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Article

# Evaluation of the Viral Diversity of Artemia Cysts from Saline Lakes of Kazakhstan through Viral Metagenomics Analysis

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**Abstract:** Artemia (brine shrimp), holds significant value as a live feed for larval fish and crustaceans, owing to their distinctive dietary requirements. However, it is vital to acknowledge that Artemia also carries potential risk as a vector of infection. We conducted a metagenomic analysis to explore the virome present in Artemia cysts collected from inland salt lakes across four distinct regions in Kazakhstan. The study identified the presence of dsDNA phages and RNA virus sequences, with a predominant representation from the *Reoviridae*, *Nodaviridae*, *Dicistroviridae*, *Picornaviridae*, *Astroviridae*, *Tombusviridae*, and *Solemoviridae* families. In general, this study has significantly enhanced our understanding of the virome of Artemia cysts in the saline lakes of Kazakhstan. It has also furnished crucial insights for the identification, surveillance, and management of viral infections that impact brine shrimp and fish species populations.

**Keywords:** Artemia (brine shrimp); artemia cysts; metagenomics analysis; viral diversity; saline lakes

## 1. Introduction

Viruses are extensively dispersed and abundant entities on Earth, particularly in both marine and freshwater environments [1–3]. The utilization of a conventional culture-based technique in the investigation of environmental viruses frequently leads to an underestimation of their prevalence and ecological importance [4]. However, the emergence of metagenomic analysis provided a reliable and efficient method for identifying genetic material from individual or pooled samples within a significantly shortened period [5,6]. Also, this methodology enables the discernment of known and novel viral species, thereby enabling the exploration of ecological associations between microorganisms and their respective natural environments [7,8]. Through metagenomics, we obtain deeper insights into the variety, dynamics, and ecological functions of viruses, enriching our knowledge of their influence on ecosystems.

In recent years, due to the significant impact of viruses on microbial communities and ecosystem functions, people have become increasingly interested in investigating the diversity of viruses in salt lakes [9–11]. Salt lakes are fascinating ecosystems characterized by high salt concentrations and unique physical and chemical properties [12]. These saline systems are distributed around the world, with prominent examples including the Great Salt Lake in Utah [13], the Dead Sea in the Middle East [14], and the Aral Sea in Central Asia [15]. Hypersaline viral communities across different salinity environments exhibit a remarkable degree of genetic consistency, indicating the formation of a cohesive community [10]. Moreover, viral populations exhibit similarities to the viruses found in hypersaline ponds and lakes that possess diverse salinity levels [9,16]. The awareness of the variety

of viruses present in saline water environments contributes to our understanding of the intricate associations between viruses and their hosts, thereby unveiling the ecological dynamics that influence these exceptional habitats.

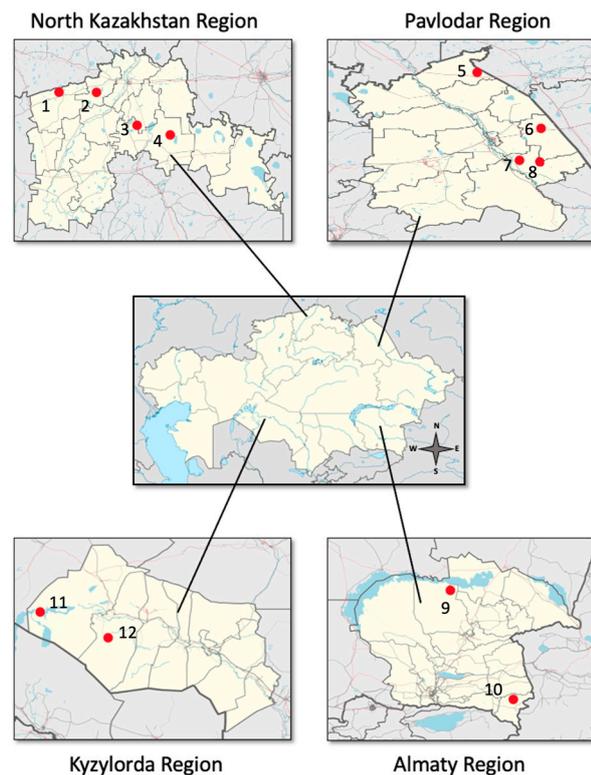
*Artemia*, often known as brine shrimp, is a small crustacean that lives in global saline water environments, and this species is of significant importance in upholding ecosystem equilibrium and productivity [17], while also providing sustenance for a wide array of aquatic organisms that rely on these habitats for survival [18–20]. *Artemia*, despite its widespread use as a live feed in diverse applications, presents a potential concern as a vector for transmitting infections into rearing systems [21,22]. Research has demonstrated that *artemia* has the likely to serve as a vector for parasites, bacteria, and viruses, thereby enabling the dissemination of these diseases over the food chain [23,24]. It is essential to understand the role of *artemia* as a possible medium for the spread of pathogens in order to establish efficient management methods and reduce the hazards associated with its utilization in aquaculture and other interconnected systems.

The primary objective of this study is to evaluate the viral diversity present in *Artemia* populations inhabiting salt lake ecosystems in four distinct locations in Kazakhstan. This will be achieved through the utilization of metagenomic analysis. The employed methodology facilitates a thorough investigation of viral populations present in salt lakes located in both the northern and southern areas. The findings of this study contribute to the advancement of knowledge regarding viral dynamics in saltwater lake ecosystems.

## 2. Materials and Methods

### 2.1. Sampling Sites and Collection

The *Artemia* cysts analyzed in this study were sourced from the Fisheries Research and Production Centre in Almaty and were collected from several salt lake ecosystems in four regions of Kazakhstan, namely North Kazakhstan, Pavlodar, Almaty, and Kyzylorda. Figure 1 and Supplementary Table S1 depict the sampling sites and their respective position coordinates, wherein the labeled numbers correspond to the names of the lakes in each region. The sampling sites in the North Kazakhstan region included Pasyunki Lake (1), Smirnovka Lake (2), Zhamantuz Lake (3), and Kalibek Lake (4). The Pavlodar region includes several salt lakes, namely Kyzyltuz Lake (5), Ashchitakyr Lake (6), TuzKala Lake (7), and Kazy Lake (8). The Almaty region encompassed two distinct geographical locations, namely Ray Lakes (9) and Tuzkol Lake (10). Similarly, the Kyzylorda region featured Tushchybas Bay (11) and Qashqansu Bay (12) in the Aral Sea as prominent spots within its territory.



