Acta Manilana 74 (2026), 01-16

ISSN: 0065-1370

Acta Manilana

journal homepage: https://actamanilana.ust.edu.ph/

Insights on the Aeromonad Phages in Meycauayan-Marilao-Obando River System (MMORS) based on Integrative Physiological and Gene-based Approach

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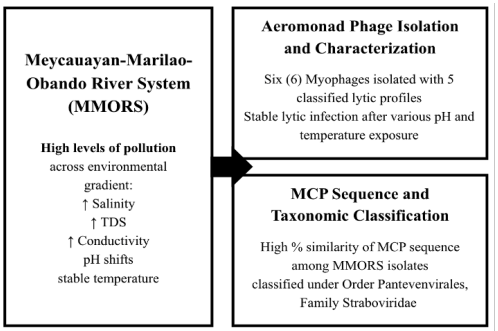
Graphical Abstract



Variation in physicochemical parameters along adjacent locations of the MMORS shaped Aeromonad phages, as identified through both physiological and genetic characterization

Abstract

Aeromonas hydrophila is a well-known fish pathogen that commonly infects aquaculture fishes. It is normally found in aquatic environments such as lakes, ponds, and sewage – with their growth more conducive in locations with high organic load. One identified location in the Philippines with high levels of pollution and anthropogenic waste input is the Marilao-Meycauayan-Obando River System (MMORS), a 52-kilometer river system located in the province of Bulacan (Luzon Is.). This study explored the presence of *Aeromonas hydrophila* phages and characterized the viruses through phenotypic and gene-based analysis. The natural existence of both *A. hydrophila* and their phages can have various implications and potential aquaculture applications. Six (6) phages were determined to have varying lytic abilities based on the nature of their isolation location and conferred stability due to their exposure to different pollutants such as fecal and organic waste, detergents, and heavy metals that can increase phage persistence. Additionally, extended marker-gene based analysis showed high sequence similarity between the isolated phages and were identified as Unclassified *Biquartavirus*, under Family *Straboviridae*, Order *Pantevenvirales*. Although limited in number, this initial description of phages from MMORS show that these locations can harbor ecologically diverse phages whose physiological and lytic characteristics can be attributed to pollution-driven variability – while highlighting the conservation of vital structural genes based on the marker gene analysis. These insights provide a foundation for advancing phage ecology research in Philippine freshwater environments and for exploring their potential applications in aquaculture and environmental health.

Corresponding author: tdgutierrez@ust.edu.ph
DOI: https://doi.org/10.53603/actamanil.74.2026.ngiw7459

Date Received: 20 November 2025
Date Revised: 17 December 2025
Date Accepted: 05 January 2026

Keywords: Phage Diversity, MMORS, physicochemical and genomic comparison

Introduction

The Marilao-Meycauayan-Obando River System (MMORS) is a 52-kilometer river system that is typically characterized by heavy pollution load, including that of heavy metals [1,2]. The river system is identified as Class C according to the Department of Environment and Natural Resources Administrative Order (DAO) 2016-08 where its water is commonly utilized for the provision of water supply, recreation, and aquaculture farming [3]. In a study conducted by Supnet et al (2020) in MMORS, several bacteria including *Bacillus* spp., *Escherichia coli*, and *Morganella morganii*, were isolated and were identified to have high Multiple Antibiotic Resistance (MAR) index, with recorded resistance against cephalosporins (Ceftriaxone and Cephalothin) and carbapenem (Ertapenem) [4]. The presence of these Antimicrobial Resistant (AMR) strains of bacteria in rivers can pose a threat, since these aquatic systems serve as major resources for the surrounding areas. In the case of the MMORS, the water system serves as a major resource for the municipalities of Marilao, Meycauayan, and Obando [4]. The excessive input of waste and pollution from anthropogenic activities in the areas also provides a conducive environment that exacerbates the growth of contaminating bacteria, thereby increasing the risk in development of AMR across the different species present in the water sources [5]. In relation, recent water monitoring report published by the Department of Environment and Natural Resources - Environmental Monitoring Bureau for Region 3 (DENR-EMB R3) showed that 12 sampling stations along the river exhibited high levels of fecal coliform contamination, all of which fail to meet the minimum levels required for fecal coliform concentration of not exceeding 100 Most Probable Number (MPN) per 100 ml (100 MPN/100mL) and 200 MPN/100 mL [6].

Aeromonas hydrophila, a Gram-negative bacterium, is ubiquitous in freshwater systems and was identified to be the causative agent of Motile *Aeromonas* Septicemia (MAS) and furunculosis in aquaculture fishes [7, 8, 9]. These infections are characterized by symptoms such as ulcerations, dropsy (swelling of the eyes), hemorrhages, and swollen organs. It is also considered an opportunistic pathogen to humans – causing several diseases such as gastroenteritis, wound and soft tissue infections. Along with this, this bacterium is also known to be prevalent in environments with high levels of waste and organic matter. Not only does this affect the quality of water in these aquatic systems but also contaminates sources that can be used as drinking water [10]. In a study by Malenab et al. (2018), they found out that 41% of fish farmers in MMORS reared *Oreochromis niloticus* (Tilapia) [11]. With the conducive environment for the growth of this bacterium, MMORS is an ideal site to study the diversity and distribution of *A. hydrophila* bacteriophages.

