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Phage phylogeny, molecular signaling, and auxiliary antimicrobial resistance in aerobic and anaerobic membrane bioreactors

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ABSTRACT

Phage emit communication signals that inform their lytic and lysogenic life cycles. However, little is known regarding the abundance and diversity of the genes associated with phage communication systems in wastewater treatment microbial communities. This study focused on phage communities within two distinct biochemical wastewater environments, specifically aerobic membrane bioreactors (AeMBRs) and anaerobic membrane bioreactors (AnMBRs) exposed to varying antibiotic concentrations. Metagenomic data from the bench-scale systems were analyzed to explore phage phylogeny, life cycles, and genetic capacity for antimicrobial resistance and quorum sensing. Two dominant phage families, *Schitoviridae* and *Peduoviridae*, exhibited redox-dependent dynamics. *Schitoviridae* prevailed in anaerobic conditions, while *Peduoviridae* dominated in aerobic conditions. Notably, the abundance of lytic and lysogenic proteins varied across conditions, suggesting the coexistence of both life cycles. Furthermore, the presence of antibiotic resistance genes (ARGs) within viral contigs highlighted the potential for phage to transfer ARGs in AeMBRs. Finally, quorum sensing genes in the virome of AeMBRs indicated possible molecular signaling between phage and bacteria. Overall, this study provides insights into the dynamics of viral communities across varied redox conditions in MBRs. These findings shed light on phage life cycles, and auxiliary genetic capacity such as antibiotic resistance and bacterial quorum sensing within wastewater treatment microbial communities.

1. Introduction

Membrane bioreactors (MBRs) are widely used for wastewater treatment (Goswami et al., 2018). MBRs can produce high-quality treated water with low effluent concentration of organic micropollutants such as such as pharmaceuticals (Goswami et al., 2018). The main application of MBRs in wastewater treatment is under aerobic conditions (Goswami et al., 2018), but interest in AnMBRs is growing due to their potential for energy recovery and lower sludge production (Smith et al., 2012). However, both aerobic and anaerobic MBRs require biofouling control to reduce energy demands of treatment and maintain high pollutant removal efficiency (Smith et al., 2012).

Phage therapy has been proposed as a mechanism to address biofouling in MBRs (Bhattacharjee et al., 2015) and for other applications in wastewater systems including controlling pathogenic bacteria, improving the digestibility and dewaterability of waste-activated sludge, and controlling filamentous bacteria by preventing foaming (reviewed by Withey et al. 2005). However, currently little is known regarding the diversity, abundance, and functions of phage and how they interact with bacteria during biological wastewater treatment. The promise of phage therapy cannot be realized without a better understanding of phage diversity, host range, and the dynamics of phage-host interactions within wastewater treatment bioprocesses and more specifically in MBRs.

Phage are said to be more abundant in water resource recovery facilities (WRRFs) than in any other aquatic environment (Fuhrman, 1999; Tamaki et al., 2012; Wommack and Colwell, 2000). Studies have used metagenomic data or meta-analysis to assess the viral communities and their auxiliary metabolic capabilities within WRRFs by analyzing the influent, activated sludge, anaerobic digester, and effluent of the treatment processes (Tamaki et al., 2012; Strange et al., 2021; Yuan and Ju, 2023). Few studies have evaluated the ecology of phage or their life cycle in membrane bioreactors, particularly under different redox conditions.

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Phage infect bacterial cells by attaching to cell surface receptors and inserting their genetic material into the bacterial cell (Clokie et al., 2011; Doss et al., 2017). After infection, phage either lyse the cell (lytic cycle) or integrate their DNA into the bacterial cell (lysogenic cycle). For the lytic life cycle, phage accumulate in the bacterial cell until phage-encoded proteins (i.e., holin and endolysin) lyse the cytoplasmic membrane of the host bacteria allowing the phage to burst out of the cell. For the lysogenic life cycle, the phage will replicate inside the host genome and could stay dormant for generations. The two life cycles play a major role in shaping microbial communities (Chevallereau et al., 2022), making it important to understand the mechanisms of how phage reproduce and switch between both life cycles.

Phage and bacteria possess the ability to communicate among themselves and with each other (reviewed by (Duddy and Bassler, 2021; Vela and Al-Faliti, 2023)). In phage, several phage-phage molecular communication systems have been observed including lysis inhibition (LIN) (Abedon, 1992; Abedon, 2019), LIN collapse (Abedon, 1992; Abedon, 2019), high multiplicity lysogeny decisions (Wadhwa, 2017), and arbitrium systems (Erez et al., 2017). Identifying the communication signals within phage is critical as it guides the switch between the lytic and lysogenic life cycles. In bacterial cells, quorum sensing is carried out with chemical signaling molecules (i.e., autoinducers) that are secreted into the environment and then sensed by other bacterial cells (Feng et al., 2013; Hu et al., 2021; Shrout and Nerenberg, 2012). Autoinducer-associated prophage induction is a type of bacteria-to-phage communication that informs lytic or lysogenic life cycles (Silpe and Bassler, 2019). These complex systems warrant further investigation to provide a mechanistic understanding of the role of microbial communication within both phage and bacteria on microbial activity, particularly in complex environments such as wastewater treatment systems.

We analyzed metagenomic data from a laboratory-scale aerobic membrane bioreactor (AeMBR) and an anaerobic membrane bioreactor (AnMBR) operated in parallel at different antibiotic concentrations (Wang et al., 2022). Bioinformatic tools were used to recover viral genes and phage community capabilities from both reactors at different antibiotic concentrations. The study provides a new perspective on the (i) phage communities, (ii) genes associated with the phage life cycles, (iii) genetic potential for antibiotic resistance and quorum sensing within phage under different redox environments and antibiotic loading conditions.

2. Materials and methods

2.1. Metagenomic data

Metagenomic data were obtained from a study of bench-scale AeMBR and AnMBR investigating antimicrobial resistance patterns as a function of antibiotic loading and redox (Wang et al., 2022). Briefly, the AeMBR and the AnMBR were operated in parallel at 25 °C and fed a synthetic feed representative of medium-strength domestic wastewater. Different antibiotic levels were applied in a stepwise fashion for a period of seven days at each of the concentrations (0, 10, and 250 μg/L). The antibiotic mixture included sulfamethoxazole (SMX, a sulfonamide), erythromycin (ERY, a macrolide), and ampicillin (AMP, a beta-lactam). Intracellular DNA (iDNA) and extracellular DNA (exDNA) were separately extracted from effluent samples from each MBR. For these samples, a 150 mL effluent sample was filtered through a 0.2 μm filter, and the filtrate was then precipitated via isopropyl alcohol and resuspended to obtain the exDNA sample. Intracellular DNA was extracted from the filter. The biomass sample was obtained from the MBRs by taking a 2 mL sample and then centrifuging at 10,000 g at 4 °C for 1 min. Both the filter and the biomass samples were extracted using Maxwell 16 LEV Blood DNA Purification Kit (Promega, Madison, WI) as previously described (Wang et al., 2022). Samples were collected at the end of each antibiotic loading period (Table 1) across the three sample types (iDNA, exDNA, and biomass) and subsequently sequenced using Illumina NextSeq platform with PE 150 bp reads (USC Genome Core, Los Angeles, CA). The raw reads were then further analyzed using a bioinformatic pipeline.

Table 1
Description of sample names, types, and antibiotic concentration.