**Figure 1.** Locations of artemia cysts collection spots in four regions of Kazakhstan. The red dots in the map represent the sampling locations allocated to each region, and the marked numbers represent the names of the corresponding lakes. Source: Wikipedia.

Sampling was conducted in the Pavlodar and Kyzylorda regions during the month of August 2021, but in the North Kazakhstan and Almaty areas, sampling happened in August 2022. In order to ensure proper preservation of the samples, they were promptly placed in a sealed icebox after collection and thereafter maintained in a biomedical freezer set at a temperature of  $-80^{\circ}\text{C}$  upon their arrival at the laboratory.

## 2.2. Viral Nucleic Acid Extraction and Sequencing

For viral nucleic acid extraction, approximately 10 mg of *Artemia* cysts were collected from each of the 12 saline lakes. These samples were then grouped based on their individual regions, resulting in the creation of four composite pools based on the sampling region. The pooled samples were homogenized using a TissueLyser (Qiagen) in conjunction with lysis buffer (Buffer RLT Plus, Qiagen) and 5 mm stainless steel beads, achieving comprehensive mixing through vigorous vortexing. Following this, the homogenized samples were centrifuged at a force of 10,000 times the acceleration due to gravity using a benchtop microcentrifuge for a duration of 5 min at a temperature of  $4^{\circ}\text{C}$  in order to remove any remnants of cyst debris. The centrifugation stage was performed twice in order to provide optimal separation. In addition, a  $0.45\ \mu\text{m}$  filter (Merck Millipore, MA, USA) was employed to exclude particles of eukaryotic and bacterial cell sizes from every pooled sample. The DNase kits (Turbo DNase from Thermo Fisher, Baseline-ZERO from Epicentre, and Benzonase from Novagen) and RNase kits (Promega, Madison, WI, USA) were utilized to enzymatically degrade unprotected nucleic acid present in the filtrates that were enriched with viral particles [25,26]. The residual viral RNA and DNA were subsequently isolated using the QIAamp viral RNA Minikit (Qiagen) in accordance with the protocol provided by the manufacturer.

Four pooled samples were produced, each containing virus sequences of both DNA and RNA, subjected to reverse transcription (RT) and double-stranded DNA construction utilizing QIASeq Stranded RNA Library Kit (Qiagen, Germany) along with a random hexamer primer at a concentration of 100 pmol.

Subsequently, four libraries were generated via the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, USA) and sequenced on the Miseq Illumina platform. The obtained raw data were submitted to the NCBI SRA database with the accession number PRJNA1000065.

### 2.3. Metagenome Data Analysis

The data processing was performed on a Linux machine located locally, utilizing the DIAMOND + MEGAN analytic combination as described in reference [27]. Initially, the raw metagenomic data obtained from each pool underwent preprocessing using Fastp software (version 0.20.0) with the following parameters: “-n 0 -l 30 -5 -r -W 5 -cut\_mean\_quality 20” [28]. This preprocessing step aimed to exclude low-quality sequencing tails and adapters. This stage involved determining the overall quantity of clean reads acquired from the four pools. To ensure contamination control, the sequencing reads were aligned to the scaffold-level genome of *Artemia sp. Kazakhstan ARC1039* (Assembly accession: ASM2916890v1) using BWA-mem v. 0.7 tools [29], and afterward removed from the dataset.

Subsequently, the quality-controlled reads were *de novo* assembled applying MEGAHIT v1.2.9, with “-k-min 21 -k-max 141 -k-step 20” [30]. In order to taxonomically annotate the assembled contigs, a comparison was made against the NCBI's nr database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz>) using DIAMOND blastx v. 0.9.24. The E-value cutoff used was  $<10^{-5}$  [31]. Subsequently, the reads were taxonomically classified, and the results were visualized using the default setting in MEGAN (MEtaGenome Analyzer, version 6.24.23, Tübingen, Germany) [27,32]. The MEGAN outputs, which were received in the form of summarised reads, were utilized to present a comprehensive overview of the taxonomic classification of the sequencing reads (Table S2). Exclusion criteria were applied to the reads originating from cellular organisms, including bacteria, archaea, and eukaryotes. The primary objective of the study was to specifically target and acquire reads resembling those of viruses.

To eliminate false positive virus hits, further analysis of virus-like reads was performed using DIAMOND Blastx (E-value  $<10^{-10}$ ) and compared with the NCBI virus reference sequence (RefSeq) database (<https://ftp.ncbi.nih.gov/refseq/release/viral/>) to identify sequences with higher similarity to viral sequences compared to non-viral sequences. The obtained results were further analyzed using MEGAN (v. 6.24.23) software, employing the lowest common ancestor (LCA) parameter. A minimum score cutoff of 100 and an e-value threshold of  $1.0E-10$  were applied during the analysis.

In order to attain a full classification of virus-like sequences within our dataset, an extensive collection of detailed information on various virus groupings was undertaken. These categories include double-strand DNA (dsDNA), single-strand positive-sense RNA (ssRNA(+)), and double-strand RNA (dsRNA). In addition, we gathered data from two key sources regarding well-documented virus hosts, including bacteria, invertebrates, vertebrates, and plants. The first material cited in this study was obtained from the ICTV website, which may be accessed at <http://talk.ictvonline.org> (Davison, 2017) [33]. The second source utilized in this study was the ViralZone database, a comprehensive repository of genetic and taxonomic data pertaining to viral diseases. The database may be accessed at the following URL: <http://viralzone.expasy.org> [34].

### 2.4. Phylogenetic Analysis

The phylogenetic analyses in our work were carried out with predicted amino acid sequences, as well as the nearest viral relatives identified using top BLASTx matches, and representative individuals from the corresponding viral species. The sequence alignment was performed using Clustal W [35], utilizing the default values. The aligned sequences underwent trimming in order to correspond to specific nucleotide orders of the viral sequences that were retrieved. The maximum-likelihood method was employed to construct a phylogenetic tree in MEGA-X (v. 10.2.6) [36]. The analysis incorporated 500 bootstrap resamples to provide statistical support. The Geneious Prime software (version 2023.2.1) was utilized to anticipate potential open reading frames (ORFs)[37].

