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# Optimization of iron flocculation for enrichment of CyHV-2 in aquaculture water

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ABSTRACT

Cyprinid herpesvirus 2 (CyHV-2) is an important infectious virus endangering the farmed *Carassius* species industry. The enrichment and identification of CyHV-2 in the water is a vital means of early warning diagnostics, which is helpful to aquatic diseases prevention and control. In this study, we employed and optimized iron flocculation to enrich the concentrations of CyHV-2 in aquaculture water. Different $Fe^{3+}$ concentrations (0.5, 1.0, and 10.0 mg/L), various membrane filters (mixed cellulose ester, glass fiber, nylon microporous filter membrane), different elution buffers containing reductants (ascorbate, citrate, DL-malate, and their combinations with EDTA or TE buffer) were compared to check the recovery yield of CyHV-2. Viral genome quantitation was determined using quantitative real-time PCR. The results showed that $Fe^{3+}$ concentration of 1.0 mg/L, the glass fiber and citrate-TE were determined to have high efficiency and stable recovery yield. To evaluate infectious CyHV-2 viral titres, crucian carps (*Carassius auratus*) were intraperitoneally injected with CyHV-2. The mortality rate was 100 % at 9 days postinoculation (dpi) with the 96.7 % detection of CyHV-2 in the pooled tissues. The histopathological phenotype showed necrotic cell death in the liver, spleen and kidney. The CyHV-2 genome was ranging from $10^{1.1}$ to $10^{7.5}$ copies/μl in the deceased fish, and $10^{1.4}$ to $10^{3.0}$ copies/μl in the feeding water at various stages from latent infection to onset of disease. The field samples from Huzhou, Zhejiang Province on June 4th and 7th had higher detection rates, 87.5 % and 100 % respectively, corresponding to higher CyHV-2 detected in the aquaculture water. CyHV-2 viral load in cultured waters tends to increase prior to fish death, which provides a new indicator for the prediction of fish diseases. In summary, we have established a unified operational standard for enrichment of CyHV-2 in the aqueous environment by iron flocculation.

## 1. Introduction

Cyprinid herpesvirus 2 (CyHV-2), also known as goldfish haematopoietic necrosis virus (GFHNV) or herpesviral haematopoietic necrosis virus (HHNV), is a linear double-stranded DNA virus (Zhu et al., 2019). It is a highly lethal pathogen for ornamental goldfish (*Carassius auratus*), crucian carp (*Carassius cuvieri*), common carp (*Cyprinus carpio*), and their hybrids (Thangaraj et al., 2021). Herpesviral haematopoietic necrosis, caused by CyHV-2 infection, predominantly occurs in the spring and autumn when the water temperature ranges from 15 to 25 ℃, with a subset of cases also reported in summer (Wei et al., 2019). Currently, CyHV-2 is prevalent primarily in *Carassius* species in different countries, and its outbreaks apparently occur when healthy carriers are exposed to different stressors (Thangaraj et al., 2021). Owing to the acute infection, herpesviruses establish lifelong, latent infections in hosts after the primary infection (Chai et al., 2020). Therefore, viral shedding in goldfish infected with CyHV-2 is deemed adequate to facilitate water-borne transmission of CyHV-2 (Zhai et al., 2024). Based on these findings, we hypothesize that CyHV-2 is omnipresent in both fish-rearing waters and subclinically infected fish, suggesting that horizontal transmission of CyHV-2 could potentially occur throughout the year. This hypothesis can be studied by monitoring CyHV-2 dynamics in the environmental

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water of aquaculture fields. Therefore, our priority is to devise a method for concentrating the virus to detect CyHV-2 from environmental water samples.

Recently, the term environmental DNA (eDNA), referring to nucleic acids extracted from environmental samples (Gomes et al., 2017), has been combined with virus concentration techniques for the monitoring of waterborne viruses (Kawato et al., 2016). Viruses are typically present at low concentrations in aquatic environments, necessitating their concentration into smaller volumes prior to downstream molecular biological applications, such as quantitative polymerase chain reaction (qPCR) (Haramoto et al., 2018). Various virus concentration techniques have been previously utilized, including polyethylene glycol precipitation (Jaykus et al., 1996), ultracentrifugation (Mehnert et al., 1997), filtration using negatively charged membranes (Katayama et al., 2002), tangential flow filtration (Wommack et al., 2010), the cation-coated filter method (Haramoto et al., 2009; Honjo et al., 2010), the electronegative membrane filtration (EMF) (Juel et al., 2021), and iron flocculation (John et al., 2011). Notably, the iron flocculation method, which relies on ferric chloride as a coagulant, is widely used for virus enrichment due to its high recovery yields of viral particles (John et al., 2011).

The iron flocculation process comprises three primary steps: the formation of Fe(III)-virus colloids, their collection, and subsequent elution. The utilization of iron flocculation has been conducted on pathogenic viruses present in water under both controlled laboratory settings and field environments, including red sea bream iridovirus (Kawato et al., 2016), betanodavirus (Nishi et al., 2016), tilapia lake virus (Taengphu et al., 2022), and white spot syndrome virus (Kim et al., 2022). It is worth mentioning that, as viral particles can be released under varying water conditions, the recovery yield during the concentration process plays a crucial role in accurately assessing natural virus levels in water. Within the iron flocculation methodology, the virus recovery yield is significantly influenced by the type of Fe(III)-virus flocculate-collecting membrane and the elution buffer employed (John et al., 2011). Consequently, to effectively utilize iron flocculation to concentrate virus from water samples, it is imperative to determine the optimal conditions tailored to each specific virus.

In this study, we developed a CyHV-2 concentration method in water environment by iron flocculation and conducted an investigation into the viral genomic recovery yield of CyHV-2 in water. Specifically, we compared different concentrations of $FeCl_3$, the types of Fe(III)-virus flocculate-collecting membranes, and the composition of the elution buffer. To assess the viral load recovered from aquaculture water, a challenge test was performed using crucian carp and the CyHV-2 expressions in the diseased fish tissues and in the water were determined. Furthermore, the iron flocculation method was utilized to concentrate CyHV-2 particles from the rearing water of farms affected by herpesviral haematopoietic necrosis disease outbreaks. The results are expected to provide an effective tool for disease surveillance.

