



Cite this: *Environ. Sci.: Water Res. Technol.*, 2023, 9, 1982

## Emerging investigator series: the role of phage lifestyle in wastewater microbial community structures and functions: insights into diverse microbial environments

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For over a century, environmental engineers have attempted to control the prokaryotic community biological wastewater treatment processes, but there is growing interest in both understanding and harnessing the activity of phages in wastewater bioprocesses. While phages are known to be present and abundant, their ecological role, potential benefits, and impacts on wastewater biological processes are not fully understood. Fundamental knowledge on how phages infect host cells from relatively simple pure culture studies alongside environmental studies from marine and soil systems can be used to predict the potential impact of phages in diverse and dynamic wastewater environments. This frontier review is focused on what is known about the molecular mechanisms by which phages infect bacteria and how that could apply to biological process control and operation within wastewater treatment systems. Here, we specifically focus on highlights from studies on the molecular mechanisms that drive lysis and lysogeny within phage cells and the impacts on the dissemination of antibiotic resistance genes and nutrient removal within a biological wastewater process.

Received 30th September 2022,  
Accepted 5th April 2023

DOI: 10.1039/d2ew00755j

rsc.li/es-water

### Water impact

This review summarizes the rapidly emerging research on how phages survive and their potential role in biological wastewater treatment systems, with a specific focus on the impact of phages on antibiotic resistance and nutrient removal systems. Continued research in this area can lead to targeted control strategies for environmental engineering processes.

## Introduction

Microbial communities represent a complex balance among eukaryotic, prokaryotic (bacteria and archaea), and viral populations that maintain a habitat through biogeochemical interactions. Environmental engineers attempt to control these microbial communities to enable pollutant degradation. Within those microbial communities, phages (viruses that infect bacteria) are the most abundant biological entity on earth, with estimates of over  $10^{30}$  phage particles on earth.<sup>1</sup> Understanding the role of phages in the environment may allow engineers to manipulate phage–bacteria interactions for the benefit of engineered processes.

In general, knowledge of phages and their role in wastewater treatment within activated sludge processes is nascent, but phage abundance as measured by flow

cytometry correlates with a variety of wastewater performance factors such as concentration of effluent, COD, and ammonia.<sup>2</sup> There have been several reviews on the impact of phages on biological wastewater systems that include methods and potential impacts of phages on microbial ecology.<sup>3,4</sup> Examples of studies on phages that are not explicitly covered in this review are the application of phages to manage sludge bulking,<sup>5</sup> control biofilms,<sup>6,7</sup> or enhance disinfection;<sup>8,9</sup> and the use of phages as surrogates for viral pathogens.<sup>10–12</sup> There are relatively fewer studies on phages in activated sludge compared with the wealth of literature available on phages in natural environments. In this frontier review, we will first present the state of knowledge on phage lifestyles (virulent *versus* temperate) and phage molecular signals. We will then provide analysis on the potential impact of these aspects of phage ecology and function on biological wastewater treatment, specifically focused on phage genetic compositions related to antibiotic resistance and nutrient removal. We conclude with a vision of how understanding phage lifestyles can ultimately be used to benefit

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environmental engineering to provide ‘bottom-up’ control of microbial communities.<sup>13</sup>

## Phage lifestyles

Phages can be either virulent or temperate (Fig. 1). Virulent phages attach to the host cell, inject their genetic material into the cell, hijack the host cell machinery to reproduce, and lyse the cell. This process is termed a lytic cycle. Temperate phages, or prophages, use lysogenic cycles, whereby the phages integrate their nucleic acids into the host organism's genome. During lysogeny, the proliferation of the phage genetic material depends on host reproduction. Phage-mediated movement of genetic information between bacteria is termed transduction. Phages conduct transduction through several mechanisms, which are often thought of as mistakes in packaging host DNA or excising phage genetic materials and are therefore traditionally thought to be rare.<sup>14</sup> Overall, the phage dynamics and life cycle impact their host organism's population dynamics and subsequently ecosystem processes.

