively cheap culture medium; and (3) the indicator organism can be quickly identified (de Brauwere et al., 2014). Fecal indicator bacteria (FIB), particularly *Escherichia coli*, are successfully and widely used indicators (Khan and Gupta, 2020). However, it often takes a long time to determine the concentration of FIB through basic experiments, and accurate information on pathogenic bacteria cannot be obtained (Khan and Gupta, 2020). Recently, some phage-based devices have shown great potential for rapid response and real-time monitoring of pathogenic bacteria.

In the electrochemical biosensor method, phages are immobilized on an electrode surface and used as biorecognition elements to detect bacteria. Phages are readily available and are more resistant to harsh environments. More importantly, their high selectivity can distinguish viable cells from nonviable cells. All these factors make phages an ideal biorecognition element (Zhou et al., 2017). At present, electrochemical impedance spectroscopy (EIS) is a commonly used method for phage-based electrochemical detection of bacteria. The detection of *E. coli* and *Staphylococcus aureus* was successfully realized using this method (Zhou et al., 2017; Richter et al., 2018). The principle of EIS involves the immobilization of phages on graphene electrodes and the measurement of their response according to charge transfer resistance (R_{ct}). EIS can provide continuous and stable detection over a wide detection range with fast response. In addition to the commonly used EIS method, the phage-based electrochemiluminescent biosensor has also been developed and applied for detecting *Pseudomonas aeruginosa* (Yue et al., 2017). Another promising method for bacterial pathogen detection using phages involves the use of phages as capture elements. By combining phage particles with specific nanoparticle, bioconjugates are obtained for detecting bacteria. Compared with traditional technology, this method takes only several minutes to complete the detection assay (Richter et al., 2018). Here, we also need to mention the potential of microfluidics. Microfluidic chips based on engineered phages have high sensitivity and rapidly detect clinical pathogenic bacteria (Dow et al., 2018). In the future, combining microfluidics with phage-based electrochemical sensors may be a major breakthrough in the detection of pathogenic bacteria.

2.1.2 Monitoring viruses in water and wastewater

The global outbreak of the novel coronavirus (SARS-CoV-2) has attracted scientists' attention, and a potential fecal-oral transmission route has been proposed (Gu et al., 2020). In general, FIB concentrations are used to indicate the presence of fecal contamination. However, the latest evidence shows that there is no significant correlation between FIB concentration and some pathogenic microorganisms, including enteric viruses (Moazeni et al., 2017). As we know, the major pathogens causing waterborne diseases are enteric viruses (Bányai et al., 2018). If an indicator cannot ensure the absence of enteric viral hazards, the purpose of water quality monitoring is not achieved. In this context, phages have been widely studied as indicator organisms for enteric viruses in wastewater treatment systems (McMinn et al., 2017; Dias et al., 2018). Membrane filtration is an important process in water and wastewater treatment. As enteric virus surrogates, phages can be used to better assess membrane performance and monitor membrane integrity. This has been reviewed previously (Wu et al., 2017). As an enteric viral indicator, phages have the following characteristics: (1) their morphology and biological properties are similar to those of enteric viruses; (2) like enteric viruses, they exist in human or animal feces; (3) compared with human enteric viruses, phages show lower average $\log_{10}$ reduction values (LRVs) and longer survival times during water treatment and under environmental pressure; (4) their culture is easier and faster than that of enteric viruses; and (5) their use is simple and safe (McMinn et al., 2017).

Currently, phage indicators are mainly divided into three groups: *E. coli* phages, *Bacteroides* spp. phages, and *Enterococcus* phages (Table 1). Previous studies have shown that the fate and transport behaviors of phages, as indicators of pathogens in water, are more similar to those of enteric viruses than to those of FIB (Dias et al., 2018). Therefore, the primary focus of current research on wastewater treatment is to evaluate these potential phage candidates. Relative abundance and tolerance are key factors for this evaluation. *Escherichia coli* phages are, more specifically, somatic coliphages (SOMPH) and F-RNA coliphages (F-RNAPH). As viral indicators, they are often the subject of discussion. In one study, the fates of three potential phages, a human-specific phage that infects *Bacteroides fragilis* (Bfl24PH), F-RNAPH, and SOMPH were monitored in multiple wastewater treatment systems. The results revealed that SOMPH had a higher detection rate, higher concentration, and better tolerance compared with the other phages (Dias et al., 2018). However, according to a meta-analysis, the average LRVs of some phages (SOMPH, F-RNAPH, MS2 coliphage, and T4 phage) were slightly different. Although the LRVs of SOMPH were lower than those of F-RNAPH, the LRVs of

