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Virucidal effects of various agents—including protease—against koi herpesvirus and viral haemorrhagic septicaemia virus

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Abstract

In a search for alternative, environmentally friendly and effective disinfecting agents, a commercially available protease—Neutrase®—was tested in this work for inactivation of koi herpesvirus (KHV) and of viral haemorrhagic septicaemia virus (VHSV). For comparison, the stability of these viral pathogens in similar configurations at various pH values and concentrations of peracetic acid or quicklime, typically used for disinfection, was tested. Therefore, virus suspensions were incubated with various concentrations of different agents for 24 hr and the titre of the remaining infectious particles was determined by virus titration. Furthermore, the treatment of both viruses, with the agents at concentrations that were previously appointed as effective, was also examined in the presence of solid material (quartz sand). All procedures investigated in this study, including the protease treatment, were able to reduce the titre of KHV and VHSV below the detection limit of the titration. Although further studies are necessary, this is the first report of the application of a protease for the inactivation of the selected fish pathogens, demonstrating the great potential of the latter for disinfection.

KEYWORDS
disinfect, koi herpesvirus, proteases, viral haemorrhagic septicaemia virus

1 | INTRODUCTION

Aquaculture plays a pivotal role as a source of high-quality food and is still growing faster than other major sectors of food production. Furthermore, inland aquaculture, based mainly on freshwater farming, contributed in 2016 51.4 million tons of food fish (64% of the world's farmed food fish production; FAO, 2018). Common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) belong to the major species produced in aquaculture (FAO, 2018) and are the most important farmed fish in Germany (Brämick, 2016).

Various pathogens, especially viruses, can cause diseases of farmed fish, resulting in high mortality rates and great economic

losses. Moreover, very often there is no cure and thus, once such a disease breaks out, the only action that can be taken is discarding diseased fish and disinfection of the affected pond, in order to prevent further spreading. However, the use of disinfectants for the control of pathogens is, especially in organic aquaculture, severely restricted. Therefore, further and new methods for combating viral diseases are needed.

Several disinfecting methods/agents are usually applied in aquaculture, for example draining, UV-irradiation, heating and incubation with quicklime (MULE, 2009). The application of quicklime is probably the most common method used in Germany (Bohl, 1999; Wedekind & Fuellner, 2015). Due to its rapid reaction with water, heat develops, causing a temperature increase as well as a pH shift to values above pH 10 (Neukirch, 2003; Roberts, 2001), thus being a very effective disinfectant by the combination of these two effects. Nevertheless, due to its highly caustic properties, it has to be handled cautiously. Used in high amounts, quick and burnt lime are toxic for aquatic plants and animals. Thus, the pH in the affected pond should be monitored and stocked with new fish only when the pH values have decreased to an acceptable level.

Peracetic acid (PAA) is another agent that is commonly used for disinfection and approved for use in organic aquaculture. It is available at concentrations ranging mainly from 5% to 15% L-1 in acidified, stabilized solutions with hydrogen peroxide and acetic acid. Due to the high reactivity of PAA, its concentration rapidly declines, especially in water rich in organic matter. Thus, water composition, temperature and characteristic of the system that should be treated have to be taken into consideration when calculating the dose (Pedersen, Jokumsen, Larsen, & Henriksen, 2015).

Finally, various chemicals were examined regarding their disinfecting properties against various viruses, including methylene blue, malachite green, Mefarol®, copper sulphate, sodium hypochloride, sodium hydroxide, formaldehyde, chloroform, iodophor, benzalkonium chloride or ethyl alcohol (Ahne, 1982; Dopazo et al., 2002; Kasai, Muto, & Yoshimizu, 2005; Neukirch, 2003). Nevertheless, the use of many of these agents in aquaculture is impossible, either due to safety risks, environmental considerations or for economic reasons.