Sample type	Redox condition	Antibiotic concentration (μg/L)	Sample code
Intracellular DNA (iDNA)	Aerobic	0	Ae_intra_0
		10	Ae_intra_10
		250	Ae_intra_250
	Anaerobic	0	An_intra_0
		10	An_intra_10
		250	An_intra_250
Extracellular DNA (exDNA)	Aerobic	0	Ae_extra_0
		10	Ae_extra_10
		250	Ae_extra_250
	Anaerobic	0	An_extra_0
		10	An_extra_10
		250	An_extra_250
Biomass	Aerobic	0	Ae_bio_0
		10	Ae_bio_10
		250	Ae_bio_250
	Anaerobic	0	An_bio_0
		10	An_bio_10
		250	An_bio_250

2.2. Bioinformatic analysis

2.2.1. Reads pre-processing, assembly, and mapping

Fastp v0.23.2 (Chen et al., 2018) was used to quality filter/trim the raw reads. Raw reads were filtered based on a PHRED quality threshold of 25 and a length threshold of at least 50. The reads were then trimmed using both a sliding window and a leading window of 4 bases with a mean PHRED quality score of 25. The reads were also deduplicated and an overlap analysis was performed for the reads to correct mismatched base pairs in overlapped regions. The filtered and trimmed reads were then assembled to contigs using Megahit v1.2.9 (Li et al., 2015) using a k-min of 21, k-max of 255, and step size of 6. The quality of the assembly was checked using QUAST v5.2.0 (Gurevich et al., 2013). The filtered and trimmed reads from fastp were also mapped to the sample's assembly using Bowtie2 v2.4.4 (Langmead and Salzberg, 2012) and then quantified using Samtools v1.13.0 (Danecek et al., 2021). Samtools provides the coverage depths of the whole contig and the coverage per position for the genes in the contigs. Assembled contigs for each of the samples are deposited on NCBI Project ID PRJNA1073904. The full bioinformatic pipeline is available at https://github.com/Delgado-Vela-Research-Group/Phage-Analysis.

2.2.2. Viral contigs identification and prophages

We adapted a previously published bioinformatic pipeline to determine the viral contigs from the assembled contigs (Hegarty et al., 2022). We established a threshold for contig length of 2300 bp based on the average N50 value from QUAST from three representative samples. Contigs longer than 2300 bp were then sorted into viral contigs based on using the consensus of three bioinformatic tools: CheckV v1.0.1 (Nayfach et al., 2021), VirSorter v1.0.6 (Roux et al., 2015), VirSorter2 v2.2.4 (Guo et al., 2021). The contig was determined viral if (1) it was complete, high, or medium from CheckV, (2) it had a score in VirSorter2 greater than 0.95, (3) it had at least two hallmark viral genes in VirSorter2, or (4) it was a category 1 or 2 in VirSorter (Hegarty et al., 2022). A contig was also considered viral if it had at least two of the following conditions (1) Category 3–6 in VirSorter, (2) low in CheckV, or (3) a score between 0.5–0.95 in VirSorter2. We also removed any contigs that had a length of more than 50,000 bp and did not contain any viral hallmark genes in VirSorter2, no viral genes with more than one host gene in CheckV, and if it had 3 times greater host genes than viral genes from CheckV. The viral contigs identified were further analyzed to determine if they were prophage using CheckV and VirSorter. From CheckV if the category provirus was deemed as “yes”, it was categorized a prophage. From VirSorter, if the viral contig is a category 1 or 2 prophage, it was categorized as a prophage.

2.2.3. *Data normalization*

The iDNA and exDNA sample reads were normalized using reads per kilobase million (rpkm) to account for differences in coverage depth and gene lengths. We calculated the rpkm by determining the number of mapped reads from the total reads for each sample. To account for coverage depth, we divided the depth of each gene in the contig by the mapped reads per million. Then to account for the gene length, we divided the normalized depth value by the gene length per 1000. Due to differing biomass concentration in the two reactors, the biomass sample reads were normalized by using rpkm per gram volatile suspended solids (rpkm/g VSS sequenced). We calculated the grams of VSS sequenced based on Eqs. (1) and (2). Mapped reads were then divided by the grams of VSS sequenced to get mapped reads per gram of sequenced VSS. The calculations to account for coverage depth and gene length were the same as the iDNA and exDNA mentioned above.

$$\begin{aligned} Y &= \frac{\text{g VSS}}{\mu\text{L DNA extract}} \\ &= \frac{\text{MLVSS (g VSS/mL Sample)} * \text{Sample Volume extracted (mL Sample)}}{\mu\text{L DNA extract}} \end{aligned} \tag{1}$$

$$\text{g VSS sequenced} = \mu\text{L DNA sequenced } * Y\left(\frac{\text{g VSS}}{\mu\text{L DNA}}\right) \tag{2}$$

2.2.4. *Gene annotations and viral taxonomy*

All gene annotations were assigned using DRAM v1.4.6 (Shaffer et al., 2020) based on the Pfam database. The coverage depths per position from Samtools (Section 2.2.3) were matched with the gene positions that had an annotation assigned to identify their coverage. The data were normalized based on the rpkm discussed in Section 2.2.3. The rpkm value of the similar annotation in the sample was summed to identify the abundance of the function in the same sample and to compare it with the other samples. From the Pfam hits we identified the proteins that were associated with the lysogenic life cycle, lytic life cycle, and potential viral-to-bacterial communication systems. We manually examined all contigs that were annotated to quorum sensing (QS) genes to ensure QS gene annotations were within a phage contig or within the phage region of the genome for prophage. If the QS gene was not less than 4000 bp away from a known phage annotation, it was not included in the analysis. The viral contigs identified from Section 2.2.3 were used as input for the taxonomy assignment. We assigned the taxonomies using PhaGCN (Shang et al., 2021), which assigns the taxonomies of the viral contigs based on the International Committee on Taxonomy of Viruses (ICTV) 2022 tables.

2.2.5. *Alpha and beta diversity*

The alpha and beta diversity within the viral communities were calculated using the vegan v2.6–4 (Oksanen et al., 2013) in R-studio (Team, R. Developement Core, 2009). We calculated several measures of alpha diversity: richness (the number of viruses found in the sample), Shannon index (a measure of the diversity of viruses in the community), and Shannon evenness index (how similar the abundance of the different species in the community). Richness was normalized based on the rpkm normalization strategy discussed in Section 2.2.5 and the Shannon indices were normalized by dividing the Shannon indices by the maximum Shannon index of the specific sample. Beta diversity was quantified using the vegandist function in the vegan package, which allowed computation of the Bray Curtis distances between samples. Data were normalized similarly to the description in Section 2.2.5 using the rpkm values of each sample.

2.2.6. *Antibiotic resistance genes (ARGs) analysis*

The viral contigs were compared against the Megares v3.0 (Bonin et al., 2023) database using nucleotide BLAST v2.11.0 to annotate the ARGs groups for all the samples. Hits with bitscores higher than 70 were used for the analysis. Data were normalized using the rpkm method discussed previously (Section 2.2.5). We manually examined the contigs deemed as prophage from Section 2.2.2 that had ARG hits. If the ARG annotation was not less than 4000 bp distance from a phage annotation, it was not included in the ARG analysis.

