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Investigation of bacterial diversity and pathogen abundances in gibel carp (*Carassius auratus gibelio*) ponds during a cyprinid herpesvirus 2 outbreak

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Abstract

Cyprinid herpesvirus 2 (CyHV-2) infection is detrimental to gibel carp health and may result in severe economic loss in freshwater aquaculture. However, information regarding the interaction of this pathogen with the aquatic environment is scarce. In this study, quantitative polymerase chain reaction (qPCR) and high-throughput sequencing were used to determine the abundances of pathogens and bacterial community compositions in two aquaculture ponds in Jiangsu Province, China. The results indicate that the concentrations of six selected pathogens were higher in the water than in the sediment and that these concentrations peaked during disease outbreak. In total, 8,326 and 18,244 operational taxonomic units were identified from water and sediment samples, respectively. The dominant phyla were Proteobacteria, Actinobacteria, Cyanobacteria, Bacteroidetes, and Chlorobi in water samples and Proteobacteria, Firmicutes, Actinobacteria, Chloroflexi, and Bacteroidetes in sediment samples. Bacterial communities were similar at the phylum level in different ponds, although significant differences were observed at the genus level. In addition, bacterial diversity was associated with environmental factors (temperature, chemical oxygen demand, NO_2^--N, NO_3^--N, and NH_4^+-N) in the pond where the outbreak occurred. Additionally, CyHV-2 abundance was positively correlated with dissolved oxygen levels and *Aeromonas* spp. abundance in pond water ($p < .01$). This study provides comprehensive insight into the mechanisms of interaction between potential pathogens and the freshwater environment of aquaculture ponds during CyHV-2 disease outbreaks. Furthermore, the results from this study can contribute to improvement of the aquatic environment and establishment of disease prevention and control measures.

KEYWORDS
cyprinid herpesvirus 2, gibel carp, high-throughput sequencing, pathogenic microorganisms, quantitative PCR

Tianming Gao and Bingjian Cui contributed equally to this work.

1 | INTRODUCTION

The gibel carp (*Carassius auratus gibelio*) is an omnivorous freshwater fish that has been commercially cultivated in China and Europe for many years; this fish is particularly popular in China, where annual production exceeds 2.9 million tons (Liu, Wu, Li, Duan, & Wen, 2017). Bacterial and fungal pathogens, such as *Aeromonas sobria*, *Aeromonas hydrophila* (Sun, Sun, Jin, & Wu, 1991), *Aeromonas veronii* (Nielsen et al., 2001), *Plesiomonas shigelloides* (Lu, Yang, Chen, Gu, & Yang, 2009), and *Saprolegnia* spp. (Cao et al., 2014), are often detected in cultured gibel carp. Several cases of cultured gibel carp with body hyperemia, swollen bellies, and red gills have been reported in Jiangsu Province since 2009, resulting in considerable economic loss (Xu et al., 2013). Cyprinid herpesvirus 2 (CyHV-2) was confirmed as the viral pathogen responsible for this disease in gibel carp (Wang et al., 2012).

Cyprinid herpesvirus 2, an epizootic virus that causes severe mortality, was first identified and reported in cultured goldfish in Japan and has subsequently developed into a global problem (Wang et al., 2012). In 2012, CyHV-2 was first isolated and identified from cultured gibel carp in China (Wang et al., 2012). Along with the motile *Aeromonas* species, CyHV-2 is now considered one of the main causative agents of fish diseases, which has been confirmed by detailed bacteriological, histopathological, and virological studies on the gill, liver, brain, kidney, and spleen of affected fish (Fichi et al., 2013; Zhang, Yan, Bing, Qin, & Qin, 2010). The virus likely remains latent, considering the extended period before the outbreak of the disease, which is often triggered by environmental variations, such as water temperature and pH (Boitard et al., 2016; Goodwin, Sadler, Merry, & Marecaux, 2009). Molecular biological techniques, including real-time Polymerase chain reaction and loop-mediated isothermal amplification (LAMP), have been effectively developed and established for the detection and quantification of CyHV-2 (He et al., 2013; Xu, Podok, Xie, & Lu, 2014). To date, studies investigating the disease caused by CyHV-2 have primarily focused on the pathogen in diseased aquatic animals, such as the CyHV-2 load and abundance of bacterial pathogens in fish in vivo (Podok, Wang, Xu, Wu, & Lu, 2014; Xu et al., 2014). However, research on CyHV-2 and bacterial pathogens in aquatic habitats, the control of which is relatively easy, is limited (Carda-Diéguez, Mira, & Fouz, 2014; Giatsis et al., 2015; Shen et al., 2018; Wang et al., 2010).

In aquatic systems, microorganisms are the principal participants in primary production, nutrient cycling, and decomposition, and are known to be closely associated with the physiological status, disease, and postharvest quality of cultured fish (Al-Harbi & Uddin, 2005; DeLorenzo, Scott, & Ross, 2001). Therefore, microorganisms are beneficial for fish propagation in ponds because microbes affect water quality, inhibit harmful pathogens, improve digestibility, and maintain the microecological balance. Disruption of the microecological balance in an aquaculture environment is one of the key reasons for disease outbreaks, and fish diseases occur when pathogenic microorganisms propagate and prevail (Murray & Peeler, 2005). Importantly, the microbial community in aquatic ponds is closely linked with disease outbreaks; various reports have shown that microbial community structures are affected by aquaculture methods and are correlated with hardness, ammonia level, total nitrogen level, and other environmental parameters (Li, Yan, Li, Xu, & An, 2016; Zheng, Tang, Zhang, Qin, & Wang, 2017). However, the mechanism underlying the interaction between environmental factors and pathogens in aquatic systems remains unknown. In this study, we analyzed the spatiotemporal dynamic changes in environmental parameters and microbial communities in an aquaculture environment during a disease outbreak and evaluated the correlation of these environmental parameters with pathogen concentrations in ponds. Our results provide a foundation for the development of adjustable preventative measures, which will guide fish propagation in freshwater ponds and prevent the proliferation of infectious organisms that can cause disease outbreaks and economic loss.

2 | MATERIALS AND METHODS

2.1 | Site description

The aquaculture farms were located in the Dafeng District (DF; 33°36′N, 120°56′E) and Sheyang County (SY; 33°46′N, 120°15′E) in Yancheng City, Jiangsu Province, which is a major gibel carp-producing area in China. Both farms use traditional extensive gibel carp cultures in large ponds of 13 to 20 ha, maintaining breeding density at 23,000 to 30,000 carp per ha. Pond water is changed annually between February and March before a new round of culture begins. The water depth of both farms was approximately 1.8 m. There was a CyHV-2 outbreak in the SY pond on July 2016, which resulted in a 70% loss in the cultured gibel carp population. Following this loss, the diseased carp were removed and the water pH was adjusted with CaO and $MgSiO_4$ to 6.5–8.5, the recommended water pH range for freshwater fish (Svobodová, Lloyd, Máchová, & Vykusová, 1993). The DF pond did not experience any disease outbreak.

2.2 | Sampling, processing, and DNA extraction

At each site, five pond water samples and five sediment samples were collected in April, July, and August 2016. In both April and July, we sampled the SY and DF ponds on two consecutive days, each between 9:00 and 10:00. In August, the SY and DF ponds were sampled on the same day, at 7:00–9:00 and 14:00–16:00, respectively. Water samples (500 ml) were obtained from approximately 30 cm below the water surface and transferred into sterile plastic bottles. Prior to collection, the sampling bottles were rinsed twice with their respective water samples. Water temperature (T), pH, oxidation–reduction potential (ORP), and dissolved oxygen (DO) levels were measured using a portable multiparameter meter (HQ40d; Hach). Chemical oxygen demand (COD), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), and ammonia nitrogen (NH_4^+-N) levels were determined following standard methods (Grashoff, Ehrhardt, & Kremling, 1976). Chlorophyll a (Chla) was extracted in 90% acetone and measured spectrophotometrically (Parsons, Maita, & Lalli, 1984). Bulk surficial

(0–10 cm) sediment samples were harvested from five separate sites per pond location using a sampling scoop. All samples were kept on ice and transported to the laboratory in a shipping box within 24 hr. The letter and number preceding the sample name represented the sampling type and month (e.g., W4DF refers to a water sample from the DF pond collected in April, while S4SY refers to a sediment sample from the SY pond collected in April).