Bacteriophages are viruses that infect bacteria and are considered to exist ubiquitously in different environments. Natural functions of bacteriophages involve controlling bacterial populations, driving bacterial evolution through infection and selective pressure, and influencing microbial biogeochemical cycle [12, 13, 14]. The viruses are inherently host-specific and their high specificity towards bacterial hosts lead to the possible application in various fields including biocontrol agents [15].

Due to the diversity of *A. hydrophila* bacteriophages, it is vital to classify and characterize all isolated phages. Ackermann (2011) emphasized the importance of classification for it will provide researchers a record of discovered phages and the individual lytic ability of these bacteria viruses [16]. It is established that phages have different applications in the industrial sector, most especially in aquaculture farming. Before its potential application in different fields, it is, therefore, important to establish the identity and the breadth of lytic infections of these viruses. Different studies have isolated, characterized, and classified *A. hydrophila* bacteriophages globally. However, there is still no current study that deals with the diversity and distribution of *A. hydrophila* phages from different aquatic sites in MMORS. Furthermore, there has not been any study that seeks to find a link between the diversity and distribution of *A. hydrophila* phages and the geographical setting of the location from which the phages have been isolated. Hence, this research intends to assess the diversity and distribution of *A. hydrophila* bacteriophages in Bulacan. Furthermore, this research specifically aims to (1) Isolate phages from rivers, ponds and sewage wastewater in MMORS, (2) determine the physiological (plaque and virion morphology, pH and Temperature stability) and genotypic (MCP sequence-based) characteristics of the isolated phages, and (3) correlate the morphology, and physiology—pH and temperature stability of the phages to the physicochemical parameters of the sites from which the phages had been isolated.

This study anticipates that the geographic setting and physicochemical parameters (pH, salinity, temperature, conductivity, and TDS) determine the diversity and distribution of *A. hydrophila* phages. Understanding the diversity and distribution of bacteriophages in MMORS is of particular importance in phylogeny, aquaculture, and other scientific endeavors. With the exploratory nature of this study, the isolation and characterization of *A. hydrophila* phages in Bulacan can be used to expand the knowledge on phage biology and phylogenetics, especially in the Philippines. This also uses polyphasic characterization of the isolated phages, aiming to correlate the environmental conditions of the isolation source to the characteristics of the phages, focusing of its lytic stability and the MCP sequence-based identity. In relation to the physicochemical parameters of the sampling site, this study will allow future researchers to know what types of *A. hydrophila* phages are present in an aquatic environment of physicochemical properties prior to the isolation.

Methodology

Study Site. The study was conducted in selected sampling sites along the MMORS. Specifically, the sampling areas were included based on their location along the MMORS, nearby municipalities, and the type of water use and classification for each of the locations. A total of six samples were collected directly from the river system traversing the three municipalities (Meycauayan, Marilao, and Obando), three from aquaculture ponds, and three from sewage areas (Fig 1). Five hundred (500) mL water samples were taken in duplicates from the water surface using a sterile glass bottle. The physicochemical parameters of each chosen sampling site were obtained and recorded using a multiprobe (ExStik® EC500) and Hach pH meter.

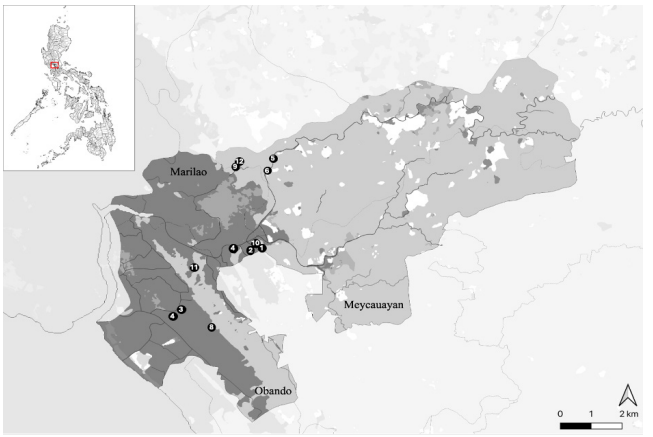


Figure 1. Map of Marilao-Meycauayan-Obando River System and Selected Sampling Sites.

Specifically, measurements for (1) temperature, (2) salinity, (3) pH, (4) conductivity, and (5) Total Dissolved Solids (TDS) was recorded in triplicates. The water samples were stored in sterile glass containers and were maintained in cold transport to maintain the quality of the samples during transfer to the laboratory.