### 3. Results

#### 3.1. Overview of Sequencing Outcomes

The present investigation involved the gathering of twelve batches of *Artemia* cysts derived from saline lakes situated in the northern and southern regions of Kazakhstan (Figure 1). The Illumina MiSeq platform was utilized to generate a substantial number of raw paired-end reads (8,108,513 in total) from the four pooled cyst samples. After starting the Fastp filtering method, a significant fraction of the raw reads, ranging from 91.8% to 93.3% (7,486,652 reads), were successfully retained. After aligning the reads with the genome of *Artemia parthenogenetica*, the average number of reads in each pool was found to be 1.85 million (95% CI: 1.22–2.48 million reads), as shown in Table 1. All raw sequences obtained during this experiment have been stored in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under the accession codes SRR25455639, SRR25455640, SRR25455638, and SRR25455641.

**Table 1.** Summary of read and contig counting of pooled cysts of *Artemia* from four regions of Kazakhstan in each data processing step.

Sampling region	raw PE reads	clean PE reads	Host-genome removed PE reads	GC%	Contigs post-assembly	Average contig length	Min contig length	Max contig length	No. of viral contigs	Percent of virus contigs	SRA accession No.
North_KZ	1,968,713	1,836,619 (93.3 %)	1,788,961 (90.9%)	44.3%	18,182	487	206	9,836	44	0.24 %	SRR25455641
Pavlodar	2,121,769	1,948,430 (91.8%)	1,941,957 (91.5%)	53.0%	5,526	468	200	9,067	24	0.43 %	SRR25455640
Almaty	2,533,969	2,330,154 (91.9%)	2,313,732 (91.3%)	48.1%	5,205	507	200	9,717	18	0.34 %	SRR25455639
Kyzylorda	1,484,062	1,371,449 (92.4%)	1,357,628 (91.5%)	42.1%	10,950	473	200	11,780	19	0.17%	SRR25455638
Mean / (95% CI)	2,027,128 (1.33–2.72 M)	1,871,663	1,850,570 (1.22 – 2. 48 M)	46.88 %	9,966	484	201	10,100	26	0.26 %	-
Total	8,108,513	7,486,652	7,402,278	-	39,863	-	-	-	105	-	-

#### 3.2. Contig Analysis

The utilization of MEGAHIT resulted in the generation of a total of 39,863 contigs with diverse lengths. The contig selection process for each individual region is summarised in Table 1. The examination of the North Kazakhstan region yielded the discovery of a grand total of 18,182 contigs. The contigs had an average length of 487 base pairs (bp), with a maximum contig length of 9,836 bp. A comprehensive set of 5,526 contigs was identified in the samples obtained from the Pavlodar region, exhibiting varying lengths spanning from 468 bp to 9,067 bp. The cyst specimens collected from the areas of Almaty and Kyzylorda produced a combined total of 5,205 contigs, with lengths ranging from 507 bp to 9,717 bp, and 10,950 contigs, with lengths ranging from 473 bp to 11,780 bp, respectively.

Following the assembly of contigs, the homology-based identification step was conducted by running BLASTx tools in Diamond and applying an e-value threshold of less than  $10^{-5}$ . And contigs originating from cellular organisms (eukaryotes, bacteria, and archaea) were omitted from the obtained results. Furthermore, contigs lacking significant similarity to any known amino acid sequences were also excluded. Subsequently, a total of 168 reads (containing phages) were identified among the remaining contigs. These putative viral contigs accounted for a proportion that varied from 0.88% to 1.77% of the total contigs found in each pool, as shown in Supplementary Figure S1. To enhance the accuracy of the dataset, two measures were implemented. Initially, we eliminated virus contigs that were identified as false positives and those exhibiting limited levels of amino acid similarity to established viral sequences. Furthermore, we retained contigs that were classified as potential viral entities while excluding those that exhibited similarities to established RNA or DNA viruses. Subsequently, the contigs that were kept passed additional analysis. Consequently, a sum of 105 contigs was identified as the best match with viral reads. The percentage of reads associated with viruses in each pool ranged from 0.17% to 0.43% (Table 1).

### 3.3. Composition of the *Artemia* Viral Community

The identification of viral sequences in our study was mostly based on homology, which resulted in a restricted range of DNA and RNA virus groups. This was determined by the examination of sequence contigs. The contigs demonstrating a significant level of similarity to the viral genome were methodically categorized into seven viral families, comprising a total of nine distinct species. In addition, our research has effectively identified a collective of seven phage families, comprising a set of 25 unique species (Tables 2, S3, and S4). Furthermore, in addition to the aforementioned findings, there have been identifications of viruses that have yet to be classified.

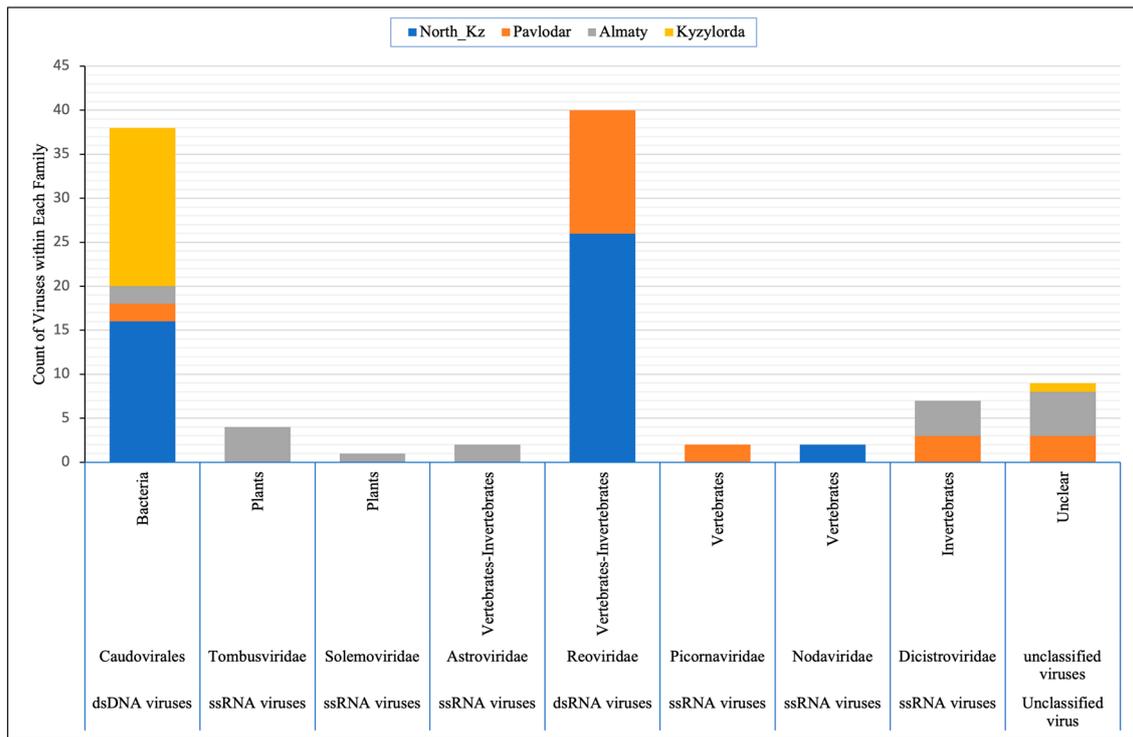
**Table 2.** Contig count for viral species, families, and orders with a range of alignment length, alignment identity percentage, and contig length.

