Chemosphere 349 (2024) 140743

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Investigating the viral ecology and contribution to the microbial ecology in full-scale mesophilic anaerobic digesters

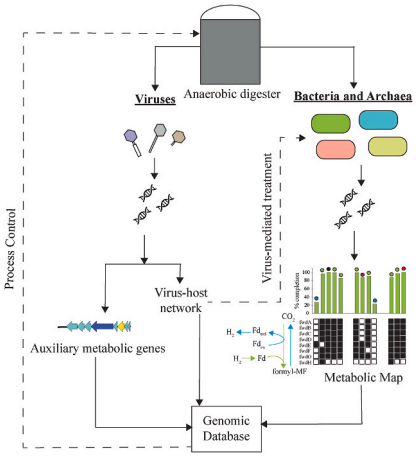
Bishav Bhattarai [a], Ananda Shankar Bhattacharjee [b,c], Felipe H. Coutinho [d], Ramesh Goel [a,*]

[a] The University of Utah, Department of Civil and Environmental Engineering, 110 S Central Campus Drive, Salt Lake City, UT, 84112, United States
[b] Department of Environmental Sciences, The University of California, Riverside, Riverside, CA, United States
[c] USDA-ARS, United States Salinity Laboratory, Riverside, CA, United States
[d] Department of Marine Biology and Oceanography, Institute of Marine Sciences, Consejo Superior de Investigaciones Científicas (ICM-CISC), Barcelona, Spain

HIGHLIGHTS

- The analyses of digester virome showed the presence of lytic and lysogenic viruses.
- *Siphoviridae* was the dominant family in mesophilic anaerobic digesters, followed by *Myoviridae* and *Podoviridae*.
- Lysogeny was prevalent in mesophilic anaerobic digesters.
- Lysogeny provides a fitness advantage to methanogens in anaerobic digesters by adding flexibility to changing substrates.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling editor: Y Yeomin Yoon

Keywords:
Viruses
Virus-host interaction
Anaerobic digester
Methanogenesis
Virus-mediated treatment
Auxiliary metabolic genes (AMGs)

ABSTRACT

In an attempt to assess the diversity of viruses and their potential to modulate the metabolism of functional microorganisms in anaerobic digesters, we collected digestate from three mesophilic anaerobic digesters in full-scale wastewater treatment plants treating real municipal wastewater. The reads were analyzed using bioinformatics algorithms to elucidate viral diversity, identify their potential role in modulating the metabolism of functional microorganisms, and provide essential genomic information for the potential use of virus-mediated treatment in controlling the anaerobic digester microbiome. We found that *Siphoviridae* was the dominant family in mesophilic anaerobic digesters, followed by *Myoviridae* and *Podoviridae*. Lysogeny was prevalent in mesophilic anaerobic digesters as the majority of metagenome-assembled genomes contained at least one viral genome within them. One virus within the genome of an acetoclastic methanogen (*Methanothrix soehngenii*) was observed with a gene (*fwdE*) acquired via lateral transfer from hydrogenotrophic methanogens. The virus-mediated acquisition of *fwdE* gene enables possibility of mixotrophic methanogenesis in *Methanothrix*

* Corresponding author.
E-mail addresses: u1201517@utah.edu (B. Bhattarai), ram.goel@utah.edu (R. Goel).

https://doi.org/10.1016/j.chemosphere.2023.140743
Received 2 September 2023; Received in revised form 14 November 2023; Accepted 15 November 2023
Available online 19 November 2023

soehngenii. This evidence highlighted that lysogeny provides fitness advantage to methanogens in anaerobic digesters by adding flexibility to changing substrates. Similarly, we found auxiliary metabolic genes, such as cellulase and alpha glucosidase, of bacterial origin responsible for sludge hydrolysis in viruses. Additionally, we discovered novel viral genomes and provided genomic information on viruses infecting acidogenic, acetogenic, and pathogenic bacteria that can potentially be used for virus-mediated treatment to deal with the souring problem in anaerobic digesters and remove pathogens from biosolids before land application. Collectively, our study provides a genome-level understanding of virome in conjunction with the microbiome in anaerobic digesters that can be used to optimize the anaerobic digestion process for efficient biogas generation.

1. Introduction

The production of excess sludge during wastewater treatment using activated sludge (AS) processes is one of the biggest drawbacks of the AS processes. Sludge production will increase more with the expansion of the industry and population (Zhang et al., 2010; Zhang et al., 2019). Sludge disposal to landfills or direct incineration is inappropriate due to the environmental impacts of these management practices (Zhang et al., 2010; Pujara et al., 2019; Istrate et al., 2021). Anaerobic digestion has been used as a popular alternative because it can reduce excess sludge, eliminate pathogens, and produce useful biofuel methane (Appels et al., 2008). Anaerobic digestion is a microbially-mediated process capable of generating renewable energy in the form of energy-rich compounds-alcohols, long-chain fatty acids (LCFA), volatile fatty acids (VFA), and methane by breaking down organic matter in the absence of oxygen (Amani et al., 2010). The capture of methane for renewable energy generation reduces its contribution to the greenhouse effect through atmospheric release (Steed and Hashimoto, 1994). The success of the anaerobic digestion process depends upon the presence of a diverse and robust microbial community in the reactor. However, the differences in the microbial growth rate and activity lead to fluctuating community composition and accumulation of intermediate products (NH_3, VFA, LCFA), disrupting the process and making it challenging to control. Along with the bacteria and archaea, the complex ecosystem of the anaerobic digester (AD) bioreactors consists of an abundant population of viruses ($\sim 10^9$ virus-like particles per mL) (Wu and Liu, 2009).

Viruses are considered key players in shaping bacterial and archaeal abundance, community composition, and ecosystem function (Breitbart, 2012; Rodriguez-Valera et al., 2009) through a) nutrient regeneration (Haaber and Middelboe, 2009; Middelboe and Jørgensen, 2006; Shelford et al., 2012), b) expression of auxiliary metabolic genes (AMGs) (Anantharaman et al., 2014; Enav et al., 2014; Thompson et al., 2011), and c) horizontal gene transfer (HGT) events (Lindell et al., 2004; Sullivan et al., 2006). The role of viruses in nutrient removal and the carbon cycle has been studied in AS systems (Chen et al., 2021; Shi et al., 2022), but AD virome has been understudied. In the AD ecosystem, some studies have attempted to highlight the diversity of viruses (Calusinska et al., 2016; Ngo et al., 2022), while some have explored the prophage induction in AD under physical and chemical stresses (Rossi et al., 2022). However, the understanding of the potential of viruses to impact the AD microbiome and metabolism is still lacking in full-scale ADs treating municipal sludge (Rossi et al., 2022; Wu and Liu, 2009; Zhang et al., 2017). Yet, there is reason to expect that viruses can exert large effects on bacterial abundance and function (Fernández et al., 2018; Shi et al., 2022; Tang et al., 2022). Additionally, the host specificity of viruses (de Jonge et al., 2019; Nobrega et al., 2018; Weinbauer, 2004) could potentially be harnessed for controlling the microbiome through the selective removal of microorganisms (Petrovski et al., 2022; Shivaram et al., 2023). Virus-mediated treatment has been successfully used in AS systems to infect and lyse filamentous bacteria to control sludge bulking (Khairnar et al., 2016; Kotay et al., 2011; Liu et al., 2015), and recent studies have emphasized creating a genomic database for future virus-mediated treatment for sludge bulking in AS systems (Chen et al., 2021). The genomic database for potentially using virus-mediated treatment to deal with process upsets in AD also lacks information.

Here, we studied the ecology of viruses in conjunction with the microbial ecology in three mesophilic ADs from full-scale WWTPs treating municipal wastewater. Full-scale ADs were selected to study the virus-host association in the microbiome acclimated to different pollutants present in real municipal wastewater. One AD chosen in this study consisted of a thermal hydrolysis pretreatment (THP) unit that hydrolyzes the sludge at high pressure and temperature before feeding them into the AD. The inclusion of AD with THP encompasses a popular technology for intensifying biosolids management in WWTPs (Gahlot et al., 2022). We extracted and sequenced the whole-metagenomic DNA and viral DNA from the isolated fraction of viruses in the digestate. The reads generated by sequencing were analyzed using bioinformatics algorithm to a) elucidate the viral community diversity and their replication lifestyle in mesophilic ADs, b) identify the presence of functional genes in viruses from mesophilic ADs that can redirect the metabolism of the host, and c) provide virus-host relationships for functional microorganisms in the AD with genomic data for the potential future design of virus-mediated treatment to tackle process upsets. This study is important as the profitability of anaerobic digestion is predicted to rise with the cost of other energy sources and increasing interest in implementing a U.S. carbon tax (Hafstead and Williams, 2020).

2. Materials and methods

2.1. Sample collection and cellular DNA extraction

Anaerobically digested sludge, hereafter called digestate, was collected from Blue Plains Advanced WWTP (DC Water, Washington DC, USA; 38.8204 N 77.0185 W), Salt Lake City Water Reclamation Facility (SLCWRF, Utah, USA; 40.813194 N 111.930806 W), and Central Valley Water Reclamation Facilities (CVWRF, Utah, USA; 40.70361293 N 111.91411557 W). One sample was collected from each ADs selected in this study. The AD in DC Water consisted of a separate unit for the THP (Cambi©). The details of the ADs used for sampling are mentioned in **Text S1**. Around 1 L of sludge samples were collected in an HDPE bottle and immediately shipped with ice to the lab at the University of Utah for further processing. Whole metagenomic DNA from sludge samples was extracted using a DNeasy PowerSoil DNA Isolation Kit (Qiagen Inc, Valencia, California) according to the manufacturer's instructions. Metagenomic DNA concentrations were measured using a Thermo NanoDrop 2000c at 260/280, and the samples with a 260/280 ratio higher than 1.80 were used for further analysis. The extracted DNA was stored at −20 °C or −80 °C until further investigation.