2.2.7. *ANOVA analysis of prophage*

The viral contigs that were considered prophage from Section 2.2.3, were further analyzed. We first calculated the percentage of prophage contigs from the total number of viral contigs per sample analyzed. Using the percentages, we performed a one-way ANOVA analysis on the iDNA and exDNA samples with the goal of identifying differences in prophage percentages between the aerobic and anaerobic samples from the two sample types (iDNA and exDNA). The ANOVA analysis was performed in R-studio (Team, R. Developement Core, 2009) to evaluate variances between all samples, only iDNA samples, and only exDNA samples.

3. Results and discussion

3.1. *Schitoviridae was more dominant under anaerobic conditions while Peduoviridae was dominant under aerobic conditions*

Metagenomic analysis allowed investigation of the functional capacity and taxonomic composition of the viral communities in the MBRs. Our assembly resulted in 212,043 contigs longer than 2300 bp, of which 1043 contigs were categorized as viral. Approximately 91 % of the viral contigs were observed in effluent iDNA and exDNA samples, as opposed to the biomass samples, likely due to insufficient sequencing depth given that the biomass community is significantly more diverse than the effluent community (Wang et al., 2022). *Schitoviridae* was the most dominant family in the effluent from the AnMBR (Fig. 1), and though more diverse, *Peduoviridae* was the most dominant family in the effluent from the AeMBR (Fig. 1). We also observed that *Schitoviridae* was almost non-existent in the AeMBR, but there was a presence of *Peduoviridae* in the AnMBR. While *Schitoviridae* accounted for 4.70 %−87.80 % of phage relative abundance in all the anaerobic samples, its relative abundance was only 0.28 %−7.02 % in 4 out of 6 of the aerobic samples. Conversely, *Peduoviridae* was present at 8.81 %−42.67 % across 5 of 6 aerobic samples and present at lower abundance (0.16 %−13.53 %) across all anaerobic samples. A beta diversity analysis (Fig. S7) did not show clustering by either sample type or antibiotic concentration.

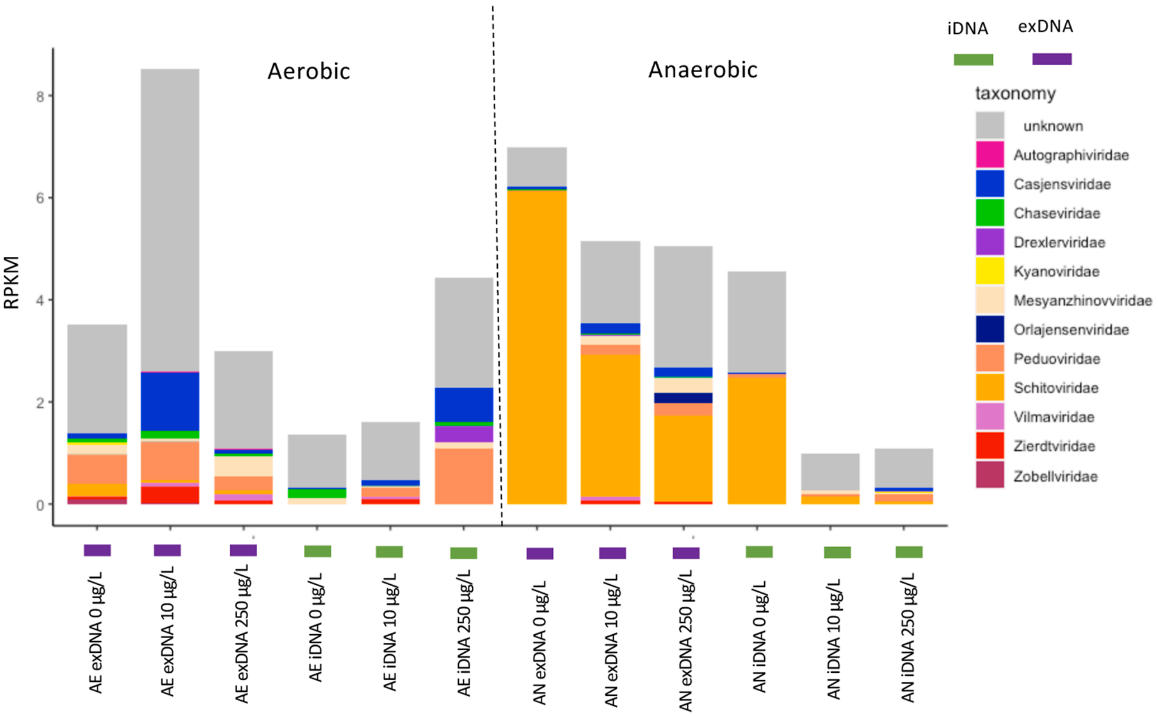

Fig. 1. Phage families of the AeMBR and the AnMBR. The figure shows the taxonomy at each antibiotic concentration level.

Our study provides evidence that the dominant viral taxa could differ between the AeMBR (*Peduoviridae* dominant) and the AnMBR (*Schitoviridae* dominant). To our knowledge, few studies have found phage associated with the two families (*Schitoviridae* and *Peduoviridae)* in wastewater environments. One study isolated phage from wastewater and found novel members of the *Schitoviridae* family with further genomic analysis showing association with the lytic life cycle (Gomes et al., 2023). No studies to our knowledge have shown the presence of *Peduoviridae* in wastewater environments. However, it has been observed in soil (Ji et al., 2023) and grass silages (Sáenz et al., 2023). Several studies have reported that the most dominant viral families in the wastewater were *Siphoviridae, Myoviridae,* and *Podoviridae* (Tamaki et al., 2012; Ballesté et al., 2022; Brown et al., 2019; Wu and Liu, 2009). However, the composition of the viral communities differs between the influent, activated sludge, anaerobic digestion, and effluent (Tamaki et al., 2012; Ballesté et al., 2022; Brown et al., 2019; Wu and Liu, 2009). We did not observe evidence of the presence of the most abundant families (*Siphoviridae, Myoviridae,* and *Podoviridae*) commonly found in activated sludge environments, from analyzing the metagenomic data from the iDNA or exDNA in the effluent of the MBRs in this study. This may be because membrane separation impacts the phage community in the effluent compared to conventional secondary effluent. Our study used recently updated ICTV tables and a classification algorithm that uses a semi-supervised learning model, but it is also relevant to note that there is a lack of consensus with respect to classification algorithms and databases for viral contigs. The application of different classification algorithms or databases could impact phage taxonomic analysis.

3.2. Phage in both redox conditions possess both lytic and lysogenic capabilities

The presence of either lytic or lysogenic genes were investigated to understand the abundance and diversity of genes related to both life cycles as a function of redox and antibiotic loading. Genes associated with lysogeny and lysis for the biomass samples were at lower abundances when compared to iDNA and exDNA samples when using our bioinformatic tools (Figs. S1 & S3). This lower coverage of lytic or lysogenic genes in the biomass samples shown in Figs. S1, and S3 in the SI, is associated with the lower overall mean coverages of all contigs (Table S1) in the biomass samples in comparison to the iDNA and exDNA samples. As described above, this may be due to the expected higher diversity in the biomass samples which could lead to lower sequencing depths. Fig. 2 shows the three most abundant lytic or lysogenic genes found in all the samples. Phage *Schitoviridae*, the most abundant phage family in the AnMBR (Fig. 1), had both lytic and lysogenic capabilities in the AnMBR. Two lytic genes (Transglycosylase SLT domain [PF01464.23], Holin of 3TMs, for gene transfer release [PF11351.11]) and one lysogenic gene (Phage integrase family [PF00589.25]) were among the most abundant.