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), causes the KHV disease (KHVD) and, due to its restricted host range, affects only common carp and koi, whereas some cyprinid species like goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*) and golden ide (*Leuciscus idus*) can likely act as its carrier (Bergmann et al., 2010, 2009). KHV virions are composed of an inner capsid, with icosahedral symmetry of approximately 100–110 nm diameter, containing the DNA genome and an amorphous layer of proteins—the tegument—enveloped by a lipid bilayer bearing viral glycoproteins (Michel et al., 2010; Miyazaki, Kuzuya, Yasumoto, Yasuda, & Kobayashi, 2008). While DNA of KHV can be found at high levels in environmental water and sediment, the virus was reported to remain infectious in such samples at 15–25°C for only up to 3 days without a susceptible host (Honjo, Minamoto, & Kawamata, 2012). Additionally, with the presence of some bacterial species in the water, it was shown that they support inactivation of the viruses (Shimizu, Yoshida, Kasai, & Yoshimizu, 2006; Yoshida, Sasaki, Kasai, & Yoshimizu, 2013).

Viral haemorrhagic septicaemia virus (VHSV), also known as Egtved virus, affects for example rainbow trout, turbot (*Psetta maxima*) and Japanese flounder (*Paralichthys olivaceus*). Over 80 different species proved to be susceptible for the disease (FLI, 2016; OIE, 2017). VHSV is a negative sense, single-stranded RNA virus with the genome encoding five structural proteins: nucleoprotein, phosphoprotein, matrix protein, glycoprotein, polymerase protein and one non-structural protein (Basurco & Benmansour, 1995; Schütze, Mundt, & Mettenleiter, 1999). Like other viruses from the rhabdovirus family, VHSV possesses a transmembrane glycoprotein responsible for the attachment to the cell membrane and for the entry into the cell by receptor-mediated endocytosis (Lecocq-Xhonneux et al., 1994).

The VHSV was reported to survive in the environment for very different periods of time, depending on temperature and the matrix surrounding the viral particles. For example, while it was deactivated in carp pond water within 48 hr, it stayed stable for up to 1 year in filtered freshwater at 4°C or for several months in sediments at the same temperature (Ahne, 1982; Hawley & Garver, 2008; Licek, 2011).

In a recent study it was determined that infectivity of KHV during in vitro replication decreases after reaching the maximum titre with progressing cell lysis (Mletzko et al., 2017). This could be due to proteolytic enzymes released from the cells that affect the proteins in the viral envelope, which in consequence perturbs virus entry into the cells and thus viral replication. Hence, the aim of this study was to examine if the commercially available proteolytic enzyme formulation, that is Neutrase®, can be used for inactivation of KHV and VHSV and thus can in future be considered as disinfecting agent. Additionally, and for comparison, two chemicals that are approved for the use in ecologic aquacultures, quicklime (CaO) and PAA, as well as the influence of various pH values were investigated in regard to inactivation of KHV and VHSV. Finally, disinfection experiments of the target viruses by the above-mentioned agents in the presence of quartz sand were performed, in order to test conditions that are slightly closer to real environmental conditions than simply disinfection in solution.

2 | MATERIAL AND METHODS

2.1 | Chemicals and solutions used

Two cell culture media—minimal essential medium Eagle (MEM, with Earle's salts, Sigma-Aldrich) and Dulbecco's modified Eagle medium (DMEM, Invitrogen Thermo Fisher Scientific)—were used in this work for cell maintenance. Antibiotic and antimycotic solution A5955 (with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml), quicklime (CaO), peracetic acid (PAA) and Accutase® were all purchased from Sigma-Aldrich. HEPES

buffer salt (Pufferan, ≥99.5%) was purchased from Carl Roth GmbH. Foetal calf serum (FCS) and non-essential amino acid solution (NEA) were obtained from Biochrom AG. Salts: sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2H_2O$) and potassium dihydrogenphosphate (KH_2PO_4), all of analytical grade, sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Carl Roth GmbH.

Phosphate-buffered saline (PBS, pH 7.4) was prepared by mixing 137 mM NaCl with 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, all dissolved in ultrapure water (H_2O_u; Millipore), autoclaved at 121°C for 20 min and stored at room temperature in darkness until use.

The protease formulation—Neutrase® (lot number: 041M1789V, Sigma-Aldrich)—that was used in this work had declared proteolytic activity of 0.84 U/g or 1.06 U/ml. The enzyme solution was filtered through a 0.2 µm polyethersulphone (PES) membrane (Sarstedt), stored at 8°C (in darkness) and diluted in the respective solution just before the application. PAA was also stored at 8°C and diluted shortly before use.

