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RESEARCH ARTICLE

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Stability of viral haemorrhagic septicaemia virus, infectious hematopoietic necrosis virus and cyprinid herpesvirus 3 in various water samples

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Abstract

Rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) are the two most common species in traditional fish farming in Germany. Their aquaculture is threatened upon others by viruses that can cause a high mortality. Therefore, this work focuses on three viruses—viral haemorrhagic septicaemia virus, infectious hematopoietic necrosis virus and cyprinid herpesvirus 3 (CyHV-3)—that endanger these species. To prevent their spread and contain further outbreaks, it is essential to know how long they can outlast in environmental waters and what affects their infectivity outside the host. Hence, the stability of the target viruses in various water matrices was examined and compared in this work. In general, all three viruses were quite stable within sterile water samples (showing mostly ≤1 log reduction after 96 hr) but were inactivated faster and to a higher extent (up to five log steps within 96 hr) in unsterile environmental water samples. The inactivation of the viruses correlated well with the increasing bacterial load of the samples, suggesting that bacteria had the greatest effect on their stability in the examined samples. In comparison, CyHV-3 seemed to be the most sensitive and maintained its infectivity for the shortest period.

KEYWORDS

aquaculture, common carp, environmental water, fish viruses, infectivity, rainbow trout

1 | INTRODUCTION

In the recent decades, fish has become increasingly important for human nutrition. It is estimated that 30% of the food fish produced worldwide is attributable to aquaculture, with a rising trend to reach 50% in 2030 (Alonso & Leong, 2013). In Germany, aquaculture is the most productive sector of fish industry with a production volume of ca. 20,600 t of fish in 2017 with rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) being the most popular species and contributing together to about 65% of annual production (Brämick, 2017). Traditionally, many fish farms in Germany are rather small and often secondary and family-run activity. Nevertheless, an outbreak of a disease, resulting in high mortalities of the affected fish population, leads to losses that can challenge their economic survival independent from the size of the fish farm. The most important viruses that can infect common carp and rainbow trout in

Germany are cyprinid herpesvirus 3 (CyHV-3), viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV). The diseases caused by these waterborne fish pathogens are spreading epidemically, can cause high economic losses and if detected have to be reported to the responsible authorities (Gotesman et al., 2013; Hawley & Garver, 2008; Kibenge & Godoy, 2016).

As there is no cure for the diseases caused by these three viruses, their spread must be contained by other strategies such as prevention and control. This includes monitoring, reporting and disinfection of an infected pond (MULE, 2009). In addition to that, it is important to know how long these viruses can retain their infectivity outside the host in the water environment, to be able to evaluate the associated risk of transmission and to keep their spread as low as possible.

Obviously, the survival of viruses in environmental water is affected by a number of different factors such as temperature, pH and/or salt content which can influence the viral structures and can lead to their inactivation. However, viruses also possess protective mechanisms, based on physical phenomena such as aggregation and adhesion that help to maintain their infectivity in water (Pinon & Vialette, 2018). As both mechanisms can result in a reversibly reduced detection of infectious virus, a temporary or apparent decrease in the viral particle numbers does not always mean that these pathogens are completely inactivated. Therefore, also such effects have to be carefully considered when studying virus survival in water matrices.

Several studies concerning the above-described viral species and their stability in water have already been conducted. Shimizu et al. (2006) investigated the infectivity of CyHV-3 in untreated river water compared with autoclaved or sterile-filtered river water. Also, Hawley and Garver (2008) investigated the stability of VHSV at different temperatures in filtered and unfiltered municipal tap water. Kamei et al. (1988) reported on IHNV in sterile-filtered and untreated freshwater samples.

Although all mentioned studies are valuable contributions to the knowledge about these fish viruses, a direct comparison between the pathogens in respective water samples is difficult due to the different approaches and set-ups used in the various investigations, for example different temperatures, water samples and pretreatments. For these reasons, the stability of VHSV, IHNV and CyHV-3 in different water samples under similar conditions was investigated in this study and directly compared with each other. In addition to a sterile cell culture medium, used as the reference matrix, a sterile-filtered water sample with defined composition, an unfiltered tap water and environmental samples collected from a carp pond and a trout hatchery (filtered and unfiltered) were used. The infectivity of the target viruses in these water samples was examined for up to 168 hr by virus titration (using an endpoint dilution method) and by the determination of viral DNA/RNA (via quantitative real-time polymerase chain reaction, qPCR or reverse transcriptase-(RT)-qPCR). In order to facilitate the interpretation of the obtained data, water parameters such as pH, hardness, conductivity and bacterial load (for unfiltered water samples) were measured for all applied water samples. Finally, the influence of serum supplementation to different water samples on the stability and/or infectivity of CyHV-3 was also investigated here.

2 | MATERIAL AND METHODS

2.1 | Chemicals and solutions used

For cell maintenance, Dulbecco's Modified Eagle Medium (DMEM, with 4.5 g/L glucose, Sigma-Aldrich) was used as cell culture medium. The antibiotic and antimycotic solution with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml (A5955, 100×, 100 ml) used for the virus titration, the Accutase® solution and the foetal calf serum (FCS) were also purchased from Sigma-Aldrich. For CyHV-3 propagation in cell disc (polystyrene, PS, 4 layers, Greiner Bio-One), *Minimal Essential Medium Eagle* (MEM, with Earle's salts, Sigma-Aldrich) was used.

HEPES buffer salt (Pufferan®, ≥99.5%), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2 \cdot 2H_2O$), magnesium sulphate ($MgSO_4 \cdot 7H_2O$) and sodium hydrogen carbonate ($NaCO_3$) were obtained from Carl Roth GmbH. Yeast extract, glucose monohydrate and agar used for the bacterial count nutrient medium (standard medium I) were all acquired from Carl Roth GmbH. The peptone from casein used for the nutrient medium was obtained from Merck.

The nutrient medium (standard medium I, pH 7.5) was prepared by mixing yeast extract (3 g/L), glucose (5.55 mM), NaCl (66 mM) agar (20 g/L) and peptone from casein (1.5 g/L) and dissolving these compounds in deionized water. It was then autoclaved at 121°C for 15 min and stored at 18°C in darkness until use.

For the preparation of standardized water with a simple and defined matrix (further referred to as iso-test water), four stock solutions (1 L) of different salts 80 mM $CaCl_2$, 20 mM $MgSO_4$, 30 mM $NaHCO_3$ and 3 mM KCl were prepared with deionized water (H_2O_d) and stored at 18°C under exclusion of light until use. When iso-test water was required, it was freshly mixed by combining 12.5 ml of each stock solution and filling up with H_2O_d to a total volume of 0.5 L. Next, the sample was sterile-filtered with a vacuum filtration system (polyether sulfone [PES], 0.22 µm, TPP AG) and aliquoted to the desired volume.