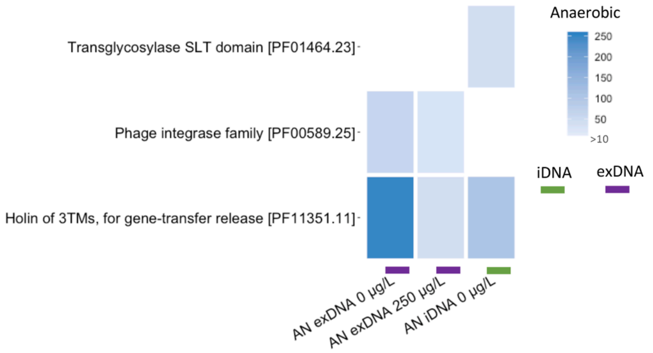

Fig. 2. The most abundant gene families related to either lysogeny or lysis identified in all the samples, overall abundance was highest in the AnMBR reactor. The green rectangle represents iDNA samples. The purple rectangle represents exDNA samples. White indicates rpkm values between 0 and 10, that is shown at higher resolution in Figs. 3 and 4.

Genes associated with the lysogenic life cycle were identified in both MBRs at the different antibiotic loadings, with no clear pattern with the antibiotic concentrations (Fig. 3). For the iDNA and exDNA samples, we identified 4 genes (Phage regulatory protein Rha [PF09669.13], Phage regulatory protein CII (CP76) [PF06892.14], Phage integrase family

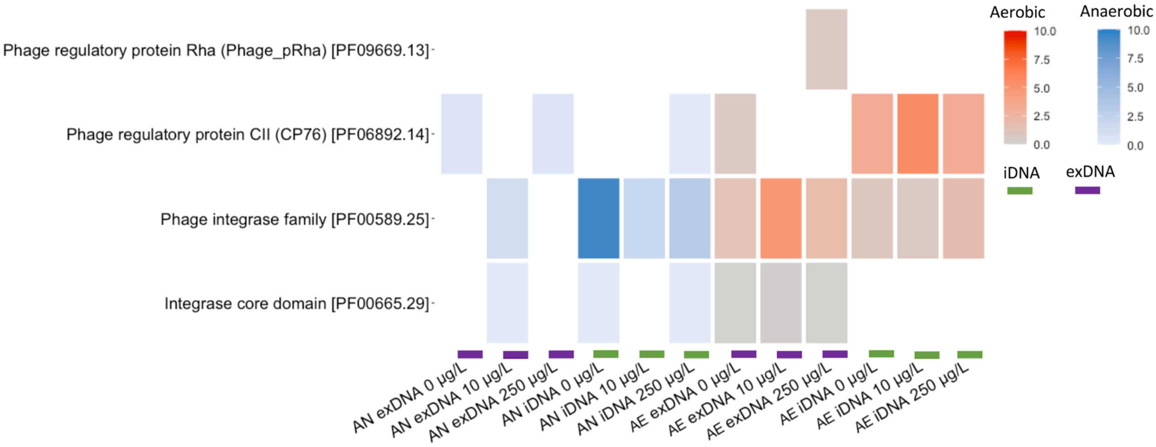

Fig. 3. Abundance (rpkm) of the gene families related to lysogeny identified in all the samples for both the aerobic (AE) and anaerobic (AN). The green rectangle represents iDNA samples. The purple rectangle represents exDNA samples.

[PF00589.25], and Integrase core domain [PF00665.29]) that have the potential to carry out lysogenic functions. We observed at least 2 lysogenic genes present in each sample. The gene Phage integrase family [PF00589.25] was observed in 10 out of the 12 samples analyzed and it was most abundant in the AN iDNA 0 μg/L condition. Phage integrases are enzymes that are used by temperate phage to promote lysogeny (Groth and Calos, 2004). The enzymes mediate the recombination between the phage DNA and the bacterial host genome to accomplish the integration of the phage into the bacterial host (Groth and Calos, 2004). Additionally, the gene encoding the Phage regulatory protein CII (CP76) [PF06892.14], was found in the MBRs. As the CII protein accumulates and reaches a threshold level, it initiates the lysogenic cycle by activating the CI repressor that blocks the promoters necessary for the Cro gene synthesis which is needed for the lytic cycle (Oppenheim et al., 2005; Richardson et al., 1989). As the expression of CII protein increases, the level of lysogenation increases as well (Hao et al., 2021). The Phage regulatory protein Rha [PF09669.13] was only found in one of the samples analyzed. The Rha protein is found in temperate phage and bacterial prophage regions (Henthorn and Friedman, 1995). Overall, several mechanisms of lysogeny were identified in both redox conditions.

Similar to lysogeny, genes associated with the lytic life cycle were identified in both MBRs at the different antibiotic loadings (Fig. 4). For the iDNA and exDNA samples, we identified 17 distinct lytic genes present in at least one of the samples. The phage lysozyme [PF00959.22] gene was found in all the samples, with the highest abundance in the AE exDNA 10 μg/L sample. We have identified 6 genes encoding for the holin protein and 10 genes encoding for the endolysin or lysozyme proteins in this study (Fig. 4). The holin protein acts by forming holes in the bacterial plasma membrane, subsequently allowing the endolysin to degrade or lyse the bacterial cell (Abedon, 2019; Wang et al., 2000). We also observed the presence of the DNA-binding transcriptional regulator Cro [PF14549.9] in the AE exDNA 0 μg/L sample. The Cro gene initiates the lytic life cycle in phage by blocking the synthesis of the CI gene in lambda phage (Abedon, 2019; Lee et al., 2018). Overall, our analysis found that the aerobic and anaerobic MBRs did not have a significant difference (p-value=0.19) in the diversity of lytic and lysogenic genes (anaerobic MBRs average was 3.8 ± 2.0 different genes, compared with aerobic MBRs 2.6 ± 2.2 in all samples).