2.2. Free virus extraction, transmission electron microscopy (TEM), and viral DNA extraction

The viral fraction from the digestate samples was isolated and concentrated according to the protocol used in similar virome studies (Bhattarai et al., 2021; Motlagh et al., 2017). Briefly, for each sample, the free viruses in the digestate were brought into the potassium citrate buffer through shaking, followed by removing the cellular fraction by centrifugation and filtration. The isolated viral fraction was purified using cesium chloride density gradient centrifugation. The purified viral

particles were observed under the TEM to assess the morphology of the extracted viral particles. The viral DNA was extracted using a phage DNA isolation kit (Norgen Biotek Corp., Canada). The details of the protocol are provided in **Text S2**. The isolation of the viral fraction allows better characterization of viral genomes without interference from other microbial and host DNA during sequencing.

2.3. DNA library preparation for sequencing

The library preparation and sequencing were performed at the HCI core facility (the University of Utah). Briefly, the library construction for the whole metagenome and viral DNA samples was performed using Nextera DNA Flex Library Prep with UDI (Illumina, CA). The constructed libraries were sequenced on an Illumina NovaSeq sequencer using NovaSeq Reagent Kit 0v1.5 (150 x 150 bp, Illumina, CA) at the HCI Core Facility (the University of Utah). The raw metagenome and metavirome were deposited in the National Center for Biotechnology Information (NCBI) database. The accession numbers for metagenome reads from DC Water, SLCWRF, and CVWRF are SRR21487928, SRR21487926, SRR21487924, respectively. Similarly, the accession numbers for metavirome reads from DC Water, SLCWRF, and CVWRF are SRR21487927, SRR21487925, SRR21487923.

2.4. Metagenome and metavirome QC and assembly

The raw paired-end metagenome and metavirome reads were checked for quality using FastQC v0.11.8 (Andrews, 2010). The read-through adapters and low-quality reads were removed using Trimmomatic v0.39 (Bolger et al., 2014). The low-quality reads included reads with lengths less than 36 bp (MINLEN:36), low-quality base calls at the start and end of reads (LEADING:3, TRAILING:3), and reads with an average Phred score lower than 28 in a sliding window of 4bp (SLIDINGWINDOW:4:28). Along with the selection of high-quality reads, the quality control parameters in Trimmomatic were benchmarked to ensure substantial retention of reads (>90% retention). The trimmed and quality-filtered reads were *de novo* assembled with a range of *k*-mer values (21, 33, 55, 77, 99, 127) using metaSPAdes v3.13.0 (Bankevich et al., 2012). The scaffolds with less than 1 Kbp length were removed from the further analysis.

2.5. Assessment of taxonomic profile of bacterial and archaeal community; construction and annotation of metagenome-assembled genomes (MAGs)

The quality-filtered whole metagenomic DNA sequences were used to determine the bacterial and archaeal community composition in the digestate using Metaphlan version 2.7.7 with default parameters (Truong et al., 2015). In Metaphlan, the quality-filtered sequences were mapped to ~1 million clade-specific marker genes to determine the relative abundance of microorganisms. MAGs were constructed, checked for quality, and assigned taxonomy as mentioned in **Text S3.**

The proportion of different genera obtained from Metaphlan was used to calculate the Shannon diversity index using the following equation (Spellerberg and Fedor, 2003).

$$H = -\sum p_i * \ln(p_i)$$

where,

H = Shannon diversity index

p_i = proportion of a specific genera.

Σ = summation (over all genera)

The MAGs of methanogens with a completeness of more than 70% and contamination of less than 5% were placed in the reference tree, including 1248 archaeal species included in the database of GTDB-tk v0.3.2 (Chaumeil et al., 2019). The branches of the tree which did not contain any MAGs of methanogens constructed in our study were collapsed in CLC Genomics Workbench v.12 (CLC Bio, Denmark). The tree was annotated with each color representing a different phylum using CLC Genomics Workbench v.12 (CLC Bio, Denmark). The functional annotation of the MAGs was performed using Prokka v1.14.6 (e−value 10^{-5}) (Seemann, 2014) and MicrobeAnnotator (default parameters) (Ruiz-Perez et al., 2021), as explained in **Text S3.**

2.6. Assessment of viral community and their auxiliary metabolism

VIBRANT v1.2.0 (Kieft et al., 2020) and VirSorter2 v2.2.2 (Guo et al., 2021) were used to recover viral scaffolds from the virome assemblies, and only scaffolds of at least 5 kbp were kept for subsequent analysis. CheckV v0.9.0 (Nayfach et al., 2021) was used to assess the quality of the recovered viral scaffolds. The taxonomy was assigned to the viral scaffolds using VPF-Class using default parameters (Pons et al., 2021). The taxonomic profiling of the viral community was performed as explained in **Text S4**. The scaffolds within the MAGs constructed using the whole metagenomic DNA sequences were also checked for viral signals using VIBRANT v1.2.0 (Kieft et al., 2020) and VirSorter2 v2.2.2 (Guo et al., 2021). The MAGs containing viral scaffold were referred to as lysogens. The viral scaffolds within the MAGs were distinguished as intact, questionable, or incomplete viral genomes using PHASTER (Arndt et al., 2016; Zhou et al., 2011). The viral genomes were annotated to identify AMGs, as mentioned in **Text S4**. The phylogenetic analysis of the AMGs was conducted, as mentioned in **Text S5**. The bacterial and archaeal hosts of the viruses were predicted using VirHostMatcher-Net using default parameters (Wang et al., 2020). The predictions with a 'score' greater than 0.95 were considered a true virus-host match.

3. Results

A total of 831.92 million (129.78 ± 22.41 million per sample) paired-end reads of length 150 bp were generated for the microbiome and virome datasets (Table S1). An average of 93.57 ± 1.09% of the reads were retained following the removal of read-through adapters and low-quality reads using Trimmomatic v0.39 (Bolger et al., 2014). A total of 726,316 (average: 242,105 per sample) and 163,086 (average: 54,362 per sample) scaffolds of size greater than 1 kbp were assembled from the microbiome and virome metagenome dataset, respectively (Table S1).

3.1. Bacterial and archaeal community in ADs

Based on the Shannon diversity index (H) calculated at the genus level of the bacterial and archaeal community, the digestate from DC Water was the least diverse (H = 1.27) compared to the digestate samples from SLCWRF (H = 1.68) and CVWRF (H = 2.93). The richness of the bacterial and archaeal community at the genus level was also less in the digestate from DC Water (12) compared to the samples from SLCWRF (79) and CVWRF (89). The microbiome in the mesophilic anaerobic digesters consisted of hydrolyzers, acetogens, acidogens, and methanogens (Fig. 1). The taxa with abundance >1% were shown in the figure. The bacterial and archaeal genera detected in the digestate from SLCWRF and CVWRF were similar, while the digestate from DC Water had a different community composition. For instance, protein hydrolyzers *Candidatus Cloacimonas* were abundant in the digestate from SLCWRF and CVWRF, while it was below the detection limit in the digestate from DC Water. Similarly, pathogens such as *Afipia* and *Dermatophilaceae* were observed in the digestate from SLCWRF and CVWRF, while they were not detected in the digestate from DC Water (Fig. 1). Among the methanogenic population, the genus *Methanosarcina* was unique to digestate from DC Water, while the genus *Methanosaeta* and genus *Methanospirillum* were detected only in the digestate from SLCWRF and CVWRF, respectively (Fig. 1). Further, acetate oxidizing genus *Tepidanaerobacter* was present in higher abundance in digestate from DC Water (Fig. 1) because a higher NH_3/NH_4^+ concentration shifts

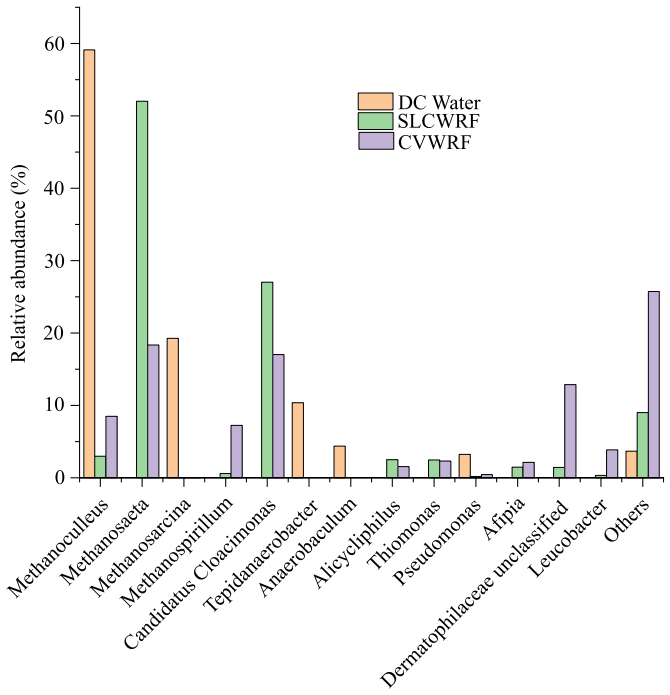


Fig. 1. Taxonomic profile of bacterial and archaeal genera in anaerobically digested sludge from Blue Plains Advanced Wastewater Treatment Plant (DC Water, Washington DC); Salt Lake City Water Reclamation Facility (SLCWRF, Utah); Central Valley Water Reclamation Facility (CVWRF, Utah). The relative abundance of the microorganisms was determined by mapping the quality-filtered sequences to ~1 million clade-specific marker genes using Metaphlan version 2.7.7 (Truong et al., 2015). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the acetoclastic methanogenesis pathway to syntrophic acetate oxidation (Schnürer et al., 1997; Schnürer and Nordberg, 2008). The genus *Methanoculleus* was observed in all three samples. The dominant methanogens observed in the digestate from DC Water, genus *Methanosarcina* and *Methanoculleus*, are mixotrophic and hydrogenotrophic methanogens, respectively (Evans et al., 2019; Maus et al., 2012; Tian et al., 2018). Mixotrophic methanogens are anaerobic archaea capable of utilizing various carbon substrates to produce methane gas (O'Brien et al., 1984), while specialist methanogens can use either of the three substrates (acetate or methanol, or CO_2).