2.2 | Water samples

The stability studies of VHSV, IHNV and CyHV-3 were performed in six different water samples: cell culture medium (DMEM supplemented with 9 vol.-% FCS), tap water, iso-test water (as described above) and sterile-filtered as well as non-filtered water samples collected from a trout hatchery and a carp pond, respectively.

The tap water was run out for 2 min and filled into a 0.5-L sterile glass bottle. As cell culture medium DMEM mixed with 25 mM HEPES, 0.5 mM $NaHCO_3$ and supplemented with 9 vol.-% FCS was used. These two samples and iso-test water were used as references

to environmental samples, aliquoted and stored frozen at −20°C until use.

The environmental waters used in this study were collected from a trout hatchery of the Institute for Fisheries (Aufseß, Germany) and a carp pond (Stockweiher). Both environmental samples were taken directly below the water surface into sterile 1-L glass bottles at the end of May and beginning of June in 2019, respectively. Next, 500 ml were removed from both water samples and sterile-filtered using a vacuum filtration system with a PES membrane (0.22 µm, TPP AG). Both filtered and non-filtered environmental samples were then aliquoted (á 30 ml) into 50-ml sterile centrifuge tubes (polypropylene, PP, Greiner Bio-One) and stored frozen at −20°C. All water samples were thawed shortly before use, incubated at room temperature for about 1 hr and then tempered to the desired temperature.

Several parameters such as water hardness, pH and conductivity were measured for each water sample type using one of the corresponding aliquots. The pH was measured at 20°C using a pH probe (SE 100 N, Knick) and pH meter (766 Calimatic, Knick). MQuant test kit (Merck) was used to determine the total hardness of the used water samples, and the conductivity measurement was performed with a conductivity probe (Le703 Conductivity, Mettler Toledo) and a conductivity meter (FiveEasy Five Go FE30/FG3, Mettler Toledo).

Determination of the bacterial count was performed for the unfiltered environmental water samples and tap water using nutrient medium.

For this, the medium was melted with slightly open bottle lid in a microwave (800 W, 5 min, L.101, Bosch) and tempered to 55°C for 1 to 2 hr. Next, 100 µl of the, if necessary prediluted, corresponding sample were placed in a Petri dish (PS, $d = 92$ mm, Sarstedt), 10 ml of the described medium was poured onto it and evenly distributed in the dish by careful pivoting. After solidification of the medium, the Petri dish was sealed with parafilm (Bemis), incubated at 25°C for 7 days and read by macroscopic count of bacterial colonies.

2.3 | Cell cultures and virus stocks

All cell lines and virus isolates used in this study for preparation of the respective virus stocks and determination of viral titre were provided by the Friedrich-Loeffler-Institut, FLI, Greifswald, Germany. The cultivation and maintenance of the cells was carried out in DMEM with 9 vol.-% FCS. The number of infectious virus particles was determined via endpoint dilution (50% tissue culture infective dose assay, $TCID_{50}$) and statistic evaluation after Reed and Muench (1938) with rainbow trout (false) (RT/F) cells for VHSV, *Epithelioma Papulosum Cyprini* (EPC) cells for IHNV and common carp brain cells (CCB) for CyHV-3 titration. For $TCID_{50}$ assays, cells were cultivated in DMEM with the addition of 100 U/ml of penicillin G, 0.1 mg/ml of streptomycin sulphate and 0.25 µg/ml of amphotericin B to avoid bacterial contamination from the unsterile samples.

RT/F cells (CCLV-RIE 88) previously declared falsely as originating from gonads of rainbow trout and assigned later to the blue sunfish (*Lepomis macrochirus*) (Schütze, 2011) are susceptible to VHSV and were used here for the preparation of the virus stock. The VHSV stock was prepared by propagation of the virus (isolate Fi13, Enzmann & Bruchhof, 1989) in cells seeded with 78,000 cells cm^{-2} in DMEM with 9 vol.-% FCS in T-75 cell culture flasks ($n = 3$; polystyrene, PS, Greiner Bio-One). Inoculated cells were incubated at 15°C for 5 days postinfection (dpi), and the replication was stopped by placing the flasks (cell and supernatant) in a freezer at −80°C. After thawing and mixing, virus suspensions were pooled, aliquoted (á 1 ml) in 1.5-ml reaction tubes (PP, Sarstedt) and stored at −80°C for further use. Determination of virus titre performed with three random aliquots resulted in a value of 3×10^8 $TCID_{50}$ ml^{-1}.

EPC cells (CCLV-RIE 173) established from fathead minnow (Schütze, 2011) that are susceptible to both IHNV and VHSV were used here exclusively for replication of the former virus. The IHNV isolate (DF 11/95) isolated by Schlotfeld et al. (Hannover, National Reference Laboratory, unpublished data) from rainbow trout was used for the virus stock preparation by replication onto EPC cells seeded with 120,000 cells cm^{-2} in T-75 cell culture flasks ($n = 3$). Inoculated EPC cells were placed at 15°C and the replication stopped after 3 dpi by placing the flasks in freezer (−80°C). After thawing and aliquoting (á 1 ml) in 1.5-ml reaction tubes, three random aliquots were used for titre determination with EPC cells resulting in 7×10^7 $TCID_{50}$ ml^{-1}.

Two different CyHV-3 virus stocks used in this study were prepared using CCB cells: CCLV-RIE 816, established by Neukirch (1999) in Hannover and the CyHV-3 Taiwan isolate (KHV-T, Dr. Peiyu Lee, Wang et al., 2015). For CyHV-3 propagation, CCB cells were seeded either with 68,000 cells cm^{-2} or 75,000 cells cm^{-2} in cell discs (PS, 4 layers, Greiner Bio-One) in MEM with 9 vol.-% FCS and incubated after inoculation with the virus for 3 dpi at 25°C. Next, cell discs with the infected cells were placed in a freezer at −80°C to stop the replication, thawed, aliquoted as described above and stored at −80°C until further use. Titration of random aliquots ($n = 3$) revealed a titre of 3×10^7 $TCID_{50}$ ml^{-1} for the first virus stock (68,000 cells cm^{-2}) and 1×10^8 $TCID_{50}$ ml^{-1} (75,000 cells cm^{-2}) for the other virus stock.

2.4 | Determination of titre and genomic material of the target viruses

Virus titration was performed by an endpoint dilution (50% tissue culture infective dose assay, $TCID_{50}$ assay) using subsequent 10-fold dilutions ($n = 8$ per each dilution step) on 96-well plates (Cellstar®, PS, F-bottom, Greiner Bio-One, Kremsmünster, Austria) with statistical analysis according to Reed and Muench (1938) as described previously by Mletzko et al. (2017) and expressed as $TCID_{50}$ ml^{-1}. Well plates with VHSV (RT/F cells) and INHV (EPC cells) were incubated at 15°C and with CyHV-3 (CCB cells) at 25°C and were examined regularly (every 5 days) microscopically for the occurrence of cytopathic effects. The final reading was carried out after 10 days of incubation. The previously calculated limit of quantification (LOQ) and the limit of detection (LOD) are 160 and 12 $TCID_{50}$ ml^{-1}, respectively.

qPCR and RT-qPCR were used to determine the viral DNA (CyHV-3) or RNA (VHSV, IHNV) in the samples. Extraction of viral DNA was performed using 200 µl of the sample with DNeasy blood and tissue kit, and 140 µl of the sample was used for isolation of viral RNA with QIAamp viral RNA mini kit (both purchased from Qiagen). Extracted DNA was stored at −20°C and RNA at −80°C until use, both in 1.5-ml reaction tubes.