The choice of the life cycle of phage can be explained by two models,

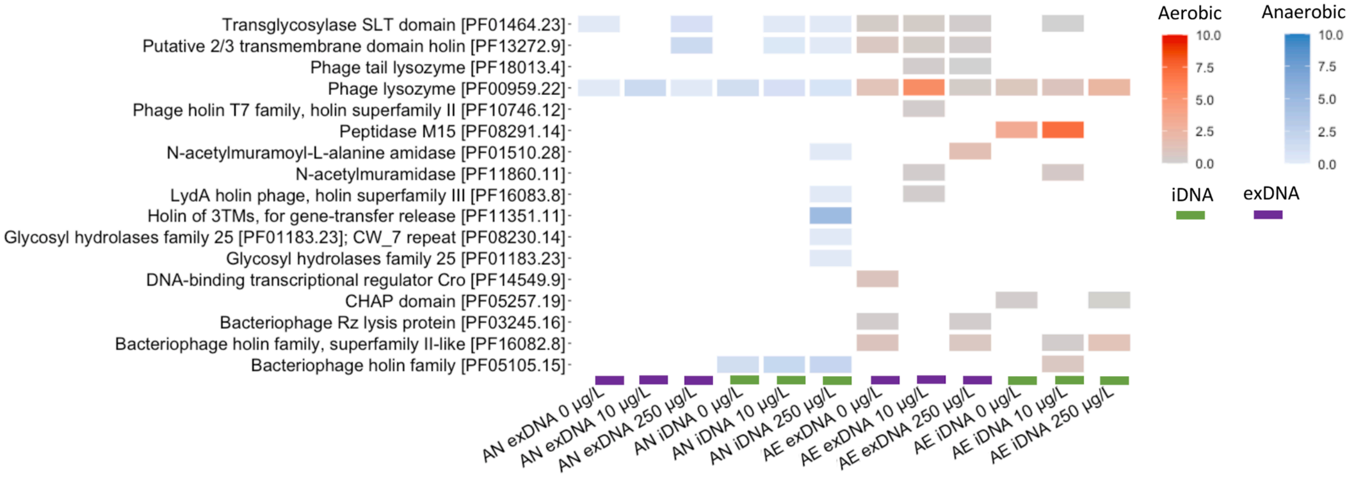

Fig. 4. Abundance (rpkm) of the gene families related to lysis identified in all the samples for both the aerobic (AE) and anaerobic (AN). The green rectangle represents iDNA samples. The purple rectangle represents exDNA samples. The genes that are associated with the holin protein are Phage holin T7 family holin superfamily II [PF10746.12], Putative 2/3 transmembrane domain holin [PF13272.9], LydA holin phage holin superfamily III [PF16083.8], Holin of 3TMs for gene-transfer release [PF11351.11], Bacteriophage holin family superfamily II-like [PF16082.8], Bacteriophage holin family [PF05105.15]. Endolysin or lysozyme genes are Transglycosylase SLT domain [PF01464.23], Phage tail lysozyme [PF18013.4], Phage lysozyme [PF00959.22], Peptidase M15 [PF08291.14], N-acetylmuramoyl-L-alanine amidase [PF01510.28], N-acetylmuramidase [PF11860.11], Glycosyl hydrolases family 25 [PF01183.23]; CW_7 repeat [PF08230.14], Glycosyl hydrolases family 25 [PF01183.23], CHAP domain [PF05257.19], Bacteriophage Rz lysis protein [PF03245.16].

‘Kill-the-Winner’ (KtW) and ‘Piggyback-the-Winner’ (PtW) (Silveira and Rohwer, 2016; Tang et al., 2022; Thingstad, 2000). The two models are thought to be driven by host density, where at higher host densities the KtW model dominates, and at lower host densities the PtW model dominates (Silveira and Rohwer, 2016; Tang et al., 2022; Thingstad, 2000). Phage interchange between the two ecological strategies to ensure their survival at different host densities and environmental conditions. We saw evidence of the presence of both lytic and lysogenic proteins in our samples, indicating that both models (KtW and PtW) were present in our samples. We also identified prophage from our viral contigs by using the CheckV and VirSorter tools (Table S2). From the total viral contigs (1043 viral contigs), CheckV had on average 8.11 ± 6.22 prophage contigs, while VirSorter had on average 7.17 ± 6.30 prophage contigs. ANOVA analysis was used to investigate differences in abundances of prophage relative to total phage abundances between the aerobic and anaerobic samples in the different sample types (iDNA & exDNA). From CheckV results, there was a significantly higher (F- value = 33.61, p-value = 0.0044, Tukey’s HSD = 0.0044) percentage of prophage in the anaerobic iDNA than in the aerobic iDNA (Tables S3 & S5). This may suggest the potential of having more phage induction in the AeMBR since the prophage were no longer in the cell. However, when using the VirSorter, we did not observe a significant difference (F-value = 0.907, p-value = 0.395, Tukey’s HSD = 0.395) between the aerobic and anaerobic prophage percentages in the iDNA samples (Tables S4 & S6). The results underscore that these tools are still being refined and further analysis is needed to identify the prophage contigs and potentially link these to their bacterial hosts.

Several studies in the literature have indicated that antibiotics act as a prophage inducer (switch the phage from being in a lysogenic state to a lytic one) (Tang et al., 2022; Motlagh et al., 2015; Zhang et al., 2020). In a bench-scale enhanced biological phosphorus removal (EBPR), it was shown that the antibiotic ciprofloxacin induced prophage resulting in the decrease of efficiency of the phosphorous removal (Silveira and Rohwer, 2016). Ciprofloxacin was also observed in another study to trigger the induction of Stx-prophage (Tang et al., 2022). However, there is not a consensus in the literature showing a positive correlation between prophage induction and antibiotic concentration (Colomer-Lluch et al., 2014a). Similarly, we did not observe a positive correlation between genes associated with lysis and antibiotic loading. It should be emphasized that our study is limited to DNA data, and we did not evaluate the expression of induction genes under higher antibiotic loading, and it is therefore possible the antibiotics were inducing cell lysis by phage.

The different redox conditions from the AeMBR and AnMBR tested could influence the dominant life cycle for phage in wastewater (Withey et al., 2005). We observed a higher diversity of the lytic proteins in the exDNA in the AeMBR in comparison to the AnMBR (Fig. 2), indicating that there is less evidence of lysis occurring in the AnMBR. However, we did not observe evidence that the different redox conditions change the presence of lysogenic genes (Fig. 3). A study by Ewert & Paynter (Ewert and Paynter, 1980) observed higher concentrations of phage titers in activated sludge compared to influent wastewater, suggesting lytic life cycles were favorable in conventional aerobic activated sludge. The importance of the lytic life cycle under aerobic conditions was also investigated in a bench-scale EBPR activated sludge process, in which four phages were isolated from the system (Khan et al., 2002). The phage showed its wide host range including in one instance an ability to infect both Gram-positive and Gram-negative bacteria. Results also showed that two of the four phage failed to infect their original hosts, which may suggest a mutation occurring on either the host or the phage that did not result in the lysis of the host cell. The study demonstrated the complex interactions and roles of phage in activated sludge. Few studies have evaluated infection mechanisms and viral life cycles in anaerobic conditions compared to aerobic environments (Withey et al., 2005; Hernández and Vives, 2020). More studies are needed to investigate the life cycle of phage under anaerobic conditions to understand the dynamics of phage, and the expression of these functions under different conditions.

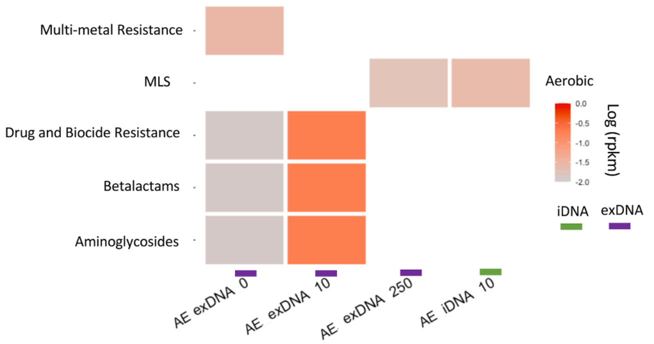

Fig. 5. Analysis of the ARG groups associated with the viral contigs for both the aerobic and anaerobic reactor. Abundances are measured using the rpkm values. Rpkm values are shown on a log scale in the figure.