## 2. Materials and methods

### 2.1. Virus

The CyHV-2 strain YC01 (Genebank: MN593216.1), isolated from diseased crucian carp, was propagated, and purified by passage in GiCF cells at 25 ℃ (Lu et al., 2019). After the development of cytopathic effect (CPE) in 90 % of the test cells, the supernatant was centrifuged at 1000 × g at 4 ℃ for 5 minutes and stored at −80 ℃ as a virus stock for subsequent use.

### 2.2. The general enrichment process of CyHV-2 in water environment by iron flocculation

According to previously reported methodologies (John et al., 2011; Zhu et al., 2019), the general enrichment process of CyHV-2 present in the spiked water via iron flocculation was illustrated in Fig. 1. First, 500 ml aquaculture water was collected and a gauze filter was utilized to remove sediment and large insoluble particles. Then 50 μl of a 10 g/L $Fe^{3+}$ solution was introduced and thoroughly mixed to initiate flocculation. The mixture was filtered twice using a negative pressure vacuum suction filter pump (Changzhou Future Technology, China) with a 0.22 μm and 0.1 μm glass fiber filter membrane. The filter membrane retaining the viral floc was placed in a Petri dish (Jet Biofil, China) and eluted by the addition of 4 ml of elution buffers. The elution was conducted at room temperature for 3 hours in a dark environment or overnight at 4 °C. Subsequently, 200 μl of the eluted virus suspension was subjected to viral genomic DNA extraction using a viral genome extraction kit (Tiangen, China) for later quantitative real-time PCR (qPCR).

### 2.3. Detection of CyHV-2 virus using PCR and quantitative real-time PCR (qPCR)

CyHV-2 virus was detected according to the standard method GB/T 36194–2018. PCR was used to detect the presence or absence of CyHV-2. Briefly, DNA from crucian carp tissues was first extracted using a universal DNA extraction kit (Tiangen, China) and PCR was performed. Primers used for PCR analysis are displayed in Supplemental Table 1. The 25 μl mixture of PCR consisted of 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 12.5 μl Taq DNA polymerase (Takara, China), 1 μl DNA template, and 9.5 μl $ddH_2O$. The PCR conditions were as follows: denaturation at 94 ℃ for 5 min; 35 cycles of denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 1 min, and extension at 72 ℃ for 1 min; finally, extension at 72 ℃ for 10 min. The PCR products were visualized using 1.5 % agarose gel electrophoresis.

The qPCR product amplified from the CyHV-2 polymerase gene sequence (Genbank AY939863) was cloned into the pMD19-T plasmid (Takara, China), and the resulting plasmid was used to establish a standard curve for the measurement of viral DNA copies in the samples. The qPCR reaction was performed in a 15 μl reaction solution containing 1 μl of template, 1 μl each of forward and reverse primers (Supplemental Table 1), 7.5 μl of TB green II premix ExTaq™ kit (RR820A, Takara, China), and 4.5 μl of $ddH_2O$. Cycling conditions were as follows: 95 ℃ for 2 min, followed by 40 cycles: 95 ℃ for 30 s, 58 ℃ for 45 s, 72 ℃ for 45 s, and 72 ℃ for 2 min.Amplification reactions were performed on a CFX96 Real-Time System (Bio-Rad, USA), and the resulting data were collected and analysed using Bio-Rad CFX Manager 2.0 software.

### 2.4. Optimization of iron flocculation among different experimental conditions

#### 2.4.1. Experimental conditions

To determine the optimal conditions for concentrating CyHV-2 in water via iron flocculation, we compared different $Fe^{3+}$ concentrations, various membrane filters, as well as various elution buffers. Specifically, we tested $Fe^{3+}$ concentrations of 0.5, 1.0, and 10.0 mg/L. Furthermore, to identify cost-effective and versatile membrane filters suitable for aquaculture water environments, we compared mixed cellulose ester (MCE, pore size: 0.22 μm, Beijing Mairuida, China), glass fiber (GF, pore sizes: 0.22 and 0.1 μm, Shanghai Xingya, China), and nylon microporous filter membranes (Nylon66, pore size: 0.22 μm, Beijing Mairuida, China). We also evaluated different elution buffers containing reductants, including ascorbate, citrate, DL-malate, and their combinations with slightly modified EDTA buffer (John et al., 2011) or 1×TE buffer, to determine the most effective buffer for resuspending viral particles from the Fe(III)-virus flocculates.

#### 2.4.2. Preparation of iron chloride solution and elution buffer

A total of 2.42 g iron chloride hexahydrate ($FeCl_3$•6 $H_2O$) was dissolved in 50 ml of distilled water to prepare a 10 g/L iron chloride stock solution for use. Iron hydroxide precipitate will form quickly if the