The DNA extracts were analysed using TaqMan qPCR for CyHV-3 according to Gilad et al. (2004) with iTaq universal probes supermix (Bio-Rad). DNA standard of CyHV-3 (after Gilad et al. [2002] with flanking sequences, prepared in our laboratory, 595 bp, accession number AF411803) was diluted stepwise (10-fold each) starting with a DNA copy number of 10^{10} to 10^{0} copies in 5 µl of nuclease-free water for molecular biology (W4502-1L, Sigma-Aldrich) for calibration. Additionally, to each row of the analysed samples, two negative controls (nuclease-free water) were prepared. The analysis was performed using a thermocycler (CFX Connect Real-Time System, Bio-Rad) with the following thermal programme: denaturation and primer hybridisation at 95°C for 5 min followed by elongation and amplification at 60°C for 30 s.

The RT-qPCR of IHNV and VHSV samples were performed using a RT-qPCR kit (iTaq universal probes one-step kit, Bio-Rad) according to Chico et al. (2006) and Hoferer et al. (2019), respectively, and the protocols recommended by the Bavarian State Office for Health and Food Safety (LGL). As the negative control nuclease-free water was used and extraction and amplification controls were prepared using intype IC-RNA (Indical Bioscience GmbH) and specific primers and probe (Sigma-Aldrich). The temperature profile applied in the thermocycler for these protocols was as follows: heating—10 min at 50°C, denaturation—3 min at 95°C, primer hybridization—15 s at 94°C, elongation and amplification—30 s at 60°C.

2.5 | Virus stability investigations in various water samples

All virus stability experiments were performed in biological triplicates using the same six water samples described above and the same set-up (50-ml PP-centrifuge tubes, temperature of 20°C, light exclusion).

For stability studies of VHSV, IHNV and CyHV-3, water and medium samples (á 30 ml) preincubated at 20°C were spiked with the corresponding virus suspension to obtain approximately 10^{6} $TCID_{50}$ ml^{-1} for VSHV and IHNV and 10^{5} $TCID_{50}$ ml^{-1} for CyHV-3. The samples were resuspended several times after spiking using a 10-ml pipette (PS, Sarstedt), and 1 ml was removed for determination of the initial virus titre and genomic material. A further aliquot (1 ml) was removed from the unsterile water samples for determination the bacterial count. Next, the samples were incubated at conditions described above for up to 168 hr for VHSV and 96 hr for IHNV and CyHV-3 and sampled (á 1 ml) at multiple time points. The sampled aliquots were stored at −80°C in 1.5-ml reaction tubes until analysis. The pH of the unsterile samples was controlled directly after the addition of virus stock and after 24 and 96 hr of incubation.

2.6 | Influence of serum supplementation to water samples on infectivity of CyHV-3

In addition to test the CyHV-3 stability over an incubation in different water and medium samples, further experiments regarding titre and stability of this virus were performed in samples with and without FCS supplementation and in cell culture medium with different serum contents. Again, all experiments were done in biological triplicates at 20°C and under light exclusion. After spiking with the appointed virus suspension (as described above) samples were resuspended, aliquots (á 1 ml) collected at selected time points and stored at −80°C until analysis.

To investigate whether protein content influences aggregation/adhesion of this virus in water, sterile-filtered samples collected from trout hatchery and carp pond (10 ml each), iso-test water and the cell culture medium (3 ml each) were used both with and without the addition of FCS (9 vol.-%) and spiked with CyHV-3 stock to reach the starting titre of 10^{5} $TCID_{50}$ ml^{-1}. In these experiments, samples were taken directly after the addition of the virus suspension and resuspension (several times with a 10-ml pipette) in 1.5-ml reaction tubes, stored at −80°C until further analysis and submitted to titre and viral DNA determination. In order to assess whether the detected differences between the virus titre values in water samples with and without serum addition are statistically significant, a one-way analysis of variance (ANOVA) (* $\alpha = 0.05$, ** $\alpha = 0.01$, *** $\alpha = 0.001$) was carried out.

Furthermore, the serum content in the cell culture medium used in this study was varied to test whether FCS contributes to the stability of the virus in this matrix. For that DMEM samples with additional 0.05%, 0.55% and 5.3%, FCS (9 ml) spiked with the CyHV-3 stock to reach approximately titres of 10^{5} $TCID_{50}$ ml^{-1} were prepared, sampled into 1.5-ml reaction tubes immediately after the addition of the virus suspension (after mixing) and after 72 and 148 hr of incubation (20°C, light exclusion). Collected samples were analysed by titration and qPCR to determine virus titre and copy number of CyHV-3. Viral titre values measured at various time points for the corresponding FCS content and at various FCS concentrations at 0, 72 and 148 hr were analysed using one-way ANOVA.

3 | RESULTS

3.1 | Characteristics of used water samples

In order to facilitate a comparative discussion of virus stability pH, conductivity and hardness were determined for all used water samples (Table 1). The highest pH of 9.2 was determined in samples collected from a carp pond, followed by tap water (pH = 8.4), the iso-test water, the samples from trout hatchery (pH = 7.9) and the cell culture medium (pH = 7.4). The conductivity of all water samples was below 1 S/cm while for the cell culture medium it was up to 25-fold higher (15 S/cm). The water hardness lay in all four measured water samples in similar range and below 300 mg_{CaCO3} L^{-1}, with the highest value determined for the sample from the trout hatchery

TABLE 1 Water hardness, pH value and conductivity of the water samples

Sample	Water hardness in $mg_{CaCO3}\ L^{-1}$	pH at 20°C	Conductivity in S/cm at 20°C
DMEM + 9% FCS	–	7.4	15.1 (±0.3%)
Iso-test water	254 (±1.8%)	7.9	0.72 (±0.6%)
Tap water	226 (±2.0%)	8.4	0.43 (±0.8%)
Water from trout hatchery	291 (±1.8%)	7.9	0.59 (±1.0%)
Water from carp pond	211 (±2.5%)	9.2	0.56 (±3.0%)

Abbreviation: FCS, foetal calf serum.

(291 $mg_{CaCO3}\ L^{-1}$) and the lowest for the one collected from a carp pond (211 $mg_{CaCO3}\ L^{-1}$). In the untreated environmental samples, the bacterial load was determined and monitored during the virus stability experiments.