3.3. *Aerobic conditions favored the abundance and presence of ARGs from the viral contigs*

ARGs from phage can be horizontally transferred to bacteria through transduction (Borodovich et al., 2022). In our analysis, we identified the ARGs from our viral contigs as these could pose a mechanism for ARG dissemination within bacterial communities (Fig. 5). We did not observe any phage-related ARGs in our AnMBR samples. In the aerobic samples, we observed that the AE exDNA 10 μg/L sample had the highest abundance of ARGs including drug and biocide resistance, betalactams, and aminoglycosides. We also observed these genes and macrolide-lincosamide-streptogramin (MLS) presence at lower abundances in the other AE exDNA samples (0 and 250 μg/L), and in one intracellular sample (AE iDNA 10 μg/L). The most abundant viral contig (Fig. S8) with an ARG was classified as *Peduoviridae* and had resistance genes of betalactams, aminoglycosides, and drug and biocide resistance. We did not observe a relationship between the increase in antibiotic concentrations and the abundance of the ARGs in the samples (Spearman’s rho= 0.56, p-value = 0.12). Only the ARG MLS was observed in the prophage identified from the viral contigs (Tables S7 & S8). However, when we compared the abundances of ARGs in viral contigs to the overall abundances of ARGs from the previous study (Wang et al., 2022), the ARGs from the viral contigs represent a small percentage (0.000182 %) of the overall abundances of ARGs across all the samples, which is consistent with a previous study that saw only 0.57 % ARGs were carried by vOTUs within anaerobic digestion ARGs (Zhang et al., 2024).

We have observed the presence of several ARGs in our viral DNA fractions. However, there could be other undetected ARGs within the viral populations which could be attributed to sequencing depth. Other studies have observed that phage carry several ARGs such as tetracycline, betalactams, ampicillin, quinolones, and erythromycin (Colomer-Lluch et al., 2014a; Balcazar, 2014; Colomer-Lluch et al., 2014b; Lood et al., 2017). In our study, phage-associated ARGs were only present in the AeMBR. Our result is consistent with the previous study (Wang et al., 2022), indicating that it is less likely for AnMBR to release ARGs (both phage- and non-phage associated) into the environment and the potential to use AnMBR to reduce the release of ARGs in full-scale wastewater treatment applications.

3.4. *Quorum sensing genes were observed in AeMBRs*

Identifying the lytic and lysogenic proteins along with identifying ARGs improves understanding of the phage life cycle and potential ARGs in the viral contigs at different redox conditions. To take our analysis a step further, we investigated quorum sensing genes within the viral contigs. There is evidence in the literature that confirms quorum sensing

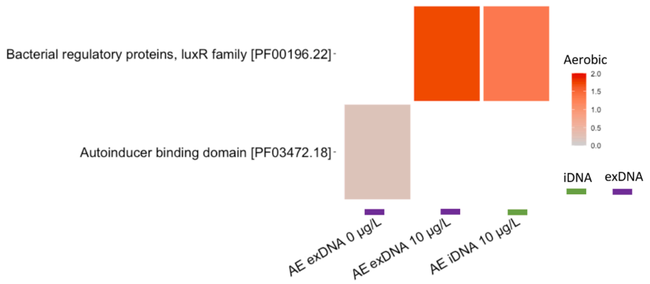

Fig. 6. Abundance (rpkm) of the gene families related to quorum sensing proteins identified in all the samples. The green rectangle represents iDNA samples. The purple rectangle represents exDNA samples.

between phage and bacteria (Vela and Al-Faliti, 2023), which could influence the switch to lytic life cycles (Duddy and Bassler, 2021; Silpe and Bassler, 2019; Laganenka et al., 2019; Tan et al., 2020). In our study, we have identified 2 genes (Bacterial regulatory proteins, *lux*R family [PF00196.22], Autoinducer binding domain [PF03472.18]) that are associated with quorum sensing capabilities from our viral contigs (Fig. 6). Similar to the ARG hits (Fig. 5), we only saw evidence of QS genes in the phage-related contigs in the AeMBR and not in the AnMBR. The most abundant QS-containing viral contig (Fig. S9) was found in the exDNA sample from the 10 ug/L conditions and contained both *lux*R and lysogenic genes but was of unknown taxonomy. The luxR protein acts as a sensor-regulator for the chemical signals produced by bacteria called N-Acylhomoserine lactones (AHLs) (Nasser and Reverchon, 2007; Subramoni and Venturi, 2009). The AHLs are produced by bacteria in a cell-density-dependent way, then the AHL signal gets detected by the luxR protein as a result the system regulates the bacterial cell functions such as biofilm formation and virulence (Nasser and Reverchon, 2007; Subramoni and Venturi, 2009; Oh and Lee, 2018). Together, these receptors indicate that phage could be responding to bacterial signals in aerobic environments.

4. Conclusion

Our study focused on the use of metagenomic analysis of viral communities in an AeMBR and AnMBR under different antibiotic loadings. Our study provides valuable insights into the diversity and functional potential of viral communities in wastewater treatment systems under different redox conditions and antibiotic exposures. We investigated different aspects of these viral communities including taxonomic composition, life cycle (lytic and lysogenic), ARGs, and quorum sensing genes. We observed that *Schitoviridae* was dominant in the AnMBR whereas phage *Peduoviridae* was more dominant in the AeMBR. Our results also indicated that regardless of the redox condition, phage had both lytic and lysogenic capabilities. Furthermore, the ARGs were only present in phage in the AeMBR effluent, supporting the results from the previous study that AnMBRs are less likely to release ARGs into the environment. This provides further validation for the potential of AnMBRs to have reduced dissemination of antimicrobial resistance.

For the potential to leverage phage for wastewater process operations, further research is needed to explore the intricate interactions between phage and their bacterial hosts in wastewater environments. The regulation of lytic and lysogenic life cycles should also be investigated under different types of wastewater environments and along the treatment process to better be able to adopt 'phage therapy' for biofouling control or other beneficial treatment outcomes. Furthermore, the mechanisms by which receptors of phage and bacteria interpret autoinducers can be further investigated to understand how they regulate the functions of the microbial communities. Overall, future research could potentially help in improving the efficiency of wastewater treatment by understanding how to use phage and bacterial quorum sensing signals to control the treatment process.

CRediT authorship contribution statement

Mitham Al-Faliti: Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Phillip Wang:** Writing – review & editing, Methodology, Data curation. **Adam L. Smith:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation. **Jeseth Delgado Vela:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data has been uploaded to NCBI under Project ID PRJNA1073904. The full bioinformatic pipeline code is available at https://github.com/Delgado-Vela-Research-Group/Phage-Analysis.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121620.

References

Abedon, S.T., 1992. Lysis of lysis-inhibited bacteriophage T4-infected cells. J. Bacteriol. 174 (24), 8073–8080. https://doi.org/10.1128/jb.174.24.8073-8080.1992.

Abedon, S.T., 2019. Look who's talking: t-even phage lysis inhibition, the granddaddy of virus-virus intercellular communication research. Viruses 11 (10), 951.

Balcazar, J.L., 2014. Bacteriophages as vehicles for antibiotic resistance genes in the environment. PLoS Pathog. 10 (7), e1004219.

Ballesté, E., Blanch, A.R., Muniesa, M., García-Aljaro, C., Rodríguez-Rubio, L., Martín-Díaz, J., Pascual-Benito, M., Jofre, J., 2022. Bacteriophages in sewage: abundance, roles, and applications. FEMS Microbes 3, xtac009.