3.2. Methanogenesis in the AD

A total of 589 bins were generated from the metagenome dataset. We recovered 270 MAGs with completeness ≥50% complete and contamination of ≤5% (Table S2). 16 MAGs were classified as Archaea, while 254 MAGs were classified as Bacteria (Table S2).

To assess the potential for methanogenesis among the archaeal MAGs, we applied a stringent condition for selecting MAGs with more than 70% completeness and contamination of less than 5%. Twelve MAGs of methanogens (completeness ≥70%; contamination ≤5%) were placed in the reference tree (Fig. S1). MAGs of acetoclastic methanogens (genus *Methanothrix*; species *Methanothrix soehngenii*); methylotrophic methanogens (family *Methanomassiliicoccaceae*; order *Methanomassiliicoccales*; genus *Methanofastidiosum*); and hydrogenotrophic methanogens (genus *Methanoculleus*; genus *Methanolinea*; family *Methanobacteriaceae*; genus *Methanobacterium*) were identified with the genes required for the respective pathways (Fig. 2). The MAG belonging to genus *Methanosarcina*, a mixotrophic methanogen, extracted from the digestate in DC Water had all genes necessary for a) hydrogenotrophic, b) methylotrophic methanogenesis pathways, and c) acetoclastic pathway (83.33% complete pathway) (Fig. 2). Studies have shown that *Methosarcina* is a mixotrophic methanogen capable of using substrates other than acetate for methanogenesis, including H_2, CO_2, methanol, and methylamine (Dworkin et al., 2006). Interestingly, four MAGs of specialist methanogens harbored genes required for multiple methanogenesis pathways (≥90% of the genes needed to complete each pathway). The four MAGs belonged to *Methanobacteriaceae* (binned_ad_5.34), *Methanolinea* (binned_ad_7.116, binned_ad_9.15), and *Methanothrix* (binned_9.58) (Table S2, Fig. 2).

The MAG of acetoclastic methanogen (genus *Methanothrix*) was observed with a complete set of genes involved in both acetoclastic and hydrogenotrophic methanogenesis (SLCWRF) (Fig. 2). Similarly, more than 90% of the genes required for acetoclastic methanogenesis were observed in the MAGs of hydrogenotrophic methanogens extracted from digestate from DC Water (family *Methanobacteriaceae* (binned_ad_5.34), and genus *Methanobacterium* (binned_ad_5.99)), SLCWRF (genus *Methanolinea* (binned_ad_7.116)), and CVWRF (family *Methanobacteriaceae* (binned_ad_9.131) and genus *Methanolinea* (binned_ad_9.15)) (Fig. 2). Since there are shared genes among different methanogenesis pathways, including the reduction of methyl-CoM to methane by the methyl-CoM reductase in the final step (Ferry, 1999), we used stringent criteria (≥90% pathway completion) along with identification of specific genes involved in respective pathways to substantiate the involvement of MAGs in a specific pathway. Despite the stringent criteria, we still observed evidence of mixotrophic methanogenesis in specialist methanogens that was different from our understanding of methanogens. The prevalence of mixotrophic methanogenesis highlights the flexibility of methanogens to changing substrates in AD.

3.3. Viral community in ADs

The TEM images of the purified viral-like particles had different morphologies. Most of the viruses observed through TEM had a long, noncontractile tail, typical of the members of the family *Siphoviridae* (Ackermann, 1998; Murphy et al., 1995) (Fig. 3A). Viruses with a contractile tail (family *Myoviridae*) and a short tail (family *Podoviridae*) were observed as well (Ackermann, 1998; Murphy et al., 1995) (Fig. 3A, ii). Out of 12,327 scaffolds with lengths greater than 5 kbp, 11,440 scaffolds (92.8%) were identified as complete viral genomes or genome fragments. The majority (92.8% of scaffolds greater than 5 kbp) of the assembled scaffolds were identified as viruses highlighting the specificity of the virus extraction protocol.

Although the diversity indices calculated at the family level were not much different in magnitude, the viral community in the digestate from DC Water was the least diverse (H = 0.92), followed by the digestate samples from SLCWRF (H = 1.16) and CVWRF (H = 1.17). *Siphoviridae* (52.1 ± 8.21%), *Myoviridae* (28.53 ± 5.29%), and *Podoviridae* (12.4 ± 5.77%) were the dominant viral families in the digestate viromes (Fig. 3B). We recovered 72 high-quality viral genomes, out of which 18 were classified as complete or high-quality viral genomes by CheckV (Nayfach et al., 2021) (Table S5). Medium-quality (237) and low-quality (8957) viral genomes with lower completeness (as low as 0.32%) and contamination (as high as 93.5%) were also detected **(Table S5)**.

A total of 123, 156, and 151 viral genomes were identified as temperate viruses from DC Water, SLCWRF, and CVWRF, respectively (Hockenberry and Wilke, 2021) (Table S6). We also checked the scaffolds within the reconstructed MAGs for viral signals. A higher proportion of lysogens (88.54–100%) with at least one viral scaffold integrated within the genome (completeness ≥50%, contamination <5%) was detected in the digestate samples. One viral genome within the MAG of the family *Tepidimicrobiaceae* (binned_ad_5.47) was identified as an intact genome with genes encoding for tail, portal, capsid, head, and integrase protein, while some MAGs contained incomplete viral genomes encoding tail, head, plate, capsid proteins (Table S7). This shows that the integrated viral genomes, upon induction, have the capability to form viral genomes by using the host cellular metabolism. Additionally, viral genomes within the MAGs of the family *Erysipelotrichaceae*

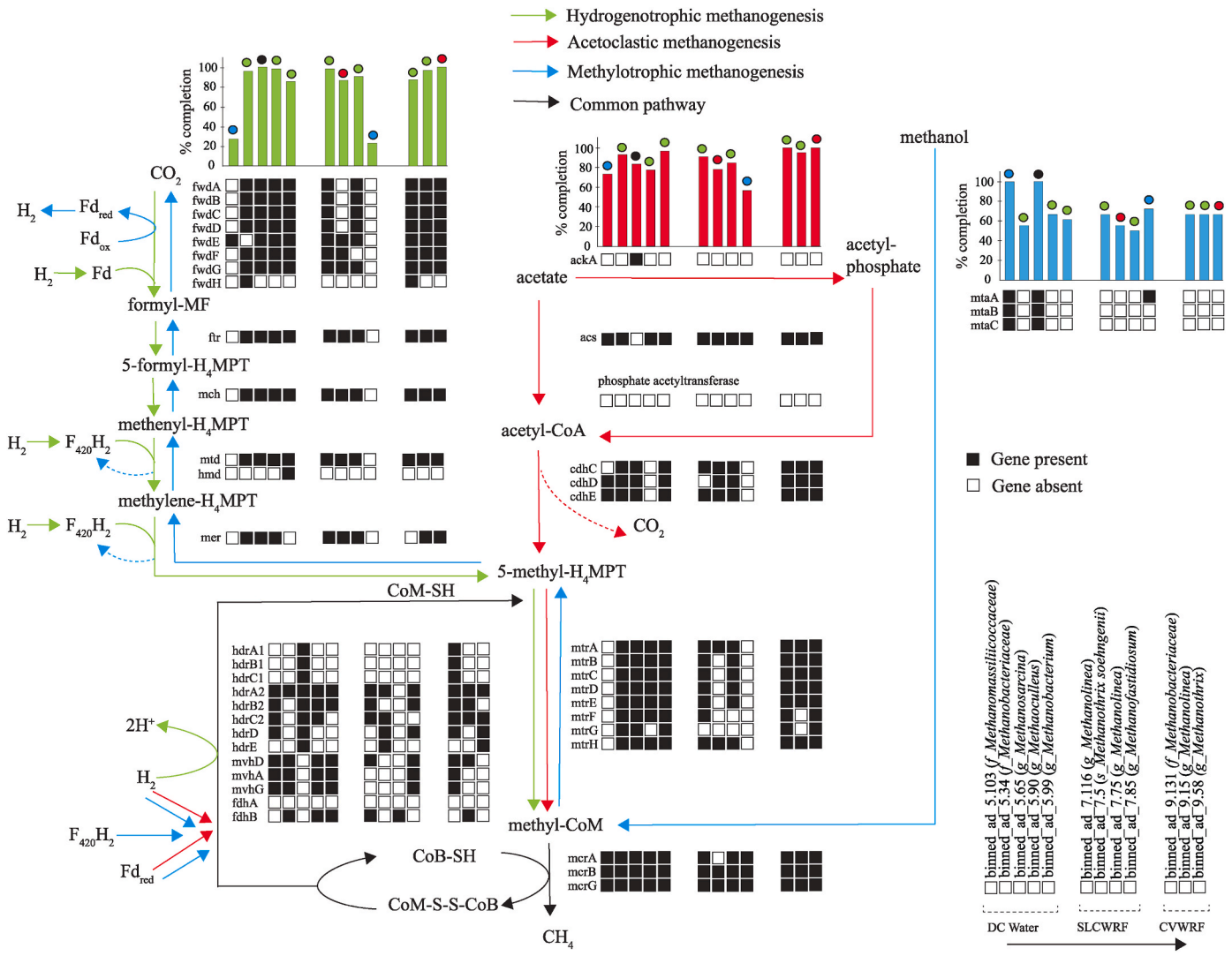


Fig. 2. Methanogenesis pathway in the metagenome-assembled genomes (MAGs) extracted from the digestate samples from three full-scale wastewater treatment facilities (Blue Plains Advanced Wastewater Treatment Facility (DC Water, Washington DC), Salt Lake City Water Reclamation Facility (SLCWRF, Salt Lake City, Utah), Central Valley Water Reclamation Facility (CVWRF, Salt Lake City, Utah). The different methanogenesis pathways are represented by different colors, as shown in the figure. The presence of the functional genes involved in the respective pathways in each MAG is represented with highlighted blocks. The percentage completion of the methanogenesis pathways is represented with bar graphs. The colored circles at the top of the bars represent the known methanogenesis metabolism (green: hydrogenotrophic; red: acetoclastic; blue: methylotrophic; black: mixotrophic) of the respective MAGs. The orientation of the MAG ID and their taxonomic identity represent the gene presence/absence blocks, and the bars are represented in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(binned_ad_5.53) and family *Paludibacteraceae* (binned_ad_5.60) contained gene encoding for lysin, an enzyme responsible for rupturing the bacterial cell wall (Table S7). This indicates that the integrated prophage can switch to the lytic lifecycle.