Region	Order	Family	Species	Alignment length <sup>a</sup> , nt	Contig identity <sup>b</sup> , %	Contig length, nt	Number of contigs	Protein Product
North_KZ	Reovirales	unclassified Reovirales	Cimodo virus	109-493	71,5-100	337-1500	26	hypothetical protein
	Nodamuvirales	Nodaviridae	Covert mortality nodavirus	112-204	87,5-90,2	337-612	2	RdRp
Pavlodar	Reovirales	unclassified Reovirales	Cimodo virus	106-153	69-100	302-459	14	hypothetical protein
	Picornavirales	Dicistroviridae	Cripavirus sp.	100-308	65,6-67,5	304-922	3	nonstructural polyprotein
		Picornaviridae	Picornaviridae sp.		107-200	72,5-75,7	322-605	2
Almaty	Picornavirales	Dicistroviridae	Dicistroviridae sp.	114-239	32,2-55,1	364-1626	4	putative replicase
	Stellavirales	Astroviridae	Microbat bastrovirus	376	37,8	1107	1	non-structural polyprotein
			Nelson Astrovirus-like 1	289	47,8	879	1	hypothetical protein
	Sobelivirales	Solemoviridae	Solemoviridae sp.	392	44,1	4992	1	hypothetical protein
Tolivirales	Tombusviridae	Tombusviridae sp.	232-679	37-41	788-6137	4	putative replicase	

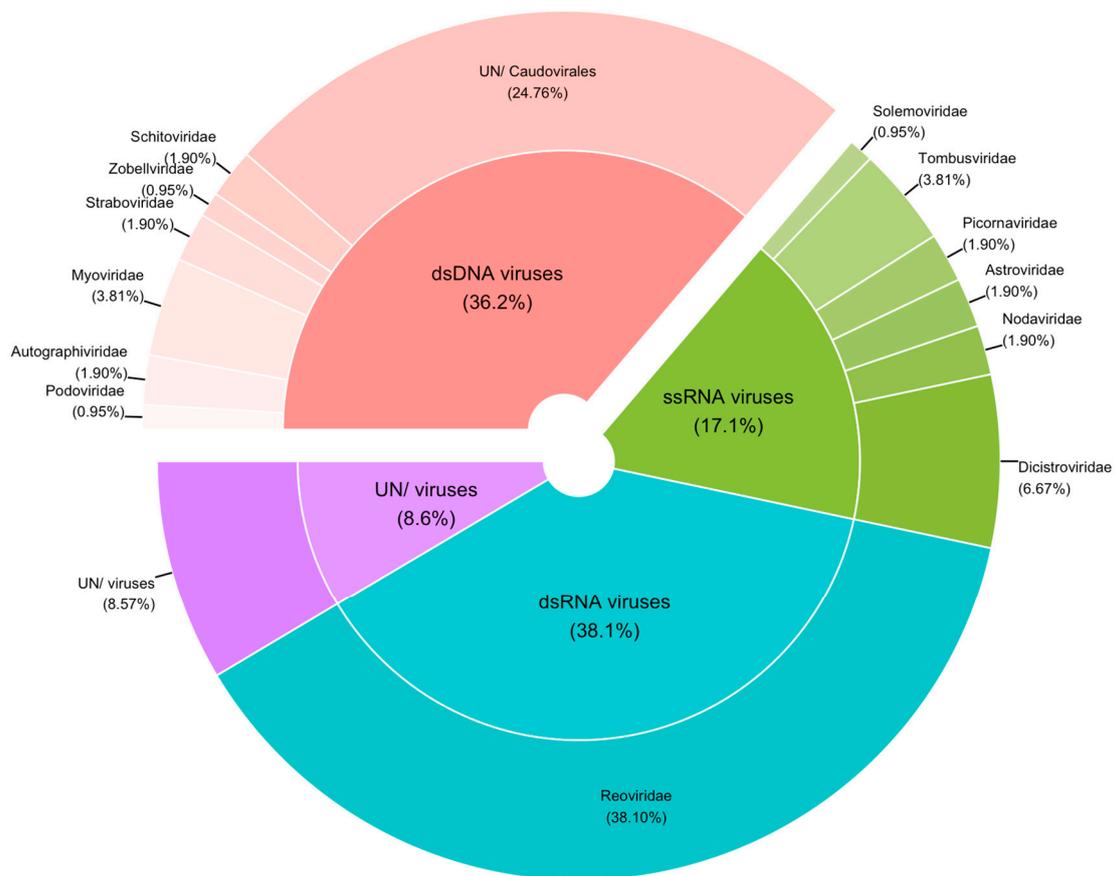
a Contigs sequences over 100 nt in length were characterized. b Determination of identity levels using BLASTx with virus reference strains from NCBI RefSeq database ( $E$ -value  $< 1 \times 10^{-10}$ ).

The classification of these viral families was established by the analysis of their unique patterns of host infection. There are seven families of double-stranded (ds) DNA viruses that have been identified, most of which host bacteria, and one of these families remains unclassified. In addition, a total of six families were formed, including single-stranded (ss) RNA viruses, with a particular focus on positive-strand viruses. It is important to state that two of these viral families have been shown to have associations with vertebrate hosts, whereas another two were recognized for their ability to infect plants. Furthermore, a specific family demonstrated a unique capacity to infect invertebrates, while the other family consisted of a virus capable of infecting both vertebrates and invertebrates. A distinct viral family is identified within the taxonomy of viruses, which is defined by its association with a non-enveloped double-stranded (ds) RNA virus. The virus exhibits a distinct capacity to infect a diverse range of hosts, including both vertebrates and invertebrates. Additionally, the presence of unclassified RNA and DNA viruses was identified (Figure 2a).