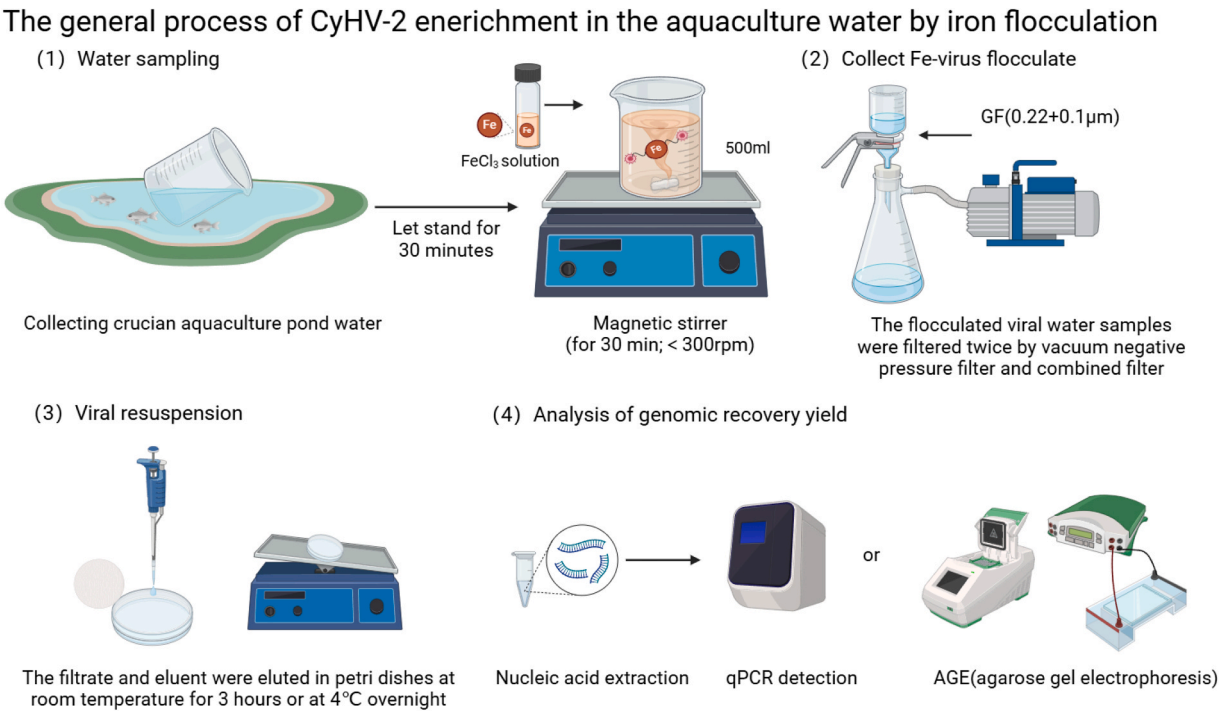


**Fig. 1.** Schematic diagram of this study. Bio Render retains all rights and ownership with regard to this content (Agreement number : DN268FKNJO).

solution is diluted (John et al., 2011). We added 25, 50, or 500 μl of 10 g/L $FeCl_3$ solution to 500 ml of UV-treated river water to prepare 0.5, 1.0, 10.0 mg/L iron chloride solution. Six kinds of suspension buffers were modified and formulated: ascorbate-EDTA buffer, citrate-EDTA buffer, DL-malate-EDTA buffer, ascorbate-TE buffer, citrate-TE buffer, DL-malate-TE buffer (Supplemental Table 2). Each reagent was added only after the previous one had completely dissolved in the solution. These solutions were stored in the dark at 4 ℃ and used within 2 days of preparation.

### 2.4.3. Comparison of CyHV-2 recovery yield by different experimental conditions

To assess the recovery yield of viral genome under varying $Fe^{3+}$ solutions, serially diluted CyHV-2 samples at concentrations of $1.0 \times 10^{6.4}$ and $1.0 \times 10^{5.3}$ copies/L were introduced into UV-treated river water (500 ml) according to the described general process of CyHV-2 enrichment. Following the pretreatment, 25, 50 and 500 μl of 10 g/L $Fe^{3+}$ solution (final concentration of 0.5, 1.0, 10 mg/L iron chloride solution) was introduced and thoroughly mixed to initiate flocculation. There was also a control group only with CyHV-2 input and without $Fe^{3+}$ solution addition. The mixture was filtered twice with 0.22 μm and 0.1 μm glass fiber filter membranes and the floc retained in the membrane was eluted by the addition of 4 ml of ascorbate-EDTA buffer.

To screen the suitable filter membranes using in iron flocculation, GF, MCE and Nylon66 were checked using the same process as above based on the optimal $Fe^{3+}$ solution of 1.0 mg/L, and the specific amount of serially diluted CyHV-2 can be found in Table 1.

To determine the elution capabilities of six distinct buffers, ascorbate-EDTA, citrate-EDTA, DL-malate-EDTA, ascorbate-TE, citrate-TE, DL-malate-TE were compared with three different concentrations of virus ($1.32 \times 10^6$ copies/μl, $1.94 \times 10^6$ copies/μl, $4.05 \times 10^6$ copies/μl) based on 1.0 mg/L iron chloride solution and GF membrane using the same process as above.

All the experiments were conducted in triplicate. The genomic copy numbers of both the input virus and the recovered virus were determined by qPCR and compared among different groups. The percent recovery was calculated as the genomic copy number of the virus recovered after filtration of the water sample × volume divided by the genomic copy number of the virus seeded into the CyHV-2 of the unseeded water sample × volume, and then multiplied by 100 %.

### 2.5. Detection of the CyHV-2 genome in the rearing water of CyHV-2 infected fish

The two-year-old crucian carps (12–15 cm) used in this study were purchased from Zhejiang Huzhou Fish Breeding Cooperative. A total of 100 crucian carps were temporarily maintained in a 200 L glass tank until the challenge experiments were conducted. The fish were acclimated for two weeks and fed with artificial feed (crude protein≥35 %, lipid≥7.5 %; Chongnong Kang, China) in the aquaculture facility of the National Aquatic Animal Pathogen Collection Center of Shanghai Ocean University. All experiments were conducted in accordance with the guidelines of the Care and Use of Laboratory Animals in China. Thirty fish were utilized for the experimental challenge test. Each fish was intraperitoneally (IP) inoculated with 0.1 mL of CyHV-2 solution containing $1.0 \times 10^4$ copies/μl. As a negative control, an additional ten fish were injected with culture medium devoid of virus. All fish were maintained at a temperature of 26±1 °C in 20 L aerated tanks. Fish deaths were daily recorded. Water samples (1 L) were collected at 0, 1, 4, 5, 9, and 10 days post-virus inoculation (dpi). Prior to DNA extraction, the rearing water was prefiltered using sterile gauze to eliminate suspended solids. Subsequently, water samples were concentrated for subsequent extraction of total DNA from culture water using iron flocculation method (GF-ascorbate-EDTA) to quantify CyHV-2 copy numbers by qPCR. Pooled samples of liver, spleen, kidney, gill, brain, and muscle tissues were individually collected from all deceased fish to extract DNA for later use to confirm CyHV-2 infection as the cause of mortality in the crucian carp. To evaluate structural and cellular changes of CyHV-2 infected fish, the histopathological examination was conducted in liver, spleen, and kidney. Tissue samples were fixed in 4 % paraformaldehyde solution, dehydrated using a series of alcohol solutions with increasing concentrations, and then embedded in paraffin wax. The embedded samples were sectioned into 4–6 mm thick slices using a microtome, stained with hematoxylin and eosin, dehydrated

again to remove excess water, and sealed for analysis under an optical microscope (Wen et al., 2021).