3.4. Host prediction of viruses shows virus infecting pathogens and functional microbes in AD

We could predict bacterial and archaeal hosts of 1025 viral genomes with a cut-off score of 0.95. (Table S8). The viruses with predicted hosts accounted for a small proportion of the total viral community (11.37 ± 1.7%) because our study was focused on the accurate prediction of hosts based on a stringent cut-off score. Nevertheless, the genomic data of the viruses isolated from the digestate can be referred to in the future for virus-host predictions when more powerful bioinformatics algorithms are developed with a database populated with digester virome.

Multiple viruses that infect hydrolytic bacteria of genera *Clostridium*, *Lactococcus*, *Ruminococcus*, *Hungateiclostridium*, *Flavobacterium*, and *Prevotella* were predicted (Fig. 3C, Table S8). The viruses predicted to infect hydrolytic bacteria were less in the digestate from DC Water (49 viral genomes) than in SLCWRF (171 viral genomes) and CVWRF (209 viral genomes), (Table S8) because hydrolysis takes place in the THP preceding the AD in DC Water. Viruses infecting acidogens and acetogens, which include bacteria of genera *Acetomicrobium*, *Desulfotomaculum*, *Bacteroides*, and *Anaerosalibacter,* were detected in DC Water, SLCWRF, and CVWRF (Fig. 3C, Table S8). The viruses that infect acetate oxidizing genus *Candidatus Cloacimonas* were only detected in the SLCWRF sample (Fig. 3C, Table S8). Viruses of four methanogenic genera (*Methanoculleus*, *Methanosarcina*, *Methanospirillum*, *Methanomassiliicoccus*) were predicted (Table S8). Similar to the microbial community composition, viruses infecting hydrogenotrophic methanogen *Methanoculleus* and mixotrophic methanogen *Methanosarcina* were observed in DC Water's digestate while they were not detected in CVWRF and rare in SLCWRF (one viral genome) samples (Fig. 3C, Table S8). The digestate from DC Water contains higher ammonium and free ammonia due to enhanced hydrolysis via THP, therefore the NH_4/

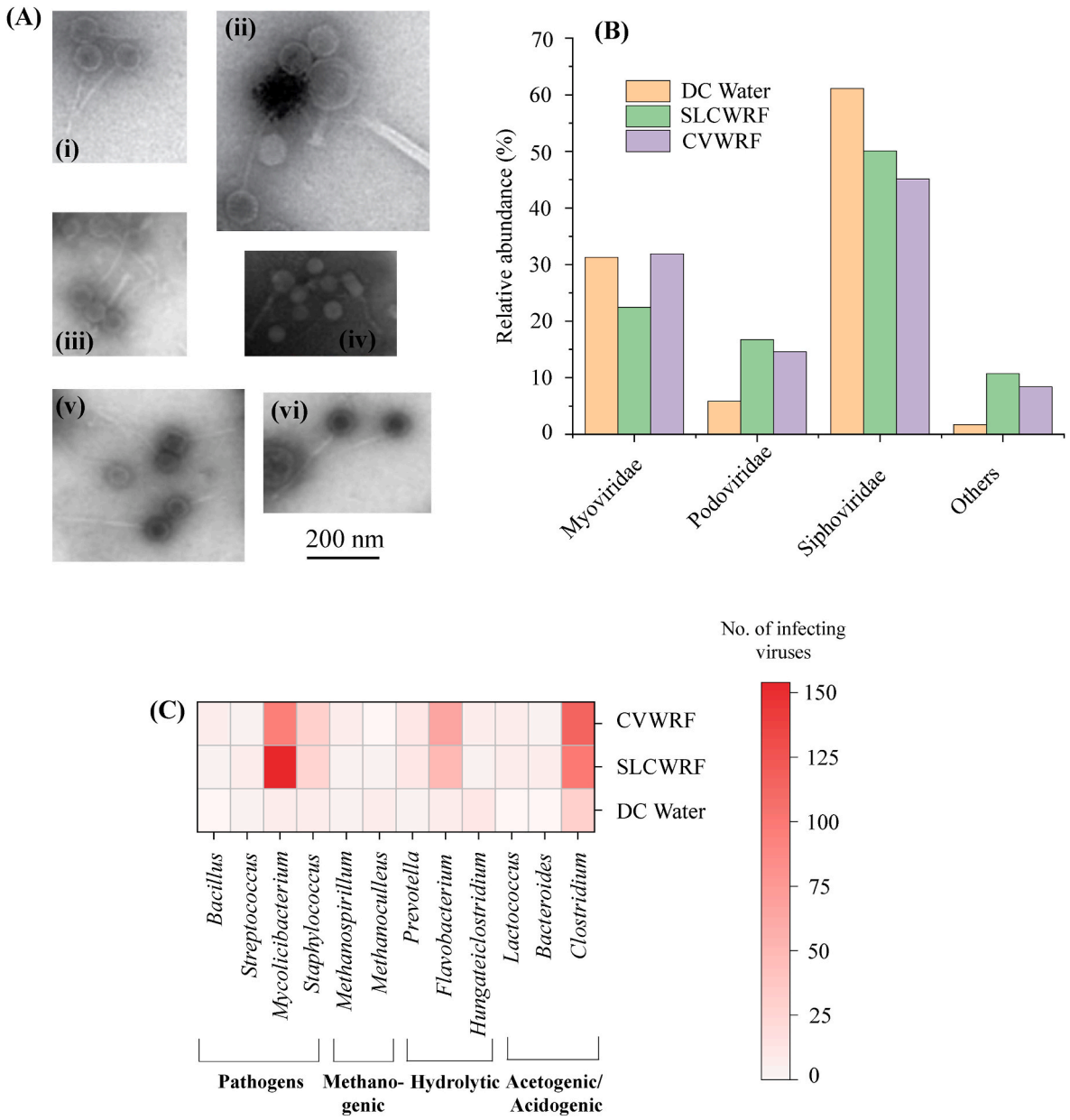


Fig. 3. **(A)** TEM images of the viruses extracted from DC Water (i, ii); SLCWRF (iii, iv); CVWRF (v, vi). **(B)** Family-level profile of the viral community in the anaerobically digested sludge from DC Water, SLCWRF, and CVWRF. **(C)** Heatmap representing the number of viruses extracted from the anaerobically digested sludge from three full-scale wastewater treatment facilities (DC Water, SLCWRF, CVWRF) infecting functional microorganisms (hydrolysis, acidogenic, acetogenic, methanogens) and pathogens.

NH_3 tolerant hydrogenotrophic methanogens were abundant in the digestate from DC Water. Viruses that infect a) methylotrophic (*Methanomassiliicoccus* only in SLCWRF) and b) acetoclastic (*Methanospirillum* in all three samples) methanogens were also detected (Fig. 3C, Table S8).

The number of viruses predicted to infect pathogens in DC Water, SLCWRF, and CVWRF were 16, 190, and 133 genomes, respectively (Table S8). Viruses infecting *Mycolicibacterium* and *Staphylococcus* were in greater abundance in SLCWRF and CVWRF's digestate (Fig. 3C). The lower detection of viruses infecting pathogens in the digestate from DC Water was consistent with the non-detection of pathogens in the microbial community.

3.5. Evidence of AMGs acquired through bacterial HGT in ADs

The viral genomes were annotated to identify AMGs. The viral genomes with 0% contamination, at least one AMG, and at least one viral hallmark gene were selected for downstream analysis (Tables S5 and S9). The viral genomes which contained either AMG or the viral hallmark gene in the extreme ends of the viral genome were not considered to eliminate the possibility of assembly artifact (Bateman, 2004). We found five viral genomes containing AMGs with abundances between 1.39 and 6.97 rpkm (Fig. 4A). A virus of the family *Siphoviridae* from SLCWRF (NODE 106; length 41.928 Kbp) was predicted to infect *Lactococcus lactis* subsp. *Cremoris* (Table S8) contained a cellulase domain-containing protein (Fig. 4A). A virus of the family *Podoviridae* (SLCWRF, NODE 1029; length 14.232 Kbp) contained a cellulase gene, while a virus of the family *Myoviridae* (SLCWRF, NODE 769; length 16.989 Kbp) contained alpha-glucosidase gene (Fig. 4A). The phylogeny of these three AMGs formed a cluster with genes from different phyla of bacteria, suggesting that the AMGs were of bacterial origin acquired in viruses, possibly via HGT (Fig. 4B). We couldn't assess the origin of the