Lytic phages are known to alter microbial communities significantly. In the deep ocean, phage infection of bacteria directly regulates carbon fluxes, the so-called viral shunt occurs because phage lysis supports heterotrophic community growth on cell debris,<sup>15</sup> illustrated by proportional heterotrophic bacteria and phage abundance in deep-sea sediments.<sup>16</sup> This phenomenon is not surprising considering phages are estimated to be about fifteen times more abundant than prokaryotes in the ocean; some studies have showed estimates up to 160 times, though the relationship between phages and prokaryotes is nonlinear and estimates depend on the location, methods, temperature, and depth.<sup>15,17,18</sup> Some researchers

have postulated that phage lytic activity supports prokaryotic growth by providing organic substrate in anaerobic digesters<sup>19</sup> and there has been evidence of this phenomenon in sediments,<sup>20</sup> although typical wastewaters are not carbon-limited compared with the deep ocean. To our knowledge, there haven't been studies on the role of phage lysis in altering carbon fluxes in activated sludge processes.

Lytic phages have been shown to carry genes associated with bacterial metabolism, auxiliary metabolic genes (AMGs). It is thought that phages carry these genes to alter the metabolic state of the host; AMGs provide fitness advantages, such as allowing expression of important metabolic pathway components during infection.<sup>21</sup> The most commonly found AMGs in phages are related to DNA synthesis, illustrating that when phages hijack the host machinery, they can shift metabolism towards phage synthesis. Some have proposed that phages carry these genes to mimic nutrient-limited metabolism, allowing host metabolism to promote phage replication.<sup>22</sup> One challenge with the evaluation of AMGs in environmental communities is the degree of confidence that the gene identified in an environmental sequencing is, in fact, on a viral fragment, and not a gene within a bacterial cell. Researchers have published guidelines for viromics, including the classification and identification of AMGs in sequencing datasets that should be applied for sequencing studies in wastewater microbial communities.<sup>23</sup>

Temperate or lysogenic phages can change the way host cells behave<sup>24–26</sup> and are a vital part of the microbial community population ecology. Temperate phages can have evolutionary impacts on their host cells, can be sources of genetic variability, and can serve as weapons against competing organisms. Temperate phages can also be a defense mechanism for the cell because bacteria will use temperate



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cyanide toxicity, and low pH in a pure culture of ammonia oxidizing bacteria.<sup>40</sup> In contrast, in a study of contaminated chromium soils, there was higher abundance of lysogenic phages than lytic phages in more contaminated samples and lysogenic phages carried metal resistance genes.<sup>41</sup> This indicates a selective pressure, as the prophage provided metal resistance to the bacteria. Together, these studies indicate that the relationship between bacterial stress and phage induction likely varies based on the individual type of phages and the functional capability the phages provide their host organism.

### Phage host range in wastewater systems is unknown

The phage host range indicates the diversity of hosts that a phage can infect. A phage with a broad host range has the machinery and capability to infect many different bacterial species. Conversely, most studied phages have a narrow host range indicating that an individual phage can only infect one host.<sup>42</sup> Host switching is where each individual phage can only infect one host, but the phage quasispecies can infect multiple species.<sup>42</sup> One meta-analysis across multiple environments suggests that there is a weak negative correlation between the phage virulence and host range; phages with a broad host range are less virulent.<sup>43</sup> Little is known about the host range for phages that dominate wastewater environments. One study indicates that phage isolates from wastewater have a broad host range, but this research was limited to a handful of isolates.<sup>44</sup> This finding was supported by a more recent metagenomic survey of several wastewater treatment plants that suggests the phage community could infect a diverse set of hosts,<sup>45</sup> and similarly a survey of sewage indicates a broad host range.<sup>46</sup> While there is a significant need to isolate and identify phages from wastewater in order to build databases,<sup>45,47</sup> culture-based methods for evaluating the host range are cumbersome and miss the abundant uncultured prokaryotic species in wastewater. Emerging bioinformatic methods including those that incorporate machine learning are very promising for evaluating host range (*e.g.*, ref. 48 and 49) and could be applied to wastewater environments.