### 2.6. Application of the iron flocculation method for CyHV-2 concentration in the field samples

In order to verify the practicability of this iron flocculation method, we concentrated CyHV-2 particles in rearing water obtained from crucian carp farms with this method. Specifically, rearing water samples were collected from aquaculture facilities located in Huzhou, Zhejiang Province (June 4th, 7th, 16th; October 13th, 14th), Fuyang, Anhui Province (June 12th), and Hefei, Anhui Province (June 27th) in 2023. These samples were then processed using the iron flocculation technique, which was based on the GF-ascorbate-EDTA protocol as previously described, with the $Fe^{3+}$ concentration of 1.0 mg/L. Subsequently, DNA extraction and quantitative analysis of CyHV-2 in the concentrated rearing water samples were performed according to the established protocols. The experiment was performed twice in triplicate.

### 2.7. Statistical analysis

All the data were collated using Microsoft Excel. Results were expressed as the average ± SEM. Firstly, the tests of normal distribution and homogeneity of variance was carried out, which confirmed that data conformed to normal distribution and homogeneity of variance. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by LSD post hoc test using SPSS software (version 25.0, USA). The level of statistical significance for variables was set at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Curves and graphs were made using GraphPad Prism (version 9.0, USA) or Microsoft Excel software (version 2020, USA).

## 3. Results

### 3.1. Optimal conditions for CyHV-2 concentration by iron flocculation

For the different initial concentration of CyHV-2 at $1.0 \times 10^{6.4}$ and $1.0 \times 10^{5.3}$ copies/μl, the recovery rates through flocculation were assessed and compared between different $Fe^{3+}$ concentrations. The recovery rates at $Fe^{3+}$ concentration of 0.5 mg/L were 69.65 ± 4.44 % and 58.51 ± 4.5 %, respectively (Fig. 2). Similarly, at $Fe^{3+}$ concentration of 1.0 mg/L, the recovery rates were 78.34 ± 17.96 % and 96.05 ± 18.54 % (Fig. 2). However, the presence of the 10.00 mg/L $Fe^{3+}$ concentration group resulted in CyHV-2 genome percentages recovered of 9.89 ± 0.02 % and 17.70 ± 1.67 % (Fig. 2). Flocculation efficiency appears to increase with increasing iron concentration, while the lowest recovery properties appear at $Fe^{3+}$ concentration of 10.0 mg/L. Finally, the optimal $Fe^{3+}$ concentration for the recovery of CyHV-2 is determined to be 1.0 mg/L.

### 3.2. Different membrane filter detection efficiency of the iron flocculation method

Based on the optimazied $Fe^{3+}$ concentration of 1.0 mg/L, the genomic recovery yields were determined in CyHV-2-spiked river water samples containing varying genome copies for different filter membranes. The relationship between the spiked and recovered CyHV-2 DNA copies was assessed by linear regression using Nylon66, GF, and MCE respectively (Fig. 3). $R^2$ value represents the fitting degree of the curve, and the value closer to 1, means the better fitting degree. We got the fitting degree of GF > Nylon66 > MCE, which exhibits GF had the excellent stability. The mean viral genomic recovery yields were 61.79 ± 6.78 % in Nylon66, 89.40 ± 11.50 % in GF, and 65.00 ± 23.50 % in MCE (Table 1). Notably, the mean CyHV-2 genomic recovery yield was significantly higher with the GF membrane compared to the Nylon and MCE membranes ($P < 0.05$). Regardless of the number of CyHV-2 copies utilized, the GF membrane filters gave the optimal enrichment effect and recovery yield (Table 1).

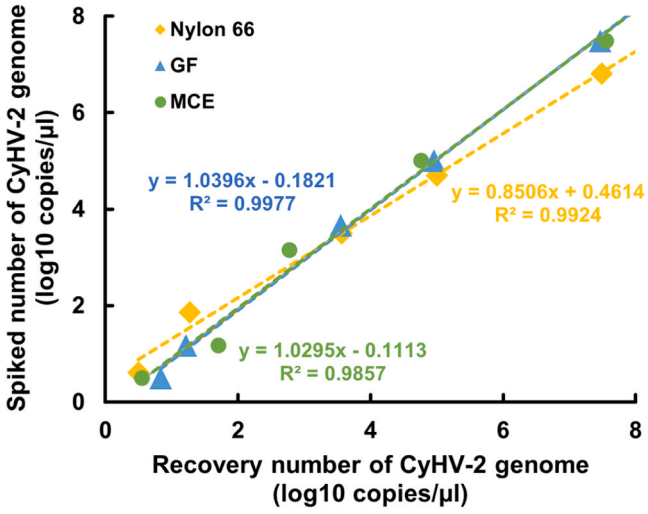


**Fig. 3.** The linear relationship between spike and genomic recovery numbers of CyHV-2 DNA copies in river water based on different types of membrane filters (Nylon66, GF, or MCE) for the collection of Fe(III)-virus flocculate. The closer R2 is to 1, the better the recovery effect.

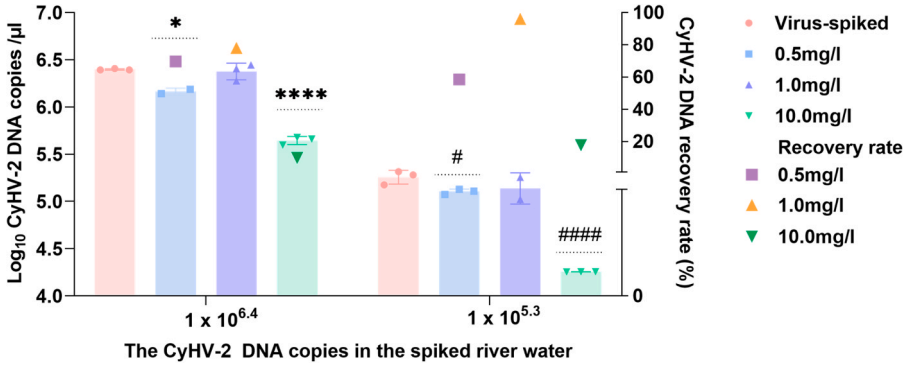


**Fig. 2.** Effect of the final concentration of $FeCl_3$ in water on the recovery rate of Fe(III)-virus concentrate. The $Fe^{3+}$ iron concentration of CyHV-2-containing river water was adjusted to 0.5, 1.0, 10.0 mg/L, respectively. Virus-spiked group indicated none $Fe^{3+}$ addition. Then, the total DNA was extracted from 500 ml of river water spiked with CyHV-2 using the iron flocculation method. The copy number of the CyHV-2 genome in the extracted DNA was measured by real-time PCR. The error bars indicate the standard error of the means (n=3). * and # indicate $P < 0.05$, **** and #### indicate $P < 0.0001$.