To determine the concentrations of microbes and virus-like particles, water samples from each pond were concentrated by filtering through a 0.22-μm mixed cellulose membrane (47 mm diameter; Millipore). Virus particles were concentrated using the cation-coated filter method (Honjo et al., 2010). Briefly, a mixed cellulose membrane with a 0.22-μm pore size was immersed in a glass beaker with 250 mM $AlCl_3$, thus forming a cation (Al^{3+})-coated filter. Water samples were then passed through the filter. The filter membranes were folded in half four times with sterile forceps and placed inside a 1.5-mL microcentrifuge tube. Sediment samples were freeze-dried and then homogenized and sieved to remove crude particles. Pretreated samples were stored at −20°C until DNA extraction.

Microbial DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's protocol. The folded filter membranes were cut into pieces to promote contact between the contents and the sodium phosphate and MT buffers. For each sediment sample, genomic DNA was extracted in triplicate from 500 mg of lyophilized sediment. Bead beating was performed on a FastPrep-24 instrument with the QuickPrep™ adapter (MP Biomedicals) for 45 s at an intensity setting of 5.5 m/s. Genomic DNA was resuspended and eluted using 50 μl of DNase/pyrogen-free water and then stored at −80°C until further molecular analyses. Raw DNA concentrations and purities were determined by measuring the absorbance at 260 and 280 nm using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific).

2.3 | Detection of aquatic pathogens

Polymerase chain reaction primers and probes used for pathogen detection in water and sediment samples have been published previously (Table A1). To obtain standard curves for qPCR, conventional PCR was conducted using the primer sets shown in Table A1. Amplicons from different primer sets were cloned into the pGEM-T Easy vector system (Promega, Madison, WI, USA) and transformed into competent cells (*Escherichia coli* DH5α). The recombinant plasmids were isolated and used to generate standard curves. The concentration of plasmid DNA was determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). Plasmids harboring 16S rDNA or targeted gene sequences were used to construct calibration standards. Copy numbers were calculated from 10-fold dilutions in the range of 10^8–10^1 copies per reaction. The product size for each target was verified by performing a BLAST search on GenBank (National Center for Biotechnology Information).

To quantify the selected pathogens in all samples, qPCR analysis using SYBR® Premix Ex Taq™ (Tli RNaseH Plus; Takara Biotechnology) was performed according to the manufacturer's instructions. The PCR mixture contained 12.5 μl of 2 × SYBR Premix Ex Taq, 0.5 μl of each primer (0.2 μM final concentration), 2 μl of template DNA, and DNase- and RNase-free deionized water. For CyHV-2, qPCR amplification (probe qPCR) was performed in a 25 μl reaction volume containing 12.5 μl of 2 × Premix Ex Taq, 0.5 μl of each primer (0.2 μM final concentration), 1 μl of FAM-labeled probe (0.2 μM final concentration), 2 μl of template DNA, and 8.5 μl DNase- and RNase-free deionized water. The PCR amplification thermal profile consisted of an initial incubation of 30 s at 95°C, followed by 40 cycles of 95°C for 5 s, 46–63°C for 30 s, and 72°C for 30 s. Fluorescence was measured at the end of each cycle at 72°C, and a melt curve analysis (65–95°C, 0.5°C/s increments) was performed at the end of the amplification. All qPCR assays were performed using the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). All qPCR samples were run in triplicate, and the corresponding negative (DNase/RNase-free distilled water) controls were included in each reaction run (Table A2).The cycle threshold (*Ct*) and efficiency were calculated from the slope of the line using the following formula:

$$\text{Efficiency} = (10^{(1/\text{slope})} - 1) \times 100.$$

2.4 | PCR amplification and 16S rDNA sequencing using Illumina Hiseq

Genomic DNA was amplified from the samples with a primer pair targeting the hypervariable V3-V4 regions of the bacterial 16S rDNA. The primers used, namely 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Caporaso et al., 2011; Muyzer, Waal, & Uitterlinden, 1993), were equipped with a 12-base-pair unique barcode to distinguish the different samples. Genomic DNA was diluted to 20 ng/μl using sterile water. All PCRs were conducted in a 30 μl reaction volume with 15 μl of Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs), 0.2 μM forward and reverse primers, and 10 ng of template DNA. Thermal cycling consisted of an initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, and a final elongation at 72°C for 5 min. The PCR products were verified using agarose gel electrophoresis and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific). The purified products were quantified using a NanoDrop 2000 UV-Vis spectrophotometer and mixed in equimolar amounts. In addition, 10-base-pair barcodes were designed for each PCR library to differentiate sequences in the mixed reactions. Sequencing libraries were generated using the NEB Next®Ultra™ DNA Library Prep Kit for Illumina Sequencing (New England Biolabs) following the manufacturer's recommendations, and index codes were added. Library quality was assessed on both a Qubit® 2.0 fluorometer (Life Technologies) and an Agilent Bioanalyzer 2,100 system (Agilent Technologies,). Libraries were sequenced by a commercial company (Novogene Science and Technology Co., Ltd) using an Illumina HiSeq platform to generate 250-base paired-end reads.

2.5 | Sequence and statistical analysis

Sequences less than 200 bp in length were removed, the noncoding areas were truncated, and paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč & Salzberg, 2011). Paired-end reads were assigned to each sample according to their unique barcodes. The assembled sequences (raw tags) were further analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software package V1.7.0 (http://qiime.org/index.html) to generate effective tags (Caporaso et al., 2010). Sequences less than 200 base pairs in length or with quality scores < 25 in the raw reads were removed. The UCHIME algorithm was used to remove chimeras (Edgar, Haas, Clemente, Quince, & Knight, 2011). High-quality clean tags were assigned into the same operational taxonomic units (OTUs) at an identity threshold of ≥97% similarity using UPARSE (Edgar, 2013). All analyses, from clustering to the generation of alpha- (within sample) and beta- diversity (between samples), were performed using QIIME and were visualized using in-house Perl scripts. Raw sequencing datasets were submitted to the NCBI Sequence Read Archive (SRA) (www.ncbi.nlm.nih.gov/sra) under the accession number SRP118729.

Statistical analysis was performed using the SPSS software package (version 20; IBM Corp., Armonk). All data are presented as the mean ± standard deviation (*SD*; $n = 3$). Canonical correspondence analysis (CCA) plots were used to analyze the relationship between bacterial community composition and environmental factors using the CANOCO 5.0 program (Microcomputer Power).

3 | RESULTS AND DISCUSSION

3.1 | Analysis of water quality

Table 1 summarizes the characteristics of water samples from the two ponds at specific sampling times. All parameters exhibited significant spatiotemporal differences ($p < .05$). The pH of the DF pond water ranged between 8.58 ± 0.04 and 9.24 ± 0.03, exhibiting a slight increasing trend over the sampling period. However, the pH of the SY pond water increased from 8.44 ± 0.08 in April to a maximum value of 11.88 ± 0.44 in July. The maximum pH of the SY pond exceeded the acceptable range for freshwater fish (pH 6.5–8.5, EIFAC). The water temperature during the sampling period ranged from 11.0 ± 0.2°C to 35.3 ± 0.2°C. DO levels varied from 2.19 ± 0.2 to 12.95 ± 2.74 mg/L, with minimum DO being measured in August in the SY pond. The DO levels therefore ranged from excessively low to oversaturation and were affected by temperature, photosynthesis, and different operations of the aeration system, particularly in summer. Chla levels in both DF and SY ponds peaked in July at 0.12 ± 0.006 and 0.19 ± 0.006, respectively. High concentrations of NH_4^+-N and NO_2^--N are toxic to aquatic organisms and can contribute to eutrophication of the receiving watersheds. The concentrations of NH_4^+-N and NO_3^--N in the SY pond exhibited a decreasing trend, whereas the NO_2^--N concentration increased to a dangerously high level, as 0.1 mg/L is the upper limit for NO_2^--N levels in breeding ponds (Svobodová et al., 1993). However, the NO_2^--N, NH_4^+-N, and NO_3^--N levels remained stable in the DF pond during the sampling period. The results suggest that the excessively low level of DO (4.41 ± 0.32 mg/L) and the dangerously high level of NO_2^--*N* (0.436 ± 0.02 mg/L) in the SY pond in July favored the disease outbreak.