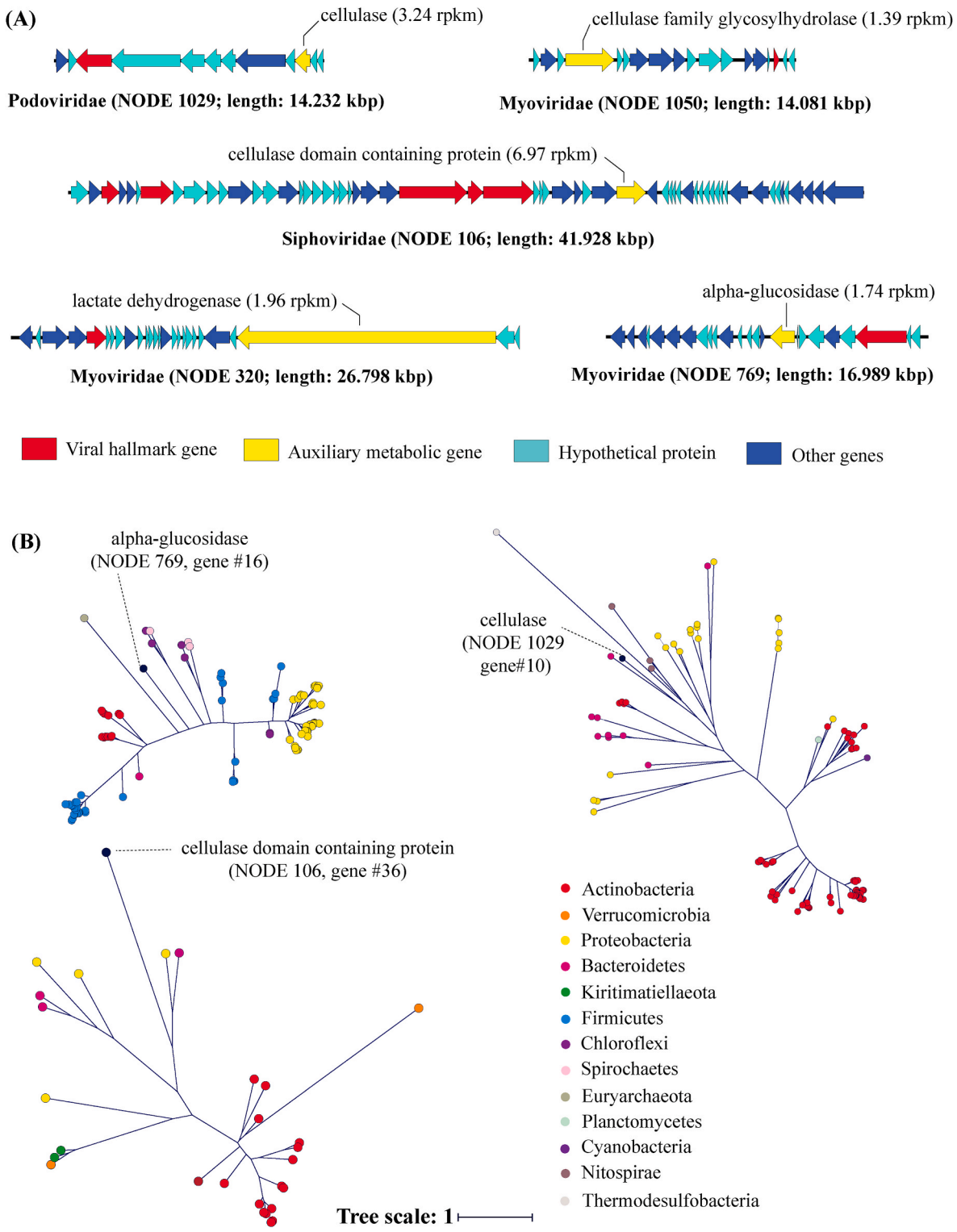


Fig. 4. **(A)** Gene map of the viral genome with AMGs. The AMGs and viral hallmark genes are color coded. **(B)** Phylogenetic analysis (unrooted trees) of the AMGs identified in viral genomes from SLCWRF'S digestate. The origin of the phylogenetically close genes is color-coded at the phylum level. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

other two AMGs, cellulase family glycosyl hydrolase and lactate dehydrogenase, in two viruses of the family *Myoviridae* (NODE 1050; length 14.081 Kbp and NODE 320; length 26.708 Kbp) (Fig. 4A) by the phylogenetic analysis. Similarly, a viral scaffold (SLCWRF, NODE 25161, length 4.298 kbp) within the MAG of *s_Methanothrix soehngenii* (binned_ad_7.5) was observed with a *fwdE* gene. The *fwdE* gene in the viral scaffold was phylogenetically similar to the *fwdE* gene from hydrogenotrophic methanogens (Fig. S2).

4. Discussion

4.1. Lysogeny is prominent in the viral community dominated by Caudovirales in mesophilic ADs

In an already complex microbiome of AD, the ecology of the viruses remains unexplored. Viruses can modulate the host community and metabolism, but we lack information about their community structure and replication lifestyle in the AD ecosystem. To fill this knowledge gap, we studied the ecology of viruses in three mesophilic ADs. The three mesophilic ADs were from full-scale WWTPs harboring microbiome acclimated to different pollutants present in real municipal wastewater.

Although the relative abundance of the respective viral families was different, all three ADs were observed with an abundant population of *Siphoviridae*, followed by *Myoviridae* and *Podoviridae*. .The morphological analysis further confirmed the predominance of *Siphoviridae*, characterized by long, noncontractile tails (Fig. 3A). Our observation was similar to the survey of viruses in methanogenic digesters (Calusinska et al., 2016; Willenbücher et al., 2022). We extended the analysis of the viral community based on their host range and compared it with the microbial community. The AD with a THP unit was characterized by a lesser microbiome and virome diversity with non-detectable protein hydrolyzers and pathogens and the viruses infecting them (Fig. 1). We postulate that the slower rate of hydrolyzers to take up the increased soluble organic matter generated by the THP caused the hydrolyzers to get outnumbered in AD, while the killing of indigenous microbes in the THP unit caused the nondetectable presence of pathogens (Luo et al., 2012; Ma et al., 2013). Similarly, the methanogens in the AD with THP were dominated by hydrogenotrophic (genus *Methanoculleus*) and mixotrophic (genus *Methanosarcina*) methanogens, while acetoclastic methanogens (genus *Methaosaeta*) were prevalent in the AD without THP (Fig. 1). The slow-growing *Methanosaeta* (Conklin et al., 2006) was more abundant in the digestate from SLCWRF due to the higher operating solids retention time (SRT) (~50 days) compared to DC Water (~23 days) and CVWRF (~15–20 days).

The AD with THP in DC Water was abundant with the genus *Methanosarcina* and *Methanoculleus* concurring with the findings in previous studies under similar conditions (Poirier et al., 2016; Zhang et al., 2021). The improved sludge mixing and pumping of a less viscous sludge coming out of the THP allowed the operation of AD with high total solids (TS) concentrations (Barber, 2016; Nielsen et al., 2011), resulting in increased degradation of nitrogenous matter and production of higher ammonium and free ammonia (Barber, 2016; Higgins et al., 2017). Among the ADs selected in our study, an average sludge TS of 9.5% was fed to the AD with THP (DC Water) (Li et al., 2020), compared to TS of AD without THP (monthly average of 2.5% in SLCWRF and 4.63% in CVWRF, respectively). The virome community in the digestate from AD with THP was also characterized by a higher presence of viruses infecting hydrogenotrophic (*Methanoculleus*) and mixotrophic (*Methaosarcina*) methanogens (Fig. 3C). All in all, *Caudovirales* were dominant in mesophilic anaerobic digesters while the presence or absence of THP unit was observed to shape the viral community in conjunction with the microbial community. Future studies can further substantiate this finding by collecting multiple samples from water reclamation facilities across the United States that are installing THP unit to intensify their biosolids management processes.

Almost all MAGs of medium to high quality (completeness $\geq$50%, contamination $<$5%) were identified as lysogens. The proportion of lysogens in mesophilic ADs (88.54–100%) was higher than what was observed in publicly available bacterial genomes (46–54%) (Kim and Bae, 2018). Some of the integrated viral genomes contained genes encoding for head, capsid, tail, portal, and plate proteins. This suggests that the integrated viruses within the bacterial genomes have the capability to form new virions when they get induced under DNA-damaging stresses, such as exposure to heavy metals and changes in pH (Motlagh et al., 2015; Nanda et al., 2015). AD ecosystems are often exposed to these prophage-inducing triggers with pH changes via VFA accumulation and a higher concentration of Cu (II) in the waste-activated sludge that it treats (Dai et al., 2007). Additionally, the presence of virulent genes, such as genes encoding for lysis, in integrated viral genomes highlights the possibility of a switch from lysogeny to a lytic lifecycle. One of the integrated viral genomes within the MAG of an acetogen *Paludibactericeae* was detected with a gene encoding for lysin. The induction of prophage followed by the switch to a lytic lifestyle can affect the functional microbiome in AD. On the other hand, prophages can create a positive impact on their host by providing super-infection immunity and exclusion (Bondy-Denomy and Davidson, 2014) in harsh ecosystems such as AD.

4.2. Mixotrophic methanogenesis in specialist methanogens, possibly via viruses

Viruses are known to redirect the host metabolism by expressing functional genes they carry in their genome. Evidence of the redirection of host metabolism has been discussed aplenty in natural aquatic ecosystems, including the redirection of host carbon metabolism from the Calvin cycle to the pentose phosphate pathway through the expression of the gene responsible for inhibition of the Calvin cycle (CP12) (Thompson et al., 2011). With virome studies lacking in wastewater treatment plants, evidence of redirection of host metabolism through viral infection is lacking.

In our study, the metabolic reconstruction of the MAGs of methanogens showed genes involved in multiple methanogenesis pathways in MAGs of specialist methanogens (Fig. 2). The evidence of mixotrophic methanogenesis in MAGs of specialist methanogens (genus *Methanothrix*, *Methanolinea*, *Methanobacterium*; family *Methanobacteriaceae*) was different from our knowledge of methanogens. The higher proportion of lysogens among methanogens (8 lysogens out of 12 MAGs selected for the reconstruction of the methanogenesis pathway) suggested that methanogens in ADs tend to carry prophages and potentially the genes responsible for methanogenesis. The MAG of *s_Methanothrix soehngenii* (binned_ad_7.5), an acetoclastic methanogen, contained a viral scaffold (NODE_25161_length_4298_cov_13.310717) with a *fwdE* gene responsible for the conversion of CO_2 to formyl-MF in the hydrogenotrophic methanogenesis pathway (Fig. 2). The presence of the viral genome within the MAG of methanogen indicates a virus-host relationship. The observation of s_*Methanothrix soehngenii* as the predicted host of the integrated viral genome further substantiated the virus-host relationship. The phylogenetic analysis showed a close relationship between the *fwdE* gene in the viral genome and the *fwdE* gene from hydrogenotrophic methanogens, indicative of HGT (Fig. S2). This suggests that viruses may contribute to mixotrophic methanogenesis in specialist methanogens via HGT. More studies are required to further substantiate the role of viruses in methanogenesis, including transcriptome analysis to see if the genes responsible for methanogenesis are being expressed or staying dormant. Regardless, this highlights that lysogeny provides a fitness advantage to the host, methanogen, by adding flexibility to the changing substrates in the AD. This evidence offers a new and vital genomic-level understanding of the biological processes in AD that will help researchers to come up with optimum operating conditions to enhance the productivity of the functional microbiome and the process.