Phage isolation and purification. Prior to phage isolation, the target host strain (*Aeromonas hydrophila* PNCM [Philippine National Collection of Microorganisms] 100089) was obtained from the UST-BEATS Collection and was cultured using Tryptic Soy Broth (TSB). Processing of samples for phage isolation was done starting with the removal of debris from the samples through centrifugation. The resulting filtrate underwent phage enrichment to isolate phages that specifically infect *A. hydrophila*. The enrichment process was done thrice to favor phage replication from the samples collected. Lastly, a spot test was performed to determine the presence of phages infecting the target host strain. Phage purification was done through a modified double layer agar method (plaque assay). The plates were incubated at 30°C overnight and the appearances of the plaques were observed. Formation of plaques across the bacterial lawn are indicative of one infective virion. Repeated process of plaque assay was performed until uniformly-sized plaques are obtained to indicate pure phage stocks.

Plaque and virion morphology. Once isolated, the average plaque size for each phage was obtained through replicated measurement of the plaque formation from each assay. A minimum of 10 plaques was measured using a digital vernier caliper (General Ultratech® stainless steel No. 147). The diameter for each plaque was obtained and reported in averaged size in millimeters (mm). Virion morphology was visualized through negative staining viewed under Transmission Electron Microscope (TEM) (JEOL JEN1220) in the Research Institute for Tropical Medicine (RITM). The electron micrograph obtained was used to define the physiological characteristics and dimensions of the phages, as well as to classify them into their respective morphotypes.

Phage Stability Testing. The stability of the isolated phages was determined by exposing the isolated phages in different pH, and temperature. To determine the pH stability, one (1) mL phage stock, with a starting concentration of 108 Plaque Forming Units (PFU) / mL, exposed to different pH (3, 5, 7, 9, and 11). One (1) M NaOH and HCl were used to adjust to the desired pH (Verma, et al., 2009). Before spot inoculation, it was made sure that the solution was neutralized (to pH 7) so as not to affect the formation of spots against the bacterial lawn. Lastly, phage stock inoculated at pH 7 served as the control. The plates were incubated overnight at 30°C. Similar procedures were performed to temperature stability wherein exposed to different temperatures (25, 37, 40, 50 and 60°C) to test their stability in low, physiological, and high temperature, respectively. After exposure at 30-minute intervals until 90 minutes, a spot test was performed, and the setup was incubated overnight at 30°C. After incubation, the spot clearings were characterized using the criteria by Clokie and Kropinski (2009) [17].

Statistical Analysis and Hierarchical Clustering. Paleontological statistics (Past3.0) and Microsoft Excel were the software used for the statistical analysis of the data [18]. The Shapiro-Wilk test was used to test the normality of the physicochemical parameters and the Kruskal-Wallis test was used to compare the median of the physicochemical parameters for each site. Additionally, capsid gene (gene 23) sequences for the isolated phages were obtained from the study of Gutierrez et al. (2024) with the corresponding accession numbers from MZ768941.1 to MZ768946.1 [19]. Bootstrap replicates of 1000 were used for the generation of the phylogenetic tree, utilizing FastME TN93 algorithm. Bootstrap (BS) of 50 – 69% were considered weakly supported, 70 – 85% were classified as moderately supported and, 86 - 100% as strongly supported clades. Characteristics of the isolation sources were mapped against the generated phylogenetic tree to create an ancestral character / hierarchical clustering tree using MESQUITE and R Studio.

Results and Discussion

Physicochemical Characterization of Selected Sampling Points. Three (3) major sampling site classifications were identified during the collection of samples in the MMORS. Specifically, samples from the main river, sewage, and pond along the MMORS were collected.

The physicochemical characteristics of each site are shown in Table 1. The plot of sampling points was used to determine whether there was a difference in the characteristics of adjacent locations, based on Principal Component Analysis (PCA) (Fig 2). The PCA of the variables per sampling site showed variations steered by (1) salinity, TDS, and conductivity, (2) pH and Temperature. The Principal Components (PCs) have computed eigenvalues (>1), resulting in 89.48% cumulative variance. PC 1 differentiates the samples with a positive association with salinity and TDS, and a negative association with conductivity. Similarly, PC2 presented samples with a positive association with pH and temperature. Based on the figure 2, different clusters were formed, discriminating the sites depending on the dominant gradient observed.

Table 1. Physicochemical Description of Collected Samples from MMORS.

Site No.	Sample Code	Salinity *ppm*	TDS *ppm*	Conductivity *S/m*	Temperature *°C*	pH -
1	Meycauayan River-A	472	667	951	84.3	7.1
2	Meycauayan River-B	543	780	1096	88.5	7.23
3	Obando River-A	457.03	736.2	635.1	88.9	7.81
4	Obando River-B	399.3	691	593.7	88.8	7.63
5	Marilao River-A	275	398	568	90.86	7.65
6	Marilao River-B	281	412	584	90.73	7.63
7	Meycauayan Pond	689	1124	1345	88.8	9.03
8	Obando Pond	0.00000803	0.00000963	13790	87.9	7.81
9	Marilao Pond	0.00000291	0.00000368	5320	92.48	9.1
10	Meycauayan Sewage	604	854	1219	85.6	8.33
11	Obando Sewage	0.00000458	0.00000593	7670	84.7	7.18
12	Marilao Sewage	0.00000274	0.00000354	5050	91.04	8.54

The different parameters tested can provide an overview of the nature of the sites along the river [20]. To note, consistent high salinity, TDS, and conductivity have been recorded for all Meycauayan and Marilao River sites, which may signal a high degree of pollution and contamination that can stem from both domestic and industrial discharge, as supported by Figure 2. Similarly, high conductivity levels and low salinity observed in Obando sites may indicate hydrological mixing between the freshwater and polluted water in the area. Based on both the physicochemical and the PCA, the main driving characteristic of site variation is the mineralization gradient, as dictated by the measured values for TDS and Salinity.