Bhattacharjee, A.S., Choi, J., Motlagh, A.M., Mukherji, S.T., Goel, R., 2015. Bacteriophage therapy for membrane biofouling in membrane bioreactors and antibiotic-resistant bacterial biofilms. Biotechnol. Bioeng. 112 (8), 1644–1654.

Bonin, N., Doster, E., Worley, H., Pinnell, L.J., Bravo, J.E., Ferm, P., Marini, S., Prosperi, M., Noyes, N., Morley, P.S., 2023. MEGARes and AMR++, v3. 0: an updated comprehensive database of antimicrobial resistance determinants and an improved software pipeline for classification using high-throughput sequencing. Nucleic Acids Res. 51 (D1), D744–D752.

Borodovich, T., Shkoporov, A.N., Ross, R.P., Hill, C., 2022. Phage-mediated horizontal gene transfer and its implications for the human gut microbiome. Gastroenterol. Rep. (Oxf) 10, goac012.

Brown, M.R., Baptista, J.C., Lunn, M., Swan, D.L., Smith, S.J., Davenport, R.J., Allen, B. D., Sloan, W.T., Curtis, T.P., 2019. Coupled virus-bacteria interactions and ecosystem function in an engineered microbial system. Water Res. 152, 264–273.

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34 (17), i884–i890.

Chevallereau, A., Pons, B.J., van Houte, S., Westra, E.R., 2022. Interactions between bacterial and phage communities in natural environments. Nat. Rev. Microbiol. 20 (1), 49–62.

Clokie, M.R., Millard, A.D., Letarov, A.V., Heaphy, S., 2011. Phages in nature. Bacteriophage 1 (1), 31–45.

Colomer-Lluch, M., Calero-Cáceres, W., Jebri, S., Hmaied, F., Muniesa, M., Jofre, J., 2014b. Antibiotic resistance genes in bacterial and bacteriophage fractions of tunisian and spanish wastewaters as markers to compare the antibiotic resistance patterns in each population. Environ. Int. 73, 167–175.

Colomer-Lluch, M., Jofre, J., Muniesa, M., 2014a. Quinolone resistance genes (qnrA and qnrS) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes. J. Antimicrob. Chemother. 69 (5), 1265–1274.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., 2021. Twelve years of SAMtools and BCFtools. Gigascience 10 (2), giab008.

Doss, J., Culbertson, K., Hahn, D., Camacho, J., Barekzi, N., 2017. A review of phage therapy against bacterial pathogens of aquatic and terrestrial organisms. Viruses 9 (3), 50. https://doi.org/10.3390/v9030050.

Duddy, O.P., Bassler, B.L., 2021. Quorum sensing across bacterial and viral domains. PLoS Pathog. 17 (1), e1009074.

Erez, Z., Steinberger-Levy, I., Shamir, M., Doron, S., Stokar-Avihail, A., Peleg, Y., Melamed, S., Leavitt, A., Savidor, A., Albeck, S., Amitai, G., Sorek, R., 2017. Communication between viruses guides Lysis–Lysogeny decisions. Nature 541 (7638), 488–493. https://doi.org/10.1038/nature21049.

Ewert, D.L., Paynter, M.J.B., 1980. Enumeration of bacteriophages and host bacteria in sewage and the activated-sludge treatment process. Appl. Environ. Microbiol. 39 (3), 576–583.

Feng, L., Wu, Z., Yu, X., 2013. Quorum sensing in water and wastewater treatment biofilms. J. Environ. Biol. 34 (2 suppl), 437.

Fuhrman, J.A., 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399 (6736), 541–548. https://doi.org/10.1038/21119.

Gomes, M., Domingues, R., Turner, D., Oliveira, H., 2023. Genomic analysis of two novel bacteriophages infecting acinetobacter beijerinckii and halotolerans species. Viruses 15, 643, 2023.

Goswami, L., Kumar, R.V., Borah, S.N., Manikandan, N.A., Pakshirajan, K., Pugazhenthi, G., 2018. Membrane bioreactor and integrated membrane bioreactor systems for micropollutant removal from wastewater: a review. J. Water Process Eng. 26, 314–328.

Groth, A.C., Calos, M.P., 2004. Phage integrases: biology and applications. J. Mol. Biol. 335 (3), 667–678. https://doi.org/10.1016/j.jmb.2003.09.082.

Guo, J., Bolduc, B., Zayed, A.A., Varsani, A., Dominguez-Huerta, G., Delmont, T.O., Pratama, A.A., Gazitúa, M.C., Vik, D., Sullivan, M.B., Roux, S., 2021. VirSorter2: a multi-classifier, expert-guided approach to detect diverse DNA and RNA viruses. Microbiome 9 (1), 37. https://doi.org/10.1186/s40168-020-00990-y.

Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29 (8), 1072–1075.

Hao, N., Agnew, D., Krishna, S., Dodd, I.B., Shearwin, K.E., 2021. Analysis of infection time courses shows CII levels determine the frequency of Lysogeny in Phage 186. Pharmaceuticals 14 (10), 998. https://doi.org/10.3390/ph14100998 (Basel).

Hegarty, B., Dai, Z., Raskin, L., Pinto, A., Wigginton, K., Duhaime, M., 2022. A snapshot of the global drinking water virome: diversity and metabolic potential vary with residual disinfectant use. Water Res. 218, 118484.

Henthorn, K.S., Friedman, D.I., 1995. Identification of related genes in phages Phi 80 and P22 whose products are inhibitory for phage growth in Escherichia Coli IHF mutants. J. Bacteriol. 177 (11), 3185–3190.

Hernández, S., Vives, M.J., 2020. Phages in anaerobic systems. Viruses 12 (10), 1091.

Hu, H., Luo, F., Liu, Y., Zeng, X., 2021. Function of quorum sensing and cell signaling in wastewater treatment systems. Water Sci. Technol. 83 (3), 515–531. https://doi.org/10.2166/wst.2020.601.

Ji, M., Fan, X., Cornell, C.R., Zhang, Y., Yuan, M.M., Tian, Z., Sun, K., Gao, R., Liu, Y., Zhou, J., 2023. Tundra soil viruses mediate responses of microbial communities to climate warming. mBio 14 (2), e03009–e03022.

Khan, M.A., Satoh, H., Mino, T., Katayama, H., Kurisu, F., Matsuo, T., 2002. Bacteriophage-host interaction in the enhanced biological phosphate removing activated sludge system. Water Sci. Technol. 46 (1–2), 39–43.

Laganenka, L., Sander, T., Lagonenko, A., Chen, Y., Link, H., Sourjik, V., 2019. Quorum sensing and metabolic state of the host control Lysogeny-Lysis switch of bacteriophage T1. mBio 10 (5). https://doi.org/10.1128/mbio.01884-19.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with bowtie 2. Nat. Methods 9 (4), 357–359. https://doi.org/10.1038/nmeth.1923.

Lee, S., Lewis, D.E.A., Adhya, S., 2018. The developmental switch in bacteriophage λ: a critical role of the cro protein. J. Mol. Biol. 430 (1), 58–68. https://doi.org/10.1016/j.jmb.2017.11.005.

Li, D., Liu, C.M., Luo, R., Sadakane, K., Lam, T.W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de bruijn graph. Bioinformatics 31 (10), 1674–1676.