Calcined and purified quartz sand with grain-size distribution between 100 and 300 µm was obtained from Sigma-Aldrich. A standard soil (type no. Sp.2.44212), characterized as clayey loam, was received from the Agricultural Research Institute LUFA.

2.2 | Cell culture

Common carp brain cells (CCB, CCLV-RIE 816, passage 83, provided by the Friedrich-Loeffler-Institut [FLI]) were maintained in 25-cm^2 cell culture flasks (T-25; Sarstedt) in MEM supplemented with 25 mM HEPES, 1 × NEA and 10% FCS at 25°C with 5% CO_2 (Neukirch, Böttcher, & Bunnajirakul, 1999). Rainbow trout cells (RT/F, CCLV-RIE 88, provided by FLI) were cultured in the same culture flasks in DMEM, supplemented with 25 mM HEPES, 0.5 M $NaHCO_3$ and 10% FCS at 20°C. For the virus titration (50% tissue culture infective dose assay, $TCID_{50}$), CCB cells as well as RT/F cells were cultivated in DMEM, supplemented with 25 mM HEPES, 0.5 M $NaHCO_3$, 10% FCS, 100 U/ml of penicillin G, 0.1 mg/ml of streptomycin sulphate and 0.25 µg/ml of amphotericin B.

2.3 | Virus stocks

KHV-TP 30 (KHV-T), isolated by Dr. Peiyu Lee (Taiwan, 2005), was provided by the Friedrich-Loeffler-Institut (FLI). KHV stocks for disinfection studies were prepared by inoculation of CCB cells seeded in MEM (as described above) or DMEM in T-75 flasks with a density of 60,000 or 75,000 cells/cm^2, respectively. Seeded cells were incubated overnight at 25°C. After discarding the supernatant, 3 ml of virus suspension was added to the cells and incubated for 1 hr at room temperature. Subsequently, 21 ml of MEM was added and cells incubated at 25°C for up to 60 hr post-infection (p.i.). The replication was stopped by placing the whole cell culture in T-flask at −80°C. Next, frozen cells and medium were thawed, aliquoted (1 ml), introduced to titre determination and frozen at −80°C for further use.

Virus stock of VHSV (isolate Fi13, FLI) was prepared by propagation onto RT/F cells in above described DMEM. Cells were seeded with a cell density of 28,000 cells/cm^2 and cultured over 2 days at 20°C. Afterwards, the medium was removed, 7 ml DMEM with 10% FCS and 50 µl virus suspension was added and cells incubated at 20°C for up to 5 days post-infection (d.p.i.). Next, the replication was stopped and the virus harvested, aliquoted, virus titre determined according to the below described procedure and stored for further analysis as described below.

2.4 | Analysis of virus suspensions

Virus titres were determined by endpoint titration via $TCID_{50}$ assay in 96-well plates according to Reed and Muench (1938) as previously described by Mletzko et al. (2017). CCB cells were utilized for determination of KHV titres, whereas RT/F cells were used to appoint titres of VHSV. The read out was performed after 10 days, defining infected wells as those in which cytolytic, cytopathic effects and/or syncytia (in the case of KHV) caused by virus infection could be observed. The results were expressed as $TCID_{50}$/ml.

The limit of detection and limit of quantification of $TCID_{50}$ assay depend on the sample volume used for the experiments and was calculated for each respective experiment.

2.5 | Investigation of stability of KHV and VHSV in cell culture medium

Prior to the disinfection experiments, the stability of KHV and VHSV in cell culture media (MEM or DMEM) was examined. Therefore, virus aliquots were stored in the dark in sterile 50-ml polypropylene (PP) test tubes (Sarstedt) at 8 and 25°C (KHV) or 8 and 20°C (VHSV), respectively, up to 44 days. Aliquots of these solutions were sampled at various time points and analysed via $TCID_{50}$ assay as described above.