## Phage–host signaling & phage–phage molecular signaling

There are several molecular signals used by phages to signal one another and establish a lytic or lysogenic lifestyle. Specific phage communication systems that warrant further discussion are: 1) lysis inhibition, 2) synchronized lysis inhibition collapse, 3) high multiplicity lysogeny decisions (HMDLs), 4) arbitrium systems, and 5) autoinducer-associated prophage induction (AAPI), summarized in Table 1.

### Lysis inhibition delays lysis

Lysis inhibition is the oldest known form of phage-to-phage communication,<sup>50</sup> first described almost 75 years ago in a study investigating the lysis of *E. coli* infected with T-even phages.<sup>51</sup> Lysis inhibition delays the lysis of infected bacteria to ensure that there are enough bacteria for phages to infect. Basically, at low bacterial abundance, lysis is delayed. The lysis inhibition system is carried out when the newly released phages from a lysed bacterial cell get re-adsorbed into an already infected bacterial cell.<sup>50,51</sup> The adsorption reaction of the phages delays the lysis of the infected bacterial cell. Delaying the lytic cycle results in larger burst sizes (more phages released) from the secondarily infected bacteria.<sup>51</sup>

To understand how lysis inhibition occurs, it is important to first understand the mechanism by which phages lyse cells. One mechanism, termed lysis from within, uses several proteins including holin and endolysin proteins.<sup>50</sup> The holin protein is responsible for making holes in the bacterial cell's membrane and allowing the endolysin, located in the cell's cytoplasm, to degrade the bacterial cell wall.<sup>50,52</sup> Lysis from within occurs when the holin protein is activated.<sup>50</sup> The activation of lysis inhibition in T-even phages occurs when secondary virions get adsorbed on the cell wall of an infected bacterial cell, triggering a signal from the R1 protein in the periplasm to the holin protein in the plasma membrane.<sup>50,53,54</sup> The R1 protein thus functions as an anti-holin. An additional anti-holin in the cytoplasm, the RIII protein, also acts as a stabilizer for the RI and holin complex.<sup>50,55</sup>

**Table 1** Summary of phage–phage and phage–host molecular signaling

	Lysis inhibition	Synchronized lysis inhibition collapse	HMDLs	Arbitrium systems	AAPI
Phages	T-even phages	T-even phages	Lambda phages	Phages phi3T and SPbeta	Vibriophage VP882
Type of communication	Phage–phage	Phage–phage	Phage–phage	Phage–phage	Phage–host
Life cycle pathway	Delayed lytic cycle	Accelerated lytic cycle	Lysogeny	Lysogeny	Lytic cycle
Molecule type	Re-adsorption of secondary phages	Adsorbing phages in lysis-inhibited populations	Multiple infecting phages	<i>aimP</i> peptide	DPO peptide
Ref.	50–55	50 and 56	39, 50 and 57–62	63–66	67–69





### Synchronized lysis inhibition collapse signals the beginning of lysis

The synchronized lysis inhibition collapse system is related to lysis inhibition; it is the accelerated lysis of phage-infected bacteria that were under the influence of lysis inhibition.<sup>50</sup> In other words, it is the lysis of previously lysis-inhibited cells. This signaling system was first observed in a study investigating the lysis of lysis-inhibited T4 phage-infected cells.<sup>56</sup> The study's authors proposed two mechanisms potentially governing synchronized lysis inhibition collapse. The first mechanism is initiated from the accumulation of adsorbed secondary virions to the lysis-inhibited bacteria, resulting in the degradation of the cell walls and a process the study's authors termed lysis from without. Lysis from without is the result of membrane disruptions that further propel lysis from within in the lysis-inhibited bacteria. The second proposed mechanism that is suggested by the study's authors is secondary traumatization, which could also cause disruptions in the plasma membrane chemiosmotic barrier that could lead to lysis from within. While the mechanisms are not known, the overall effect of synchronized lysis inhibition collapse is accelerating lysis.