The river system is composed of three main rivers (Meycauayan, Marilao, and Obando rivers) and is considered as one of the country's most polluted rivers. This river system is known to traverse a heavily populated and highly industrialized region (as seen in Figure 1) and is traced to discharge directly into Manila Bay. Meycauayan river, bordered by 26 urban barangays (32.10 km^2), merges with Marilao river, which crosses 16 neighboring barangays (33.74 km^2). Obando river, meanwhile, traverses 11 barangays across the municipality with an area size of 52.10 km2. Land-Use Land-Cover (LULC) determination for the locations showed that most of the sites are surrounded by domestic and industrial areas [21]. Due to its proximity from the namesake municipalities, the discharge and runoffs observed show high concentrations of heavy metals like lead, zinc, copper, and manganese in river sediments [11, 22] . This extensive mineralization, along with the recorded conductivity and salinity levels for each site show poor water quality, most especially along the Meycauayan and Marilao river segments, and show severe levels of contamination that are affecting aquaculture farming, and industrial water supply. Basic physicochemical profiling showed several distinct characteristics across rivers, ponds, and sewage effluents, and can influence microbial and phage communities in the area.

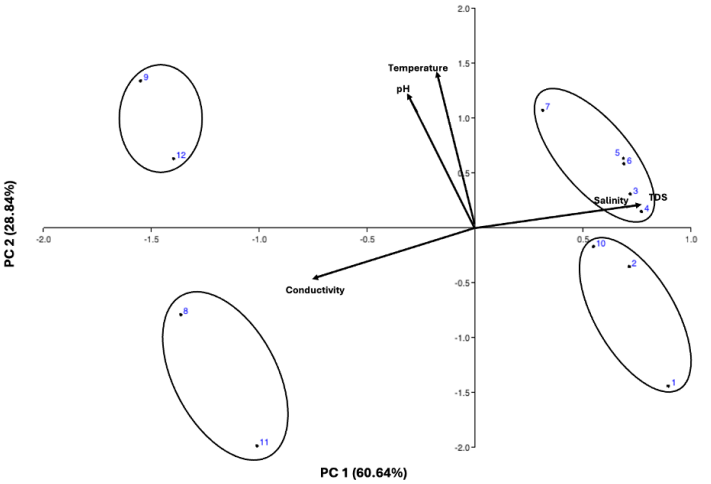
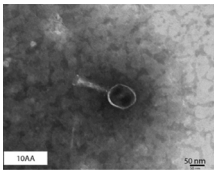
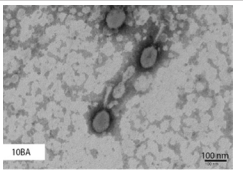
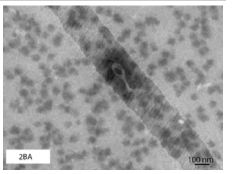


Figure 2. Principal Component Analysis (PCA) of MMORS Sampling Sites. Clusters formed based on similarity in physicochemical characters are encircled.

Bacteriophage Isolation. A total of six phages has been isolated from the set of 12 collected samples across MMORS, namely phages *Aeromonas* virus (av) 2AB and av2AC from Site 1, av2BA from Site 2, av10AA and av10AB from Site 5, and av10BA from Site 6. No phages have been isolated from the samples collected along Sites 3 and 4 (Obando River). The lack of phages isolated from this region despite its connection to the other parts of the system can be caused by the hydrological events, such as the flow, current and circulation of the water in the river [23]. Aside from these, the presence of phages is connected to the prevalence of the bacterial host in the environment. It was also observed that no phages were isolated from all selected fishponds. It is possible that there are limited number of phages that can infect the *A. hydrophila* strain used, or the runoff or contamination of antibiotics may have affected the population of *A. hydrophila*, which can correlate to the absence of bacteriophages. Lastly, no phages were also isolated from all sewage samples collected. The physicochemical characteristics of the sewage waters collected may indicate less pollution compared to the two rivers, Meycauayan and Marilao Rivers. The sites collected also experience dry outs during the dry season due to long and continuous exposure to higher temperatures. This condition may have generally affected the presence of the target bacteria in the selected areas, wherein the target hosts may have limited or inhibited the growth of *A. hydrophila*.