2.6 | Influence of buffering capacity of the cell culture medium on experiments with CaO

As the main disinfecting effect during application of CaO results from pH shift to pH >10, the buffering capacity of the cell culture media and PBS (used for virus inactivation experiments) was tested. Therefore, various concentrations of CaO, ranging from 10^{-2} to 10^{-6} mM, were prepared in ultrapure water, in DMEM with 10% FCS and in PBS solution (pH 7.4) and the respective pH values were measured.

2.7 | Examination of various agents for inactivation of virus particles

Generally, the influence of various agents for deactivation of KHV and VHSV was examined by incubation of the respective virus suspensions with the selected reagents at various concentrations (n = 3) in 1.5-ml PP reaction tubes (Sarstedt) for 24 hr at selected

temperatures. Next, samples were submitted to $TCID_{50}$ assay for determination of their infectivity after the treatment (end titre, T_{end}).

For investigation of the pH influence on viral stability, the pH of 1 ml virus suspension in DMEM (as described above) was adjusted by adding sterile filtered 1 M NaOH or 1 M HCl in ultrapure water (H_2O_u) to reach pH values of 3.0, 4.5, 6.0, 7.4, 9.0, 10.5, 12.0 and the assays were incubated for 24 hr at 8°C. The virus suspension with a pH of 7.4 (standard pH for cultivation) was used as a control and its virus titre designated as start titre. If the pH of a sample deviated from 7.4, it was adjusted to this value prior submission to $TCID_{50}$ assay by addition of predetermined volumes of NaOH (1 M) or HCL (1 M).

For experiments with PAA, 800 µl of the respective virus suspension were supplemented with 200 µl of various PAA dilutions (in H_2O_u, sterile filtered) to reach final concentrations of this agent ranging from 0.003 to 0.3 vol.-% for KHV and 0.0005 up to 1 vol.-% PAA for VHSV, incubated for 24 hr at 8°C and submitted to virus titration.

In order to test KHV inactivation with quicklime under possibly controlled conditions, CaO was prediluted in cell culture medium (DMEM with 10% FCS) to reach final concentrations of this agent for the incubation with the virus ranging from 10^{-4} and 1.5×10^{-2} M. Next, 900 µl of CaO solutions were mixed with 100 µl of the KHV suspension, incubated for 24 at 8°C and submitted to virus titration. The control samples were prepared by mixing of the respective volumes of the virus suspension used for the inactivation and the cell culture medium.

In order to investigate the potential of a proteolytic enzyme for inactivation of KHV (n = 2) and VHSV (n = 3), Neutrase® formulation was prediluted in PBS and 100 µl of the prediluted enzyme added to 900 µl of the respective virus suspension to achieve final concentrations ranging from 0.0004 to 8 mU/ml. Positive controls were prepared by mixing of 100 µl of PBS with 900 µl of the respective virus suspension. All samples were incubated for 24 hr at 8°C. Additionally, negative controls with cells but without virus or disinfectant were pipetted on each $TCID_{50}$ plate and monitored for 10 days for comparison.

2.8 | Disinfection of virus in the presence of quartz sand

Prior to the disinfection experiments with KHV and quartz sand, the removal of the virus from solution due to the sorption on sand and standard soil was examined. Therefore, 100 mg of solid material was weighed into five 1.5-ml reaction tubes and autoclaved for 20 min at 121°C. Subsequently, 1 ml of a virus suspension with five subsequent 10-fold dilutions, ranging from approximately from 10^2 to 10^6 $TCID_{50}$/ml were added and the samples were incubated for 1 hr at 25°C (500 rpm, ThermoMixer C; Eppendorf). For a comparison, samples containing the respective virus dilutions (1 ml) were placed in empty 1.5-ml PP tubes and treated comparably to those with quartz sand or soil. After 1-hr incubation at 25°C (500 rpm), the sample tubes were centrifuged for 5 min at 5,000 *g* at room temperature, the supernatants were collected and the virus titres determined via $TCID_{50}$ assay. For evaluation, virus titres—introduced and remaining after the incubation—were recalculated using the respective dilution factors and the concentrations of the virus particles as well as standard deviations prior and after incubation with the solid material were calculated.