3.2 | Quantitative evaluation of selected pathogens in pond water and sediment

We detected and quantified *Aeromonas hydrophila*, *A. sobria*, *A. veronii*, *Plesiomonas shigelloides*, CyHV-2, and *Saprolegnia* spp., which are often detected in cultured gibel carp, from both pond water and sediment samples during and after an outbreak of infectious hematopoietic necrosis. Concentrations of these selected pathogens in water and sediment samples were determined using qPCR-based analysis from two freshwater aquaculture ponds (Figure 1). The abundances of all six pathogens presented similar trends in both water and sediment samples, with an increase from April to July and a decrease from July to August. The copy number per liter of water (range, mean) was as follows: *A. hydrophila* (5.18–7.27 $\log_{10}$, 5.87 $\log_{10}$), *A. sobria* (5.67–6.99 $\log_{10}$, 6.40 $\log_{10}$), *A. veronii* (5.71–8.72 $\log_{10}$, 7.37 $\log_{10}$), CyHV-2 (2.74–5.45 $\log_{10}$, 4.12 $\log_{10}$), *P. shigelloides* (3.41–4.89 $\log_{10}$, 4.21 $\log_{10}$), and *Saprolegnia* spp. (7.30–10.54 $\log_{10}$, 8.13 $\log_{10}$). The copy number per gram of sediment (range, mean) was as follows: *A. hydrophila* (3.49–5.65 $\log_{10}$, of 4.49 $\log_{10}$), *A. sobria* (3.63–6.41 $\log_{10}$, 5.34 $\log_{10}$), *A. veronii* (5.41–8.33 $\log_{10}$, 7.08 $\log_{10}$), CyHV-2 (2.1–3.75 $\log_{10}$, 2.83 $\log_{10}$), *P. shigelloides* (2.35–3.99 $\log_{10}$, 2.91 $\log_{10}$), and *Saprolegnia* spp. (3.59–6.17 $\log_{10}$, 5.3 $\log_{10}$). Maximum values were observed during July, which is when the CyHV-2 outbreak occurred in the SY pond. In general, the concentrations of pathogenic microorganisms were lower in the sediment samples than in the water samples. High levels of pathogens, including CyHV-2, were detected in the DF pond, which was free from disease. Spearman's correlation analysis was performed to determine the level of correlation between the abundances of CyHV-2 and the other pathogens in both water and sediment samples from the two ponds. A significant correlation was observed between CyHV-2 and *Aeromonas* spp. ($p < .01$; Table A3). To investigate the mechanism of interaction between environmental factors and pathogens, Spearman's correlation coefficients between the concentrations of selected pathogens in the water samples and the physicochemical properties of the pond water were analyzed (Table A4). A positive correlation between DO level and CyHV-2 concentration was observed in both DF and SY water samples. Furthermore, CyHV-2 concentration was also correlated with pH in the SY pond and with the levels of NO_2^--N and NO_3^--N in the DF pond. This finding provides further insight into the mechanism of interaction between environmental factors and pathogens. Identification and evaluation of aquaculture pathogens will facilitate the development of countermeasures to reduce pathogen contamination in ponds, and therefore, prevent subsequent outbreaks.

Although pathogens are only minor components of the microbial community in pond environments, these microorganisms

TABLE 1 Physicochemical properties of the pond waters

	DF			SY		
Parameter	Apr	Jul	Aug	Apr	Jul	Aug
T (°C)	11.2 ± 0.2^{a}	32.9 ± 0.8^{b}	35.3 ± 0.2^{c}	11.0 ± 0.2^{a}	31.3 ± 0.4^{d}	30.3 ± 0.1^{d}
pH	8.58 ± 0.04^{a}	8.77 ± 0.07^{a}	9.24 ± 0.03^{b}	8.44 ± 0.08^{a}	11.88 ± 0.44^{c}	7.63 ± 0.06^{d}
ORP (mV)	143 ± 8^{a}	181 ± 4^{b}	151 ± 2^{a}	220 ± 27^{c}	216 ± 8^{c}	199 ± 3^{ac}
DO (mg/L)	3.71 ± 0.43^{a}	10.04 ± 0.47^{b}	12.95 ± 2.74^{c}	4.26 ± 0.27^{a}	4.41 ± 0.32^{a}	2.19 ± 0.2^{a}
COD (mg/L)	109 ± 16^{a}	65 ± 6b^{c}	74 ± 12^{b}	46 ± 7^{c}	60 ± 5^{bc}	55 ± 1^{bc}
Chla (mg/L)	0.060 ± 0.006^{a}	0.12 ± 0.006^{b}	0.060 ± 0.01^{a}	0.073 ± 0.007^{a}	0.19 ± 0.006^{c}	0.057 ± 0.003^{a}
NO_2^--N (mg/L)	0.086 ± 0.02^{a}	0.023 ± 0.01^{b}	0.054 ± 0.02^{ab}	0.042 ± 0.01^{b}	0.436 ± 0.02^{c}	0.728 ± 0.02^{d}
NO_3^--*N* (mg/L)	0.17 ± 0.01^{a}	0.12 ± 0.01^{b}	0.14 ± 0.02^{c}	0.29 ± 0.01^{d}	0.12 ± 0.01^{b}	0.13 ± 0.01^{bc}
NH_4^+-N (mg/L)	0.062 ± 0.02^{a}	0.069 ± 0.02^{a}	0.063 ± 0.03^{a}	0.087 ± 0.02^{a}	0.035 ± 0.03^{b}	0.05 ± 0.02^{c}

Note: All data are presented as the means ± *SD*; *n* = 3. DF and SY represent the ponds from Dafeng District and Sheyang County, respectively. Different lowercase superscript letters (a–d) indicate significant differences between the two sites ($p < .05$).

can survive, propagate, and cause disease outbreaks. Our observations clearly indicate that a high concentration of CyHV-2 was present in both the DF and SY ponds during the outbreak period in July. *Aeromonas* spp. and *Plesiomonas* spp. are ubiquitous and dominant in fresh and brackish water. Gastroenteritis and septicemia can be caused by *A. hydrophila*, *A. sobria*, and *A. veronii*, all of which are associated with fish epizootics. *Aeromonas* spp. is the most frequently detected bacteria that cause fish disease and have caused severe outbreaks in recent decades (Nielsen et al., 2001). Infections caused by motile *Aeromonas* species are often reported in ponds and have been detected in cultured gibel carp, common carp, grass carp, and Nile tilapia. External signs may vary according to fish species but are generally similar to those observed in CyHV-2 infections. A recent study reported a 0.01% relative abundance of *Plesiomonas* spp. in the feces of healthy gibel carp; however, this value increased to 13.76% following CyHV-2 infection. *Plesiomonas* is an opportunistic pathogen in gibel carp that can potentially be used as a biomarker for CyHV-2 infection (She et al., 2017). *Saprolegnia* is one of the main genera of oomycetes that is responsible for fungal infection of freshwater fish. Concerns have been raised regarding the occurrence of fungal infections caused by the genus *Saprolegnia* (Cao et al., 2014). Comparative studies have been conducted based on the morphological, physiological, and molecular characteristics of *Saprolegnia* spp., identifying two pathogenic species in silver crucian carp and zebrafish and providing insights into the control of saprolegniasis (Ke, Wang, Gu, Li, & Gong, 2009). As fish live in an aquatic environment, these organisms are directly exposed to many types of microorganisms, including potential pathogens (Wang et al., 2010). Although aquaculture is becoming the fastest growing food-producing industry, this sector currently faces substantial challenges with respect to the control of infectious diseases (Defoirdt, 2014). The above observations and evaluation of aquaculture pathogens will facilitate the development of countermeasures to reduce pathogen contamination in ponds and potentially prevent outbreaks of infectious diseases.

3.3 | Changes in bacterial community composition in different ponds

The composition and diversity of the bacterial community in water and sediment samples were investigated using high-throughput sequencing of the V3-V4 region of 16S rDNA. In total, 1,132,590–1,283,220 effective reads and 8,326–18,244 OTUs were obtained from both water and sediment samples, after Hiseq sequencing analysis and filtering operations. These sequences were assigned to 10 different phyla or groups. The rarefaction curves tended to approach the saturation plateau, indicating that the samples were reliably representative (Figure A1). Good's coverage estimations reveal that 97.9–99.2% of the species were detected in all samples, suggesting that the sequencing depth was sufficient for community analysis.