### 3.3. Assessment of viral recovery at different elution buffers

The elution capabilities of six distinct buffers were compared by iron flocculation method with three different concentrations of virus ($1.32 \times 10^6$ copies/μl, $1.94 \times 10^6$ copies/μl, $4.05 \times 10^6$ copies/μl), each exhibiting satisfactory elution efficacy (Fig. 4). By the amount of recovered CyHV-2 DNA copies, citrate-TE demonstrated the most effective solubility, and citrate-EDTA buffer was found to be less susceptible to oxidative yellowing and more stable in the liquid phase during the process. The elution efficiency of DL-malate-EDTA was comparable to that of Ascorbate-EDTA, albeit slightly inferior to ascorbate-TE and DL-malate-TE (Fig. 4). Buffers containing ascorbate, which is prone to oxidation within a brief period, must be freshly prepared and utilized within 48 hours. These buffers should be stored in a dark environment and maintained at 4℃. Conversely, buffers containing DL-malate and citrate components possess longer shelf lives and can be stored at room temperature.

### 3.4. Assessment of CyHV-2 infectivity for the rearing water of CyHV-2-infected fish

To evaluate infectious CyHV-2 viral titres, the recovered DNA copies in the rearing water, the DNA copies and cytopathic effects in different infected tissues were determined. The CyHV-2 genome was initially detected in the rearing water at 0 dpi, with a copy number of $10^{2.4}$ copies/μl. Subsequently, the viral copy number peaked at 1 dpi, reaching $10^{3.0}$ copies/μl. However, a rapid decline was observed at 4 dpi, with a copy number of $10^{1.4}$ copies/μl. Notably, the copy number exhibited a subsequent surge at 5 dpi, registering $10^{2.6}$ copies/μl. The viral load in the water decreased at 9 dpi and remained largely unchanged at 10 dpi (Fig. 5A). Fish began to exhibit mortality following IP injection with CyHV-2 at 5 dpi. The mortality profile within the injection-infected cohort progressively accumulated over time, resulted in a 100 % mortality rate observed at 9 dpi (Fig. 5B). Promptly following the demise of the fish, liver, spleen, kidney, gill, brain, and muscle tissues were harvested and pooled for qPCR and PCR analysis. The qPCR quantitation of the CyHV-2 genome in the deceased fish revealed concentrations ranging from $10^{1.1}$ to $10^{7.5}$ copies/μl of DNA (Fig. 5B). At 9 dpi, mass mortalities of carp were recorded, coinciding with a decrease in the viral load in the water. By 10 dpi, all crucian carp succumbed to the infection, and the viral load in the water remained relatively unchanged (Fig. 5A, B).

PCR assays indicated that CyHV-2 was detected positive in 29 samples based on the 30 dead fish, representing a positive rate of 96.7 % (Fig. 5C). Comparative histopathological analyses of liver, spleen and kidney were conducted between the deceased fish and the uninfected control fish (Fig. 5D). Hepatopancreas from infected fish exhibited hepatocyte swelling, nuclear vacuoles, vacuolation, necrosis, congestion. Similarly, splenocytes showed karyopyknosis, vacuolation, necrosis, and extensive inflammatory cell infiltration. Kidney tissue from infected crucian carp displayed glomerular swelling, nuclear vacuoles, and extensive vacuolation. In contrast, the tissue sections from the uninfected group showed normal tissue architecture. These results firmly support the conclusion that all deceased fish succumbed to CyHV-2 infection, exhibiting characteristic symptoms.

### 3.5. Application of the iron flocculation method for the rearing water of CyHV-2-infected fish and CyHV-2 concentration in field samples

The presence of CyHV-2 particles in the aquaculture water of crucian carp was confirmed using the GF-Ascorbate iron flocculation method. The qPCR analysis revealed that water samples collected from Huzhou, Zhejiang Province on June 4th and 7th exhibited a higher viral load, with concentrations approaching $10^4$ copies/μl and peaking at $10^{4.1}$ copies/μl (Fig. 6). The viral copy numbers of other water sample were significantly lower, with the lowest concentration of approximately $10^{0.6}$ copies/μl (Fig. 6). Notably, during this period, a massive morbidity and mortality of crucian carp were observed. Some dead carps were randomly chosen to test CyHV-2 infection per sampling occasion (Table 2). Fish samples from Huzhou, Zhejiang Province on June 4th and 7th had higher detection rates, 87.5 % and 100 % respectively (Table 2). Sample from Huzhou, Zhejiang Province on Oct 14th had only 50 % detection rate, while other samples had no positive detection rates.

## 4. Discussion

The concentration and recovery technology of viral particles in environmental water samples has become increasingly valuable in human health research, especially in the field of water-borne diseases caused by enteroviruses (Cashdollar and Wymer, 2013; Haramoto et al., 2018). Enteroviruses are present in relatively small numbers in water, yet they can persist in aquatic environments for extended periods (Haramoto et al., 2018). Owing to their exceptionally low infectious dose and robust resistance to commonly used disinfectants, certain viruses pose an elevated risk of human exposure to infection, thus threatening public health. Currently, the concentration and recovery technology of viral particles in environmental water samples has become the core issue in the field of aquatic environment research, facilitating enhanced comprehension of virus distribution and transmission mechanisms within water bodies, thereby enabling the formulation of effective prevention and control strategies. Further exploration and optimization of virus detection technology to enhance its application efficiency in environmental water samples represents a crucial research direction within the field of aquatic environment research (Jacquet et al., 2010).