A significant proportion of the viral sequences were linked to the dsRNA viruses within the *Reoviridae* family, making up 38.1% of the overall reads of virus-like sequences. The second largest virus group is dsDNA viruses, especially phages classified as *Caudoviricetes*, which consist of six virus families and unclassified Caudovirales. These accounted for 36.2% of the overall viral reads. Furthermore, an analysis of the viral sequence reads revealed that 17.1% of them were associated with ssRNA viruses. These ssRNA viruses belonged to several families, including *Dicistroviridae* (6.67%), *Tombusviridae* (3.81%), *Astroviridae* (1.9%), *Picornaviridae* (1.9%), *Nodaviridae* (1.9%), and *Solemoviridae* (0.95%). The viral reads that accounted for the remaining 8.6% were classified as unclassified viruses, as depicted in Figure 2b.



(a)



(b)

**Figure 2.** Distribution of virus sequence reads within a specific virus family. (a) The distribution of several virus families among taxonomic classes. The  $y$ -axis of the graph depicts the number of individual viruses belonging to each viral family, while the  $x$ -axis displays the taxonomic categorization and corresponding viral family. (b) Proportion of virus sequences in different virus families within the classification.

### 3.4. Distribution of Viral Families in Lakes of Different Regions

Through careful analysis of the data obtained, we found that some patterns of viral diversity of *Artemia* cysts among different regions emerge in Table 2 and Figure 2a. The *Artemia* cysts from the Almaty region are notable for their exceptional viral variety, which encompasses four distinct viral species from different families: *Dicistroviridae*, *Tombusviridae*, *Astroviridae*, and *Solemoviridae*. In materials from the Pavlodar region, which is known for its hypersaline ecosystems, there have been observations of four distinct viral species belonging to three viral families: *Reoviridae*, *Picornaviridae*, and *Dicistroviridae*. The subject of our inquiry now turns to the saline lakes found in the North Kazakhstan region, where two viral species can be identified belonging to the viral families *Reoviridae* and *Nodaviridae*. On the other hand, the Kyzylorda region is notable for its high occurrence of contigs that are classified within bacteriophage families. It is noteworthy that, other than an unidentified DNA virus, no other viral contigs were identified in this study. After doing a thorough comparative examination of viral diversity in different places, a significant observation becomes apparent. Although viral species and families exhibit diverse compositions, the overall amount of diversity is very low.

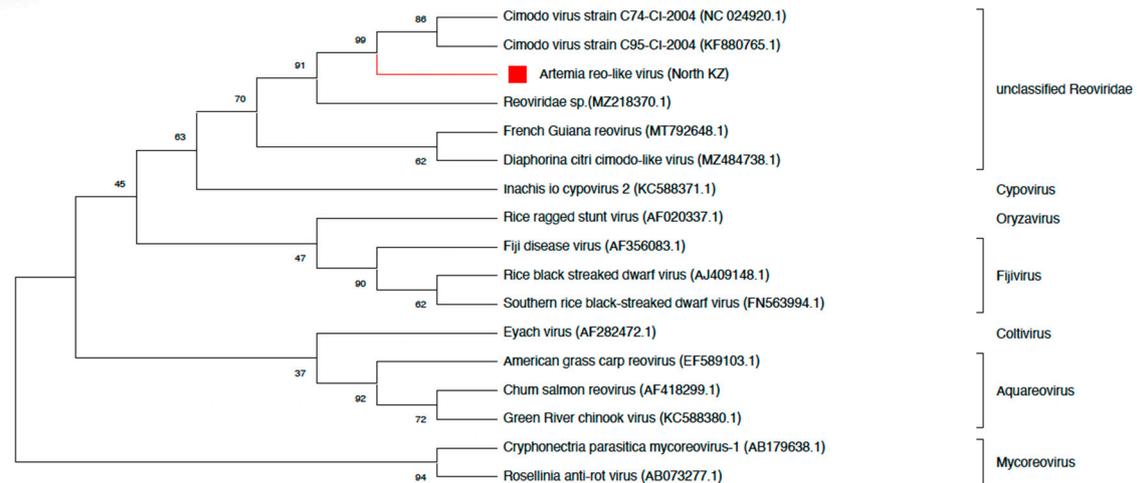
### 3.5. Characteristics of Selected Viruses

After characterizing the host properties, the dataset followed a process of excluding plant viral reads (*Tombusviridae*, *Solemoviridae*), bacteriophages, previously unidentified virus reads, and those that provide too few sequence reads (*Astroviridae*). This facilitated a shift in our attention towards a comprehensive examination of specific viruses associated with both vertebrate and invertebrate hosts, which are classified under the viral families *Reoviridae*, *Nodaviridae*, *Dicistroviridae*, and *Picornaviridae*. As part of this study, we performed an analysis and comparison of the partial genome sequences of these viruses with their closest relatives using phylogenetic analysis.

- *Reoviridae*

The viral family *Reoviridae* is categorized within the taxonomic order *Reovirales*. The viruses under consideration exhibit a non-enveloped, segmented double-stranded RNA genome and have the ability to infect a diverse array of hosts, encompassing mammals, birds, reptiles, fish, insects, and plants [38]. It is worth noting that the *Reoviridae* family has been found to be highly abundant in the pooled *Artemia* cysts collected from the North Kazakhstan and Pavlodar regions. Close relatives of unclassified *Reoviridae*, specifically the Cimodo virus (CMDV), were discovered during our investigation. CMDV was isolated from mosquitoes and consists of a total of 12 genomic segments [39]. The virus that has been recognized is classified as *Artemia* reo-like virus. A total of 40 contigs belonging to the *Artemia* reo-like virus were identified from the pooled samples, originating from two distinct regions. It has been observed that a distinct resemblance exists between the *Artemia* reo-like virus and the CMDV. The amino acid identities between them display a range from 71.5% to 100% in the North Kazakhstan region and 65.6% to 100% in the Pavlodar region, highlighting the genetic similarity between them.