### HMDLs lead to lysogeny when there is high MOI for lambda phages

A molecular signaling system that is observed within lambda phages is the HMDLs.<sup>50,57</sup> HMDLs are a result of multiple phage infections to bacteria and results in lysogeny. HMDLs are different from lysis inhibition where the latter leads to lytic life cycles, and it is a result of secondary virion adsorption, not phage infections.<sup>50,57</sup> Several experimental studies have observed this phenomenon, in which multiple

lambda phage infections lead to a lysogenic life cycle.<sup>58–62</sup> To briefly describe the mechanism for the switch between lysis and lysogeny in lambda phages, it is regulated by the CI and Cro proteins and three promoters,  $P_R$ ,  $P_L$ , and  $P_{RM}$ .<sup>44</sup> Cro controls the  $P_R$  and  $P_L$  promoters (strong promoters) that induce the lytic cycle.<sup>44</sup> This occurs because Cro dimers bind to operators with high affinities that eventually results in the blockage of the CI synthesis and initiates the lytic cycle in the lambda phages.<sup>44</sup> In the lysogenic cycle, the opposite occurs and the CI dimers have a high affinity to bind with operator proteins that that will repress the promoters for the synthesis of the Cro proteins.<sup>44</sup> Together, these communication signals ensure the initiation and maintenance of the lysogenic life cycle in lambda phages at high MOIs.<sup>44</sup>

### Arbitrium system leads to lysogeny

An arbitrium system is one of the more recently discovered forms of phage–phage communication systems summarized in Fig. 2.<sup>63</sup> The arbitrium system uses a phage-specific peptide code that directs the phage life cycle to lysogeny, similar to HMDLs. The system is encoded by three phage genes which are: *aimP*, *aimR*, and *aimX*. The gene *aimP* produces the peptide signal that gets received by the AimR receptor in phages, leading to lysogeny. The AimX receptor is an inhibitor of lysogeny, and when it is active the phage displays lytic decisions. At low cell densities, the AimR receptor as a dimer activates the AimX protein leading to the lytic cycle (inhibition of lysogeny).<sup>63,64</sup> During that time, AimP gets secreted into the medium and after multiple cycle infections it reaches a threshold concentration enabling it to bind to the AimR receptor which in turn