Table 1 Phages commonly used to evaluate water quality

Phages	Monitoring water quality	Tracking human faecal sources	Reference
Coliphages	Somatic coliphages (e.g. phages of *E. coli* strain WG5)		Dias et al., 2018
	F-RNA coliphages (geno-groups I is more accurate.)	F-RNA coliphages (geno-groups II and III)	Hagedorn et al., 2011; Haramoto et al., 2015; Amarasiri et al., 2017
Bacteroides phages	*B. fragilis* (e.g. RYC2056, GB124) phages	*B. fragilis* (e.g. GB124, and HSP40) phages	Jofre et al., 2014; Diston et al., 2015; Hodgson et al., 2017; McMinn et al., 2017
	B. thetaiotaomicron (e.g. GA-17 and ARABA 84) phages	*B. thetaiotaomicron* (e.g. GA-17 and ARABA 84) phages	Jofre et al., 2014; Diston and Wicki, 2015
	CrAssphage	CrAssphage	Ahmed et al., 2018; Wu et al., 2020
	Phages of *Bacteroides* HB-73	Phages of *Bacteroides* HB-73	Vijayavel et al., 2010
Enterococcus phages	*E. faecalis* (e.g. AIM06 and SR14) phages	*E. faecalis* (e.g. AIM06 and SR14) phages	Wangkahad et al., 2017; Chyerochana et al., 2020
	E. faecium (e.g. ENT-49 and ENT-55) phages	*E. faecium* (e.g. MW47) phages	Vijayavel et al., 2014; Purnell et al., 2018

both phages were higher than those of norovirus GII and enteroviruses. Only the MS2 coliphage had lower LRVs than those of norovirus GII and enteroviruses, suggesting that it is suitable as a validational and operational monitoring indicator (Amarasiri et al., 2017). Another test on the resistance of four F-RNAPH genogroups (GI–GIV) during wastewater treatment showed the same results; only GI F-RNAPH had better tolerance than enteric viruses (human adenoviruses and GI and GII noroviruses). Thus, GI F-RNAPH were considered the best indicator of the fate of virus pathogens (Haramoto et al., 2015). However, the number of F-RNAPH is lower than that of SOMPH in most water environments, which may be a limiting factor (Jofre et al., 2016). In this case, the simultaneous detection of two phages is the best choice. One method for this involves the construction of the *E. coli* strain CB390 by introducing the plasmid Famp into *E. coli* WG5 (the strain recommended by the International Organization for Standardization to detect SOMPH). *Escherichia coli* CB390 can detect not only F-RNAPH and SOMPH simultaneously but also a higher number than other strains used for this purpose (e.g., the C-3000 strain) (Guzmán et al., 2008). Another limitation of coliphages as indicators is long incubation times (>8 h to yield results). A recently described procedure, named *Bluephage*, provides some insight. First, an *E. coli* strain with the overexpression of β-glucuronidase and knocked-out *uidB* and *uidC* (the key genes for transporting the glucuronic acid in cells) was constructed. During SOMPH infection, lysed host cells release β-glucuronidase. This enzyme can interact with the substrate previously added to the medium, resulting in localized color changes and thus rapidly detecting SOMPH under 3.5 h (Muniesa et al., 2018). Based on the *Bluephage* approach, the newly constructed *E. coli* strain CB12 (*E. coli* CB390 is the original strain) can be used to detect F-RNAPH and SOMPH simulta-

neously in different samples through the expression of F-pili. Compared with those of the wild-type strain CB390, the detection result and speed of the *E. coli* strain CB12 were significantly improved (Toribio-Avedillo et al., 2019). Although many studies on *Enterococcus* phages (e.g., phages of *E. faecalis* AIM06 and SR14) have also shown that they have high concentrations and strong tolerance, most of them were limited to tropical or subtropical regions (McMinn et al., 2017; Wangkahad et al., 2017). Therefore, some research needs to be carried out in more regions to further evaluate them.

Other researchers have also noted that a virus widely existing in the human gut metagenomes, cross-assembly phage (crAssphage), can be a reliable indicator (Dutilh et al., 2014). One crAssphage (ΦCrAss001) has been isolated from human fecal material and identified as a phage of *Bacteroides intestinalis* (Shkoporov et al., 2018). In the following, we list several advantages of crAssphage as a virus indicator, which have been confirmed by some studies: (1) it has higher concentrations than some enteric viruses in sewage and can be detected in sewage almost worldwide (Edwards et al., 2019; Bivins et al., 2020; Farkas et al., 2020); (2) it has a stronger environmental durability than FIB, allowing it to more accurately reflect virus removal rates (Wu et al., 2020); (3) it is closely related to some human viral pathogens (human adenovirus and human polyomavirus) in sewage treatment systems, which allows it to better reflect fecal pollution (Crank et al., 2020; Wu et al., 2020). However, obtaining a pure culture of crAssphage from environmental samples is difficult, which also makes its detection reliant on molecular methods, such as qPCR (Bivins et al., 2020). Table 2 shows primers and probes for detecting and quantifying crAssphage via qPCR assay. Although molecular methods have high sensitivity and specificity, they cannot distin-guish between infectious and nonviable viruses during the