All sequences were classified from phylum to genus according to the OTU cluster and annotation. As shown in Figure 2, although the dominant phyla in water and sediment samples were similar, relative abundances differed based on sampling month and pond. Relative abundances were higher in sediment than in water, consistent with data reported previously (García-Moyano, Gonzalez-Toril, Aguilera, & Amils, 2012; Zeng et al., 2010). The predominant phyla detected in water samples (average proportions) were Proteobacteria (28.5%), Actinobacteria (27.3%), Cyanobacteria (24.8%), Bacteroidetes (12.1%), and Chlorobi (1.2%), accounting for more than 90% of the reads; the remaining OTUs mainly belonged to Verrucomicrobia, Chloroflexi, Firmicutes, and Acidobacteria. The sediment samples were dominated by Proteobacteria (40.5%), Firmicutes (14.1%), Bacteroidetes (9.8%), Chloroflexi (9.5%), Actinobacteria (8.8%), Cyanobacteria (3.6%), and Acidobacteria (3.4%), representing 90% of the reads. Phyla constituting < 0.05% of the reads were classified as "others". The relative abundances of the main phyla varied between the two ponds, particularly among Proteobacteria, Actinobacteria, Cyanobacteria, and Bacteroidetes. There was a significant increase in the abundances of Actinobacteria and Proteobacteria during the disease period in the SY pond water and sediment, respectively,

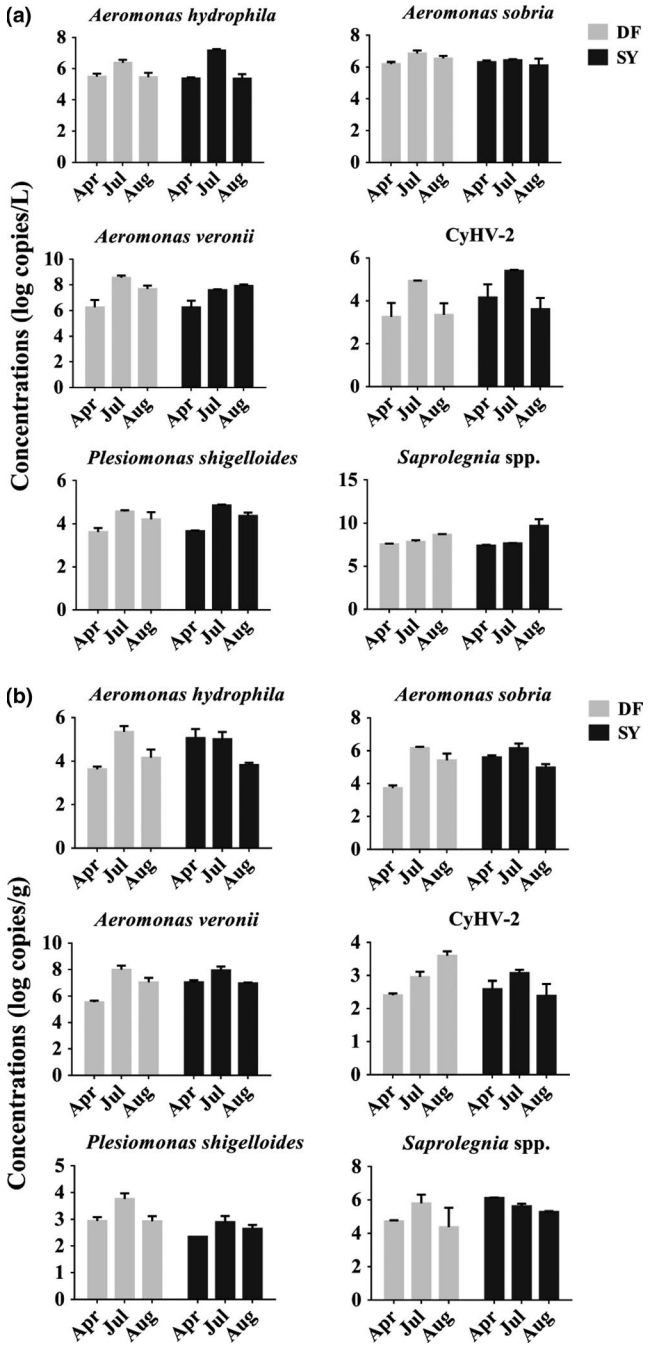

FIGURE 1 Concentrations of pathogenic microorganisms in pond waters (a) and sediments (b)

whereas Cyanobacteria and Firmicutes exhibited the opposite trend. These results are consistent with those of studies reporting that Proteobacteria, Actinobacteria, and Bacteroidetes were generally abundant in aquaculture ponds (Zhang et al., 2013; Zhou, Wang, Tang, & Dai, 2013).

In total, 1,225 genera were identified, including 519 from the water samples and 706 from the sediment samples. Other genera, constituting < 0.05% of the reads, were classified as "others". The composition and abundance of dominant genera differed significantly between water and sediment in each pond and were affected by seasonal variations and the pond environment. The dominant genera in the water samples were *Flavobacterium*, *Limnohabitans*, *Mycobacterium*, and *Ilumatobacter*, accounting for 50% of the total reads. Among these genera, the relative abundance of both *Mycobacterium* and *Ilumatobacter* increased significantly during the disease period in the SY pond where the opposite trend was observed in the same month in the DF pond. The following genera dominated the sediment samples: *Sulfuricurvum*, *Thiobacillus*, *Clostridium*, *Povalibacter*, *Gaiella*, *Methylobacter*, and *Bacillariophyta*, most of which belonged to anaerobic and potential pathogen groups. Furthermore, populations of *Clostridium* and *Thiobacillus* decreased slightly in the sediment of the SY pond in July, whereas these genera exhibited an increasing trend in the DF pond in this period. Hierarchically clustered heat map analysis was performed, based on the bacterial community profiles at the genus level, indicating the top 29 most abundant genera in both water and sediment samples (Figure A2). These results further indicate that the populations of *Candidatus Pelagibacter* (Proteobacteria) in the SY pond water and those of *Dechloromonas* (Proteobacteria), *Litorilinea* (Chloroflexi), and *Cetobacterium* (Fusobacteria) in the SY pond sediment were significantly elevated during the disease outbreak period in July. Furthermore, six genera were abundant in sediment samples, but were rarely identified in pond water: *Sulfuricurvum* (ε-Proteobacteria), *Methanosaeta* (Euryarchaeota), *Thiobacillus* (β-Proteobacteria), *Clostridium* sensu *stricto* (Firmicutes), *Methylobacter* (γ-Proteobacteria), and *Desulfatiglans* (δ-Proteobacteria). *Mycobacterium*, *Flavobacterium*, and *Clostridium*, belonging to the phyla Actinobacteria, Bacteroidetes, and Firmicutes, respectively, were dominant in the water and sediment samples, and are common opportunistic pathogens in the aquatic environment. Proteobacteria, Actinobacteria, and Cyanobacteria were more abundant during the disease outbreak period than during the healthy period in the aquaculture ponds.

3.4 | Richness and diversity of 16S rDNA

The bacterial richness and diversity of all samples from both ponds are shown in Table 2. At 97% gene similarity, 1,126–1,507 and 2,917–3,983 OTUs were identified in the water and sediment samples, respectively. The results show that the number of OTUs, abundance-based coverage estimator (ACE), Chao 1 index, and Shannon diversity index were higher in the sediment samples than in the water samples, suggesting that the diversity of the bacterial community was higher in the sediment than in the water. A clear significant difference ($p < .05$) was observed between the indexes calculated for DF and SY, indicating regional differences between the two ponds. There was no significant difference in any of these measures among the three sampling time points for the DF water. However, the richness and diversity indexes of sediment samples from DF decreased ($p < .05$) from April to July. Additionally, we observed a clear increase ($p < .05$) in the bacterial richness and diversity indexes for the water samples from the SY pond from April to July, compared to a significant decrease ($p < .05$) during the same time period in the