Further experiments were performed with selected concentrations of disinfectants and KHV in the presence of quartz sand, in order to prove that the disinfection is also successful in the presence of this matrix. For this purpose, 100 mg of autoclaved quartz sand in 1.5-ml reaction tubes were incubated with 500 µl KHV suspension (1.5×10^6 $TCID_{50}$/ml), and mixed with 1 ml of three different disinfectant solutions resulting in following final concentrations: (a) 0.01 M CaO, (b) 0.1 vol.-% PAA, (c) 53 mU/ml Neutrase® or 1 ml cell culture medium without disinfectant as positive KHV control (d). A negative control was prepared by mixing 1.5 ml DMEM (as described above) with quartz sand but without virus (e). Next, these samples were incubated for 24 hr at 8°C.

At the same time, cell culture flasks were seeded with 60,000 cells/cm^2 of CCB in MEM (as described above under Section 2.2) and incubated overnight at 25°C with 5% CO_2. After that, the medium supernatant was removed and the 1.5 ml of DMEM with silica sand either with or without virus or disinfectants were transferred into the seeded cell cultures. Finally, the volume was adjusted to 8 ml with DMEM and the quartz sand distributed by soft panning of the flasks. The cells were incubated at 25°C and monitored for syncytia or cytopathic effects caused by the KHV infection over 10 days.

3 | RESULTS

3.1 | Stability of KHV and VHSV in cell culture medium

The infectivity of KHV incubated in two different cell culture media: (a) MEM—buffered with the bicarbonate buffer system, used mainly for cell maintenance; (b) DMEM—buffered with HEPES, utilized for titre determination, was examined at two different temperatures: 8 and 25°C (used for its in vitro replication) and up to 44 days prior to further experiments. The KHV titre was determined at different time points and expressed as $TCID_{50}$/ml as shown in Figure 1a. The same investigation, covering 30 days, was performed for VHSV in DMEM at 8 and 20°C (applied for propagation of VHSV; Figure 1b). As depicted in the Figure 1, the reduction of the titre of both viruses progressed relatively slowly in DMEM at 8°C, resulting in comparably high $TCID_{50}$ values for up to ca. 20 days. However, when incubated in the bicarbonate-buffered MEM at 8°C, the reduction of the KHV titre was about 2.9-fold faster in comparison to an incubation in DMEM. Moreover, the stability of both viral pathogens in DMEM at elevated temperatures was greatly reduced. The infectivity of KHV decreased by a factor of nearly 10 within only 5 days of incubation in DMEM at 25°C. The reduction of VHSV titre in the same medium at 20°C was slower, resulting in a comparable drop within 10 days of