4.3. Potential of viruses to assist in hydrolysis and control of process upsets in AD

The studies on anaerobic digester viromes have largely focused on their diversity with little emphasis on their auxiliary metabolism (Calusinska et al., 2016; Ngo et al., 2022) unlike the viruses from AS processes. AMGs involved in carbon and nutrient removal have been identified in viruses from AS processes (Chen et al., 2021; Shi et al., 2022). The advancement in viral metagenomics has started to expand our understanding of AMGs in viruses from anaerobic digesters. A recent

study found one evidence of gene encoding an ADP-ribosyltransferase (ADPRT) in viral genome from anaerobic digester (Willenbücher et al., 2022). Here, we identified AMGs of bacterial origin in viral genomes from mesophilic anaerobic digesters (Fig. 4A and B). The viral genome with cellulase-domain-containing protein shows that it is capable of assisting in the fermentation of cellulose that its host (*Lactococcus lactis*) (Table S8) isn't recognized to do on its own (de Vos, 1999; Tarraran and Mazzoli, 2018). Other AMGs of bacterial origin were cellulase and alpha-glucosidase, which catalyze the hydrolysis of cellulose and starch, respectively (Segel, 1975). Taken together, the AMGs related to hydrolysis in AD virome indicate the potential implication of the viral infection in the hydrolysis of the sludge in the anaerobic digestion process while their activity still needs to be assessed. Future studies can incorporate metatranscriptomics analysis to assess if the viruses are assisting the hosts or not.

Viruses also serve as a potential tool to combat process upsets in WWTPs through virus-mediated treatment. Some demonstrated studies to infect and lyse filamentous bacteria in the AS process (Khairnar et al., 2016; Kotay et al., 2011; Liu et al., 2015). However, the applicability of virus-mediated treatment in AD is underexplored, with less knowledge and genomic information on the virus-host relationship in the complex AD ecosystem. Here, we predicted viruses infecting acidogenic and acetogenic bacteria (Fig. 3C, Table S8). In cases of a kinetic imbalance between acid production by acidogenic and acetogenic bacteria and consumption by methanogenic archaea, excess buildup of VFAs can lead to an over-acidification/souring problem. The drastic pH drop due to over-acidification favors acidogenic bacteria with a broader pH range (4.0–8.5), while the sensitive methanogens (pH 6.8 to 7.2) are impacted (Izrail S. Turovskiy & P. K. Mathai, 2006; Michael H. Gerardi, 2003) ultimately affecting the microbial growth and metabolic pathways in the anaerobic digestion process. Using viruses as an antibacterial tool for virus-mediated treatment against specific functional microbes could help restore the AD microbiome to its steady state, which ensures process stability. Similarly, a higher number of viruses infecting pathogens (339 viral genomes), such as *Mycolicibacterium* and *Staphylococcus*, were detected across the three digestate samples (Fig. 3C). Like viruses infecting acetogenic and acidogenic bacteria for over-acidification control in ADs, viruses infecting pathogens can remove pathogens from the sludge before they get processed into biosolids for land application. Eliminating pathogens from the sludge is essential, considering the risk it poses to human and animal health after land application. Selecting an appropriate solids retention time (SRT) is one of the popular approaches for sludge stabilization. However, it has been shown that increasing SRT favors pathogen removal but does not significantly improve biogas production (Chen et al., 2012; de la Rubia et al., 2006; Riau et al., 2010). In the two-stage AD from CVWRF, the second-stage digester, with an SRT of more than 20 days, is primarily for the reduction of pathogens and odor reduction rather than for generating renewable gas. The additional digester adds to the SRT, making it inefficient and uneconomical. In the modern era of wastewater treatment intensification, using viruses for pathogen removal can eliminate the need for longer SRT while maintaining the same biogas yield. Bench-scale studies are required to determine the optimal phage-to-host ratios for removing the required proportion of the problematic microbes, similar to the approach used for controlling biomass bulking (Kotay et al., 2011).

Our result sheds light on the AMGs and virus-host network of the AD virome. We add to the genomic database of AD viruses that can be referred to for the potential use of genetically modified viruses for virus-mediated treatment in AD, similar to some demonstrated applications in controlling biofilm in WWTPs (Motlagh et al., 2016). However, the range of hosts the virus can infect must be carefully studied.

5. Conclusion

Despite the fact that viruses are key players in controlling microbial ecology, viral ecology in the diverse microbiome of ADs have been largely understudied. Here, we shed light on the ecology and auxiliary metabolism of viruses from ADs. Lysogeny was prevalent in mesophilic ADs and the viruses contained AMGs related to sludge hydrolysis and methanogenesis. By expressing these AMGs, viruses could potentially regulate and redirect the metabolism of functional microbiome involved in the anerobic digestion process. Next step should be focused on transcriptomic analysis to explore the activity and expression of AMGs. Additionally, we provide a genomic database of viruses and their hosts in full-scale mesophilic ADs acclimated to different pollutants present in real municipal wastewater. The genomic database can be used for virus-mediated treatment to control process upsets caused by undesired microbes in ADs. Our study adds to the understanding of AD virome that will help in developing next-generation technology in wastewater engineering to optimize the anaerobic digestion process.

Author contribution

BB conducted the sampling, metagenomics analysis and prepared the first draft of this manuscript. AB and FC helped BB with metagenomics analysis. All the sampling and analysis work was conducted under the direct supervision of RKG.

Funding

The funding from this project was provided by the United States National Science Foundation with grant number 1804158. However, the views and data discussion expressed are those of the authors and do not necessarily reflect any role of the funding agency. FHC was supported by a Juan de la Cierva - Incoporación fellowship (Grant IJC2019-039859-I). A portion of the sequencing cost was covered by a small grant to BB by the Global Change & Sustainability Center at the University of Utah under its student grant program.

Availability of data and materials

The raw sequences and metadata were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA). The project number is PRJNA872498.

Ethics approval and consent to participate

Not applicable.

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.

Acknowledgments

We thank Dr. Haydee De Clippeleir (Blue Plains Advanced Wastewater Treatment Plant, DC Water); Cas Knies (Salt Lake City Water Reclamation Facility, SLCWRF); and Dr. Phillip Heck (Central Valley Water Reclamation Facility, CVWRF) for helping us with samples from the respective wastewater treatment facilities. We would also like to thank Sharon Burton (CVWRF) and Jose Rubalcaba (SLCWRF) for providing information on the ADs under operation from the respective treatment facilities.

List of abbreviations

AD	Anaerobic digester
AMG	Auxiliary metabolic genes
THP	thermal hydrolysis pretreatment

WWTP	Wastewater treatment plants
LCFA	long-chain fatty acids
VFA	volatile fatty acids
TAN	total ammonia nitrogen
AS	activated sludge
CDS	coding sequences
SLCWRF	Salt Lake City Water Reclamation Facility
CVWRF	Central Valley Water Reclamation Facility
SRT	solids retention time
TS	total solids
TEM	transmission electron microscopy
HGT	horizontal gene transfer
MAG	metagenome-assembled genome

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140743.

References

Ackermann, H.-W., 1998. Tailed Bacteriophages: the Order Caudovirales, pp. 135–201. https://doi.org/10.1016/S0065-3527(08)60785-X.

Amani, T., Nosrati, M., Sreekrishnan, T.R., 2010. Anaerobic digestion from the viewpoint of microbiological, chemical, and operational aspects — a review. Environ. Rev. 18 (NA), 255–278. https://doi.org/10.1139/A10-011.

Anantharaman, K., Duhaime, M.B., Breier, J.A., Wendt, K.A., Toner, B.M., Dick, G.J., 2014. Sulfur oxidation genes in diverse Deep-sea viruses. Science 344 (6185), 757–760. https://doi.org/10.1126/science.1252229.

Andrews, S., 2010. FastQC: a Quality Control Tool for High Throughput Sequence Data.

Appels, L., Baeyens, J., Degrève, J., Dewil, R., 2008. Principles and potential of the anaerobic digestion of waste-activated sludge. Prog. Energy Combust. Sci. 34 (6), 755–781. https://doi.org/10.1016/j.pecs.2008.06.002.

Arndt, D., Grant, J.R., Marcu, A., Sajed, T., Pon, A., Liang, Y., Wishart, D.S., 2016. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res. 44 (W1), W16–W21. https://doi.org/10.1093/nar/gkw387.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V. M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.v., Sirotkin, A.v., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19 (5), 455–477. https://doi.org/10.1089/cmb.2012.0021.

Barber, W.P.F., 2016. Thermal hydrolysis for sewage treatment: a critical review. Water Res. 104, 53–71. https://doi.org/10.1016/j.watres.2016.07.069.

Bateman, A., 2004. The Pfam protein families database. Nucleic Acids Res. 32 (90001), 138D–141. https://doi.org/10.1093/nar/gkh121.

Bhattarai, B., Bhattacharjee, A.S., Coutinho, F.H., Goel, R.K., 2021. Viruses and their interactions with bacteria and archaea of Hypersaline Great Salt Lake. Front. Microbiol. 12 https://doi.org/10.3389/fmicb.2021.701414.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Bondy-Denomy, J., Davidson, A.R., 2014. When a virus is not a parasite: the beneficial effects of prophages on bacterial fitness. J. Microbiol. 52 (3), 235–242. https://doi.org/10.1007/s12275-014-4083-3.

Breitbart, M., 2012. Marine viruses: Truth or Dare. Ann. Rev. Mar. Sci 4 (1), 425–448. https://doi.org/10.1146/annurev-marine-120709-142805.