To further investigate the genetic links between the *Artemia* reo-like virus and closely related reference sequences, comprehensive phylogenetic research was undertaken. The study involved creating a phylogenetic tree based on the sequence of a particular segment of the *Artemia* reo-like virus genome. The partial contig sequence (length 1500 nt) was selected because of its high amino acid identity and covering over 80% of the hypothetical protein segment 6 of CMDV. The result showed that there is a closer relatedness between the *Artemia* reo-like virus and CMDV (Accession No. KF880765.1) (Figure 3). Our data suggested that *Artemia* reo-like virus belonged to the unclassified *Reoviridae* family based on the result of a homology similarity and phylogenetic analysis.



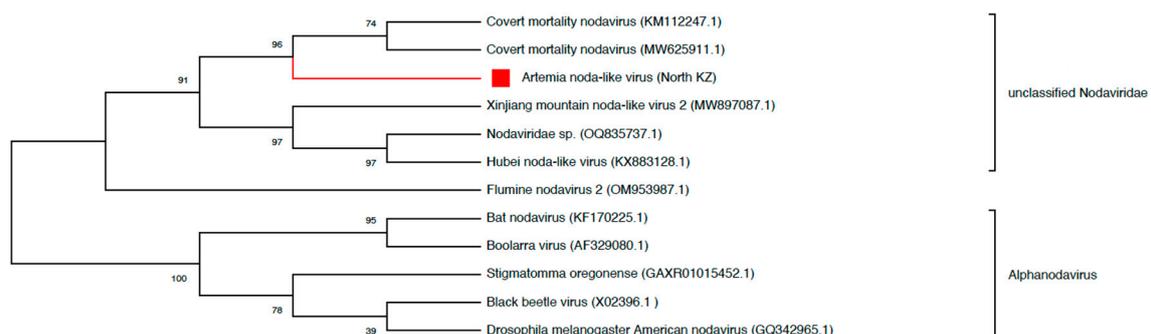
**Figure 3.** Phylogenetic relationships of the Artemia reo-like virus. A phylogenetic tree was constructed using a maximum likelihood method, utilizing the alignment of the conserved portion of the partial hypothetical protein of Artemia reo-like virus (segment 6) and a set of representative sequences from phylogenetically related viruses. The numerical values assigned to the branches of the tree diagram represent bootstrap support levels. The virus found in this specific investigation is prominently indicated using a red square.

- Nodaviridae

The family Nodaviridae is comprised of small, non-enveloped RNA viruses with positive-sense, single-stranded genomes. This family can be further divided into two separate genera known as *alphanodavirus* and *betanodavirus*. These viral species demonstrate a remarkable ability to infect a wide range of hosts, including insects, fish, and crustaceans [40].

In the course of our inquiry, we encountered a couple of viral sequences belonging to the *Nodaviridae* family, which we have designated as an Artemia noda-like virus, in Artemia cysts from the North Kazakhstan region. During the BLASTx analysis, an identity level of amino acid similarity, specifically 90%, was observed between the contig in query and the sequence of the Covert Mortality Nodavirus (CMNV). The analyzed sequences had similarities to the RNA-dependent RNA polymerase gene (RdRp) of CMNV isolated from Larimichthys, with the contigs covering approximately 11%–20% of the Open Reading Frame 1 (3132nt) of CMNV (Accession No. MW625911.1).

Following the previous evaluation of similarities, a thorough examination of the phylogenetic relationships was conducted (Figure 4). This investigation employed the contig sequence of the Artemia noda-like virus, focusing on the longest length, along with other viruses, particularly those resembling the CMNV. Based on phylogenetic research utilizing the partial genome sequence of Artemia noda-like virus, it was observed that the genome of this virus exhibited clustering with other viruses belonging to the CMNV in unclassified *Nodaviridae*.

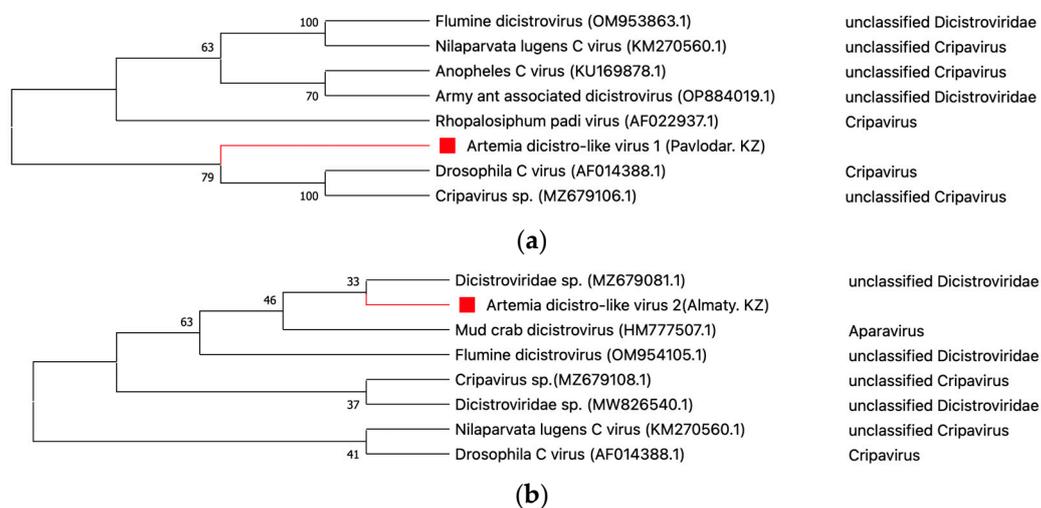


**Figure 4.** Phylogenetic relationships of *Artemia noda*-like virus. The construction of the phylogenetic tree utilized a maximum likelihood methodology, employing the alignment of the RdRp partial genome of the *Artemia noda*-like virus alongside a carefully chosen set of representative sequences from phylogenetically similar viruses. The numerical values assigned to the branches of the tree diagram represent bootstrap support levels. The virus found in this specific investigation is prominently indicated using a red square.

- **Dicistroviridae**

The *Dicistroviridae* family comprises a group of small, non-enveloped RNA viruses with single-stranded genetic material that demonstrates a host range that includes insects and crustaceans. The family can be classified into two genera, namely *Cripavirus* and *Aparavirus*. The viruses belonging to the *Dicistroviridae* family exhibit tiny icosahedral geometries and feature positive-sense RNA genomes that are typically 8–10 kilobases in size [41,42].