Table 2 Primers and probes for crAssphage for the qPCR assay

qPCR Assay	Primer/Probe	Sequence 5′ - 3′	Reference
CPQ_056	Forward primer	CAGAAGTACAAACTCCTAAAAAACGTAGAG	Stachler et al., 2017; Wu et al., 2020
	Reverse primer	GATGACCAATAAACAAGCCATTAGC	
	Probe	[FAM]AATAACGATTTACGTGATGTAAC[MGB]	
CPQ_064	Forward primer	TGTATAGATGCTGCTGCAACTGTACTC	Stachler et al., 2017
	Reverse primer	CGTTGTTTTCATCTTTATCTTGTCCAT	
	Probe	[FAM]CTGAAATTGTTCATAAGCAA[MGB]	
	Forward primer	AGGAGAAAGTGAACGTGGAAACA	García‑Aljaro et al., 2017
	Reverse primer	AACGAGCACCAACTTTAAGCTTTA	
	Probe	[FAM]AGGATTTGGAGAAGGAA[MGBNFQ]	
	Forward primer	GAATCTAAAGGTGCTCTTAATCCTATGAT	Liang et al., 2018
	Reverse primer	CCTACATTTTGAGTAAGACAAAAGTCAAG	
	Probe	[FAM]TGCCTATTGTTGCTCAAG[MGB]	
	Forward primer	GGTAAGAATATTACTGAATATCCTACTTG	Cinek et al., 2018
	Reverse primer	CAATCATGTTCATCAATAAAYGCTTCA	
	Probe	[FAM]ATGATATTAATTATCTTACTGGAGATGAACCTACAAGACAAAC[BHQ]	

detection process (Hamza and Bibby, 2019). Thus, there is a need to improve the molecular technology or to find a standardized culture method to better evaluate crAssphage as a virus indicator.

2.1.3 Tracking human fecal sources

Pathogenic microorganisms in the water are mostly transmitted through human excreta rather than through animal excreta (Soller et al., 2010). These microorganisms endanger public health safety. Therefore, it is especially important to track the source of human fecal waste. By identifying the source of fecal pollution, we can more accurately conduct risk assessments and specify management strategies (Soller et al., 2010). Microorganisms can be used for this purpose, known as microbial source tracking (MST). Some indicator microorganisms cannot provide accurate information on pollution sources; therefore, human-specific MST markers are urgently needed. Current bacterial markers (e.g., *Bacteroides* HF183) lack host specificity, whereas low concentrations of viral markers (e.g., human adenovirus and human polyomavirus) have insufficient sensitivity (Ahmed et al., 2018). Therefore, more attention needs to be focused on the search for ideal markers.

In recent studies, phage markers have shown higher specificity and sensitivity. Currently, there are three main human-specific MST markers based on phages (Table 1). The first is F-RNAPH, a type of RNA virus. F-RNAPH resemble the norovirus and hepatitis A virus. FRNAPH are mainly divided into four geno-groups (I–IV). Only geno-groups II and III are mostly detected in human excreta (Hagedorn et al., 2011). The second is a phage infecting specific *Bacteroides* strains. *Bacteroides thetaiotaomicron* GA-17 and *Bacteroides fragilis* GB124 exist only in human excreta. *Bacteroides fragilis* RYC2056 is widely expressed in human and animal excreta. Phages infecting GA-17 or GB-124 are considered to indicate human excreta, whereas broad-host-range phages are tested for tracking human and animal excreta (Jofre et al., 2014; Farkas et al., 2020). CrAssphage is a *Bacteroides* phage highly abundant in human feces. Although crAssphage is also found in some samples contaminated with animal feces, higher amounts of crAssphage is present in samples with human fecal pollution (García‑Aljaro et al., 2017). To verify whether crAssphage is suitable for source discrimination, molecular qPCR assays were performed to compare crAssphage with existing bacterial markers (such as HF183/BacR287). The concentrations of crAssphage and these bacterial markers in the same sewage samples were similar, but crAssphage had certain advantages in terms of specificity and accuracy. In multiple studies, crAssphage exhibited high sensitivity (100%) (Stachler et al., 2017; Ahmed et al., 2018). This ideal sensitivity, high specificity, and abundance compels us to believe that it has great potential. The third type of human-specific MST markers is the *Enterococcus* phage, which has been successfully used in many areas to track pollution sources (Purnell et al., 2011; Wangkahad et al., 2017; Chyerochana et al., 2020). In addition to high specificity and sensitivity, the culture technique of *Enterococcus* phages is relatively simple and economical (Wangkahad et al., 2017). Thus, *Enterococcus* phages may be more suitable as MST tools in some remote areas. Because there are regional differences in the species and abundance of human intestinal microorganisms, it is best to choose a

suitable MST tool according to the region. Additionally, the combination of multiple MST tools often provides more information (Ahmed et al., 2018).

2.2 Active control of harmful bacteria by phage-based technology

2.2.1 Treatment of pathogenic bacteria found in wastewater

The shortage of water resources is an urgent problem to be solved. The safety of drinking water is also receiving more attention. It has been reported that about one-ninth of the people in the world have no access to safe drinking water (Jassim et al., 2016). A major reason is the large number of pathogens in sewage, which spread through water and lead to the outbreak of various infectious diseases. At present, the advanced technology of wastewater treatment systems is highly effective for the removal of pathogenic bacteria, but it is difficult to achieve a widespread use of these systems in most developing countries. High-throughput sequencing provides a way to understand the composition of microbial communities in sewage, and it was found that the phage community is an important component (Li et al., 2015). As safe, efficient, and low-cost biological control agents, phages can be used to treat specific pathogenic bacteria in sewage. Phage-based technology also reduces the use of chemical reagents (Mathieu et al., 2019).