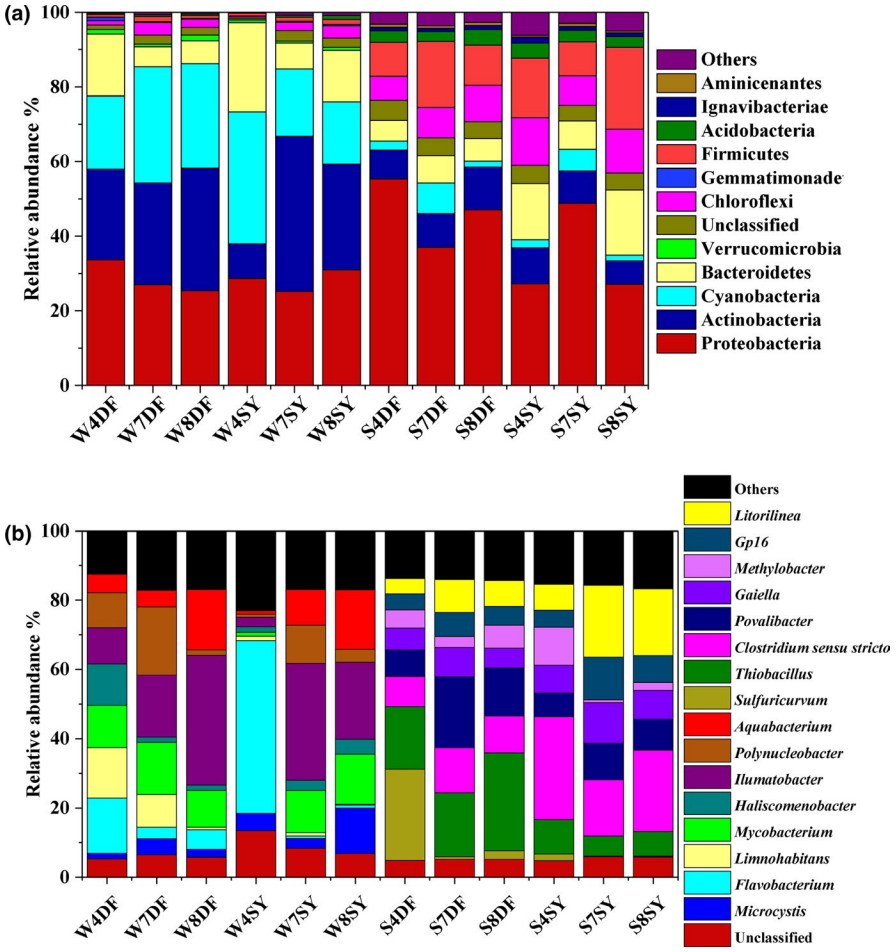

FIGURE 2 Bacterial community composition in water and sediment samples of freshwater polyculture ponds (a) at the phylum level and (b) at the genus level. The relative abundance is presented as the percentage of total effective bacterial sequences per sample

DF pond. Based on the Chao 1 index and Shannon index, bacterial diversity was significantly lower in the intestinal microbiota of sick fish than in the intestinal microbiota of healthy fish, supporting the hypothesis that CyHV-2 infection reduced bacterial diversity in the intestine (She et al., 2017). To evaluate the effects of environmental factors on bacterial diversity, Spearman's correlation coefficients

TABLE 2 Bacterial indexes of water and sediment samples in fish polyculture ponds (97% identity)

Sample ID	OTUs	ACE	Chao 1 index	Shannon diversity index	Simpson diversity index	Good's coverage (%)
W4DF	1,376 ± 67^{a}	1851 ± 128^{a}	1791 ± 141^{a}	7.46 ± 0.05^{a}	0.99 ± 0.00^{a}	98.9 ± 0.00
W7DF	1,341 ± 51^{a}	1,730 ± 63^{ab}	1646 ± 54^{ab}	7.54 ± 0.03^{a}	0.98 ± 0.00^{a}	99.0 ± 0.00
W8DF	1,393 ± 58^{a}	1849 ± 102^{a}	1785 ± 116^{a}	7.21 ± 0.09^{a}	0.98 ± 0.00^{a}	98.9 ± 0.01
W4SY	1,126 ± 98^{b}	1,455 ± 124^{b}	1,401 ± 130^{b}	5.23 ± 0.5^{b}	0.84 ± 0.00^{b}	99.2 ± 0.00
W7SY	1507 ± 46^{a}	1980 ± 76^{a}	1902 ± 80^{a}	7.19 ± 0.03^{a}	0.98 ± 0.00^{a}	98.8 ± 0.00
W8SY	1,417 ± 50^{a}	1,820 ± 64^{a}	1732 ± 65^{a}	7.72 ± 0.03^{a}	0.99 ± 0.00^{a}	99.0 ± 0.00
S4DF	3,983 ± 98^{a}	4,720 ± 112^{a}	4,596 ± 98^{a}	9.25 ± 0.1^{abd}	0.99 ± 0.02^{a}	98.1 ± 0.00
S7DF	3,512 ± 82^{b}	4,213 ± 119^{b}	4,083 ± 106^{b}	9.09 ± 0.05^{bcd}	0.99 ± 0.00^{ab}	98.3 ± 0.00
S8DF	3,760 ± 152^{ab}	4,455 ± 189^{ab}	4,268 ± 170^{b}	9.20 ± 0.09^{bcd}	0.99 ± 0.00^{ab}	97.9 ± 0.00
S4SY	3,912 ± 96^{a}	4,557 ± 267^{ab}	4,458 ± 116^{a}	9.46 ± 0.07^{a}	0.99 ± 0.00^{ab}	98.3 ± 0.00
S7SY	2,939 ± 49^{cd}	3,467 ± 170^{c}	3,384 ± 71^{c}	8.98 ± 0.05^{c}	0.99 ± 0.00^{b}	98.7 ± 0.00
S8SY	2,917 ± 60^{d}	3,446 ± 200^{c}	3,355 ± 85^{c}	9.03 ± 0.07^{cd}	0.99 ± 0.00^{ab}	98.7 ± 0.00

Note: All data are presented as the means ± standard deviation (*SD*), $n = 5$; WDF and WSY represent the water samples of ponds. SDF and SSY represent the sediment samples of the ponds, respectively. Lowercase superscript letters (a–d) indicate significant differences between the two sites ($p < .05$).

Abbreviations: OTUs, operational taxonomic units, which were determined with a 3% width; ACE, abundance-based coverage estimator; Chao 1 index, richness estimate.

between the bacterial diversity indexes of the water samples and the physicochemical properties of pond water were analyzed (Table A5). A significant correlation was observed between the diversity indexes in the SY pond and pond water temperature, COD, and levels of NO_2^--N, NO_3^--N, and NH_4^+-N, indicating that the overall increasing trends of bacterial diversity in the water samples from the SY pond are possibly associated with environmental factors.

3.5 | Variation of the bacterial community structure before and during the disease outbreak

Venn diagrams were used to profile the shared and unique bacterial communities among sample groups (Figure A3). In the DF pond, 1,501 and 4,252 common OTUs were identified in the water and sediment samples, respectively, which accounted for 42.9% and 42.4% of the OTUs, respectively. In the SY pond, 1,425 and 3,074 common OTUs were identified in the water and sediment samples, respectively, which accounted for 40.4% and 33.0% of OTUs, respectively. Among these OTUs, 11.1% and 14.2% were unique to the water samples of the DF and SY ponds during the disease period, respectively, which decreased to 6.8% and 5.8%, respectively, in the sediment samples. No distinct variation was observed in the number of unique OTUs of the ponds during the disease period. The number of special OTUs was higher in the water samples than in the sediment samples, although overall higher OTU numbers were observed in the sediments.

The similarity of bacterial communities among different sampling times was estimated using principal coordinates analysis (PCoA) at an OTU level of 0.03 (Figure 3). The first principal coordinate (PC1) of the weighted analysis accounted for 57.33% and 47.96% of the variation in water and sediment samples, respectively. This result suggests that the bacterial communities detected in April were different from those detected in July and August. The PC2 explains 19.23% and 18.23% of the information, respectively. These results suggest that time is one of the main factors that affects the composition of the bacterial community.

Canonical correspondence analysis (CCA) was used to determine the correlation between the bacterial composition in the water samples and different environmental variables, including CyHV-2 abundance. As shown in Figure 4, the CCA plot was obtained using the OTUs in combination with main driving variables (pH, temperature, COD, ORP, DO, Chla, NO_2^--N, NO_3^--N, and NH_4^+-N). Temperature, pH, DO, and Chla exerted the greatest influence on the bacterial community composition of pond water in July and August and were positively correlated with axis 1 (p = .001). Axis 2 correlated positively with ORP, NO_2^--N, and NO_3^--N (p = .001; Figure 4a). These results are consistent with those of a previous study that reported that NH_4^+-N, NO_2^--N, and PO_4^{3-} in aquaculture water were positively correlated with the bacterial communities of sick fish (She et al., 2017). Bacterial communities in aquaculture water are diverse and dynamic due to different environmental parameters. Thus, diverse microbial taxa may facilitate host metabolism and nutrient cycling. The CCA diagram indicates that Chlorobi and Acidobacteria were positively correlated with ORP and NO_2^--N, but inversely correlated with COD. Compared to other phyla, the presence of Proteobacteria and Cyanobacteria was positively correlated with NO_3^--N. Actinobacteria and Chloroflexi were possibly affected by pH and DO (Figure 4b). CyHV-2 abundance explained only 0.6% of the data and therefore was excluded from the driving factors. These results suggest that, during a CyHV-2 outbreak, environmental factors have greater influence over the structures of bacterial communities in ponds, and can be used as control measures for effectively regulating the microbial ecosystem.