The current study has detected sequences of *Artemia* dicistro-like viruses in both the Pavlodar and Almaty regions. Within the pooled sample of *Artemia* originating from the Pavlodar region, a limited number of sequences (*Artemia* dicistro-like virus 1) displayed the highest resemblance to the *Cripavirus* genus. These sequences demonstrated an amino acid identity ranging from 65% to 68% for nonstructural polyprotein genes of *Cripavirus* sp. The *Artemia* dicistro-like virus 1, which has close phylogenetic clustering with members of the *Cripavirus* genus (Figure 4a), has been discovered to occupy approximately 17% of the ORF1 (5343 nt) of the drosophila C virus, which has a length of 9264 bp (Accession No. AF014388.1). On the other hand, the genomic sequence of *Artemia* dicistro-like virus 2 was detected in the Almaty region, exhibiting the highest resemblance to *Dicistroviridae* sp. The amino acid sequence similarity between the two entities varied from 32% to 55%. In phylogenetic analysis, the queried sequence was subjected to clustering with the *Dicistroviridae* sp. (Accession No. MZ679081.1) (Figure 4b).



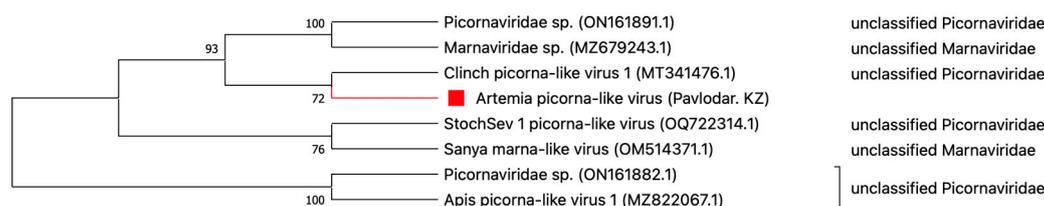
**Figure 5.** Phylogenetic relationships of *Artemia* dicistro-like virus 1 (a) and *Artemia* dicistro-like virus 2 (b). The nonstructural polyprotein genes of *Artemia* dicistro-like virus 1 (Pavlodar, KZ) and *Artemia* dicistro-like virus 2 (Almaty, KZ) were aligned with representative selected sequences from phylogenetically related viruses using a maximum likelihood approach to create the phylogenetic tree. The numbers indicated on the branches are bootstrap support values. Notably, a red square clearly highlights the virus that was found in this particular investigation.

- **Picornaviridae**

The family *Picornaviridae* comprises viruses that exhibit small, non-enveloped particles that possess a linear single-stranded RNA (+) genome with a size ranging from 7.1 to 8.9 kilobases (kb). The present genome exhibits polyadenylation and is comprised of a single ORF that encodes a

polyprotein. These viruses exhibit significant diversity and possess a broad spectrum of hosts, infecting not just human beings but also several vertebrate and invertebrate species [43,44].

*Artemia picorna*-like viral sequences, closely resembling an unidentified virus from the *Picornaviridae* family, were identified in the Pavlodar region. Two contigs were generated by assembling the acquired sequences of *Picornaviridae*-like viruses. The initial contig exhibited a 76% amino acid identity, covering 54% of the partial polyprotein gene of *Picornaviridae sp.* (Accession No. ON162097.1). The second contig exhibited a 73% amino acid identity in the incomplete RdRp gene, spanning a length of 605 base pairs, which corresponds to 54% of the RdRp gene of *Picornaviridae sp.* (Accession No. ON161882.1). In order to further our comprehension of the evolutionary correlation, we performed a phylogenetic analysis utilizing the most extensive contig sequence (Figure 6). Consequently, the *Artemia picorna*-like virus exhibited clustering with the unclassified *Picornaviridae* Clinch *picorna*-like virus (Accession No. MT341476.1).



**Figure 6.** Phylogenetic relationships of *Artemia picorna*-like virus. The construction of the phylogenetic tree involved the implementation of a maximum likelihood method. This method utilized the alignment of the incomplete RdRp gene of the *Artemia picorna*-like virus with a set of carefully chosen representative sequences from viruses that are phylogenetically related. The numerical numbers depicted on the branches signify bootstrap support values. Significantly, the virus found in this specific study is prominently emphasized within a red square.

#### 4. Discussion

Metagenomic research, coupled with homology-based data processing, has provided evidence for all three categories of viruses, encompassing both single-stranded and double-stranded RNA/DNA viruses. The viral groups under consideration consist of a combined total of 15 viral families and 35 viral species. These groups can infect a wide range of hosts, including vertebrates, invertebrates, plants, and microbes. The present study highlights the dynamic diversity observed mostly in dsDNA viruses, with a specific emphasis on the abundance of bacteriophages. The seven viral families in this specific category, together representing 36.2% of viral families, underscore their basic importance within the viral ecology. The prevalence of phages was not a surprise, since earlier studies have demonstrated the predominance of phage families in comparison to other viruses in the saltwater ecosystem [9,10,16,45]. However, it is crucial to acknowledge that bacteriophages did not constitute the primary focus of our investigation.

- **Reoviridae**

The findings of our analysis have shed light on the dominant viral group, the *Reoviridae* family, which accounts for 38% of the overall viral readings. As previously mentioned, this particular family is classified under a category of dsRNA viruses renowned for their capacity to infect a diverse array of hosts [38]. Significantly, our research has observed a plausible occurrence of cross-species transmission involving the *Artemia* reo-like virus detected in pooled *Artemia* cysts originating from the northern region of Kazakhstan. Significantly, the present virus has a considerable degree of genetic resemblance to the hypothetical protein of the Cimodo virus, which belongs to the *Reoviridae* family and was originally discovered in mosquitoes inhabiting an African rainforest. CMDV is purportedly responsible for the classification of a new genus in the *Spinareovirinae* subfamily [39]. The veracity of this association was additionally corroborated through the use of phylogenetic research, as depicted in Figure 3. Multiple study articles have provided evidence for the presence of diverse reo-like viruses, such as the Hubei reo-like virus, the French Guiana reovirus, and the

Shenzhen reo-like virus [46–48]. A recent study documented the discovery of the diaphorina citri cimodo-like virus within the Asian citrus psyllid insect [49]. Moreover, the cimodo-like virus in brine shrimp has been detected in some countries' brine shrimp cysts, including Russia, China, Iran, and the United States [50].