Isolation of specific phages for specific pathogens is important. Table 3 summarizes some isolated, effective phages. Some relatively important bacterial pathogens are highlighted, such as *Acinetobacter baumannii*, which is an important agent of nosocomial infections. However, few researchers have noticed the spread of this clinical pathogen in sewage. ΦAB2 is a phage isolated from hospital sewage. It exhibits strong lysis and can infect most of the known *A. baumannii* strains (Lin et al., 2010). Therefore, it is considered an important means to eliminate drug-resistant *A. baumannii*. According to data, water-borne diseases are mainly caused by microbial pathogens, among which *Vibrio cholerae* is important (Yen et al., 2017). After a continuous culture of *V. cholerae* and its specific phages, a mathematical model was established to explore the population dynamic changes of phages and bacteria (Wei et al., 2011). The results revealed that phages are a good tool to limit the *V. cholerae* flora, and a mixture of two types of phages had a better effect than a single phage. Another major disease is dysentery caused by *Shigella*, which only requires a very low dose to infect humans. The combination of three phages (pSf-1, pSb-1, and pSs-1) can effectively inhibit *Shigella* infection (Jun et al., 2016). Some bacteria have evolved a series of mechanisms to resist phages. Thus, more effective methods are required to deal with the problem of bacterial resistance. We will discuss this in detail in section 3.2.

2.2.2 Controlling bloom-forming cyanobacteria by cyano-phages

Cyanobacteria are prokaryotes that often cause water blooms and green or red tides. Bloom-forming cyanobacteria can produce cyanotoxins, which are harmful for the surrounding wildlife and aquatic farming animals, and

Table 3   Isolation of phages for specific pathogens

Isolated phages	Sample source	Classification of phages	Pathogenic bacteria	The effect achieved	Reference
Coliphages	Zayandehrood River	*Podoviridae* *Myoviridae*	*Escherichia coli*	A mixture of coliphages can reduce the most probable number (MPN) of coliforms in sewage by 22 times.	Maal et al., 2015
AB2	Wastewater and raw sewage	*Podoviridae*	*Acinetobacter baumannii*	AB2 can make *A. baumannii* strains almost completely lysis.	Lin et al., 2010
pSs-1	Environmental water in South Korea	*Myoviridae*	*Shigella*	The combination of phages can control all strains of *Shigella*.	Jun et al., 2016
ICP1 ICP2 ICP3	Stool samples from Bangladeshi cholera patient	*Myoviridae* *Podoviridae* *Podoviridae*	*Vibrio cholerae*	A mixture of three ICP phages can target the elimination of *Vibrio cholerae* and provide a phage cocktail therapy to prevent cholera.	Yen et al., 2017
sww297 sww65 sww275	Raw wastewater from Tunisia	*Podoviridae* *Siphoviridae* *Siphoviridae*	*Salmonella*	A mixture of three phages can eliminate *Salmonella*.	Turki et al., 2012
LK1	Sewage	*Podoviridae*	*Citrobacter freundii*	LK1 have a relatively narrow host range and better tolerance, which make it control the growth of *Citrobacter freundii* well.	Chaudhry et al., 2014
MAG1 MAG4	Sewage from WWTP	*Myoviridae* *Podoviridae*	*Pseudomonas aeruginosa*	A mixture of MAG1 and MAG4 can lyse almost all bacterial cells.	Kwiatek et al., 2017
vB_Klp_5 vB_Klox_2	Sewage from Mtkvari River	*Podoviridae* *Podoviridae*	*Klebsiella*	One or more phages were capable of lysing a large part of strains.	Karumidze et al., 2013

threaten human health through its accumulation in the food chain (Jassim and Limoges, 2013). The excessive presence of these cyanobacteria in the aquatic environment has caused huge economic losses, prompting the search for more effective methods to control them. As viruses that infect cyanobacteria, cyanophages have strong specificity and high lysis efficiency; thus, attracting the attention of many researchers. In recent years, many cyanophages have been successfully isolated, such as PaV-LD, and Ma-LMM01 (Gao et al., 2012; Yoshida-Takashima et al., 2012). Remarkably, a homologous gene of cyanobacterial *nblA* (a key gene for degrading phycobilisomes) was found in some cyanophages (Gao et al., 2012; Yoshida-Takashima et al., 2012). These cyanophages can significantly reduce phycobilisome proteins and destroy the thylakoid structure of cyanobacteria. They have shown good prospects for the control of harmful blooms.

However, there are some problems in the practical application of cyanophages. For example, the emergence of host-resistant mutants and reduction of cyanophage infectivity caused by sunlight irradiation (Jassim and Limoges, 2013). One possible solution is to mix a high concentration cyanophages with a buffer solution in a special capsule. When the cyanophages are released under pressure in water, they infect the surrounding cyanobacteria. Additionally, cyanobacteria infected by cyanophages are purposefully placed in the water. When cyanophages "break out of the shell," they have a stronger lysing ability, which allows them to bring under control some resistant cyanobacteria (Jassim and Limoges, 2017). However, it is unclear whether these approaches are affected by changes in the water flow and severe weather, and further research is needed to improve them. What is certain is that they are crucial for the biological control of cyanobacteria.