Phage Phenotypic Characterization. For the phenotypic characterization of bacteriophages, two methods were performed covering the morphological and infective ability of the phages. Phage morphology was done to provide preliminary classification of the isolates based on the formation of plaques and the virion structure. The initial determination of phage isolates was dependent on the ability of the phages to produce clear plaques. The clearer plaques that a phage can produce indicate its activity and the susceptibility of the target host to the virus, wherein formation of complete clearing indicates complete lysis of the bacterial cells [24, 25]. The phages isolated in this study have produced clear plaques, with sizes ranging from 0.315 to 0.546 mm.

Table 2. Electron micrographs and representative virion measurements of *A. hydrophila* phages 10Aa, 10BA, and 2BA isolated from Marilao and Meycauayan rivers.

			
Virion Structures (nm)	**10AA**	**10BA**	**2BA**
Head length	106	100	100
Head diameter	75	83	60
Tail length	133	106	122
Total length	240	206	222
Tail diameter	17	18	18
Neck/collar	Present	Present	Present
Terminal Spike/Knob	Spike	Spike	Unclear
Morphotype Classification	Myovirus	Myovirus	Myovirus

One factor affecting plaque size is the adsorption rate where the lysis timing of the phages which then affects the burst period of the phage and its diffusion to other bacterial cells [26]. The size of the plaques is also related to the virion morphology; wherein larger phages tend to diffuse slower compared to smaller phages causing differences in the plaques formed – with larger phages (morphotypes Myoviruses and Siphoviruses) creating smaller plaques, and smaller phages showing larger plaques (Podovirus morphotype). Based on the study of Jurczak-Kurek et.al (2016), all phages that were isolated under the study which had a plaque diameter of < 1mm were determined to be Myoviruses [27]. To confirm this, representative isolates were viewed under TEM, as seen in Table 2.

Based on Table 2, phages 10AA, 1BA, and 2BA exhibit tailed phage morphologies, indicative of phages under class *Caudoviricetes*. Based on the tail length, the phages can be classified as Myoviruses, all with tail length greater than 40 nm (> 40 nm) [28]. Additionally, the phages were characterized to have the presence of a contractile tail with the diameter of 17-18 nm. Electron micrographs of isolated phages showed other visible structures such as having neck/collar tail spikes/knobs. All three phages exhibit a presence of a neck while phage 10AA showed a presence of a collar. Phages 10AA, and 10BA exhibited needle-like tail accessories or tail spikes. Tail spikes/knobs are utilized in phage interaction with the host cell surface. It provides stabilization to avoid bending and curving of the virion, and the driving force needed for the adhesion of the phage to the host surface. The spikes/knob is inserted into the host cells followed by the release of the phage proteins and genetic material [29]. The tail tube or "core" is covered with a sheath; a part of the tail tube that lacks this covering is an empty space or "neck" that separates the head from the tail [28]. The necks of some Myoviruses such as in T4 phage, are accessorized by the "collar" and "whiskers", that are made up of fibritin. This molecule aids in the attachment of the tail fibers during the assembly process. These structures are also utilized as sensory structures that regulate the retraction of the tail fibers under poor conditions to prevent infection [30].

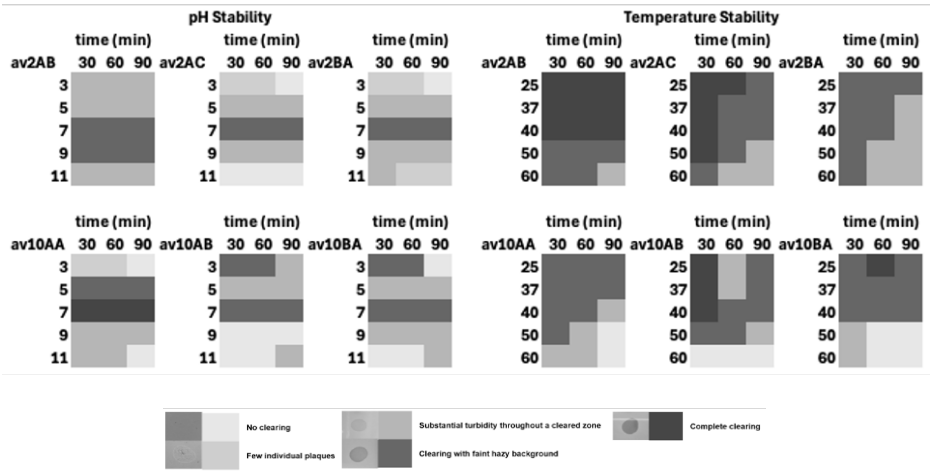


Figure 3. Phage Stability Heat Map based on Spot Assay: (A) pH Stability, and (B) Temperature Stability.

With initial morphological classification of the phages, further characterization was performed to determine the infective ability and stability of the phages in different conditions. The stability of phages in different water conditions, such as pH and temperature, are important factors in administration of phages in the target species during phage [8, 31, 32]. The summary of phage stability in pH and temperature is presented as a heat map, shown in Figure 3. The gradient for characterization was used to semi-quantitatively express the changes in the phage activity after each exposure test.