Lood, R., Ertürk, G., Mattiasson, B., 2017. Revisiting antibiotic resistance spreading in wastewater treatment plants–bacteriophages as a much neglected potential transmission vehicle. Front. Microbiol. 8, 2298.

Motlagh, A.M., Bhattacharjee, A.S., Goel, R., 2015. Microbiological study of bacteriophage induction in the presence of chemical stress factors in enhanced biological phosphorus removal (EBPR). Water Res. 81, 1–14.

Nasser, W., Reverchon, S., 2007. New insights into the regulatory mechanisms of the LuxR family of quorum sensing regulators. Anal. Bioanal. Chem. 387, 381–390.

Nayfach, S., Camargo, A.P., Schulz, F., Eloe-Fadrosh, E., Roux, S., Kyrpides, N.C., 2021. CheckV assesses the quality and completeness of metagenome-assembled viral genomes. Nat. Biotechnol. 39 (5), 578–585. https://doi.org/10.1038/s41587-020-00774-7.

Oh, H.S., Lee, C.H., 2018. Origin and evolution of quorum quenching technology for biofouling control in MBRs for wastewater treatment. J. Membr. Sci. 554, 331–345.

Oksanen, J.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'hara, R.B.; Simpson, G.L.; Solymos, P.; Stevens, M.H.H.; Wagner, H. Package 'Vegan'. *Community ecology package, version* 2013, 2 (9), 1–295.

Oppenheim, A.B., Kobiler, O., Stavans, J., Court, D.L., Adhya, S., 2005. Switches in bacteriophage lambda development. Annu. Rev. Genet. 39, 409–429.

Richardson, H., Puspurs, A., Egan, J.B., 1989. Control of gene expression in the P2-related temperate coliphage 186: VI. Sequence analysis of the early lytic region. J. Mol. Biol. 206 (1), 251–255. https://doi.org/10.1016/0022-2836(89)90539-1.

Roux, S., Enault, F., Hurwitz, B.L., Sullivan, M.B., 2015. VirSorter: mining viral signal from microbial genomic data. PeerJ. 3, e985. https://doi.org/10.7717/peerj.985.

Sáenz, J.S., Rios-Galicia, B., Rehkugler, B., Seifert, J., 2023. Dynamic development of viral and bacterial diversity during grass silage preservation. Viruses 15 (4), 951.

Shaffer, M., Borton, M.A., McGivern, B.B., Zayed, A.A., La Rosa, S.L., Solden, L.M., Liu, P., Narrowe, A.B., Rodríguez-Ramos, J., Bolduc, B., 2020. DRAM for distilling microbial metabolism to automate the curation of microbiome function. Nucleic Acids Res. 48 (16), 8883–8900.

Shang, J., Jiang, J., Sun, Y., 2021. Bacteriophage classification for assembled contigs using graph convolutional network. Bioinformatics 37 (Supplement_1), i25–i33.

Shrout, J.D., Nerenberg, R., 2012. Monitoring bacterial twitter: does quorum sensing determine the behavior of water and wastewater treatment biofilms? Environ. Sci. Technol. 46 (4), 1995–2005.

Silpe, J.E., Bassler, B.L., 2019. A host-produced quorum-sensing autoinducer controls a phage lysis-lysogeny decision. Cell 176 (1), 268–280 e13.

Silveira, C.B., Rohwer, F.L., 2016. Piggyback-the-winner in host-associated microbial communities. NPJ Biofilms Microbiomes 2 (1), 1–5. https://doi.org/10.1038/npjbiofilms.2016.10.

Smith, A.L., Stadler, L.B., Love, N.G., Skerlos, S.J., Raskin, L., 2012. Perspectives on anaerobic membrane bioreactor treatment of domestic wastewater: a critical review. Bioresour. Technol. 122, 149–159.

Strange, J.E., Leekitcharoenphon, P., Møller, F.D., Aarestrup, F.M., 2021. Metagenomics analysis of bacteriophages and antimicrobial resistance from global urban sewage. Sci. Rep. 11 (1), 1600.

Subramoni, S., Venturi, V., 2009. LuxR-family 'Solos': bachelor sensors/regulators of signalling molecules. Microbiology 155 (5), 1377–1385.

Tamaki, H., Zhang, R., Angly, F.E., Nakamura, S., Hong, P.Y., Yasunaga, T., Kamagata, Y., Liu, W.T., 2012. Metagenomic analysis of DNA viruses in a wastewater treatment plant in tropical climate. Environ. Microbiol. 14 (2), 441–452.

Tan, D., Hansen, M.F., de Carvalho, L.N., Røder, H.L., Burmølle, M., Middelboe, M., Svenningsen, S.L., 2020. High cell densities favor lysogeny: induction of an H20 prophage is repressed by quorum sensing and enhances biofilm formation in vibrio anguillarum. ISME J. 14 (7), 1731–1742.

Tang, X., Fan, C., Zeng, G., Zhong, L., Li, C., Ren, X., Song, B., Liu, X., 2022. Phage-host interactions: the neglected part of biological wastewater treatment. Water Res., 119183

Team, R. Developement Core. A Language and Environment for Statistical Computing. http://www.R-project.org 2009.

Thingstad, T.F., 2000. Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. Limnol. Oceanogr. 45 (6), 1320–1328.

Wadhwa, D. Design principle of lysis/lysogeny decision vis-a-vis multiplicity of infection. *BioRxiv* 2017, 146308.

Vela, J.D., Al-Faliti, M., 2023. Emerging investigator series: the role of phage lifestyle in wastewater microbial community structures and functions: insights into diverse microbial environments. Environ. Sci.: Water Res. Technol. 9 (8), 1982–1991.

Wang, I.N., Smith, D.L., Young, R., 2000. Holins: the protein clocks of bacteriophage infections. Annu. Rev. Microbiol. 54 (1), 799–825.

Wang, P., Zarei-Baygi, A., Delgado Vela, J., Smith, A.L., 2022. Metagenomic analysis of the antibiotic resistance risk between an aerobic and anaerobic membrane bioreactor. ACS ES&T Water 3 (7), 1865–1873.

Withey, S., Cartmell, E., Avery, L.M., Stephenson, T., 2005. Bacteriophages–potential for application in wastewater treatment processes. Sci. Total Environ. 339 (1–3), 1–18.

Wommack, K.E., Colwell, R.R., 2000. Virioplankton: viruses in aquatic ecosystems. Microbiol. Mol. Biol. Rev. 64 (1), 69–114.

Wu, Q., Liu, W.T., 2009. Determination of virus abundance, diversity and distribution in a municipal wastewater treatment plant. Water Res. 43 (4), 1101–1109.

Yuan, L., Ju, F., 2023. Potential auxiliary metabolic capabilities and activities reveal biochemical impacts of viruses in municipal wastewater treatment plants. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.2c07800.

Zhang, J., Lu, T., Song, Y., da Rocha, U.N., Liu, J., Nikolausz, M., Wei, Y., Richnow, H.H., 2024. Viral communities contribute more to the lysis of antibiotic-resistant bacteria than the transduction of antibiotic resistance genes in anaerobic digestion revealed by metagenomics. Environ. Sci. Technol. 58 (5), 2346–2359.

Zhang, Y., Liao, Y.T., Salvador, A., Sun, X., Wu, V.C., 2020. Prediction, diversity, and genomic analysis of temperate phages induced from shiga toxin-producing Escherichia Coli strains. Front. Microbiol. 10, 3093.