2.2.3 Treatment of activated sludge bulking and foaming

In the process of wastewater treatment using an activated sludge system, if filamentous microorganisms proliferate excessively, they can form flocs and cause sludge bulking that can destroy the sedimentation of solid particles. Additionally, owing to the presence of surfactant in the aeration tank, the growth of filamentous microorganisms often forms thick viscous foams that are difficult to remove (Aracic et al., 2015). The long-term existence of these foams greatly affects the operation of the activated sludge system. Chemical and physical methods may eliminate beneficial microorganisms in the sludge; therefore, they are not recommended. In this case, phage-based technology has been proposed as an attempt to deal with the sludge bulking and foaming problems (Mathieu et al., 2019).

Haliscomenobacter hydrossis is an important filamentous bacterium that causes sludge bulking. In a group of studies, the bacterium was mixed with normal sludge for a period of time. When phages specific to *H. hydrossis* were added to the sludge, the settling speed of sludge was increased, and the final settling height was lower than that without phage addition (Kotay et al., 2011). This demonstrates the potential of the phage to control sludge bulking. In addition, the removal efficiency of chemical oxygen demand and nutrition did not decrease significantly after the addition of the phages, and other microorganisms were not affected, further proving the feasibility of phage-based technology. Excessive proliferation of filamentous bacteria is considered the main reason for the foam problem (Liu et al., 2015). Polyvalent phage treatment is even more attractive because most foams contain at least two types of filamentous bacteria, which prevents the monovalent phage from achieving a good effect. Two phages, GTE7 and GTE2, which lyse multiple host bacteria, can pose a threat to the stable existence of foams (Petrovski et al., 2011a,b). This suggests that the screening polyvalent of phages as an effective method to control filamentous bacteria and bulking sludge should be widely studied. At present, the isolation of polyvalent phages is a thorny problem. In a study, two sequential multiple-host approaches were proposed (Yu et al., 2016). Unlike simultaneous multiple-host enrichment methods, sequential multiple-host approaches use only one host to enrich the phage in each step. The two sequential multiple-host methods proved to be more effective than previous methods in isolating polyvalent phages. Additionally, Liu et al. used isolated *Gordonia* phages to perform a test in a simulated aeration tank system and found that phages can not only survive stably but also significantly reduce the sludge sedimentation volume (Liu et al., 2015). This indicates that phages can survive and work not only in laboratory systems but also in aeration tank environments. However, a challenge in activated sludge is the decrease in phage concentration owing to off-target adsorption. In practical applications, this may be a common problem faced by phage-based technology. We will discuss this in Section 3.1.

2.2.4 Treatment of biofilm pollution using phages

In order to survive, some bacteria develop complex biofilm communities in natural environments and during infections. Biofilms are usually composed of many types of bacteria, and the synergistic effect can help them resist the invasion of antibacterial agents and other bacteria (Pires et al., 2017). This undoubtedly increases their stability and makes the traditional sterilization methods ineffective. Membrane bioreactors, which represent a new wastewater treatment technology, are also challenged by bacterial biofilms. The existence of bacterial biofilms causes the net flow rate of wastewater through the membrane surface to continuously decrease, significantly impacting the normal operation of the device (Wu et al., 2017). In recent years, phage-based technology has attracted the attention of

researchers, especially with the emergence of more and more antibiotic-resistant bacteria (ARB). The potential of phages in alleviating membrane fouling has been previously reviewed (Wu et al., 2017). Here, we discuss and propose methods that may help enhance the effectiveness of phages.

In one study, the biofilm formed by *Delftia tsuruhatensis* on a membrane filter was treated with the lytic phage DTP1 isolated from a wastewater treatment system. The results showed that the membrane flux increased by 70% (Bhattacharjee et al., 2015). The effect of phage DTP1 on controlling biofilm formation and increasing water flux was confirmed. In another study, *E. coli* phage P2 was used to clean up a nanocomposite membrane contaminated with ARB. The combination of phage and modified nanocomposite membranes can solve the fouling problem of bacteria and proteins simultaneously, thereby increasing the membrane flux. Additionally, it could be observed through scanning electron microscopy that phages effectively prevented the formation of biofilms on the membrane surface (Ayyaru et al., 2018).

The interaction between phages and bacteria on biofilms is a complex process. Bacteria can generate a variety of strategies to avoid phages-hunting, such as the use of extracellular polymeric substances. Currently, phages with the ability to encode polysaccharide depolymerases seem to easily find the host receptor, being more effective in controlling biofilms (Pires et al., 2017). The development of synthetic biology provides us with inspiration. An overexpressed DspB (a type of polysaccharide depolymerase) T7 phage was constructed and used to infect biofilms. The results showed that the engineered phage reduced bacterial biofilm cells by 97.997% and was more efficient than the wild-type phage (Lu and Collins, 2007). Genetically modified phages provide a new strategy to treat biofilm pollution, but they also have certain limitations. Limited knowledge of many phage genomes and their gene functions hinders the development of engineered phages to some extent (Motlagh et al., 2016). Fortunately, metagenomics can help us mine polysaccharide depolymerases. In addition, it is difficult for only one phage to effectively treat biofilm pollution. Phage cocktails and polyvalent phages are important means of expanding the phage host range, thereby improving the biofilm removal efficiency. This has been reviewed previously (Mathieu et al., 2019). There are also methods that can be combined with phages usage to control biofilms more effectively, such as quorum sensing inhibitors (QSIs), quorum quenching (QQ) enzymes, and nanotechnology. To our knowledge, the use of quorum sensing (QS) among bacteria to transmit molecular signals is important for biofilm formation. QSIs and QQ enzymes can inhibit signal synthesis or degrade signal molecules and thus interfere with QS. Furthermore, this strategy can also enhance bacterial susceptibility to phages, which can improve the efficacy of the phage-based technology (Rémy

et al., 2018). Other researchers have focused on applying nanoparticles to biofilms. The results showed that nanoparticles could effectively disperse biofilms and help phages to reach relatively inaccessible locations within biofilms, thus enhancing the efficacy of phages (Li et al., 2017). In general, a combination of these new technologies with phages may be an effective means to control biofilm pollution in the near future.