3.2 | Stability of viruses in selected water samples

The stability of the target viruses in the described water samples, that is sterile cell culture medium, iso-test water (sterile), tap water (unsterile) and environmental water samples from a carp pond and a trout hatchery (both sterile-filtered and unfiltered) was investigated for up to 168 hr (20°C, in darkness) via quantification of the viral titres and their genomic material. In order to facilitate the comparison of the stability of the three viruses, only one incubation temperature was selected for the described above experiment. As CyHV-3 in vitro replication is performed in our laboratory at 25°C, while the replication of VHSV and IHNV gives the best results at 15°C, we chose the temperature which lays between these two points for these investigations.

The VHSV titres during the incubation in the described samples are shown in Figure 1. While in cell culture medium the titre stayed more or less constant for over 168 hr, a linear reduction reaching about two and three orders of magnitude after 168 hr was observed in iso-test water and unsterile tap water, respectively (Figure 1a). The bacterial count in tap water sample increased continuously (from 10^2 to 10^6 colony forming units [CFU] ml^{-1}) up to 96 hr (further time points not determined). In sterile-filtered environmental water samples, the virus titre decreased only slightly during the whole incubation period (below one log step and about one log step in water from trout hatchery and carp pond, respectively [see Figure 1b,c]). In contrast, if these environmental samples were applied unfiltered, virus titres reached the limit of detection of the titration assay already within 24 hr (Figure 1b,c), corresponding to a reduction of 4.5 log steps. At the same time, the bacterial count increased strongly within the first 24 hr of incubation and then remained almost constant till the end of the experiment at 9.5×10^5 and 4.4×10^5 CFU/ml, respectively (Figure 1b,c). The analysis of viral genomic RNA in all

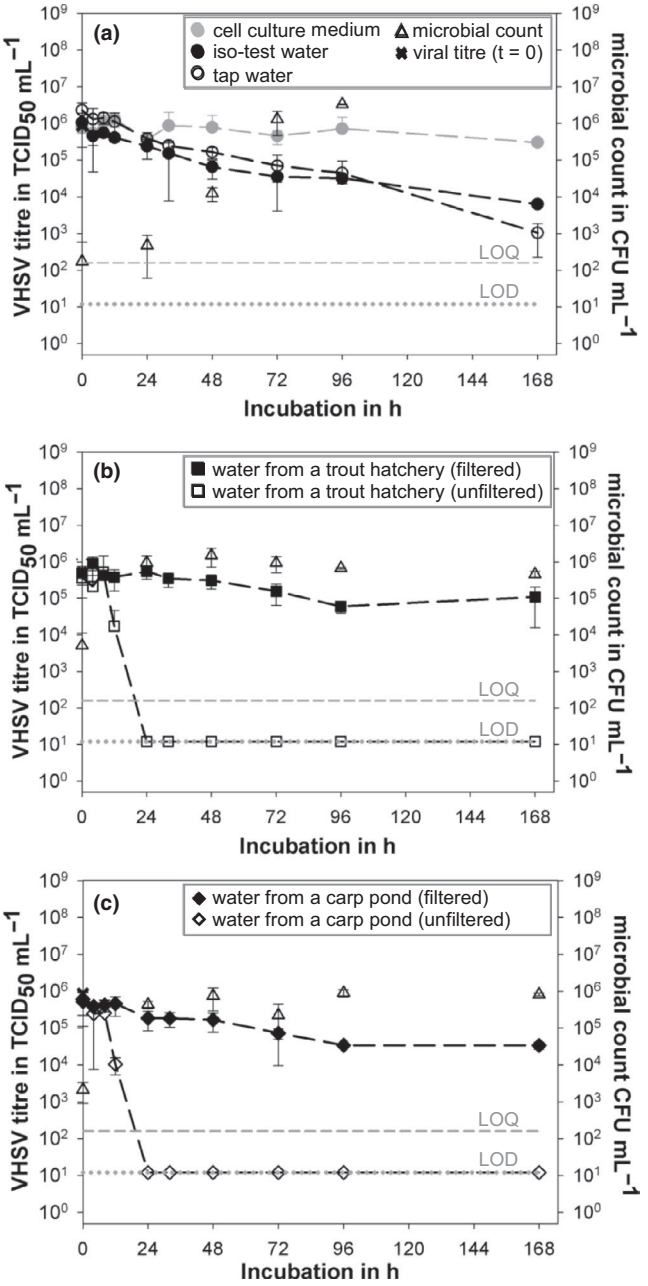

FIGURE 1 Stability of VHSV in different water samples and microbial count of the unfiltered water samples over an incubation time of 168 hr. Legend: (a) (●) Cell culture medium (DMEM + 9% FCS), (●) iso-test water and (○) tap water. (b) (■) Sterile-filtered and (□) unfiltered water from a trout hatchery. (c) (◆) Sterile-filtered and (◇) unfiltered water from a carp pond. (△) microbial count, (✕) theoretical virus titre at $t = 0$ ($titre_{theo}$), calculated based on the applied dilution and the titre of the used virus stock. (- - -) LOQ, (····) LOD. Each data point refers to a triplicate ($n = 3$). Virus titres were estimated at different time points by $TCID_{50}$ assay and expressed as log_{10} [Colour figure can be viewed at wileyonlinelibrary.com]

samples revealed constant copy numbers in all sterile samples and the tap water whereas a reduction of up 6 C_t values after 168 hr occurred in the unsterile environmental samples, corresponding approximately to two orders of magnitude of RNA_{VHSV} copy number (Supporting Information Figure A).