Although various detection methods based on different principles have been reported (Haramoto et al., 2018), these methods generally lack sufficient sensitivity. When directly applied to water sample

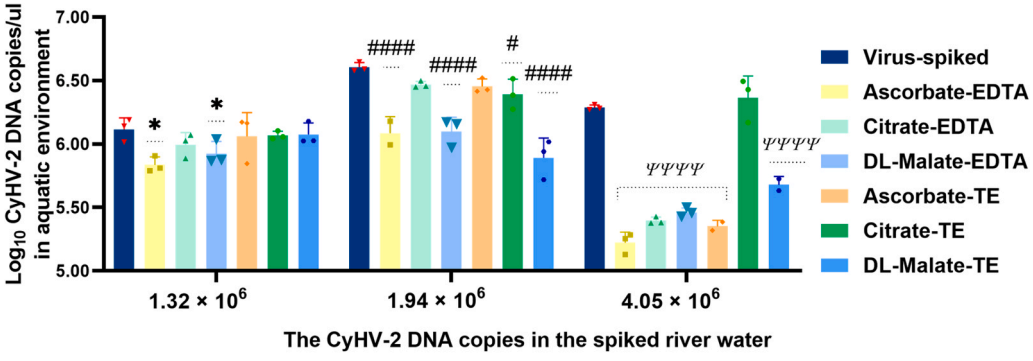


**Fig. 4.** The recovery effect of different reductant buffers (Ascorbate-EDTA, Citrate-EDTA, DL-malate-EDTA, Ascorbate-TE, Citrate-TE, DL-malate-TE) for the elution during iron flocculation. Spiked viral genome copy was $1.32\times10^6$ copies/μl, $1.94\times10^6$ copies/μl, $4.05\times10^6$ copies/μl respectively. *, $^{\#}$ and $^{\Psi}$ indicate $P < 0.05$.

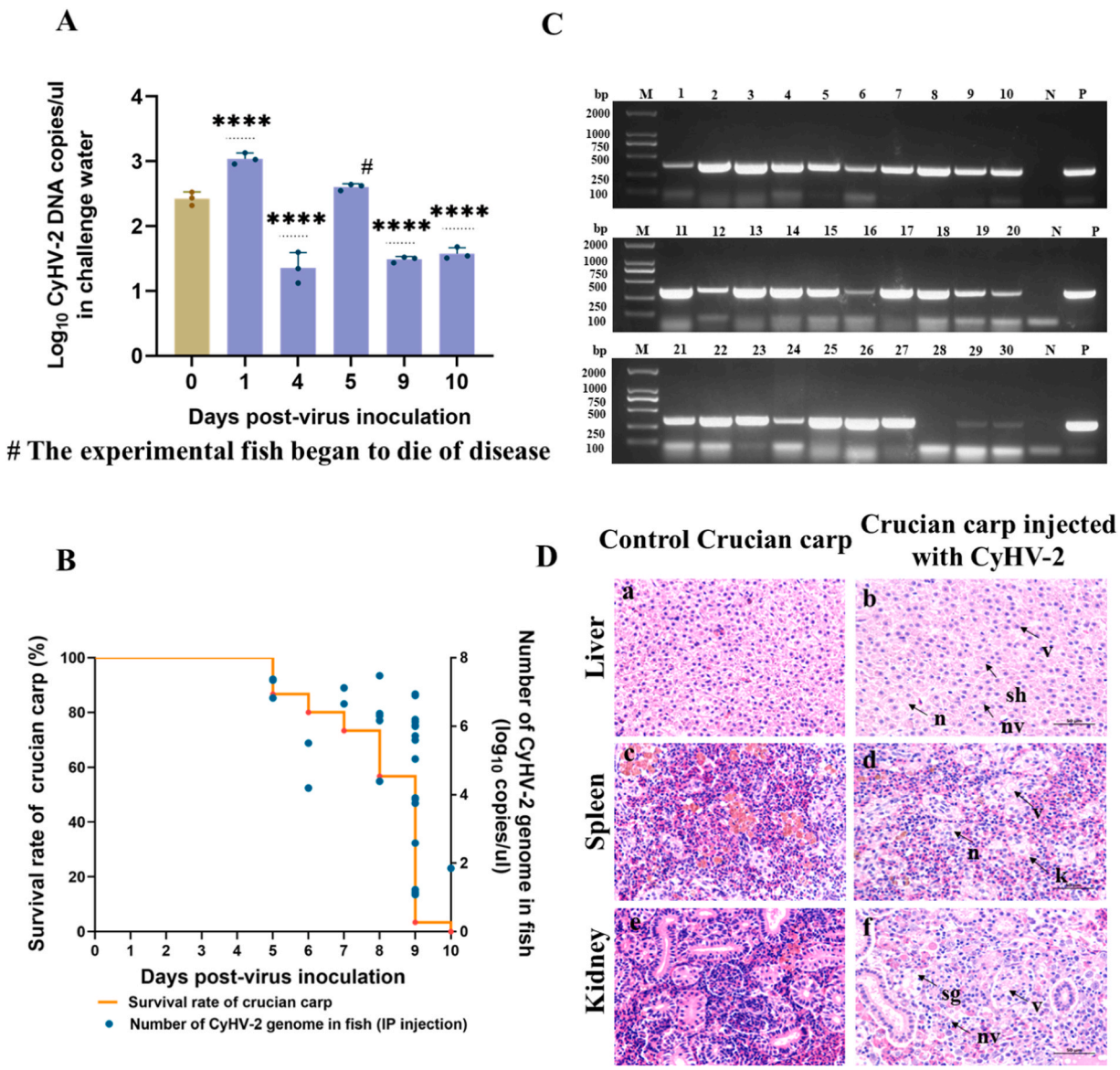


**Fig. 5.** **A.** Detection of the CyHV-2 genome in the rearing water of CyHV-2-infected crucian carps. The total DNA of 500 ml of rearing water was extracted by the iron flocculation method and the copy number of the CyHV-2 genome was measured by real-time PCR at 0, 1, 4, 5, 9, 10 days post-virus inoculation. *$P < 0.05$. **B.** Cumulative mortalities of crucian carp infected with CyHV-2 and detection of CyHV-2 genome of infected crucian carp. The total DNA was extracted from liver, spleen, kidney, gill, brain and muscle of dead crucian carp, and the genome copy number after CyHV-2 infection was detected by quantitative real-time PCR. **C.** Agarose gel electrophoresis images of PCR products using CyHV-2Hel F/R primers shown Cyprinid herpesvirus 2 infection in the dead fish tissues. Lane M: DL 2000 bp marker; Lane 1–30: every single fish sample; Lane N: negative control; Lane P: Positive control. **D.** Histopathological changes of the liver, spleen and kidney from the infected and healthy crucian carps. Scale bar = 50 μm. k: Karyopyknosis v: vacuolation; sg: swelling of glomerular; nv: nuclear vacuole; sh: swelling of hepatocytes; se: swelling of hepatocytes; n: necrosis.