3 Limitations of phage-based technology in wastewater treatment

3.1 Phage concentrations decrease in practical applications

Accomplishments in phage-based technology have mostly been confined to the laboratory. In practical applications, unpredictable situations often affect the utility of phages. The harsh environment may cause damage to phages or off-target adsorption (Fig. 2). This is a unignorable problem for phage-based technology.

On the one hand, we need to monitor the concentration of phages released into the environment. By monitoring changes in kinetic curves of phages concentrations, we can determine the best time point and delivery concentration to guide the application of phages. On the other hand, we can develop various methods to delay the decrease in phage concentration. One method is to encapsulate phages into liposomes, which can prevent a decrease in phage concentration before reaching the target bacteria (Mathieu et al., 2019). Microfluidics can help to encapsulate phages of different sizes into liposomes with reasonable efficiency and uniform titer (Leung et al., 2018). Another study used bacterial endospore as a protective shell for phages. Compared with free phages, endospores showed higher resistance to harsh environments, such as high temperature and extreme pH (Gabiatti et al., 2018). In summary, more design schemes are needed in the future to solve the problem of the decrease in phage concentration in practical applications.

3.2 Evolution of bacterial immune systems

Under the pressure of phage infection, bacterial immune mechanisms continue to evolve, generating multiple resistance strategies. Currently, this is a major limiting factor in the application of phage therapy. The immune mechanisms evolved by bacteria are diverse, such as restriction-modification systems, CRISPR-Cas systems, and abortive infection anti-phage systems. Additionally, prophage-encoded defense systems can prevent infection by other lysogens (Hampton et al., 2020). Although phages generate multiple escape strategies during long-term interaction with bacteria, it is difficult for traditional phage therapy to produce optimum effects in the presence of these immune systems (Fig. 2).
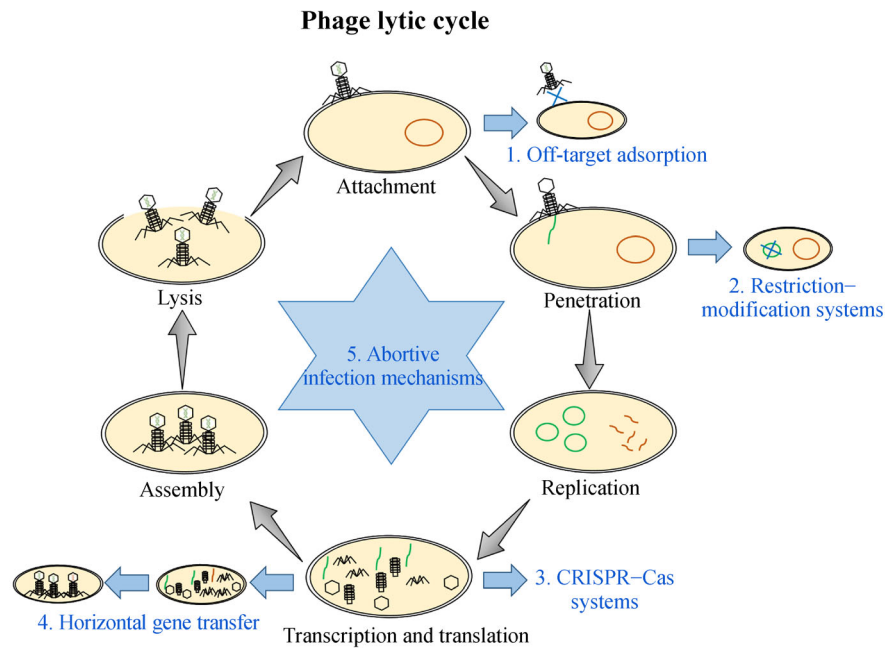
Phage lytic cycle

Fig. 2 The limitations of phages in controlling wastewater contamination. 1. Phage concentration decrease due to off-target adsorption. 2. Restriction-modification systems can recognize and destroy phage DNA. 3. CRISPR-Cas systems provide bacteria with adaptive immunity against phages. 4. Phages can transfer ARGs and virulence genes between bacterial communities. 5. Abortive infection mechanisms provide population protection in different stages of the lytic cycle.

Phage cocktails combine phages with complementary characteristics to expand the host range and reduce the resistance of host bacteria (Yen et al., 2017). In a study, polyvalent phage cocktails were used to treat ARB in activated sludge. Compared with narrow host range phage cocktails and single phages, these cocktails had a better inhibitory effect (Yu et al., 2017). Additionally, it was more difficult for bacteria to develop resistance to them. In future research, the formula and dose of phage cocktails need to be optimized to kill pathogenic bacteria more effectively. In addition to strict lytic phages, some studies have also started to explore the potential of temperate phages. The genome of temperate phages can be modified by synthetic biology to make them lytic and use them as lytic phages to control bacteria. This way, we can expand the arsenal against bacterial resistance (Monteiro et al., 2019). When a single type of therapy is ineffective, the combination of phage therapy and antibiotics can often compensate for the deficiencies of the single-type therapy and effectively reduce bacterial resistance.