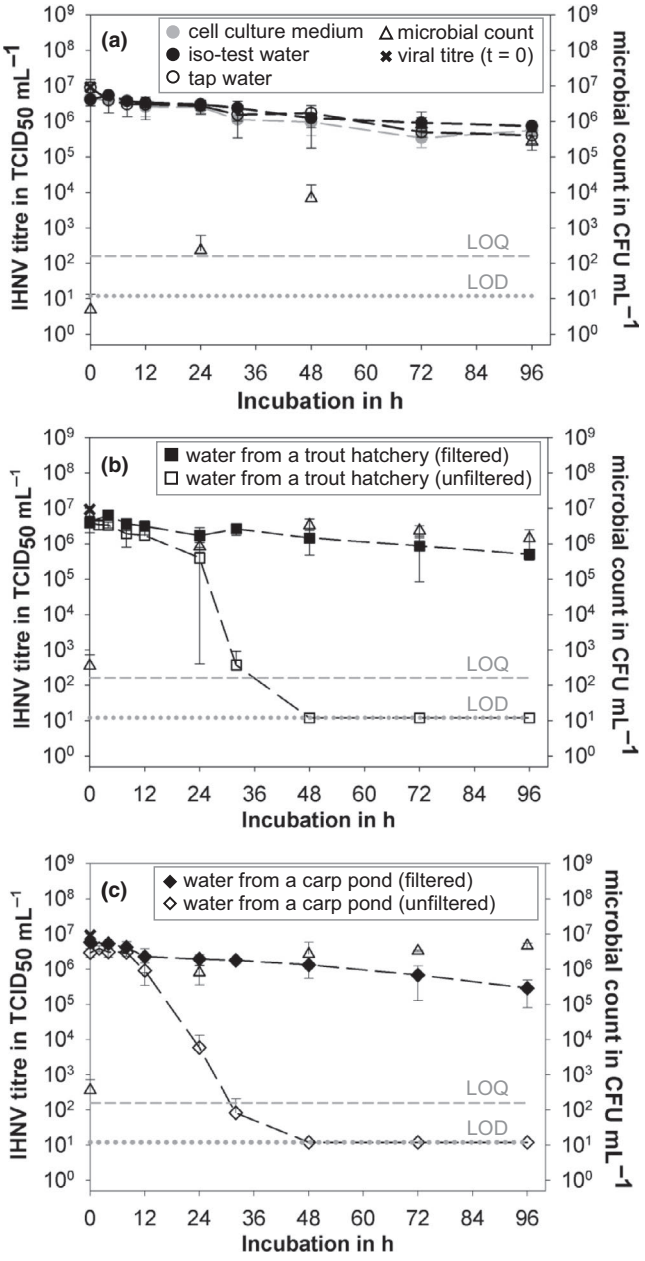

FIGURE 2 Stability of IHNV in different water samples and microbial count of the unfiltered water samples over an incubation time of 96 hr. Legend: (a) (●) Cell culture medium (DMEM + 9% FCS), (●) iso-test water and (○) tap water. (b) (■) Sterile-filtered and (□) unfiltered water from a trout hatchery. (c) (◆) Sterile-filtered and (◇) unfiltered water from a carp pond. (△) microbial count, (✕) theoretical virus titre at $t = 0$ ($titre_{theo}$), calculated based on the applied dilution and the titre of the used virus stock. (- - -) LOQ, (····) LOD. Each data point refers to a triplicate ($n = 3$). Virus titres were estimated at different time points by $TCID_{50}$ assay and expressed as log_{10} [Colour figure can be viewed at wileyonlinelibrary.com]

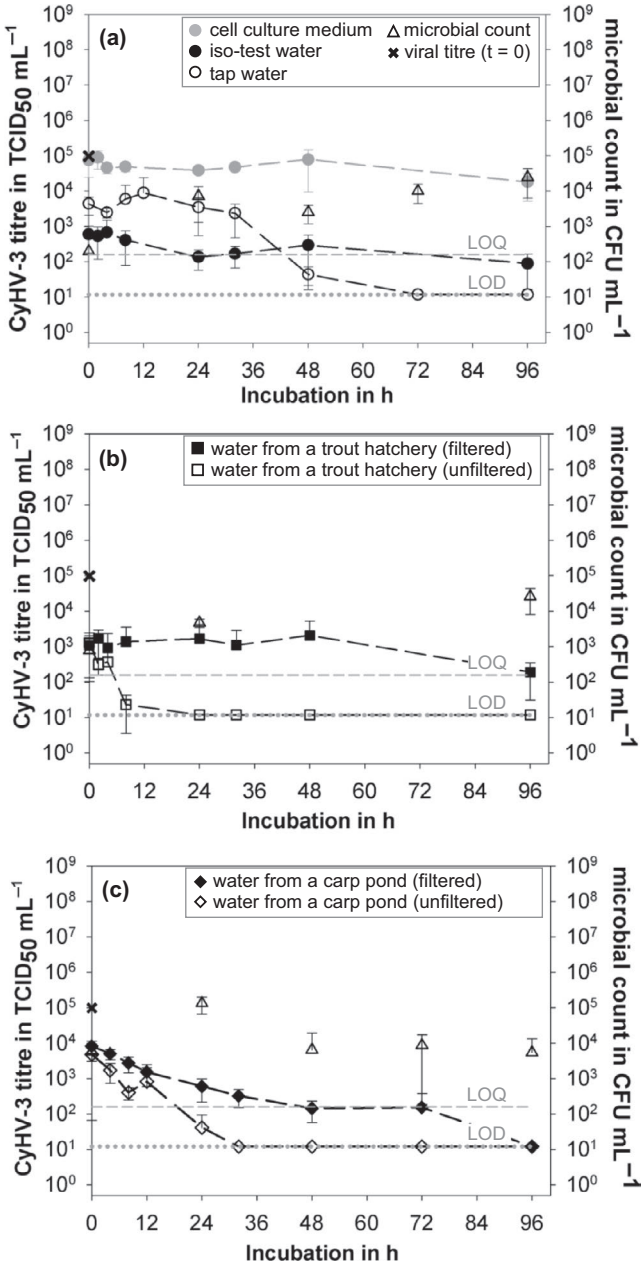

FIGURE 3 Stability of CyHV-3 in different water samples and microbial count of the unfiltered water samples over an incubation time of 96 hr. Legend: (a) (●) Cell culture medium (DMEM + 9% FCS), (●) iso-test water and (○) tap water. (b) (■) Sterile-filtered and (□) unfiltered water from a trout hatchery. (c) (◆) Sterile-filtered and (◇) unfiltered water from a carp pond. (△) microbial count, (✕) theoretical virus titre at $t = 0$ ($titre_{theo}$), calculated based on the applied dilution and the titre of the used virus stock. (- - -) LOQ, (····) LOD. Each data point refers to a triplicate ($n = 3$). Virus titres were estimated at different time points by $TCID_{50}$ assay and expressed as log_{10} [Colour figure can be viewed at wileyonlinelibrary.com]

In order to set the starting titre of IHNV in a comparable manner to 10^6 $TCID_{50}$ ml^{-1} as for VHSV, higher volumes of the virus stock as for VHSV and later for CyHV-3 had to be used. This resulted in a slightly pink colouring of the unfiltered environmental water samples due to the phenol red indicator contained in the cell culture medium in the stock solution. After 48 hr, a shift from light pink to yellowish orange was observed indicating a change in pH from slightly alkaline to slightly acidic.

The development of the IHNV virus titres over the incubation time in the investigated samples is depicted in Figure 2. Again, in all sterile

samples and unsterile tap water the IHNV titre decreased by only approximately one order of magnitude within 96 hr (Figure 2a–c), while after already 48 hr of incubation in unsterile environmental waters it dropped below the limit of detection, corresponding to a reduction of over 4.6 log steps (Figure 2b,c). Again, the bacterial count in the unfiltered environmental water samples raised strongly within 48 hr and then remained almost constant till the end of the experiment (3.3×10^6 and 2.7×10^6 CFU/ml for water samples from trout hatchery and carp pond, respectively—see Figure 2b,c). The viral RNA analysis revealed a constant number of RNA_{IHNV} copies in all sterile samples and tap water and a reduction of ca. two orders of magnitude in unsterile environmental samples (over 6 C_t-values—see Supporting Information Figure B).