Calusinska, M., Marynowska, M., Goux, X., Lentzen, E., Delfosse, P., 2016. Analysis of ds <scp>DNA</scp> and <scp>RNA</scp> viromes in methanogenic digesters reveals novel viral genetic diversity. Environ. Microbiol. 18 (4), 1162–1175. https://doi.org/10.1111/1462-2920.13127.

Chaumeil, P.-A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2019. GTDB-Tk: a toolkit to classify genomes with the Genome Taxonomy Database. Bioinformatics. https://doi.org/10.1093/bioinformatics/btz848.

Chen, Y., Fu, B., Wang, Y., Jiang, Q., Liu, H., 2012. Reactor performance and bacterial pathogen removal in response to sludge retention time in a mesophilic anaerobic digester treating sewage sludge. Bioresour. Technol. 106, 20–26. https://doi.org/10.1016/j.biortech.2011.11.093.

Chen, Y., Wang, Y., Paez-Espino, D., Polz, M.F., Zhang, T., 2021. Prokaryotic viruses impact functional microorganisms in nutrient removal and carbon cycle in wastewater treatment plants. Nat. Commun. 12 (1), 5398. https://doi.org/10.1038/s41467-021-25678-1.

Conklin, A., Stensel, H.D., Ferguson, J., 2006. Growth kinetics and competition between Methanosarcina and Methanosaeta in mesophilic anaerobic digestion. Water Environ. Res. 78 (5), 486–496. https://doi.org/10.2175/106143006X95393.

Dai, J., Xu, M., Chen, J., Yang, X., Ke, Z., 2007. PCDD/F, PAH and heavy metals in the sewage sludge from six wastewater treatment plants in Beijing, China. Chemosphere 66 (2), 353–361. https://doi.org/10.1016/j.chemosphere.2006.04.072.

de Jonge, P.A., Nobrega, F.L., Brouns, S.J.J., Dutilh, B.E., 2019. Molecular and evolutionary Determinants of bacteriophage host range. Trends Microbiol. 27 (1), 51–63. https://doi.org/10.1016/j.tim.2018.08.006.

de la Rubia, M.A., Perez, M., Romero, L.I., Sales, D., 2006. Effect of solids retention time (SRT) on pilot scale anaerobic thermophilic sludge digestion. Process Biochem. 41 (1), 79–86. https://doi.org/10.1016/j.procbio.2005.03.073.

de Vos, W.M., 1999. Gene expression systems for lactic acid bacteria. Curr. Opin. Microbiol. 2 (3), 289–295. https://doi.org/10.1016/S1369-5274(99)80050-2.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), 2006. The Prokaryotes.

Enav, H., Mandel-Gutfreund, Y., Béjà, O., 2014. Comparative metagenomic analyses reveal viral-induced shifts of host metabolism towards nucleotide biosynthesis. Microbiome 2 (1), 9. https://doi.org/10.1186/2049-2618-2-9.

Evans, P.N., Boyd, J.A., Leu, A.O., Woodcroft, B.J., Parks, D.H., Hugenholtz, P., Tyson, G. W., 2019. An evolving view of methane metabolism in the Archaea. Nat. Rev. Microbiol. 17 (4), 219–232. https://doi.org/10.1038/s41579-018-0136-7.

Fernández, L., Rodríguez, A., García, P., 2018. Phage or foe: an insight into the impact of viral predation on microbial communities. ISME J. 12 (5), 1171–1179. https://doi.org/10.1038/s41396-018-0049-5.

Ferry, J.G., 1999. Enzymology of one-carbon metabolism in methanogenic pathways. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 23 (1), 13–38. https://doi.org/10.1111/j.1574-6976.1999.tb00390.x.

Gahlot, P., Balasundaram, G., Tyagi, V.K., Atabani, A.E., Suthar, S., Kazmi, A.A., Štěpanec, L., Juchelková, D., Kumar, A., 2022. Principles and potential of thermal hydrolysis of sewage sludge to enhance anaerobic digestion. Environ. Res. 214, 113856 https://doi.org/10.1016/j.envres.2022.113856.

Gerardi, Michael H., 2003. The Microbiology of Anaerobic Digesters. John Wiley & Sons, Inc.

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.

Haaber, J., Middelboe, M., 2009. Viral lysis of Phaeocystis pouchetii: implications for algal population dynamics and heterotrophic C, N and P cycling. ISME J. 3 (4), 430–441. https://doi.org/10.1038/ismej.2008.125.

Hafstead, M.A.C., Williams, R.C., 2020. Designing and evaluating a U.S. Carbon tax adjustment mechanism to reduce emissions uncertainty. Rev. Environ. Econ. Pol. 14 (1), 95–113. https://doi.org/10.1093/reep/rez018.

Higgins, M.J., Beightol, S., Mandahar, U., Suzuki, R., Xiao, S., Lu, H.-W., Le, T., Mah, J., Pathak, B., DeClippeleir, H., Novak, J.T., Al-Omari, A., Murthy, S.N., 2017. Pretreatment of a primary and secondary sludge blend at different thermal hydrolysis temperatures: impacts on anaerobic digestion, dewatering and filtrate characteristics. Water Res. 122, 557–569. https://doi.org/10.1016/j.watres.2017.06.016.

Hockenberry, A.J., Wilke, C.O., 2021. BACPHLIP: predicting bacteriophage lifestyle from conserved protein domains. PeerJ 9, e11396. https://doi.org/10.7717/peerj.11396.

Istrate, I.R., Galvez-Martos, J.L., Dufour, J., 2021. The impact of incineration phase-out on municipal solid waste landfilling and life cycle environmental performance: case study of Madrid, Spain. Sci. Total Environ. 755, 142537.

Khairnar, K., Chandekar, R., Nair, A., Pal, P., Paunikar, W.N., 2016. Novel application of bacteriophage for controlling foaming in wastewater treatment plant- an eco-friendly approach. Bioengineered 7 (1), 46–49. https://doi.org/10.1080/21655979.2015.1134066.

Kieft, K., Zhou, Z., Anantharaman, K., 2020. VIBRANT: automated recovery, annotation and curation of microbial viruses, and evaluation of viral community function from genomic sequences. Microbiome 8 (1), 90. https://doi.org/10.1186/s40168-020-00867-0.

Kim, M.-S., Bae, J.-W., 2018. Lysogeny is prevalent and widely distributed in the murine gut microbiota. ISME J. 12 (4), 1127–1141. https://doi.org/10.1038/s41396-018-0061-9.

Kotay, S.M., Datta, T., Choi, J., Goel, R., 2011. Biocontrol of biomass bulking caused by Haliscomenobacter hydrossis using a newly isolated lytic bacteriophage. Water Res. 45 (2), 694–704. https://doi.org/10.1016/j.watres.2010.08.038.

Li, B., Romero, A., Wadhawan, T., Tobin, M., Manning, E., Higgins, M., Al-Omari, A., Murthy, S., Riffat, R., de Clippeleir, H., 2020. Recuperative thickening for sludge retention time and throughput management in anaerobic digestion with thermal hydrolysis pretreatment. Water Environ. Res. 92 (3), 465–477. https://doi.org/10.1002/wer.1230.

Lindell, D., Sullivan, M.B., Johnson, Z.I., Tolonen, A.C., Rohwer, F., Chisholm, S.W., 2004. Transfer of photosynthesis genes to and from *Prochlorococcus* viruses. Proc. Natl. Acad. Sci. USA 101 (30), 11013–11018. https://doi.org/10.1073/pnas.0401526101.

Liu, M., Gill, J.J., Young, R., Summer, E.J., 2015. Bacteriophages of wastewater foaming-associated filamentous Gordonia reduce host levels in raw activated sludge. Sci. Rep. 5 (1), 13754 https://doi.org/10.1038/srep13754.

Luo, K., Yang, Q., Li, X., Yang, G., Liu, Y., Wang, D., Zheng, W., Zeng, G., 2012. Hydrolysis kinetics in anaerobic digestion of waste activated sludge enhanced by α-amylase. Biochem. Eng. J. 62, 17–21. https://doi.org/10.1016/j.bej.2011.12.009.

Ma, J., Frear, C., Wang, Z., Yu, L., Zhao, Q., Li, X., Chen, S., 2013. A simple methodology for rate-limiting step determination for anaerobic digestion of complex substrates and effect of microbial community ratio. Bioresour. Technol. 134, 391–395. https://doi.org/10.1016/j.biortech.2013.02.014.

Maus, I., Wibberg, D., Stantscheff, R., Eikmeyer, F.-G., Seffner, A., Boelter, J., Szczepanowski, R., Blom, J., Jaenicke, S., König, H., Pühler, A., Schlüter, A., 2012. Complete genome sequence of the hydrogenotrophic, methanogenic archaeon Methanoculleus bourgensis strain MS2 ^{T}, isolated from a sewage sludge digester. J. Bacteriol. 194 (19), 5487–5488. https://doi.org/10.1128/JB.01292-12.