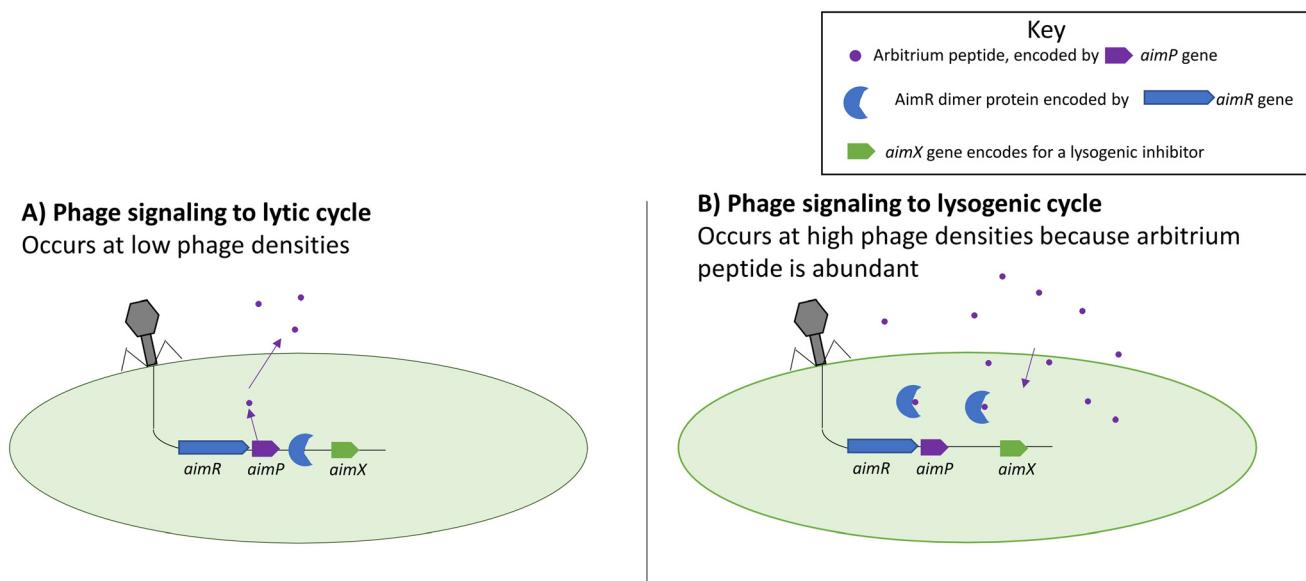


Fig. 2 Functions of the aim complexes during lytic (left) and lysogenic (right) cycles. Arbitrium peptides prevent the AimR protein from binding to the genome and prevent lysogeny. Adapted with permission from ref. 63 Springer Nature, copyright 2017.



inactivates the AimR dimer and results in the silencing of AimX leading to lysogeny.<sup>63,64</sup>

It was shown that different phages produce different peptide signals.<sup>63,64</sup> The peptide signals from phages Phi3T and SPbeta are SAIRGA and GMPRGA, respectively. For the Phi3T phage, the peptide signal, SAIRGA, results in conformational changes to its AimR receptor leading to its dissociation and lysogeny. However, in the SPbeta phage, the peptide signal GMPRGA stabilizes the AimR receptor in a dimeric state leading to lysis.<sup>63,64</sup> These differences show how different phages could use the same molecular signaling system for distinct outcomes.

The phi3T phage also exhibits a second communication system called the Rap $\phi$ -Phr $\phi$  system.<sup>65</sup> This bacterial quorum sensing mediated system works together with the arbitrium system to weaken the host defense systems during the lytic cycle. Rap $\phi$  is a receptor that can inhibit the ComA-P and Spo0F-P regulators in *Bacillus*. The ComA-P regulator is responsible for the production of antimicrobials, whereas the Spo0F-P regulator is responsible for sporulation or biofilm formation. The downregulation of the host defense through Rap $\phi$  enhances the efficiency of the lytic cycle. It is also postulated that during lysogeny, this communication system is used to enhance the fitness of the host bacteria by not wasting resources in the production of biofilm or antimicrobials.

### Autoinducer-associated prophage induction (AAPI)

The autoinducer-associated prophage induction (AAPI) system is a bacteria-virus communication system rather than a virus-virus communication system presented in previous sections. It was shown that vibriophage VP882 encodes a receptor that detects an autoinducer from *Vibrio cholerae* which then controls the life cycle decision for the phage.<sup>67</sup> *V. cholerae* has a well-characterized quorum sensing system that uses the autoinducer 3,5-dimethylpyrazin-2-ol (DPO) and a protein receptor VqmA,<sup>68</sup> needed for virulence and biofilm formation.<sup>68,69</sup> Phage VP882 has a similar receptor to that of *V. cholerae* called VqmA<sub>phage</sub>.<sup>68,69</sup>

There are two pathways for induction in phage VP882. The first pathway is through the activation of the VqmA<sub>phage</sub> receptor by binding with bacterial autoinducer DPO. This will upregulate the quorum-triggered inactivator of the CI protein (Qtip), which will bind to the CI repressor protein and initiate the lytic life cycle through the activation of the Q antiterminator protein. The second pathway is DNA damage. This will result in the cleavage of the CI repressor in a RecA-dependent manner that leads to the upregulation of the Q antiterminator protein. VqmA<sub>phage</sub> can bind to promoters of its own and of the host, while VqmA only binds to its own promoters.<sup>48,50</sup> In essence, phage VP882 can use the QS system used by its host and also uses the phage's own pathways to infer the decisions on lysis or lysogeny.<sup>48,50</sup>