3.3　Phage-mediated horizontal gene transfer

Horizontal gene transfer (HGT) is the major process for disseminating virulence genes and ARGs among bacteria (Touchon et al., 2017). Mobile genetic elements (MGEs) play an important role in bacterial HGT, usually including insertion sequences, integrons, transposons, plasmids, and phages (Lekunberri et al., 2017a). Phage-mediated HGT can be divided into two categories: generalized transduction and specialized transduction (Fig. 3). Specialized transduction is limited to temperate phages. Owing to the incorrect excision of a lysogen, a phage genome comprises both its own genes and host genes. Generalized transduction packs random fragments of the host DNA into the viral capsid; this can be done by lytic phages (Touchon et al., 2017). For lytic phage-based technology, generalized transduction is a factor that must be considered (Fig. 2).

The transfer of bacterial virulence genes usually involves *pac*-type phages as MGEs. However, some studies have shown that *cos*-type phages can also transfer virulence genes to different genera (Chen et al., 2015). This provides us with a new understanding of the gene transfer mechanism. Recently, more studies have focused on the dissemination of ARGs. Some studies based on the virome or phageome have shown that phages frequently carry ARGs, suggesting that phages could act as reservoirs of ARGs (Calero-Cáceres et al., 2019). Phage particles harboring ARGs were also detected using qPCR in a variety of environments (Table 4). To understand the role of phages harboring ARGs, Yang et al. collected 36 water samples from the Funan River in Sichuan, China. They found that the abundance of ARGs carried by phages is higher in samples collected from hospital discharge points
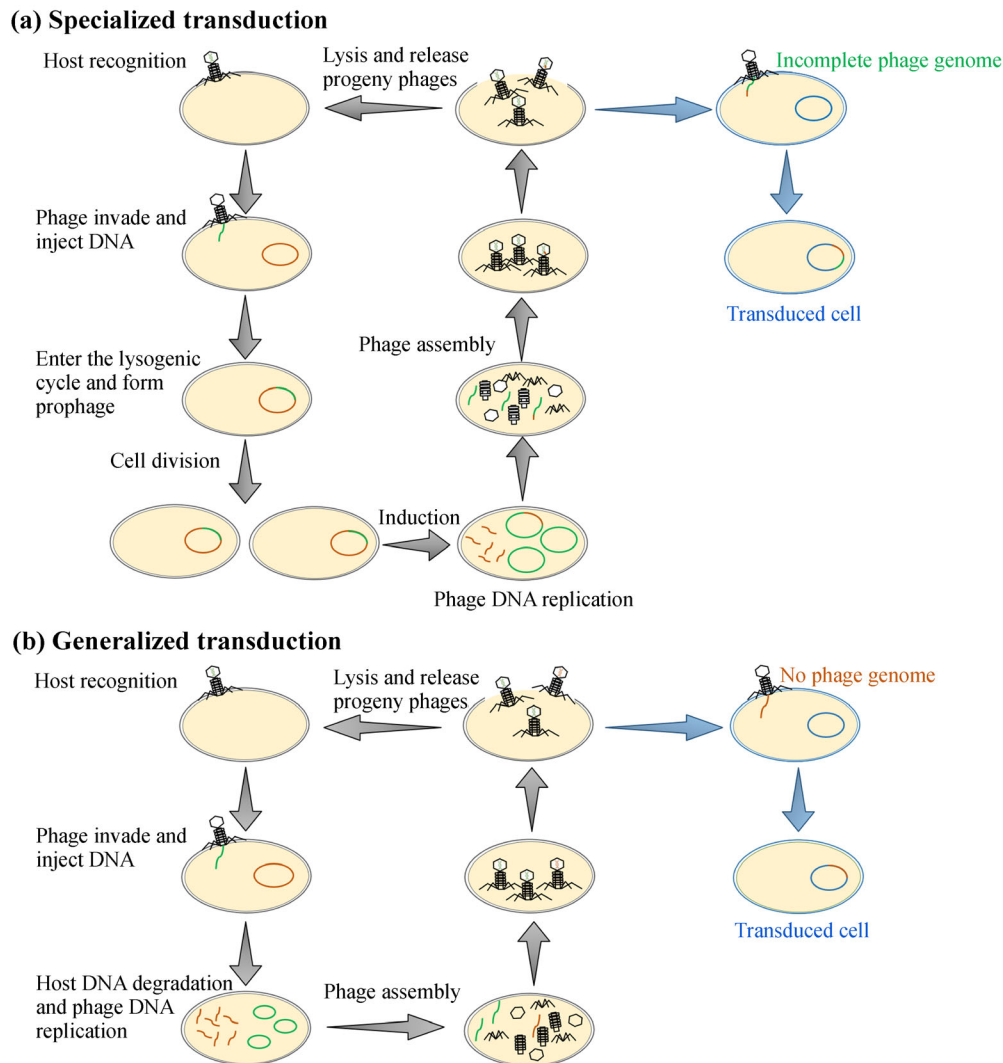
(a) Specialized transduction