detection, they can easily lead to false negative results, greatly impacting the accuracy and reliability of the analysis. In this context, optimizing pre-processing steps is particularly critical. An ideal pretreatment method should effectively concentrate the virus particles while eliminating potential interfering substances, thereby improving the detection accuracy. However, the traditional pretreatment methods are often unsatisfactory in terms of virus concentration effect, recovery rate and stability. The recovery rate varies significantly between different methods, and even the results of the same method are less comparable between different laboratories (Ruan et al., 2020). In order to improve the recovery rate and stability of viruses in water samples, it is necessary to select suitable pretreatment methods for different water samples and establish a unified operating standard. By continuously optimizing the pre-treatment process, more precise and reliable results in virus detection can be expected, which will contribute to aquatic environmental research as well as prevention and control of waterborne diseases.

The methods for concentrating virions in water have their own advantages and disadvantages; filter membrane filtration-elution method can handle large volumes of water samples and is simple to operate, but the filtration membrane is expensive; ultrafiltration and centrifugation require specialized ultrafiltration or centrifugal equipment, which can achieve a higher concentration effect, but are limited by the volume of treated water sample, and the costs associated with equipment maintenance cannot be ignored. The effect of flocculant precipitation or concentration is unsteady, requires a long time, and involves complicated operations. In view of the advantages and disadvantages of the above methods, our team conducted an in-depth exploration during preliminary studies and successfully achieved stable enrichment and detection of CyHV-2 in ultra-pure water (Zhai et al., 2024). However,

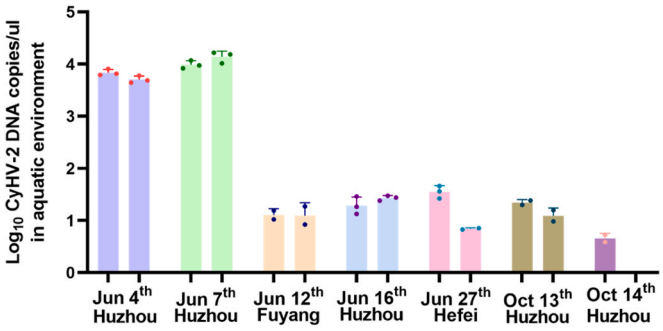


**Fig. 6.** Detection of the CyHV-2 genome in rearing water samples of aqua farms via iron GF-Ascorbate-EDTA flocculation method. The detections were performed with two parallel replicates and three technical replicates. $^{*}P < 0.05$.

confronted with the complexity of various water samples, the lack of unified operating standards and mature technical routes remains an urgent problem to be solved. Based on our original research, we further refined each treatment step, and established suitable pretreatment methods according to the characteristics of different water samples. These methods aim to improve the concentration efficiency of the virus particles while reducing interference factors during operation processes and establish a unified operating standard to ensure the accuracy and repeatability of the experimental results. These improvement measures have resulted in the establishment of a mature technical route, which strongly supports the detection and enrichment of CyHV-2 in aquatic environments. The establishment of this technical route not only helps to improve the efficiency and accuracy of disease prevention and control in aquaculture, but also provides a useful reference for the development of related research fields.

In this research, the mechanism of iron concentration in water in the process of flocculation and filtration of virions was systematically discussed. The results showed that the suitability of iron ion concentration had a significant effect on virus enrichment. When the concentration of iron in the water is low, the flocculation ability of iron ion is limited, and most of the virions in the water cannot be adsorbed and enriched effectively, thus reducing the enrichment efficiency. Conversely, when the concentration of iron in the solution is too high, it will not only lead to the blockage of the filter membrane, increase the difficulty of filtration, prolong the time cost of enrichment operation, but also have a negative impact on the elution effect of the eluent, and then interfere with the subsequent viral DNA extraction process. Additionally, high iron concentrations caused a dark brown appearance in the solution which hindered subsequent qPCR detection and reduced detection result accuracy. This conclusion is consistent with the results obtained by development of red sea bream iridovirus concentration method in seawater by iron flocculation (Kawato et al., 2016) in their study on the enrichment of RSIV virus in seawater by iron flocculation, further confirming the importance of optimal control of iron concentration in water in the process of virion enrichment.

Under current technical conditions, membrane filtration enrichment method is an effective means of virus concentration in water. The Nylon66 organic microporous filter membrane applied in our previous study encountered challenges in dealing with the complex actual aquaculture water body. When the content of suspended matter, inorganic salt or soluble organic matter in the water sample was high, the filtration flow rate of the filter membrane would be severely limited, resulting in low filtration efficiency. Consequently, the filter membrane is solely suitable for relatively clear tap water or circulating water equipped with a filtration system; otherwise, it necessitates intricate pretreatment procedures to clarify turbid water samples. Based on the determination of the concentration of iron in the solution, a comparative study was made on the filter membrane with different materials. In this experiment, glass fiber filter membrane showed a better enrichment effect compared to Nylon66 organic microporous filter membrane and mixed cellulose filter membrane. In addition, the glass fiber filter membrane also has many advantages such as high filtration efficiency, no need to replace frequently, and low price. The MCE filter membrane achieved a high recovery rate under the same condition, but the recovery effect varied significantly and was unstable with addition of different copy numbers of viruses. It is worth noting that if the water body contains a large amount of sediment, it needs to be settled or prefiltered with sterile gauze. However, GF filter membrane also has some limitations because of its strong water absorption, the amount of eluent needs to be increased and GF membrane is easily damaged when exposed to moisture. Although its suspension effect is not affected in the filtration process, it still needs to be paid attention to when used.