All the phages had consistent activity against the different temperature gradients tested, with noted optimal activity at 37°C, with decreasing activity in exposures across the timepoints. This suggests that even though the phages can produce plaques beyond its optimum temperature, longer exposure from temperatures beyond the optimum range affects the stability of the phages and the capability to produce clear spots. On the other hand, variable phage activity was observed when exposed to different pH, with a recorded stability on pH 7.

Similar to the results of this study, phage stability test done by Jurczak-Kurek et al. (2016) showed that phages isolated started to produce turbid plaques when exposed at temperatures higher [28]. The higher temperatures can alter structure of the virions that cause breaking or detachment of phage components such as the tails and tail fibers and can lead to impaired phage infectivity. The decrease in the structural integrity of the phages due to the prolonged exposure to beyond optimal temperatures may have caused the observed turbidity [34, 35]. Similarly, exposure to suboptimal conditions of pH resulted in turbidity or lessened phage activity. Phages are typically stable at neutral to slightly alkaline conditions (pH 6 – 8). At acidic pH, phage structures and enzymes tend to denature while higher pH inactivates and destabilizes phages irreversibly [36].

According to the review by Silva et al. (2014), several important factors affect phage infection including changes in pH and temperatures [37]. Phage stability should be determined to obtain a range of effective pH and temperature ranges for the application of these viruses in different settings.

Phages isolated from polluted or sewage environments were observed to be more stable than phages obtained from less harsh environments. Fluctuation of conditions in the environment (i.e., pH and temperature) can modulate phage stability and affect viral structure and infection and can also depend on the persistence of the hosts in those environments [37].

Phylogenetic Analysis and Hierarchical Clustering of MMORS Phages. The phages under this study have been sequenced for the marker gene encoding for Major Capsid Protein (MCP) or *gene 23* for phages with Myovirus morphotypes. The sequences used in this study was obtained from the work by Gutierrez et al (2024) wherein the sequences from the MMORS phages were analyzed together with other Aeromonad phages from the Philippines [19]. In this study, comparison and identification of the MMORS phages was done, as shown in Figure 4A and B. Local alignment of the sequences obtained for the MMORS phages showed high similarity of the sequences, with phages av2AB and av10BA, and av10AB and av10AA, as seen in Fig 4A. Although a similarity score of 1 has been shown with phages av2AB and av10BA, the phages were isolated from different sampling sites (Meycauayan and Marilao Rivers, respectively) and difference in the phage lytic stability has also been observed. On the other hand, av10AB and av10AA may present putatively conspecific relationships of the phages.

To further determine the placement of phages and its taxonomic classification, a phylogenetic tree was generated alongside Aeromonad phages isolated from the Philippines and other phage references from GenBank, NCBI. Compared to the local comparison of the sequences shown in 4A, the expanded analysis provided a better resolution of the clades, inclusive of the isolated phages in this study. Of note, similar grouping was observed for phage av10AA and av2AB, while placements of av2AC and av2BA have delineated, showing av2BA more closely related to reference phages AS4, GomatiRiver_11, and AhFM11 based on the capsid gene [39, 40, 41]. The specific cluster of bacteriophages, along av2BA, is known to have a taxonomic lineage of Order Pantevenvirales, Family Straboviridae [42]. To date, phages under this clade are still unclassified under the Straboviridae. In relation, phage av2AC, av10AB, av10AA, av2AB, and av10BA all cluster under the unclassified genus Biquartavirus under Family Straboviridae – with phage 2AC can be considered as basal organism for the clade. The Major Capsid Protein (MCP) gene or *g23* is considered as one of the marker genes that can be used to provide preliminary identification of phages. These are generally conserved across phage species and analysis of which can provide an overview of the taxonomic classification of phages. Aside from the phage virion morphology done during the physiological analysis, *g23* sequencing can aid in determining the diversity and distribution of the phages in the environment. This marker sequence can provide a reliable standard in basic characterization of phages to supplement the physiological characterization of these viruses. However, the resolution of genus-level classification can ultimately be established through analysis of the whole genome of the isolated phages [43].