(b) Generalized transduction

Fig. 3 Phage-mediated horizontal gene transfer mechanisms. (a) After the invasion of temperate phages into their hosts, their DNA (green) is injected and replicated along with the replication of host DNA. In this process, prophages may package some DNA fragments (brown) of the host. Under spontaneous induction or environmental stress, the lysogenic cycle is terminated. Phages enter the lytic cycle and release progeny phages, which can invade other host cells and transfer genes. (b) Generalized transduction packs random fragments of the host DNA (brown) into the viral capsid. When progeny phages are released, they transfer genes between bacteria.

and wastewater treatment plants (WWTPs), indicating that sewage may be an important source of ARGs (Yang et al., 2018). Another study also found that the abundance of β-lactamase genes in phages is higher in sewage samples (Zhang et al., 2019). This makes it necessary to consider the role of sewage in promoting transduction by phages. Metagenome analysis is another commonly used method to study ARGs. In one study, virome analysis was carried out in diverse samples, including human feces, pig feces, sewage, freshwater, and seawater (Lekunberri et al., 2017a). The results showed that although human-related phage genomes rarely carry ARGs, they are quite different in other natural environments. The abundance of ARGs carried by phages was the highest in sewage. In fact, the number of phages and bacteria in sewage is indeed higher than that in other environments, which greatly increases the frequency of their interactions; this makes phages an excellent vehicle for ARGs transfer between bacterial communities.

It is noteworthy that some ARGs can still be detected in the final effluent of WWTPs (Su et al., 2017). They may spread throughout the ecosystem via agricultural runoff or animal behavior, eventually threatening human health. Phages carrying ARGs have been detected in some vegetables and meat products (Larrañaga et al., 2018). Moreover, one study showed that ARGs in phages were extremely persistent and could exist for a long time even after treatment with strong acids, bases, or disinfectants

Table 4 The situation of phage particles harboring ARGs

Test sample source	Types of ARGs carried by phages	Reference
Soil	$sul1$, $qnrA$, $armA$, $bla_{CTX-M-1}$, bla_{OXA-48}, bla_{TEM}	Larrañaga et al., 2018
The Funan River	$aac(6')$-lb-cr, $aph(3')$-$IIIa$, bla_{CTX-M}, $ermF$, $sul1$, $sul2$	Yang et al., 2018
Sewage from WWTP	bla_{TEM}, bla_{OXA-48}, bla_{PSE}, bla_{CTX-M}, bla_{KPC}, bla_{CMY-2}, bla_{NDM-1}	Zhang et al., 2019
Chicken feces	$aac(6')$-lb-cr, $aph(3')$-$IIIa$, bla_{CTX-M}, $ermB$, $ermF$, $floR$, $mcr-1$, $qnrS$, $sul1$, $sul2$, $tetM$, $vanA$	Yang et al., 2020
Human fecal samples	bla_{TEM}, $bla_{CTX-M-1}$, $bla_{CTX-M-9}$, $armA$, $qnrA$, $qnrS$, $sul1$, bla_{OXA-48}, $mecA$	Brown-Jaque et al., 2018
A river receiving treated wastewater discharges	$qnrS$, $ermB$, $sul1$, $tetW$, bla_{TEM}, bla_{NDM}, bla_{KPC}, $vanA$	Lekunberri et al., 2017b
Pig fecal	$sul1$, bla_{TEM}, $ermB$, $qnrA$, $qnrS$, $fexA$, $floR$, $aac(6)$-Ib-cr, cfr, $tetM$, bla_{CTXM-1}	Wang et al., 2018
Water and sediment	bla_{TEM}, $sul1$, bla_{CTX-M}, $qnrA$, $qnrS$, $mecA$	Calero-Cáceres et al., 2017

(Calero-Cáceres and Muniesa, 2016). We need to reexamine the role of phages in water pollution control. Therefore, the application of phage-based technology to control water pollution is potentially limited. Interestingly, a molecular imprinting-based biosensor may help in diagnosing and monitoring phage-mediated HGT (Ertürk and Lood, 2018). Furthermore, the latest research also shows that an algal-based wastewater treatment system can reduce the frequency of phage-mediated HGT (Cheng et al., 2020).

4　Conclusions

The treatment and reuse of wastewater have always been hot research topics. However, in the past few decades, only a few novel methods have been proposed for these purposes. Phage-based technology has shown great potential. It can be used not only to monitor pathogens in water but also as an effective tool to track pollution sources. In addition, for recalcitrant bacteria (bloom-forming cyanobacteria, pathogenic bacteria in wastewater, filamentous bacteria in activated sludge, and bacteria forming biofilm communities) in various wastewater environments, phages are considered as "bacterial killer." Additionally, we emphasize that phages can promote the spread of ARGs and virulence genes among bacterial communities. This is often overlooked, but it is an important problem that limits the application of phage-based technology. In response to the challenges, such as the resistance developed by bacterial immune systems and decreased phage concentrations, we have also proposed a series of possible solutions. Before phage-based technology can be applied to wastewater treatment, further research is needed on the community structure and interaction mechanisms of microorganisms in wastewater.

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