After collecting the Fe(III)-virus floc, it is necessary to dissolve the precipitate in order to release the virus for subsequent detection. The composition and pH of the eluent have significant influences on the efficiency of virus recovery (John et al., 2011). We first used ascorbic acid buffer to re-suspend virus particles from iron virus floc, and successfully formulated an eluent suitable for Fe(III)-CyHV-2 virus floc precipitate. However, the eluent has obvious shortcomings: it needs to be freshly prepared before used, the formulation is complicated and time-consuming, and the validity period is short. It needs to be stored at 4 ℃ away from light, which brings inconvenience to storage. To address these issues, we have refined the composition of the eluent. The results showed that the filtration effect was better than that of ascorbate-EDTA eluent. It is noteworthy that the two newly developed eluents can be stored under room temperature conditions, and their shelf life exceeds three weeks. However, the specific duration of their validity requires further investigation. This improvement not only simplifies the preparation process of eluent, improves the convenience of use, but also extends the validity period, providing a more stable and reliable eluent selection for virus detection.

To assess the sensitivity of our method across diverse water environments, we incorporated as few as $10^1$ copies/μl of CyHV-2 into virus-free river water. Despite the extremely low viral levels, we were able to detect viral enrichment through iron flocculation, although there is also a risk of detection at such low concentrations. In the course of the fish infection experiment and the analysis of samples collected from farmers' ponds, a discernible pattern emerged between viral load and host disease incidence. Specifically, crucian carp exhibit sensitivity to CyHV-2 infection when the virus copy number in aquaculture water ranges from $10^3$ copies/μl to $10^4$ copies/μl. This finding aligns with our team's prior research on goldfish, which indicated a similar sensitivity to CyHV-2 infection at virus concentrations as low as $10^4$ copies/μl (Zhai et al., 2024).

The selection of ferric chloride as a flocculant was primarily based on its widespread application in marine and freshwater virology, encompassing the removal of viruses such as fish nordavirus in fish cultured seawater (Nishi et al., 2016), viral hemorrhagic septicemia virus genotype IVa in seawater (Ryu et al., 2023), white spot syndrome virus in seawater (Kim et al., 2022), red snapper iridovirus in seawater (Kawato et al., 2016), tilapia virus (Taengphu et al., 2022). Using virus tagging and recovery experiments, we verified the efficiency and reliability of iron flocculation technology for CyHV-2 virus in concentrated aquaculture water bodies. Furthermore, in this paper, we established a unified operating standard and optimized a proven technical route. This technology is not only suitable for efficient CyHV-2 recovery under laboratory conditions but can also be widely applied to the virus detection in actual aquaculture and open water bodies. Iron flocculation can be suitable for extremely low levels of virus concentration close to the minimum detection limit of qPCR analysis. However, there exists a risk of false positives if the virus concentration in the water body is excessively low. In addition, according to the challenge test with whiteleg shrimp, resuspended viruses induced infection in shrimp and resulted in approximately 100 % cumulative mortality (Kim et al., 2022), which suggesting that iron flocculation minimally or negligibly

impacts viruses' activity, which has hygienic significance.

At present, the dynamic changes of CyHV-2 and the natural infection mechanism remain poorly understood, especially the literature on its horizontal transmission is very limited. Our findings indicated a close correlation between the dynamic changes of viruses in the water and their associated morbidity and mortality rates. Application of the iron flocculation method for the rearing water of CyHV-2-infected fish and CyHV-2 concentration in field samples. In our attack experiment, we observed that the crucian carp infected with CyHV-2 virus had a certain incubation period of 0 dpi, and there was a certain concentration of virus in the water body at 1 dpi, but the crucian carps did not get sick immediately. Interestingly, while the viral load in the water decreased at 4 dpi, but the viral load in the aquaculture water suddenly increased at 5 dpi followed by the successive deaths of the infected crucian carp until the conclusion of the experimental period. Following the removal of the deceased fish from the water, the viral load decreased, and there was no significant change in the viral concentration 24 hours later. These findings suggested that the virus shedding level in aquaculture water can serve as an indicator of the infection stage. And the technology has the ability to monitor the dynamics of viruses in the aquatic environment of aquaculture in real time. Specifically, we observed an upward trend in the viral load in the water environment when the fish were exposed to external stimuli. However, over time, due to natural degradation and other processes, the viral load in the water gradually decreases. It is worth noting, however, that during the period of viral pathogenesis, the viral load in the water rises significantly again due to enhanced viral activity and accelerated replication. Furthermore, based on our observations, the virus is capable of horizontal transmission, can detach from the host, and survive in the aquatic environment for a duration. However, the virus primarily resides within the host, and the viral load diminishes as the host population in the water decreases. The iron flocculation method enables the monitoring of dynamic changes during the virus-prone period and throughout the entire breeding process, facilitating early detection and effective prevention of the virus prior to an outbreak. The detection of CyHV-2 in the culture water environment based on iron flocculation can be used as one of the reasonable diagnostic methods for necrosis of hematopoietic organs in crucian carp. Moreover, non-destructive sampling can be performed across various culture objects, enabling detection before, during and after the virus outbreak. Early warning of virus outbreaks can help reduce aquaculture losses, maintain the health of the water environment, and avoid water waste.

## 5. Conclusion

Integrating the above methods with rapid pathogen detection technology to investigate the viral load in aquaculture water environment and the patterns of host disease will aid in elucidating the correlation between CyHV-2 infection and environmental factors, thus establishing virus outbreak early warning systems. This technique was employed to investigate the correlation between aquatic chemical indices and virus replication, thereby offering a data reference for the prevention and control of aquatic diseases. In aquatic animal disease surveillance, eDNA-based viral analysis in water is important for both source tracing and transmission modeling. Therefore, the determination of CyHV-2 concentration in the aquatic environment through iron flocculation is anticipated to serve as an effective tool for disease surveillance. However, the detection and concentration of viruses in water is still a current challenge, and continuous research and exploration are needed to ensure the safety of the aquatic environment, humans, and animals. In summary, we have established unified and efficient operating standards and technical routes for various water samples, thus providing strong technical support for virus detection, prevention, and control in the aquaculture industry. In future studies, we will continue to optimize the method and broaden its application scope to better facilitate the sustainable development of the aquaculture industry.

## Ethical approval

The study was conducted in accordance with the guidelines on the care and use of animals for scientific purposes set up by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Ocean University, Shanghai, China.

## CRediT authorship contribution statement

**Patarida Roengjit:** Validation. **Liqun Lu:** Funding acquisition. **Hao Wang:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Binghui Pang:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yufeng Si:** Writing – review & editing, Data curation. **Ying Zhan:** Methodology, Investigation. **Zhaoyang Ding:** Validation.

## Declaration of Competing Interest

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

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102351.

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