Finally, the results concerning the stability of the third virus, that is CyHV-3, are shown in Figure 3. The infectivity of the CyHV-3 in cell culture medium again stayed almost constant for over 96 hr and showed a linear reduction in the iso-test water by approximately one order of magnitude. In contrast to the other two viruses, the CyHV-3 titre in tap water decreased by more than 2 log steps to reach the detection limit of the titration assay after 72 hr (Figure 3a). At the same time, the bacterial count of the tap water sample increased from 1.98×10^2 to 2.5×10^4 CFU/ml within 96 hr. The reduction of the CyHV-3 titre in the sterile-filtered sample from a trout hatchery was comparable with the one in the iso-test water (Figure 3b), while a linear reduction of the virus titre was observed in the sterile-filtered water from a carp pond (over 2 log steps after 48 hr). A sharp drop of viral titre, by 2 log steps within 8 hr and below the limit of detection, was observed in the unfiltered environmental sample from a trout hatchery, whereas in the unfiltered sample from a carp pond the same reduction was recorded only after 24 hr. The bacterial count in the environmental sample from a trout hatchery increased continuously (from 7.8×10^2 to 2.6×10^4 CFU/ml) within 96 hr and stayed almost constant during the experiment in unfiltered water from a carp pond (5.5 10^3 CFU/ml).

It was remarkable that the planned starting titre of 10^5 $TCID_{50}$ ml^{-1} of CyHV-3, which were supposed to be adjusted by adding the corresponding dilution of the stock, could be confirmed exclusively in the cell culture medium, while in all other tested samples the resulting titre was one to two orders of magnitude lower than expected, already at the beginning of the experiment. This effect was most pronounced in the iso-test water (ca. 2 log steps).

The analysis of genomic CyHV-3 DNA (Supporting Information Figure C) correspondingly revealed a reduction of one (tap water), two (trout hatchery) and up to more than three (carp pond) orders of magnitude in viral DNA copy number observed in the unfiltered water samples, while no effect could be registered for the sterile water samples used in this work.

3.3 | Influence of serum on the detected infectivity of CyHV-3

Due to the previous reports, showing that the addition of bovine serum albumin (BSA) to water can improve stability of viruses or prevent aggregate formation (Kocan et al., 2001; Wadu-Mesthrige et al., 1996), further experiments to test whether the addition of FCS to various water samples can influence the stability of CyHV-3 in the corresponding matrix were performed in this work. First, four different water samples—cell culture medium, iso-test water, unfiltered water from a carp pond and unfiltered water from a trout hatchery—with and without addition of FCS (9 vol.-%) were spiked with the virus suspension and sampled directly afterwards. Despite the finding of lower CyHV-3 starting titres in the above-described stability experiments in all water samples (except cell culture medium) right after the addition of the virus, in this set-up only in iso-test water the titre was significantly lower without FCS supplementation (1.1 log step, one-way analysis ANOVA, p-value = 0.0053, $\alpha = 0.01$, see Figure 4). No statistically significant influence of the FCS supplementation on the CyHV-3 titre was observed for the other three water samples.

In a second experimental set-up, the cell culture medium (DMEM) with three different concentrations of FCS (0.05, 0.55, 5.3 vol.-%) was spiked with CyHV-3 suspension to reach—at least theoretically—roughly 10^5 $TCID_{50}$ ml^{-1}, resuspended and sampled directly and after 72 and 148 hr (Figure 5). The determination of the virus titre in the medium sample with 0.05 vol.-% FCS showed 2.4×10^4 $TCID_{50}$ ml^{-1} just after spiking and thus about 1 log step below the starting titre. This value is significantly lower than the value measured at the same time point in medium with 5.3% FCS (**$p = .008$, ANOVA, $\alpha = 0.01$). On the other hand, the starting titre in the samples with higher serum content was only about 0.5 log

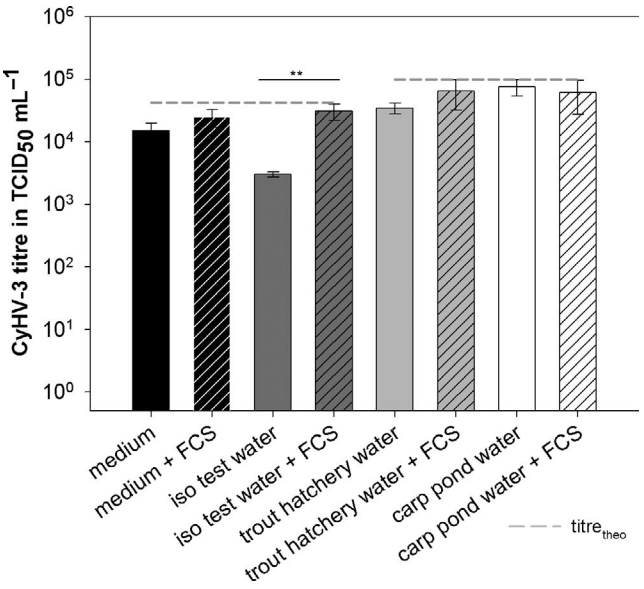

FIGURE 4 CyHV-3 virus titres of different water samples with and without FCS in $TCID_{50}$ ml^{-1}. Legend: (– – –) theoretical virus titre at $t = 0$ ($titre_{theo}$), calculated based on the applied dilution and the titre of the used virus stock. Each sample was prepared in triplicate ($n = 3$). Virus titres were estimated at different time points by $TCID_{50}$ assay and expressed as log_{10}. ** (one-way ANOVA, $p < \alpha = 0.01$)

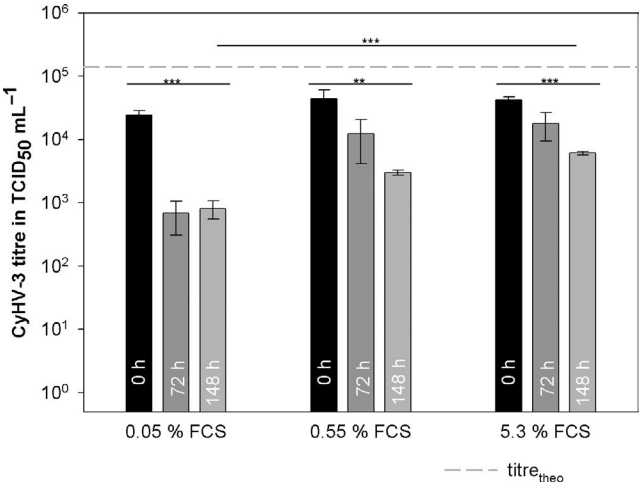

FIGURE 5 Stability analysis of CyHV-3 in medium samples with different serum content (FCS) at different time points within 148 hr. Legend: (▬) 0 hr, (▬) 72 hr, (▬) 148 hr, (- - -) theoretical virus titre at $t = 0$ ($titre_{theo}$), calculated based on the applied dilution and the titre of the used virus stock. Each sample and time point were prepared in triplicate ($n = 3$). Virus titres were estimated at different time points by $TCID_{50}$ assay and expressed as log_{10}. Statistical analysis—one-way ANOVA: $^{*}p < \alpha = 0.05$, $^{**}p < \alpha = 0.01$, *** $p < \alpha = 0.001$

steps below the set value. At all serum contents, the concentration of infectious CyHV-3 particles decreased over time. While the lowest reduction (0.8 log steps after 148 hr, $^{***}p = .00057$, ANOVA, $\alpha = 0.001$) was observed for samples with the highest (5.3 vol.-% FCS) applied serum content, the titre of the virus in the samples with the lowest FCS concentration (0.05 vol.-%) decreased within 72 hr by 1.5 logs ($^{***}p = .00003$, ANOVA, $\alpha = 0.001$) and then remained constant, slightly above the limit of quantification.