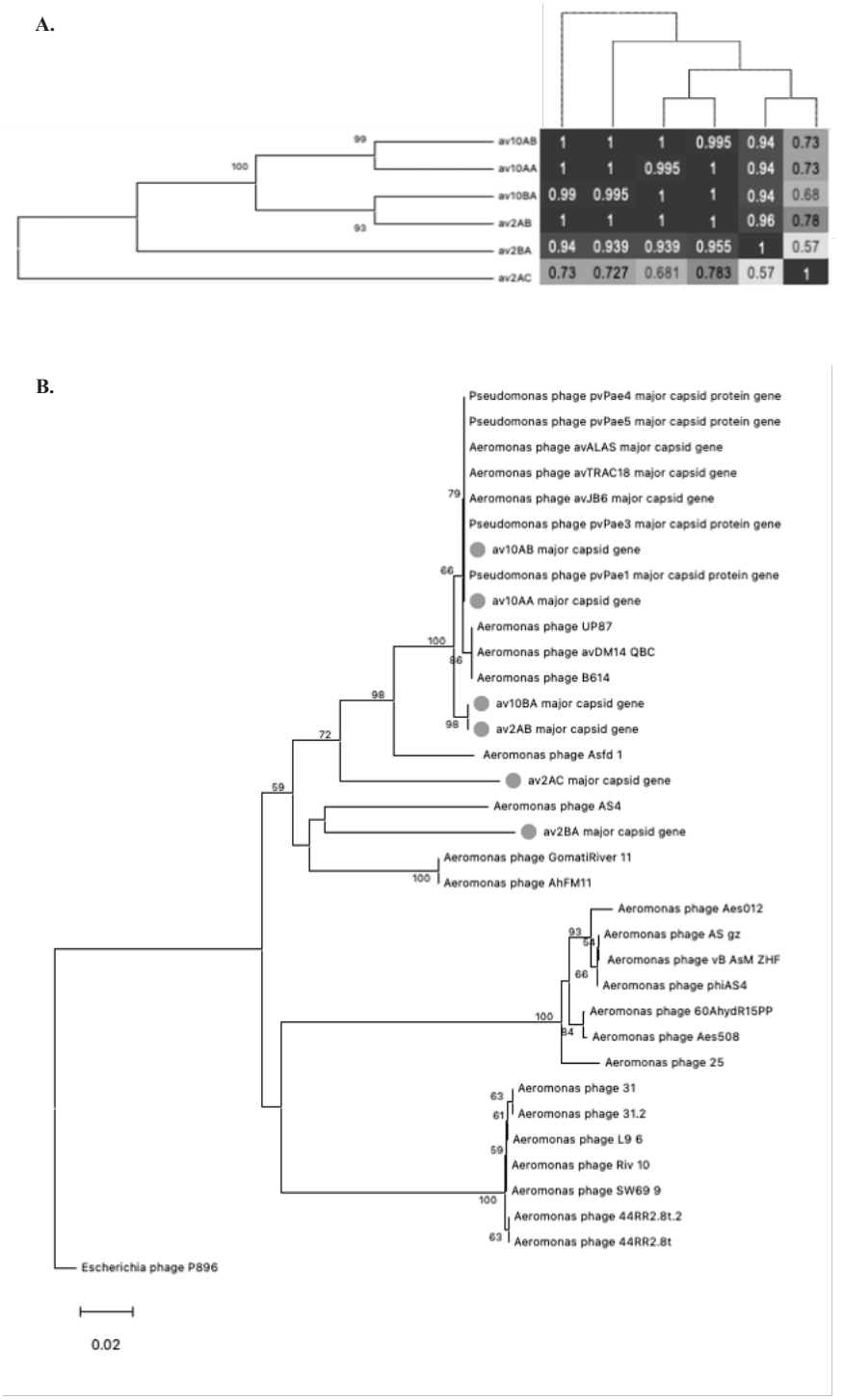


Figure 4. Major Capsid Protein (MCP) analysis of MMORS phages: (A) Sequence Similarity of 6 MMORS phages; (B) Minimum Likelihood tree generated with reference sequences from GenBank.

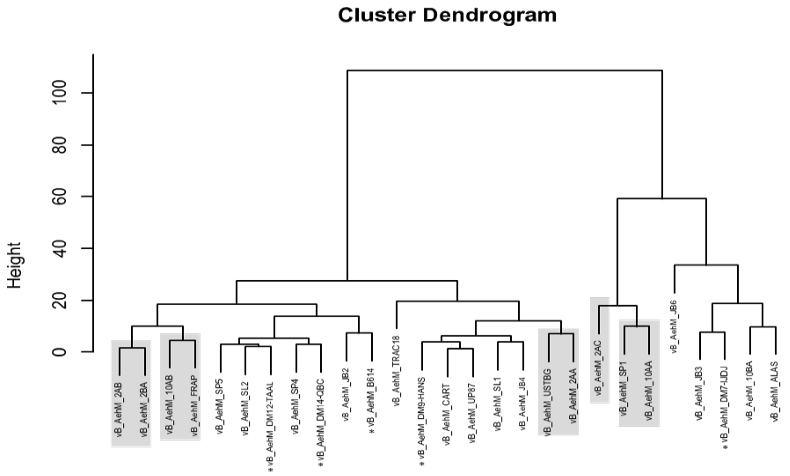


Figure 5. MMORS phages in hierarchical clustering together against other Aeromonad bacteriophages in the UST BEATS HAPPhi Collection.

Correlation of Physicochemical and Genomic Characteristics of MMORS phages. Based on the results of this study and from the preliminary analysis done in the paper by Gutierrez et al (2024), initial comparison of the MMORS phages clusters them along with other Aeromonad phages isolated from different water systems in the Philippines [19].

In terms of the physiological characteristics of the phages, the summarized lytic ability of the phages was analyzed, resulting in the cluster dendrogram seen in Figure 5. Clusters of the same branch are considered phages with the same phenotypic characteristics. Based on the figure, 5 phages with different lytic characteristics were isolated in this study – with varying stability and ability against the tested parameters and based on the morphological data collected.