Many studies have focused on the diversity and composition of the complex intestinal microbiota in fish using high-throughput sequencing techniques (Carda-Diéguez et al., 2014; Giatsis et al., 2015; Wu et al., 2012). Water is the shared environment between microbes and aquatic animals in aquaculture systems, suggesting that the association of the host intestinal microbiota with the environmental microbiota in aquatic systems is stronger than that observed for terrestrial systems (De Schryver & Vadstein, 2014). Although the aquatic environment of fish habitats is important for fish health, this environment has not been studied in detail. In this study, we used Illumina sequencing to characterize the shifts in bacterial community structures in both water and sediment during and after an outbreak of CyHV-2 infection in gibel carp. The bacterial community compositions in the water samples differed substantially from those in the sediment. We observed that the bacterial compositions of these communities were similar at the phylum level, although there were differences at the genus level. In addition, the relative abundances varied considerably with CyHV-2 infection. The complex microbiota in the aquaculture environment is known to depend on the

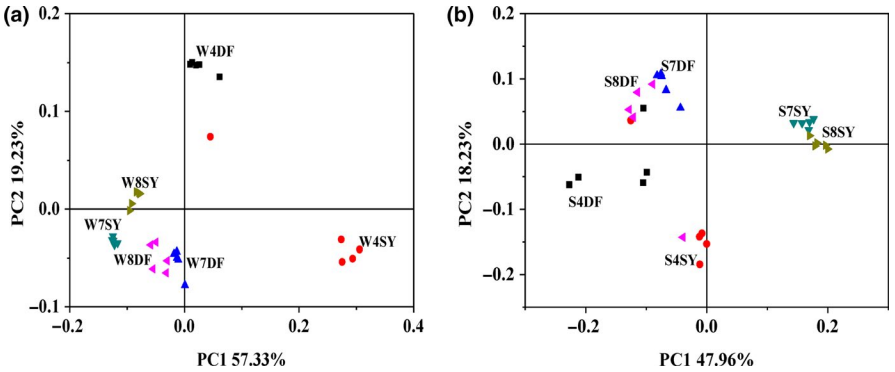

FIGURE 3 Principal coordinate analysis (PCoA) of water samples (a) and sediment samples (b) from the DF and SY ponds via weighted UniFrac

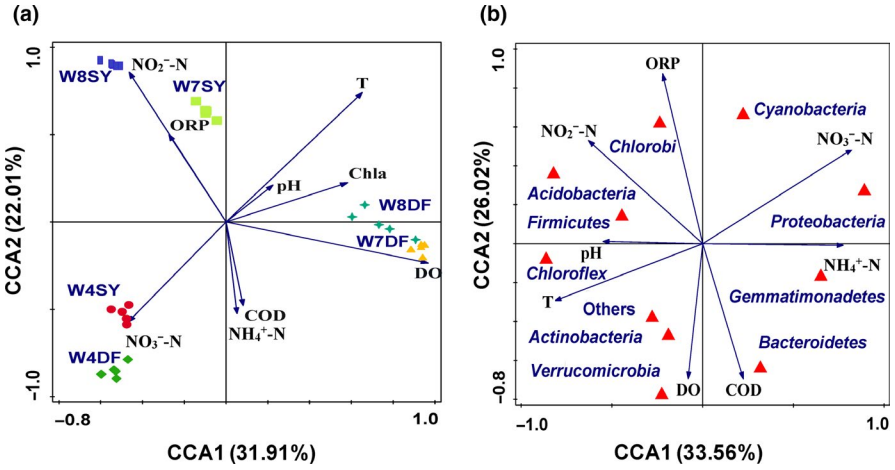

FIGURE 4 Canonical correlation analysis (CCA) of the relationships between bacterial communities and environmental variables in aquaculture water. Environmental variables are indicated with arrows

functional and ecological balance between host, environment, and dietary factors (even causative agents). Thus, perturbations in this balance can alter the microbial diversity and abundances of certain bacteria and can exert beneficial or harmful effects on fish. Previous studies have indicated that the bacterial community composition in the aquaculture environment or fish gut is influenced by aquaculture methods (Li et al., 2016; Qin et al., 2016; Zheng et al., 2017), stocking densities (Fan, Barry, et al., 2017), nutrient input (Fan, Chen, et al., 2017), host development (Li et al., 2017), and seasonal changes (Fan et al., 2016). An additional important factor that affects the changes in microbial communities is disease outbreaks. In our study, Proteobacteria, Cyanobacteria, and Actinobacteria were the most important phyla and accounted for 25.2–27.1%, 27.2–41.6%, and 18.0–31.1% of the OTUs, respectively. γ-Proteobacteria is the predominant class of Proteobacteria. The main members of this class, *Aeromonas*, *Shewanella*, *Vibrio*, *Legionella*, and *Plesiomonas* spp. are opportunistic pathogens, that can induce fish epizootic disease under stress. A previous study showed a shift in the bacterial community structure to a relatively high abundance of γ-Proteobacteria due to the presence of Sudan grass in ponds (Qin et al., 2016). Cyanobacteria are the most common phytoplanktonic organisms and are capable of producing cyanotoxins. The proliferation of Cyanobacteria and Actinobacteria was found to be affected by the peak breeding period of fish (Fan, Barry, et al., 2017). Furthermore, bacterial community composition was significantly correlated with abiotic environments in the aquaculture ponds. The relative abundances of Actinobacteria and Cyanobacteria in July and August were higher than those in April, which might be due to high temperature, accumulation of nutrient salts, and variance in DO. The highest abundance of Actinobacteria in this study was observed in July, which is consistent with the results of bacterioplankton community analysis in tilapia ponds (Fan et al., 2016). Actinobacteria synthesize important antibiotics and have been used as potential probiotics in aquaculture systems (Das, Ward, & Burke, 2008). However, this phylum contains the genus *Mycobacterium*, which is an opportunistic pathogen and is highly abundant in aquaculture ponds. The occurrence of mycobacteria in natural water, water reservoirs, and ponds has been investigated, especially in freshwater fish cultures, where these bacteria cause chronic disease (Mrlik et al., 2012). Cyanobacteria, which are usually found in eutrophic freshwater, were significantly enriched in the aquaculture ponds during the months of July and August, which may explain the increase in pH. The bacterial blooms and toxins produced by *Microcystis* and *Synechococcus* have been shown to be harmful to aquatic animals and humans (Huang, Liu, Li, & Wang, 2014). Therefore, genera within Actinobacteria and Cyanobacteria that may be potentially associated with CyHV-2 infection, require further investigation. We used only one primer pair targeting the hypervariable V3-V4 regions of the bacterial 16S rDNA for PCR prior to high-throughput sequencing; therefore, sequences of archaea, fungi, and other microorganisms were not identified. In the future, metagenomic sequencing can be used to explore the microbial community structure in detail to further clarify the relationship between microbial diversity and pathogens in aquatic environments.

4 | CONCLUSION

In this study, we analyzed the aquatic environment, concentration of pathogens, and bacterial communities in both water and sediment during a CyHV-2 infection of two aquatic ponds in Jiangsu Province, China. We further investigated the mechanism connecting these three factors. Our results demonstrated that during the disease period, both the concentrations of selected pathogens and bacterial diversity in the water of the SY pond significantly increased and were positively associated with DO, pH, and other water parameters. In addition, the bacterial communities showed distinct responses to environmental factors during the disease outbreak. These results indicate that both CyHV-2 and other bacterial pathogens and bacterial community changes are affected by internal and external factors, such as water quality and disease outbreak. Furthermore, water quality, especially levels of DO and NO_2^--N, are closely associated with disease occurrence. However, it is difficult to determine the factors that induce outbreaks of infectious diseases. Thus, appropriate measures must be adopted to not only improve the aquatic environment and maintain microecological balance, but also regularly monitor the dynamics of major pathogens for prevention of disease outbreaks in the future.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Tianming Gao and Bingjian Cui conceived and designed the experiments and wrote this manuscript. Xiao Kong participated in the High-seq experiments. Zhihui Bai involved in critical revision of the manuscript. Xuliang Zhuang and Zhi Qian provided ideas.