Middelboe, M., Jørgensen, N.O.G., 2006. Viral lysis of bacteria: an important source of dissolved amino acids and cell wall compounds. J. Mar. Biol. Assoc. U. K. 86 (3), 605–612. https://doi.org/10.1017/S0025315406013518.
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. https://doi.org/10.1016/j.watres.2015.04.023.
Motlagh, A.M., Bhattacharjee, A.S., Goel, R., 2016. Biofilm control with natural and genetically-modified phages. World J. Microbiol. Biotechnol. 32 (4), 67. https://doi.org/10.1007/s11274-016-2009-4.
Motlagh, A.M., Bhattacharjee, A.S., Coutinho, F.H., Dutilh, B.E., Casjens, S.R., Goel, R.K., 2017. Insights of phage-host interaction in Hypersaline ecosystem through metagenomics analyses. Front. Microbiol. 8 https://doi.org/10.3389/fmicb.2017.00352.
Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A., Summers, M.D. (Eds.), 1995. Virus Taxonomy. Springer Vienna. https://doi.org/10.1007/978-3-7091-6607-9.
Nanda, A.M., Thormann, K., Frunzke, J., 2015. Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. J. Bacteriol. 197 (3), 410–419. https://doi.org/10.1128/JB.02230-14.
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.
Ngo, V.Q.H., Enault, F., Midoux, C., Mariadassou, M., Chapleur, O., Mazéas, L., Loux, V., Bouchez, T., Krupovic, M., Bize, A., 2022. Diversity of novel archaeal viruses infecting methanogens discovered through coupling of stable isotope probing and metagenomics. Environ. Microbiol. https://doi.org/10.1111/1462-2920.16120.
Nielsen, H.B., Thygesen, A., Thomsen, A.B., Schmidt, J.E., 2011. Anaerobic digestion of waste activated sludge-comparison of thermal pretreatments with thermal inter-stage treatments. J. Chem. Technol. Biotechnol. 86 (2), 238–245. https://doi.org/10.1002/jctb.2509.
Nobrega, F.L., Vlot, M., de Jonge, P.A., Dreesens, L.L., Beaumont, H.J.E., Lavigne, R., Dutilh, B.E., Brouns, S.J.J., 2018. Targeting mechanisms of tailed bacteriophages. Nat. Rev. Microbiol. 16 (12), 760–773. https://doi.org/10.1038/s41579-018-0070-8.
O'Brien, J.M., Wolkin, R.H., Moench, T.T., Morgan, J.B., Zeikus, J.G., 1984. Association of hydrogen metabolism with unitrophic or mixotrophic growth of Methanosarcina barkeri on carbon monoxide. J. Bacteriol. 158 (1), 373–375. https://doi.org/10.1128/jb.158.1.373-375.1984.
Petrovski, S., Batinovic, S., Rose, J.J.A., Seviour, R.J., 2022. Biological control of problematic bacterial populations causing foaming in activated sludge wastewater treatment plants—phage therapy and beyond. Lett. Appl. Microbiol. 75 (4), 776–784. https://doi.org/10.1111/lam.13742.
Poirier, S., Desmond-Le Quéméner, E., Madigou, C., Bouchez, T., Chapleur, O., 2016. Anaerobic digestion of biowaste under extreme ammonia concentration: identification of key microbial phylotypes. Bioresour. Technol. 207, 92–101. https://doi.org/10.1016/j.biortech.2016.01.124.
Pons, J.C., Paez-Espino, D., Riera, G., Ivanova, N., Kyrpides, N.C., Llabrés, M., 2021. VPF-Class: taxonomic assignment and host prediction of uncultivated viruses based on viral protein families. Bioinformatics 37 (13), 1805–1813. https://doi.org/10.1093/bioinformatics/btab026.
Pujara, Y., Pathak, P., Sharma, A., Govani, J., 2019. Review on Indian Municipal Solid Waste Management practices for reduction of environmental impacts to achieve sustainable development goals. J. Environ. Manag. 248, 109238.
Riau, V., de la Rubia, M.Á., Pérez, M., 2010. Temperature-phased anaerobic digestion (TPAD) to obtain class A biosolids: a semi-continuous study. Bioresour. Technol. 101 (8), 2706–2712. https://doi.org/10.1016/j.biortech.2009.11.101.
Rodriguez-Valera, F., Martin-Cuadrado, A.-B., Rodriguez-Brito, B., Pašić, L., Thingstad, T.F., Rohwer, F., Mira, A., 2009. Explaining microbial population genomics through phage predation. Nat. Rev. Microbiol. 7 (11), 828–836. https://doi.org/10.1038/nrmicro2235.
Rossi, A., Morlino, M.S., Gaspari, M., Basile, A., Kougias, P., Treu, L., Campanaro, S., 2022. Analysis of the anaerobic digestion metagenome under environmental stresses stimulating prophage induction. Microbiome 10 (1), 125. https://doi.org/10.1186/s40168-022-01316-w.
Ruiz-Perez, C.A., Conrad, R.E., Konstantinidis, K.T., 2021. MicrobeAnnotator: a user-friendly, comprehensive functional annotation pipeline for microbial genomes. BMC Bioinf. 22 (1), 11. https://doi.org/10.1186/s12859-020-03940-5.
Schnürer, A., Nordberg, Å., 2008. Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. Water Sci. Technol. 57 (5), 735–740. https://doi.org/10.2166/wst.2008.097.
Schnürer, A., Svensson, B.H., Schink, B., 1997. Enzyme activities in and energetics of acetate metabolism by the mesophilic syntrophically acetate-oxidizing anaerobe Clostridium ultunense. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 154 (2), 331–336. https://doi.org/10.1111/j.1574-6968.1997.tb12664.x.
Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30 (14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
Segel, I.H., 1975. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady–State Enzyme Systems. Wiley.
Shelford, E., Middelboe, M., Møller, E., Suttle, C., 2012. Virus-driven nitrogen cycling enhances phytoplankton growth. Aquat. Microb. Ecol. 66 (1), 41–46. https://doi.org/10.3354/ame01553.
Shi, L.-D., Dong, X., Liu, Z., Yang, Y., Lin, J.-G., Li, M., Gu, J.-D., Zhu, L.-Z., Zhao, H.-P., 2022. A mixed blessing of viruses in wastewater treatment plants. Water Res. 215, 118237 https://doi.org/10.1016/j.watres.2022.118237.
Shivaram, K.B., Bhatt, P., Applegate, B., Simsek, H., 2023. Bacteriophage-based biocontrol technology to enhance the efficiency of wastewater treatment and reduce targeted bacterial biofilms. Sci. Total Environ. 862, 160723 https://doi.org/10.1016/j.scitotenv.2022.160723.
Spellerberg, I.F., Fedor, P.J., 2003. A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon–Wiener' Index. Global Ecol. Biogeogr. 12 (3), 177–179.
Steed, J., Hashimoto, A.G., 1994. Methane emissions from typical manure management systems. Bioresour. Technol. 50 (2), 123–130. https://doi.org/10.1016/0960-8524(94)90064-7.
Sullivan, M.B., Lindell, D., Lee, J.A., Thompson, L.R., Bielawski, J.P., Chisholm, S.W., 2006. Prevalence and evolution of core Photosystem II genes in marine cyanobacterial viruses and their hosts. PLoS Biol. 4 (8), e234. https://doi.org/10.1371/journal.pbio.0040234.
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. 226, 119183 https://doi.org/10.1016/j.watres.2022.119183.
Tarraran, L., Mazzoli, R., 2018. Alternative strategies for lignocellulose fermentation through lactic acid bacteria: the state of the art and perspectives. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 365 (15) https://doi.org/10.1093/femsle/fny126.
Thompson, L.R., Zeng, Q., Kelly, L., Huang, K.H., Singer, A.U., Stubbe, J., Chisholm, S. W., 2011. Phage auxiliary metabolic genes and the redirection of cyanobacterial host carbon metabolism. Proc. Natl. Acad. Sci. USA 108 (39). https://doi.org/10.1073/pnas.1102164108.
Tian, H., Fotidis, I.A., Kissas, K., Angelidaki, I., 2018. Effect of different ammonia sources on aceticlastic and hydrogenotrophic methanogens. Bioresour. Technol. 250, 390–397. https://doi.org/10.1016/j.biortech.2017.11.081.
Truong, D.T., Franzosa, E.A., Tickle, T.L., Scholz, M., Weingart, G., Pasolli, E., Tett, A., Huttenhower, C., Segata, N., 2015. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nat. Methods 12 (10), 902–903. https://doi.org/10.1038/nmeth.3589.
Turovskiy, Izrail S., Mathai, P.K., 2006. Wastewater Sludge Processing. John Wiley & Sons, Inc.
Wang, W., Ren, J., Tang, K., Dart, E., Ignacio-Espinoza, J.C., Fuhrman, J.A., Braun, J., Sun, F., Ahlgren, N.A., 2020. A network-based integrated framework for predicting virus–prokaryote interactions. NAR Genomics and Bioinformatics 2 (2). https://doi.org/10.1093/nargab/lqaa044.
Weinbauer, M.G., 2004. Ecology of prokaryotic viruses. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 28 (2), 127–181. https://doi.org/10.1016/j.femsre.2003.08.001.
Willenbücher, K., Wibberg, D., Huang, L., Conrady, M., Ramm, P., Gätcke, J., Busche, T., Brandt, C., Szewzyk, U., Schlüter, A., Barrero Canosa, J., Maus, I., 2022. Phage genome diversity in a biogas-Producing microbiome analyzed by Illumina and Nanopore GridION sequencing. Microorganisms 10 (2), 368. https://doi.org/10.3390/microorganisms10020368.
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. https://doi.org/10.1016/j.watres.2008.11.039.
Zhang, D.Q., Tan, S.K., Gersberg, R.M., 2010. Municipal solid waste management in China: status, problems and challenges. J. Environ. Manag. 91 (8), 1623–1633. https://doi.org/10.1016/j.jenvman.2010.03.012.
Zhang, J., Gao, Q., Zhang, Q., Wang, T., Yue, H., Wu, L., Shi, J., Qin, Z., Zhou, J., Zuo, J., Yang, Y., 2017. Bacteriophage–prokaryote dynamics and interaction within anaerobic digestion processes across time and space. Microbiome 5 (1), 57. https://doi.org/10.1186/s40168-017-0272-8.
Zhang, X., Zhang, M., Liu, H., Gu, J., Liu, Y., 2019. Environmental sustainability: a pressing challenge to biological sewage treatment processes. Current Opinion in Environmental Science & Health 12, 1–5.
Zhang, L., Gong, X., Wang, L., Guo, K., Cao, S., Zhou, Y., 2021. Metagenomic insights into the effect of thermal hydrolysis pre-treatment on microbial community of an anaerobic digestion system. Sci. Total Environ. 791, 148096 https://doi.org/10.1016/j.scitotenv.2021.148096.
Zhou, Y., Liang, Y., Lynch, K.H., Dennis, J.J., Wishart, D.S., 2011. PHAST: a fast phage search tool. Nucleic Acids Res. 39 (Suppl. l), W347–W352. https://doi.org/10.1093/nar/gkr485.