Similar mechanisms of bacterial QS-dependent phage induction have been demonstrated with other hosts and in the environment. For instance, a study of groundwater microbial communities found increasing abundance of viruses, and correspondingly decreased abundance of bacteria, with the additional acyl homoserine lactones (AHLs), indicating that the mixture of AHLs in the study led to phage induction.<sup>70</sup> That study identified the mechanisms of AHL-mediated phage induction in an *E. coli* lambda phage, both with exogenously added AHLs and in a co-culture with AHL-producing *P. aeruginosa* cultures. There are several additional examples of phages carrying QS system genes,<sup>71-73</sup> but the role of these genes in guiding lysis and lysogeny decisions is unknown. As indicated with the vibriophage and phi3T communication systems mentioned above, it would be beneficial for phages to use the host QS signals to guide lysis or lysogenic cycles. An additional example is related to QS degradation; one study identified acylhydrolase genes, which encode enzymes that degrade AHLs, within a phage genome.<sup>74</sup> Again, the fitness benefit of carrying these genes by phages is unknown. The carrying costs of these functions within a small genome indicates that phages likely derive a benefit from either 'listening in' or directly manipulating bacterial QS signals.

Though we still lack an understanding of phage diversity and its role in wastewater treatment systems, these varied and complex signaling systems are likely important for understanding phage lifestyle. Phages are likely using bacterial signals and phage-phage signaling systems in tandem with varied outcomes. Understanding what is regulating phage behavior is critical for applying phages to enhance wastewater treatment and other environmental engineered processes.

## Role of phage lifestyle and signaling in wastewater systems

### Phage within wastewater systems carry antibiotic resistance genes

The importance of phage-bacteria interactions as drivers for human and environmental health is only recently appreciated. Phages impact human health because they can carry genes that encode antimicrobial resistance (AMR) and transduction events can lead to the spread of resistance in the environment.<sup>75,76</sup> Transduction can be generalized, specialized, and lateral.<sup>14</sup> In generalized transduction, host DNA is mistakenly taken for viral DNA and mispackaged; any portion of the host genome can be packaged this way. Specialized transduction is rarer because it is the result of aberrant excision that joins a segment of viral and bacterial DNA; only portions of the host genome would undergo specialized transduction. Both are the result of missteps that are uncommon; therefore, there are generally low frequencies of host gene transfer due to generalized and specialized transduction. For this reason, transduction was thought to not be the dominant mechanism of horizontal gene transfer



events. The recent discovery of a third mechanism of transduction, lateral transduction,<sup>77</sup> has added more complexity to the role of phages in horizontal gene transfer events because it results in much more frequent gene transfer events, at least 1000-times more frequent than generalized and specialized transduction.<sup>78</sup> Lateral transduction is the result of a delayed excision of viral DNA—replication occurs before excision. Studies of lateral transduction are nascent and limited to model systems, so the role of this mechanism in the environment is unknown.

Though the role of lateral transduction in environmental systems is unknown, high frequency transduction is of particular concern for the case of AMR, especially if resistance genes are transferred from non-pathogenic environmental bacteria to disease causing pathogenic bacteria. It is important to note that transduction is not the only, or primary, potential form of transfer of AMR, and one analysis of all forms of mobile genetic elements identified transposable elements as dominant carriers of antibiotic resistance genes.<sup>79</sup> The same analysis found resistance genes to be significantly enriched in phage-like elements. Given our new understanding of the mechanisms of transduction, consideration of phages for AMR spread is needed because although AMR is a natural bacterial defense mechanism, this function can have deadly consequences on human health when acquired by pathogenic organisms.