Although the study only presented five different phages, the variations observed can be related to the influence of environmental conditions. Generally, phages exposed to extreme environmental parameters can confer greater stability compared to other phages. The phages' ability to persist in polluted waters can depend on the type of pollutant present in the water [35]. Typically, more alkaline pH and higher organic matter can be seen in polluted areas, and these components can help maintain the structural integrity and infectivity of the phages. Heavy metals, detergents and other compounds can also have different effects on stability which can ultimately influence phage survival [44]. Lastly, presence of fecal matter and other organic compounds in the water can influence the persistence of phages and can allow them to propagate in the environment due to constant exposure to their bacterial hosts [45]. In this case, MMORS continues to be one of the most polluted riverine systems in the country [6]. The constant input from varying land use systems (domestic and agriculture sewage discharge) can directly influence the microbial communities present in different points or locations along the system.

Further analysis of the phages showed local clustering, regardless of their isolation source. Since the sequence only provides a chosen marker gene, this tends to be mainly strictly conserved across the species. In the case of the MCP, it is highly conserved and always expected to be found in all phage species as it serves as a vital phage structural protein. In the context of its classification, descendants of T4 supercluster (exemplified by the well-studied T4 coliphage), the *g23* structure is mainly conserved and is encoded from a wide variety of T4-related phages, regardless of their environmental source. This shows the possible cosmopolitan distribution of these Aeromonad phages and can point to the high evolutionary conservation of the gene, and the broad ecological presence of *A. hydrophila*-infecting phages. The conservation of the genes would mean low mutation rates, and its detection across phage across different environments can be that the phylogenies created through these genes reflect more of the phages' evolutionary descent rather than environmental origin. The selected marker gene can be used, therefore, identifying phage populations based on major evolutionary clades but may not always reflect habitat-driven divergence. It can provide reliable information for preliminary identification of the phages, however, full resolution of its taxonomic classification and its correlation to the environmental sources can be limited. This unique grouping, hence, can suggest an evolutionary history of Aeromonad phages that dominate over local environments, rather than the reflection of habitat-based divergence.

This study shows the limitations of using conserved structural genes in phage taxonomic classification and evolutionary studies, as single markers are not optimized for detecting subtle changes influenced by ecological conditions. Though using signature genes provide a snapshot of the diversity and is important in providing preliminary data on identity along with the phenotypic characteristics of phages, it is vital to structure a holistic analysis (from phenotypic lytic characterization to Next-Generation Sequence [NGS]-based analysis) to provide a better resolution of phage-host diversity. River systems such as the MMORS are rich, albeit untapped, locations of microbial biodiversity studies in the country – despite their vital ecological functions. MMORS was identified to be a major provider of ecosystem services in the area mainly concentrating on aquaculture farming and water supply in the surrounding areas in Bulacan. With the enhanced proliferation of this bacterium in polluted environments, it can allude to the presence of various bacterial viruses that can still be studied. Aside from the therapeutic potential of phages, they serve as natural predators of pathogenic bacteria. Furthermore, the presence of specific phages may offer a new method for monitoring of river health, most especially in relation to fecal and total coliform contamination in these sources. The use of bacteriophages as both biocontrol and bioindicator agents to assess river health can be a future application to be considered to improve management and monitoring of river systems, and may focus on different bacterial contaminants, including *Escherichia coli* and *Vibrio cholerae* [46, 47].

Conclusion

Bacteriophages were isolated from the Marilao-Meycauayan-Obando River System; however, phages were only isolated in Meycauayan river and Marilao river due to the probable persistence of the target host, *Aeromonas hydrophila*, in those areas. Although representing a small group of phages with varying lytic abilities, the viruses tend to be relatively stable across the parameters tested – which can be related to the presence and the phages' exposure to different pollutants and organic materials from the samples collected along MMORS. Moreover, the phages exhibited both conserved (local) and divergent placement of lineage within Family Straboviridae, Order Pantevenvirales upon analysis of the Major Capsid Protein (MCP) gene. The result from this paper underscores the richness of Philippine freshwater phage diversity and emphasizes the importance of integrative and holistic approaches to phage-host studies.

Acknowledgements

The authors would like to thank the Department of Science and Technology – Science Education Institute (DOST-SEI) Accelerated Science, and Technology Human Resource Program (ASTHRDP) for TADG.

Conflict of Interest

The authors declare no conflict of interest for this study.

Author Contributions

Conceptualization: DMDP, RDSP, PDB, PYSB, HGJ, INM; Methodology: PDB, PYSB, HGJ, INM, TADG; Data Collection: PDB, PYSB, HGJ, INM, TADG; Analysis and Interpretation of Data: PDB, PYSB, HGJ, INM, TADG; Original Draft Preparation: TADG , DMDP, PDB, PYSB, HGJ, INM, ; Review and Editing of Draft: TADG , DMDP, RDSP. All authors have read and agreed to the final version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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