4 | DISCUSSION

4.1 | Infectivity/stability of the target viruses in different water samples

The primary concern of this work was to compare three exemplary fish viruses with regard to their stability in different water samples under otherwise identical conditions. Typical water parameters as pH (7.4–9.2), hardness (211–291 mg_{CaCO3} L^{-1}) and conductivity (water samples <1 S/cm, the medium 15 S/cm) were of minor influence. The marginal influence of water hardness on virus stability complies well with the data published by Pietsch et al. (1977), who examined the influence of water hardness on the stability of IHNV and showed that no significant reduction of its titre occurred in samples with water hardness values ranging between 20 and 250 mg/L. As water hardness of the samples used in this work lay approximately in this range, no effect on the stability of IHNV was expected and that could be confirmed by the obtained data. While Pietsch et al. (1977) reported that IHNV is very sensitive to pH values around 5 and 9, a significantly faster inactivation of this virus in a water sample from carp pond with pH of 9.2 was not observed in this study. This might be due to the pH shift to 8.3 resulting from spiking with the virus solution (in cell culture medium pH = 7.4). It cannot be ruled out that the faster inactivation of CyHV-3 in the filtered sample from a carp pond registered here might be connected to the higher pH value of this water in comparison with other tested samples. On the other hand, in a previous study of our group no reduction of the CyHV-3 titre was observed at pH values between 5 and 9 within 24 hr in cell culture medium (Amtmann et al., 2019), but longer incubation at a pH around 9 was not tested and therefore the influence to the infectivity of CyHV-3 cannot be excluded.

As a slightly faster (by 2.8 log steps within 96 hr in water from carp pond in comparison with 0.8 log steps in the water sample from trout hatchery) inactivation in sterile-filtered water samples with elevated pH was observed only for CyHV-3, no clear correlation between characteristics of the water samples, such as pH or water hardness, and the stability of the three target viruses can be established based on the data collected here.

The by far most effective influence on viral stability and infectivity was the unsterile condition of water samples. In all sterile-filtered water samples, the concentration of infectious virus particles decreased relatively slowly. In cell culture medium, a reduction by 6%, 76% and 88% of infectious virus particles for VHSV, CyHV-3 and IHNV, respectively, was measured after 96 hr of incubation. After the same time in iso-test water, higher reductions accounting to 82%, 85% and 97% were determined for IHNV, CyHV-3 and VHSV, respectively. The highest decrease of the virus titres (99.9%) in sterile-filtered water samples was registered for CyHV-3 (96 hr) in the water from a carp pond, followed by the reduction of VHSV (99.4%) measured in iso-test water after 168 hr. In contrast, in unsterile environmental samples the inactivation increased dramatically and virus titres below 1% were measured in most cases already after the first 24 hr. So, in both unfiltered environmental samples the infectivity of VHSV declined by >4 log steps within the first 24 hr. Based on the extrapolation of a regression, a comparable reduction could be expected in the corresponding sterile-filtered samples, only after 37 and 25 days in water from a trout hatchery and carp pond, respectively (Figure 1b,c). Thus, VHSV was inactivated 12 to 16 times faster in the presence of bacteria. Similar observations were previously published in the literature. For example, Hawley and Garver (2008) described a six times faster inactivation of VHSV (three log steps) in unfiltered compared with filtered freshwater samples and Mori et al. (2002) found the same ratio for the VHSV titre reduction in unfiltered to filtered saltwater. Even more rapid inactivation of VHSV in unsterile environmental waters reported in this study might be due to a different virus strain or the characteristics of the environmental samples used here.

Similar to VHSV, the incubation of IHNV with unfiltered environmental water resulted in a pronounced decrease of the virus titre, 5 to 6 times faster than in the corresponding sterile samples

(Supporting Information Figure B). This corresponds well with the data presented by Kamei et al. (1988), where a titre reduction of three log steps within 3 days was registered for IHNV in an untreated freshwater sample, while no such effect was observed in sterile-filtered fresh water even after 14 days.

For CyHV-3, more substantial inactivation in comparison with the other two viruses was observed in unsterile tap water samples. In this matrix, the CyHV-3 virus titre dropped by two log steps (≥99% reduction) already after 48 hr of incubation and the limit of detection of the titration assay was reached within 72 hr. Same as for the other two viruses, the titre of CyHV-3 decreased faster in unfiltered environmental samples reaching the reduction by over 99% within 24 hr in both waters.

Though time consuming and cumbered with relatively high uncertainty, the inactivation of the viruses can be monitored with highest fidelity via virus titration as this allows to estimate the number of still infectious virus particles and not only the presence of their DNA or RNA. However, the faster and easier PCR tests for viral RNA or DNA are good substitutes, the detection of viral genetic material does not necessarily mean that the virus is still infectious. Moreover, if the protocol used for such procedures are not carefully considered and selected for specific purpose, also PCR analysis might show negative results, while infectious virus is present in the analysed sample. For easily understandable reasons, the degradation of viral genetic materials is less severe than the loss of functionality. Most probably, the observed reduction in viral RNA or DNA is a consequence of the degradation of viral particles resulting in exposure of its genetic material and following degradation. Thus, an inactivation of the infectivity of a viral particle, which can have numerous reasons, is much more likely than the loss of its genomic material due to a severe degradation of the particle. Nevertheless, in this study the severe decrease of the virus titres always correlated well with the observed reduction of genomic material of the investigated viruses.