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

All data are included in the main manuscript apart from the raw sequencing datasets which are available at the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under accession number SRP118729.

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APPENDIX

TABLE A1 Real-time PCR primers and probe sequences used in the assay

	Primer sequences (5'→3')	Amplicon size (bp)	Tm (°C)	Reference
Aeromonas hydrophila	aerAF: CAAGAACAAGTTCAAGTGGCCA aerAR: ACGAAGGTGTGGTTCCAGT	309	59	Wang et al., 2003
Aeromonas sobria	ASA1F: TAAAGGGAAATAATGACGGCG ASA1R: GGCTGTAGGTATCGGTTTTCG	249	59	Wang et al., 2003
Aeromonas veronii	F: GAGGAAAGGTTGGTAGCTAATAA R: CGTGCTGGCAACAAAGGACAG	658	60	Dorsch, Ashbolt, Cox, & Goodman, 1994
Plesiomonas shigelloides	F: GCGAGCGGGAAGGGAAGAACC R: GTCGCCCCAAACGCTAACTCATCA	435	63	Herrera, Santos, Otero, & Garcia-Lopez, 2006
Cyprinid herpesvirus (CyHV–2)	F: TCGGTTGGACTCGGTTTGTG R: CTCGGTCTTGATGCGTTTCTTG Probe-FAM-CCGCTTCCAGTCTGGGCCACTACC-BHQ1	170	58	Goodwin, Merry, & Sadler, 2006
Saprolegnia spp.	161F: GTCAAATACCCAACTGCTTG 854R: CTATTAATCATTACCTCGGTGTGC	693	60	Kestrup, Thomas, Rensburg, Ricciardi, & Duffy, 2011
Cyanobacteria	CYA359F: GCGGTAATTCCAGCTCCAA CYA781R: GACTACWGGGGTATCTAATCCCWTT	422	62	Nübel, Garcia-Pichel, & Muyzer, 1997
Universal bacteria	Eub338: ACTCCTACGGGAGGCAGCAG Eub518: ATTACCGCGGCTGCTGG	200	60	Fierer, Jackson, Vilgalys, & Jackson, 2005

TABLE A2 The linear relation and amplification efficiency of qPCR

	R^2	linear relation	Eff.	Samples Ct Range
Aeromonas hydrophila	.993	$Y = -3.020 \times \log(X) + 40.66$	114.3%	19.29–27.74
Aeromonas sobria	.985	$Y = -3.489 \times \log(X) + 47.31$	93.5%	28.44–35.63
Aeromonas veronii	.995	$Y = -3.736 \times \log(X) + 48.19$	85.7%	16.62–23.36
Plesiomonas shigelloides	.998	$Y = -3.634 \times \log(X) + 49.34$	88.3%	33.03–35.42
CyHV–2	.991	$Y = -3.095 \times \log(X) + 37.61$	110.4%	28.52–36.32
Saprolegnia spp.	.968	$Y = -3.424 \times \log(X) + 46.46$	103.0%	25.64–27.76
Cyanobacteria	.992	$Y = -3.043 \times \log(X) + 36.43$	113.2%	14.39–16.09
Total bacteria	.990	$Y = -3.298 \times \log(X) + 38.76$	101.0%	13.38–14.06

TABLE A3 Correlation analysis between CyHV-2 and pathogens

		Correlations						
		Aeromonas hydrophila	***Aeromonas sobria***	***Aeromonas veronii***	***Plesiomonas shigelloides***	***Saprolegnia* spp.**	***Cyanobacteria***	**CyHV–2**
Aeromonas hydrophila	R	1.000	0.864**	0.814**	0.327	0.687**	0.454*	**0.738****
	P		0.000	0.000	0.077	0.000	0.012	0.000
Aeromonas sobria	R		1.000	0.963**	0.280	0.535**	0.201	**0.742****
	P			0.000	0.133	0.002	0.288	0.000
Aeromonas veronii	R			1.000	0.366*	0.479**	0.011	**0.723****
	P				0.047	0.007	0.953	0.000
Plesiomonas shigelloides	R				1.000	0.026	−0.280	0.294
	P					0.891	0.134	0.115
Saprolegnia spp.	R					1.000	0.439*	0.565**
	P						0.015	0.001
Cyanobacteria	R						1.000	0.184
	P							0.332
CyHV–2	R							1.000
	P							

*Significant at $p < .05$.
**Significant at $p < .01$.

TABLE A4 Correlation analysis between pathogens and water physicochemical properties

	AH	AS	AV	C2	PS	S.spp
DF						
pH	0.050	0.400	0.417	0.083	0.467	0.917**
T	−0.117	0.633	0.467	−0.033	0.567	0.900**
ORP	0.717*	0.817**	0.717*	0.550	0.617	0.183
DO	0.517	0.850**	0.900**	0.667*	0.800**	0.517
COD	−0.500	−0.917**	−0.783*	−0.317	−0.600	−0.567
NO_2^--N	−0.600	−0.867**	−0.950**	−0.817**	−0.900**	−0.333
NO_3^--N	−0.756*	−0.798**	−0.899**	−0.824**	−0.832**	−0.437
NH_4^+-N	0.183	0.150	0.333	0.150	0.250	0.167
SY						
pH	0.736*	0.285	−0.435	0.837*	0.471	−0.460
T	0.603	0.385	0.452	0.603	0.891**	0.477
ORP	0.550	0.500	−0.483	0.200	−0.109	−0.383
DO	0.633	0.350	−0.717*	0.767*	0.117	−0.567
COD	0.377	0.251	0.527	0.444	0.798**	0.552
NO_2^--N	−0.117	0.133	0.883**	−0.283	0.477	0.883**
NO_3^--N	−0.262	−0.025	−0.633	−0.321	−0.720*	−0.574
NH_4^+-N	−0.201	−0.159	−0.728*	−0.226	−0.672*	−0.569

*Significant at $p < .05$.
**Significant at $p < .01$.

TABLE A5 Spearman correlation coefficients between the bacterial diversity indexes of water samples and physicochemical properties pond water

	OTUs	ACE	Chao 1 index	Inv_Simpson	Shannon
DF					
pH	0.143	0.070	0.150	−0.874**	−0.397
T	−0.140	−0.170	−0.100	−0.819**	−0.420
ORP	0.029	−0.189	−0.096	−0.240	0.471
DO	−0.018	−0.182	−0.086	−0.468	0.350
COD	0.002	0.198	0.098	0.497	−0.227
NO_2^--N	0.232	0.382	0.332	0.493	−0.121
NO_3^--N	0.027	0.220	0.178	0.500	−0.254
NH_4^+-N	0.082	0.054	0.079	0.007	0.357
SY					
pH	0.286	0.245	0.202	−0.493	−0.595*
T	0.728**	0.627*	0.547*	0.421	0.226
ORP	0.143	0.186	0.171	−0.526*	−0.382
DO	0.111	−0.036	−0.089	−0.631*	−0.643**
COD	0.700**	0.587*	0.527*	0.532*	0.357
NO_2^--N	0.443	0.400	0.371	0.918**	0.839**
NO_3^--N	−0.700**	−0.609*	−0.525*	−0.501	−0.310
NH_4^+-N	−0.674**	−0.754**	−0.720**	−0.510	−0.406

Abbreviations: OTUs, operational taxonomic units, which were determined with a 3% width; ACE, abundance-based coverage estimator; Chao 1 index, richness estimate.
*Significant at $p < .05$.
**Significant at $p < .01$.