Wastewater treatment plants are thought to be an important potential transmission route for AMR in the environment.<sup>80–82</sup> This is further exacerbated by the constant influx of antibiotics going into wastewater treatment plants at sub-lethal concentrations.<sup>83,84</sup> In a study of phage isolates from wastewater, half of the isolated phages were found to carry beta-lactamase resistance. Twenty percent of the isolated phages were not only carrying beta-lactamase genes but were transducing phages and were able to infect a variety of *E. coli* indicator strains.<sup>85</sup> This study represents only a snapshot of culturable phages that infect *E. coli*, but sequencing tools are expected to uncover a broader scope of the potential phages that harbor AMR. Another qPCR-based study found beta-lactamase resistance to be in some instances more prevalent (detected in more samples) in the phage DNA fraction than in the bacterial DNA fraction.<sup>86</sup> Thus, phages may play an important role in the spread of AMR in wastewater treatment systems.

Sequencing is a powerful tool to develop an understanding of the diversity of AMR in a sample. However, particularly within the phage fraction, calculating the relative abundance of resistance genes in samples is challenging. Within bacteria, gene read counts can be normalized to a single copy bacterial gene. But, within a phage sequencing pool there is a lack of standardization in protocols to make the data quantitative or semi-quantitative. The application of internal standards holds promise in this regard,<sup>87</sup> but for the time being many studies have focused on the richness of resistance genes found within the phage fraction. For

instance, one study of phage diversity within swine wastewater confirmed a potential diverse set of resistance genes within phage DNA, including the *mcr-1* gene which confers resistance to a last resort antimicrobial, colistin.<sup>88</sup> Another study identified tetracycline resistance within the phage sequences of sewers.<sup>46</sup> Though it is known that phages along with other mobile genetic elements can play a role in horizontal gene transfer, the relative importance of phages compared to these other mechanisms is unknown. The discovery of new and more frequent transduction mechanisms underscores the need to revisit the role of phages in AMR transfer in the environment. The adoption of quantitative sequencing methods for phages, along with benchmarking standards for accurately characterizing a gene to be in the bacterial *versus* viral fractions,<sup>23</sup> could help us understand the relative importance of phages compared with other agents for the spread of AMR in the environment.

### Phages are known to carry genes related to nutrient removal and related processes

Molecular evidence suggests that abundant phages in marine and soil environments can contain AMGs related to nitrogen,<sup>89,90</sup> sulfur,<sup>90–92</sup> and carbon<sup>15,93–95</sup> cycling. Given the relative genome sizes of phages, it is somewhat counterintuitive that there is a fitness benefit to carrying AMGs related to nutrient metabolism and shuttling. Although cycling of these nutrients by bacteria is well-studied, the role of phages on nutrient cycling is not as well characterized. Moreover, few studies have evaluated the AMG content of phages in wastewater environments, but it has been shown that organisms related to nutrient cycling (sulfur, nitrogen, and phosphorus accumulating organisms) are all prominent hosts for phages.<sup>45</sup>

Sulfur cycling organisms are consistently found to host phages within activated sludge systems. Notably in one study, all sampled wastewater treatment plants (6) had phages with hosts related to sulfate reduction, and an organism related to sulfide-based denitrification (*Thiothrix*), indicating that phages have a prominent impact on sulfur cycling in the treatment plants from that study.<sup>45</sup> This was supported by another metagenomic survey,<sup>96</sup> and a more recent study also identified carbon and sulfur-related AMGs within the phage genome in sewer metagenomes.<sup>46</sup> The prominence of phages with sulfur cycling hosts and AMGs within wastewater is in line with a study of publicly available datasets illustrating that phages carrying sulfur cycling-related AMGs were widespread in many ecosystems.<sup>92</sup> That study illustrated that in some cases, phage-directed sulfur oxidation was expressed at higher levels than host sulfur oxidation. This demonstrates the potential importance of phages in biogeochemical cycling of sulfur. Understanding whether this aspect extends to other nutrients (nitrogen and phosphorus) is a research area ripe for further study.







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