Moreover, the earlier result implies a plausible ecological interdependence between brine shrimp and mosquitoes, maybe facilitated by an unknown intermediary species. The complex interaction of viruses linked to aquatic animals highlights their ability to move across various species within aquatic ecosystems. According to these observations, additional extensive investigation is imperative to ascertain the potential transmission of these viruses to fish and other animals reliant on brine shrimp or inhabiting similar environments. It is imperative to comprehend the potential effects of these interactions and transmission pathways in order to get insight into the broader implications for aquatic ecosystems and their inhabited species.

- Nodaviridae

The present study not only emphasizes the widespread occurrence of the *Reoviridae* family but also reveals the intriguing existence of the *Nodaviridae* family inside the brine shrimp population. In the context of our research, we have identified a viral strain classified under the *Nodaviridae* family, specifically referred to as the *Artemia noda*-like virus, present within the *Artemia* population residing in North Kazakhstan. By employing phylogenetic analysis and homology-based identification techniques, we have identified sequences present in the *Artemia noda*-like virus that exhibit similarities with the covert mortality nodavirus (CMNV), an unclassified virus belonging to the *Nodaviridae* family. Previous studies have demonstrated that CMNV possesses the ability to cause several types of pathology to the tissues and organs of prominent farmed crustaceans [51,52]. Moreover, CMNV has exhibited its capacity to surpass species boundaries and infect a wide array of cultivated and wild fish species, obtained from various habitats including prawn ponds or drainage channels that have been impacted by CMNV [53,54]. This statement emphasizes the inherent capacity of CMNV to surpass taxonomic divisions, therefore highlighting its ability to infect both vertebrate and invertebrate taxa.

The identification of the *Artemia noda*-like virus in the northern region of Kazakhstan prompts inquiries regarding species interactions and the potential involvement of *Artemia* in the transmission of the virus. The discernible correlation between brine shrimp and other aquatic organisms underscores the necessity for comprehensive investigation in order to reveal the implications for aquatic ecosystems and various taxa in the North Kazakhstan region. Furthermore, an examination of CMNV-like sequences within the *Artemia* population has the potential to provide valuable knowledge regarding the virus's ability to adapt and its effects on both invertebrates and vertebrates.

- Dicistroviridae

The identification of *Dicistroviridae* genome sequences among *Artemia* cysts originating from the Pavlodar and Almaty regions serves as a noteworthy illustration of their capacity to thrive in various ecological habitats. It is postulated that the occurrence of an unclassified *Cripavirus* within *Artemia* cysts found in the salt lakes of the Pavlodar region could potentially contribute to the partial impact of these viruses on the indigenous invertebrate fauna in the indicated region. In contrast, the *Artemia* dicistro-like virus 2 found in the Almaty region exhibits lower levels of amino acid similarity when compared to the unclassified *Dicistroviridae*. Based on published research studies, the presence of the *Dicistroviridae* family has been observed in aquatic invertebrates inhabiting saltwater habitats. Two examples of viruses include the Crustacea picorna-like virus N14, which was found in the South China Sea, and the Bivalvia picorna-like virus D23, which was discovered in the East China Sea [55]. Nevertheless, the coverage levels of the genomes belonging to the *Dicistroviridae* family exhibited comparatively low values in both the regional pooled samples. Further investigation is necessary to ascertain the classification of these viruses within the *Dicistroviridae* family.

- Picornaviridae

The identification of members belonging to the *Picornaviridae* family within the Pavlodar region contributes to the advancement of our knowledge regarding the diversity of viruses. The family *Picornaviridae* is considered one of the most prominent and substantial collections of ssRNA viruses [43,44]. Significantly, there has been significant growth in the *Picornavirales* order in recent years. This expansion can be attributed to the identification of previously unidentified picornaviruses using advanced sequencing techniques, such as next-generation sequencing, across a wide range of animals, including vertebrates, arthropods, algae, humans, insects, and plants [55,56]. The investigation conducted by our research examined the detection of an unidentified virus belonging to the *Picornaviridae* family in the samples collected from the Pavlodar region. Although, the virus has a restricted number of contigs, posing a significant challenge in accurately identifying the true viral species.

Moreover, the prevalence of plant viruses, vertebrate viruses, and unidentified viruses is seen in almost all pooled samples. This statement highlights the critical necessity for additional investigative studies aimed at understanding the complex viral dynamics present among populations of *Artemia*. Nevertheless, it is important to acknowledge that certain crustacean host viruses that have been identified as detrimental to aquacultures, such as the white spot virus, yellow head virus, viral nervous necrosis (VNN), and Lymphocystis disease virus (LCDV), were not identified in the samples examined throughout the period of this investigation [22,57,58].

In addition, it is vital to point out the viral diversity that has been discovered within the examined populations of *Artemia* cysts. The limited diversity observed in this study may be influenced by various factors, including specific sampling locations, salinity degrees, timing of sampling, temperature conditions, and sample size [9,10,16,45]. As previously stated, the collecting sites for *Artemia* cysts in the North Kazakhstan and Pavlodar areas are located in the northern part of Kazakhstan, whereas the Almaty and Kyzylorda regions are located in the southern part of the country (Figure 1). It is important to note that the temperatures and seasonal circumstances in these regions also exhibit variations based on their respective geographical locations. The availability of sample collection locations in the southern region is comparatively limited in comparison to the northern region. Future studies should take into account these considerations, such as collecting larger sample sizes and minimizing variations in sample site factors, in order to conduct a full assessment of viral diversity in these ecosystems.

## 5. Conclusions

Overall, this pioneering research offers novel and significant perspectives on the artemia virus communities residing in saline aquatic environments across four distinct regions of Kazakhstan. Furthermore, these investigations serve as a foundation for further research endeavors aimed at exploring the evolutionary dynamics of viruses within their natural reservoir hosts. Additionally, they enable the anticipation of the potential impact of viral diseases on fish and crustacean aquaculture, thereby facilitating the advancement of sustainable production in this field.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Information about the sampling location in the four regions (sampling site number, sampling site, sampling location, sampling dates); Table S2: Overview of Sequencing Reads Classification at the Domain Level (DIAMOND Blastx and MEGAN-LCA) Figure S1: Proportion of the classification level of sequencing reads for different regions; Table S3: Summary of the total count of viral readings obtained from 15 distinct viral families and unclassified viruses; Table S4: Overview of contigs of Bacteriophage species identified using BLASTx in *Artemia* cysts collected from four regions

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