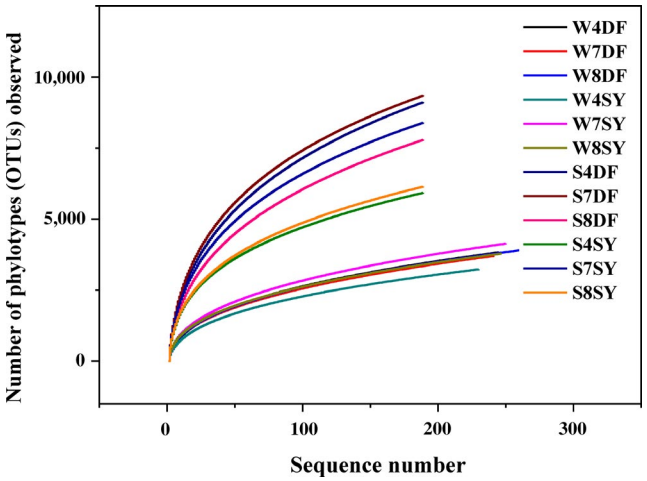

FIGURE A1 Rarefaction analysis of the 12 water and sediment samples. The curves of OTUs clustered at 97% sequence identity across different samples

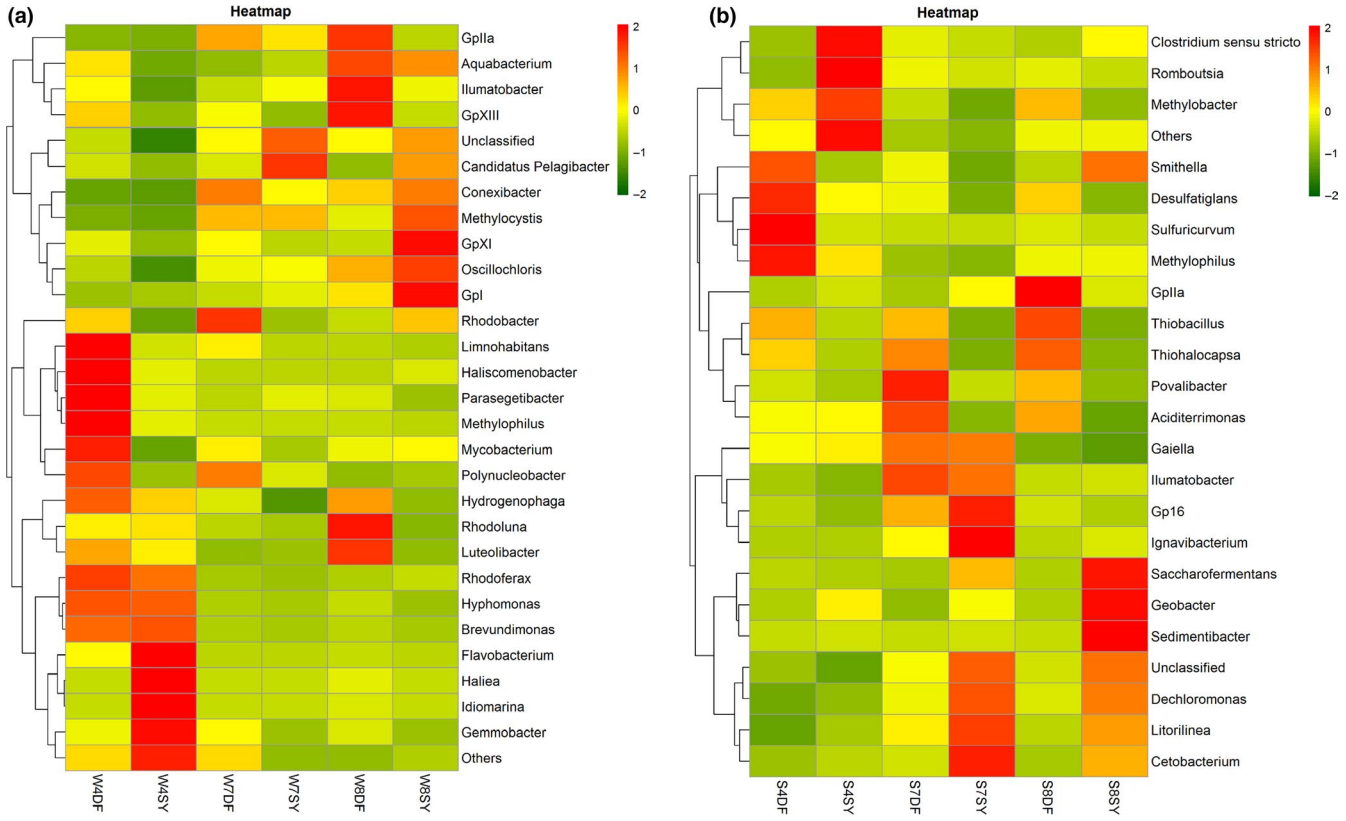

FIGURE A2 Heat map and clustering of bacteria at the genus level: water (a) and sediment (b). The relative frequencies are indicated via color intensity with the legend at the top right corner

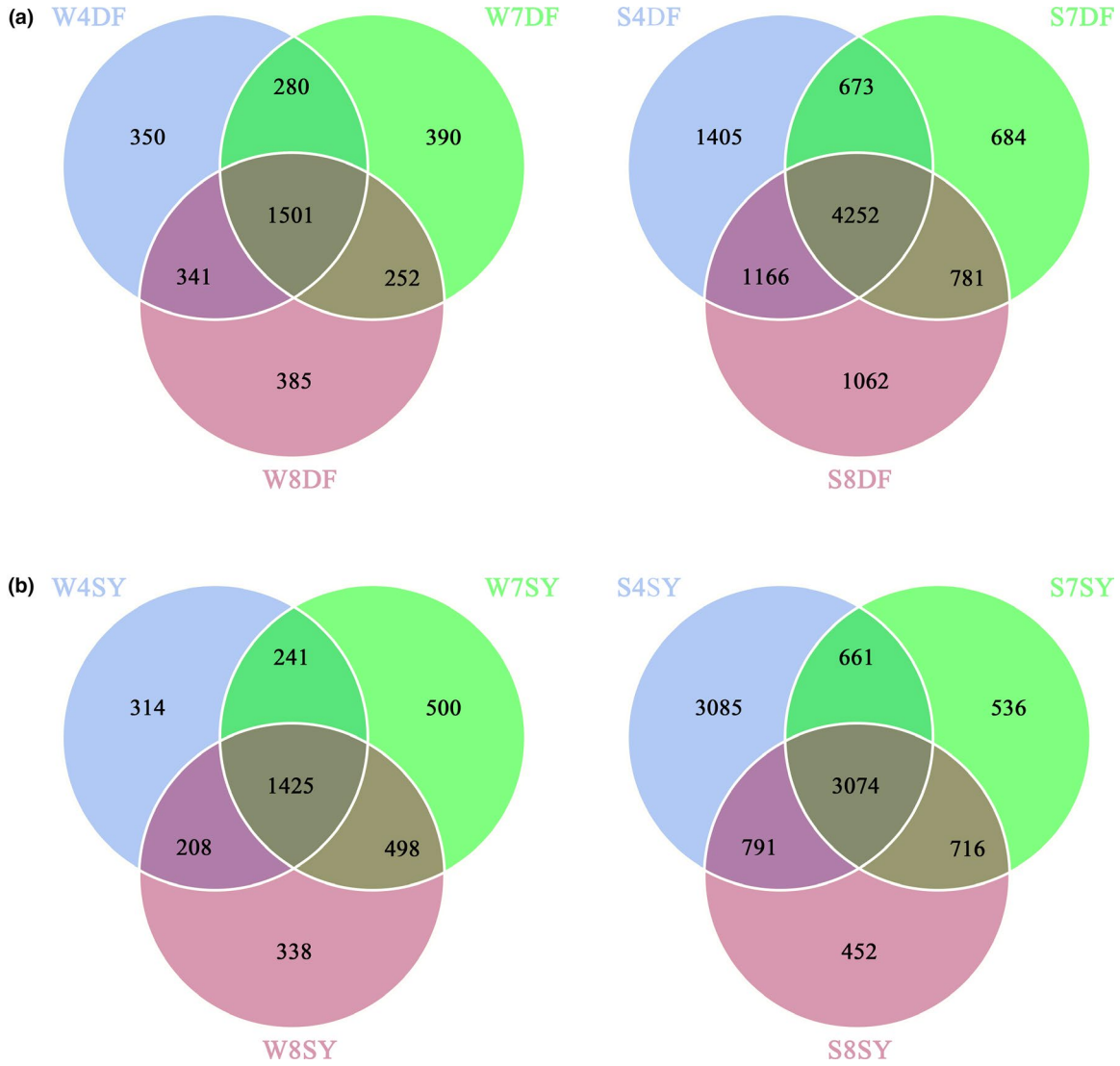

FIGURE A3 Shared OTU analysis of the different libraries. The Venn diagram shows the unique and shared OTUs (at 3% distance level) in different libraries, (a) the pond water and sediment from DF, (b) the pond water and sediment from SY