Even though we did not examine the influence of bacteria in the environmental water samples on viral inactivation in detail nor were the participating species characterized, it is reasonable to assume that the bacterial load was the prevalent factor being responsible for the effective destruction of viruses observed in this study. Comparable observations have been made by other groups, for example Hawley and Garver (2008) and Mori et al. (2002). Yoshimizu et al. (1986, 1992), Yoshimizu and Kimura (1976), Yoshimizu and Yoshio (1999) described similar results and were able to identify bacteria with antiviral effects. According to their studies, bacteria belonging to the genera *Vibrio*, *Aeromonas* and *Pseudomonas*, that were found in the intestinal tract of salmonids and also the environmental waters, contribute to IHNV inactivation in environmental waters. This antiviral influence probably became more pronounced during the course of our experiments since we observed that the bacterial load in unsterile samples increased over time drastically. For CyHV-3, an increased degradation of the infectious viral particles could be correlated with the bacterial load in the unfiltered water samples. This is in good agreement with the data published by Shimizu et al. (2006) who described bacteria with antiviral activity against CyHV-3 that caused a reduction of CyHV-3 titre by 4 log steps within a maximum of 32 hr. Furthermore, Yoshida et al. (2013) were able to isolate several types of bacteria that promote the inactivation of CyHV-3—including the genera *Enterobacteriaceae*, *Aeromonas*, *Achromobacter* and *Pseudomonas*, found in the intestinal tract of common carp and koi. Furthermore, these two bacterial genera are known to produce metalloproteases (Janda & Abbott, 2010; Wandersman, 1989) that can contribute to the degradation of envelope proteins of viruses, thereby inactivating them. In our previous work, a reduction of 3 and 4 log steps within 24 hr of CyHV-3 and VHSV titres, respectively, could be achieved by the application of Neutrase®, a metalloendoprotease from *Bacillus amyloliquefaciens*, to the samples (Amtmann et al., 2019). Thus, it was demonstrated that a correlation exists between a proteolytic enzyme belonging to metalloendoproteases and the decrease in titres of these two fish viruses.

Surprisingly, in all examined samples with the exception of cell culture medium, lower than expected (by at least 1 log step) CyHV-3 titres were measured already at the start of the incubation. A pipetting error can be excluded, since a nearly identical and expected virus DNA load could be verified in all samples at the beginning of the experiment (see Supporting Information Figure C). This also suggests that the infectious viral particles were not removed from the water samples by adhesion to the surface of the sample container, at least at this time point, as the same samples were used for determination of virus titre and its genomic material.

Such reduction in virus titre at the start of the incubation was not observed for VHSV and IHNV. As the same water samples, beside the iso-test water, and the same procedures were used for all investigations performed in this study, it is unclear what was the reason for that. However, such effects were repeatedly observed in our other studies performed with CyHV-3 (data not shown here) when performing virus dilution in other matrices than cell culture medium. Thus, it is likely that these differences between CyHV-3 and VHSV/IHNV are due to their different sensitivity to environmental parameters and for example stronger tendency of the first one to build aggregates.

4.2 | Influence of serum supplementation on the infectivity of CyHV-3

It was previously shown that the addition of BSA to water can improve the stability of viruses in this matrix or even resolve previously formed virus aggregates (Kocan et al., 2001; Wadu-Mesthrige et al., 1996). As CyHV-3 seems to be inactivated more rapidly than VHSV and IHNV, further experiments with serum supplementation were performed for this virus.

Supplementation of the iso-test water and environmental samples used in this work with FCS (9 vol.-%) was carried out in order to investigate whether the protein concentration in the water matrix can prevent aggregation of viral particles and therefore help to avoid the detection of lower than expected virus titres directly

after addition to such water samples (Figure 4). However, no significant effect was observed in medium or in the filtered environmental samples within such short periods of time. The CyHV-3 titre in the iso-test water with serum supplementation was about 1 log step above the titre of the same matrix without serum addition. Therefore, we suppose that in case of this matrix the addition of FCS might have prevented the aggregation of the virus. For the other water samples, such an effect was not significant. The apparent, fast decrease of the virus infectivity in this matrix can possibly be explained by the fact that it does not contain any other substances that could naturally stabilize/protect or prevent aggregation of CyHV-3. Furthermore, Gutierrez et al. (2010) showed in a study with rotavirus that bivalent ions, such as Mg^{2+} and Ca^{2+}, have aggregation-promoting behaviour on viruses. Since the matrix of the iso-test water contains mainly ions, it is not unlikely that the aggregation of virus particles was the reason for the low titre of CyHV-3 detected directly after addition of the virus to this sample. Thus, the addition of serum to iso-test water could have a particularly strong effect on the stabilization of CyHV-3 in this sample, whereas the components naturally occurring in water samples from a trout hatchery and a carp pond could themselves exert this stabilization and the addition of serum would thus have only a minor influence on it.

Furthermore, varying serum content in cell culture medium spiked with CyHV-3 proved the stabilizing properties of proteins on this virus (Figure 5). Consequentially, the inactivation of the virus was the slowest in the samples containing the highest concentration of FCS (5.3 vol.-%) and led to the least reduction of the titre. Thus, it can indeed be speculated that the components of the serum can increase the stability of CyHV-3 in aquatic matrices and preserve its infectivity.

These observations correspond well with the data published by Wadu-Mesthrige et al. (1996) who found that the addition of BSA dissolved the aggregates formed by the tobacco mosaic virus in water under neutral pH conditions. Moreover, also Kocan et al. (2001) described that no reduction in viral titre of VHSV for 15 days was observed after adding 10 vol.-% serum to salt water.

Hence, it can be concluded that serum and therein present proteins, especially serum albumin, can have a stabilizing effect on virus particles and prevent their aggregation in water samples with particularly low protein content. The results shown here suggest that even small amounts of proteins can have such an effect on the investigated viruses in water.

5 | CONCLUSION

In order to understand mechanisms responsible for the spread of viral infections in aquaculture, it is of prime importance to compare individual viral species, influencing parameters and their combinations on a standardized basis. Therefore, the direct comparison of three important viruses, that is CyHV-3, VHSV and IHNV, which can infect carp and trout, respectively, has been conducted. The central influence on the infectivity of all three viruses turned out to be the bacterial load of the water. All other parameters considered in this study were of minor influence. The titres of these viruses were strongly reduced (between two and five log steps) in unfiltered environmental samples within a maximum of 48 hr and up to 16 times faster than in the same sterile-filtered environmental samples. This is an indirect prove that the presence of bacteria in water contributes to inactivation of these viruses. The correlation between increasing bacterial loads in unsterile environmental samples with the decreasing virus titres supports this theory. Even though bacterial populations can contribute to the inactivation of viruses in environmental waters, due to the difficult control of such processes under environmental conditions, other methods for inactivation/containment of viruses have to be applied in order to prevent transmission and/or outbreak.

The direct comparison of three viruses confirmed the assumption that individual species differ in their behaviour and in their sensibility towards environmental parameters. IHNV demonstrated twice the stability in both unfiltered environmental samples in comparison with VHSV. The surprising effect of significantly lower virus titres directly after addition to the water samples was only observed with CyHV-3 and suggests that this virus tends to form aggregates more readily in comparison with the other two species. This can lead to an underestimation of the number of infectious viral particles of CyHV-3 in such a matrix. However, the aggregate formation does not necessarily reduce viral infectivity as they can be resolved again for example when protein concentration increases in the water. This has to be considered when performing tests in practice.

To the best of our knowledge, this is the first study reporting the stability of CyHV-3, VHSV and IHNV—that endanger the two most important fish species in German aquaculture—in various water samples under similar conditions.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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