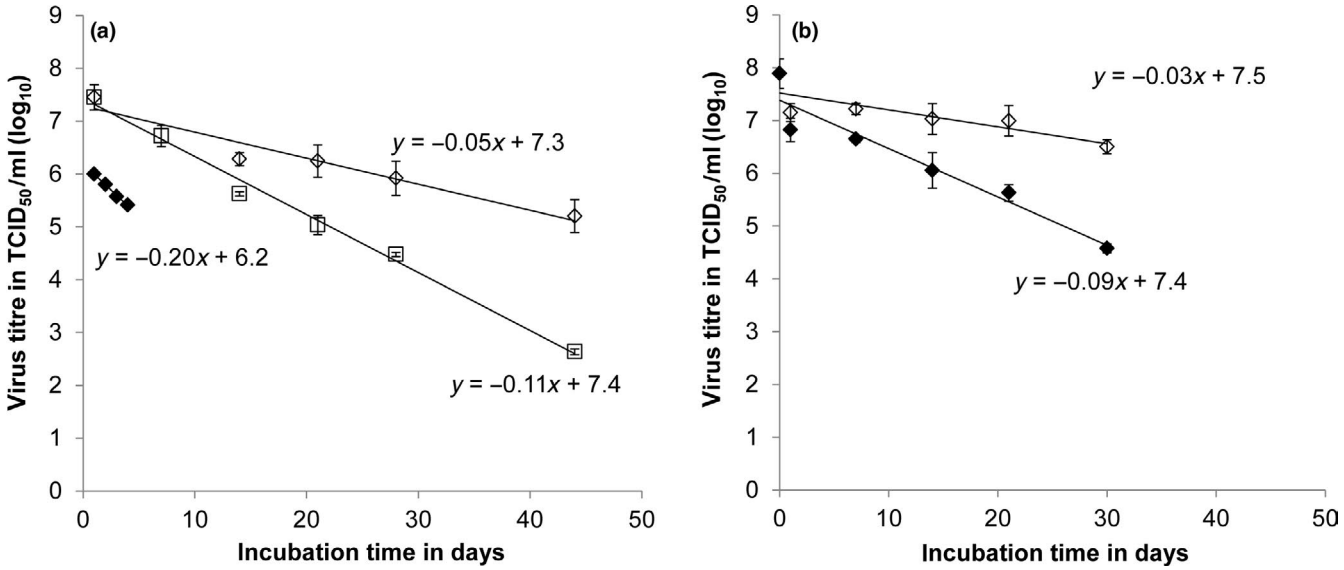

FIGURE 1 Stability of KHV (a) and VHSV (b) in different media and temperatures. (□) Virus in MEM medium at 8°C; (◊) virus in DMEM at 8°C; (♦) virus in DMEM at 25°C (KHV) or 20°C (VHSV). Virus titre was estimated at different time points by $TCID_{50}$-assay and expressed as log_{10}

incubation and lower slope (factor 2) in comparison to that of KHV at 25°C.

3.2 | Determination of the buffering capacity of cell culture medium in regard to CaO

In order to evaluate the buffering capacity of cell culture media used for titre determination as well as present in virus suspension used for inactivation procedures, solutions with various concentrations of CaO in DMEM were prepared, their pH measured and compared to the pH reached by adding the same amount of CaO to ultrapure water. Results are displayed in Table 1. As expected, in ultrapure water, pH values changed in relation to the CaO concentration in the solution, while in DMEM medium, with its higher buffering capacity, a substantial pH shift was registered only at concentrations of CaO higher than 10^{-3} M. Thus, no substantial influence on virus stability due to a pH shift can be expected when performing inactivation experiments in this cell culture medium with CaO at concentrations lower than that.

3.3 | Deactivation of viral particles in suspension

The influence of the 24 hr application of various agents, such as pH, PAA formulation, quicklime and a proteolytic enzyme formulation—Neutrase®—on the infectivity of KHV and VHSV in suspensions at various temperatures was investigated. Results are presented in Figure 2a–d.

The results of 24-hr incubations of KHV and VHSV in suspensions at various pH values are shown in Figure 2a. In order to adjust the pH, acid (HCl) or base (NaOH), solutions were added to the virus suspension in cell culture media, resulting in slightly different virus titres prior to the incubation. Due to the high uncertainty of the titre determination via end dilution method ($TCID_{50}$ assay), no relevant titre reduction could be observed for either KHV or VHSV when incubated in medium with pH ranging from 6 to 9. At pH of 10.5, a titre reduction at least by two orders of magnitude was observed for KHV and VHSV. At pH values as low as 3 and 4.5 or as high as 12, no infectious viral particles could be detected for either of the two viruses.

TABLE 1 pH of cell culture medium and ultrapure water at various concentrations of quick lime

Concentration of CaO[a] [M]	Measured pH values	
	Ultrapure water	DMEM with 10% FCS
10^{-2}	12.6	12.3
10^{-3}	11.7	7.8
10^{-4}	10.7	7.5
10^{-5}	10.0	7.5
10^{-6}	8.8	7.5

Abbreviation: FCS, foetal calf serum.
[a]CaO—quick lime.

Incubation of KHV and VHSV at 8°C (24 hr) with concentrations of PAA ≥ 0.1 vol.-% (pH of the medium of 4.2) also resulted in deactivation of viral particles (see Figure 3b and Table 2 for pH values of corresponding PAA solutions). At PAA concentrations ≤0.01 vol.-% (with pH between 6.9 and 7.3), the reduction of titres of both pathogens was about or lower than two magnitudes. When applying concentrations ≥0.1 vol.-%, no infectious particles of either of the viruses were detected.

Incubation of KHV with CaO concentrations below 0.01 M at given conditions showed no titre reduction, while when treated in 0.015 M CaO medium no infectious viral particles of KHV could be detected after the incubation.

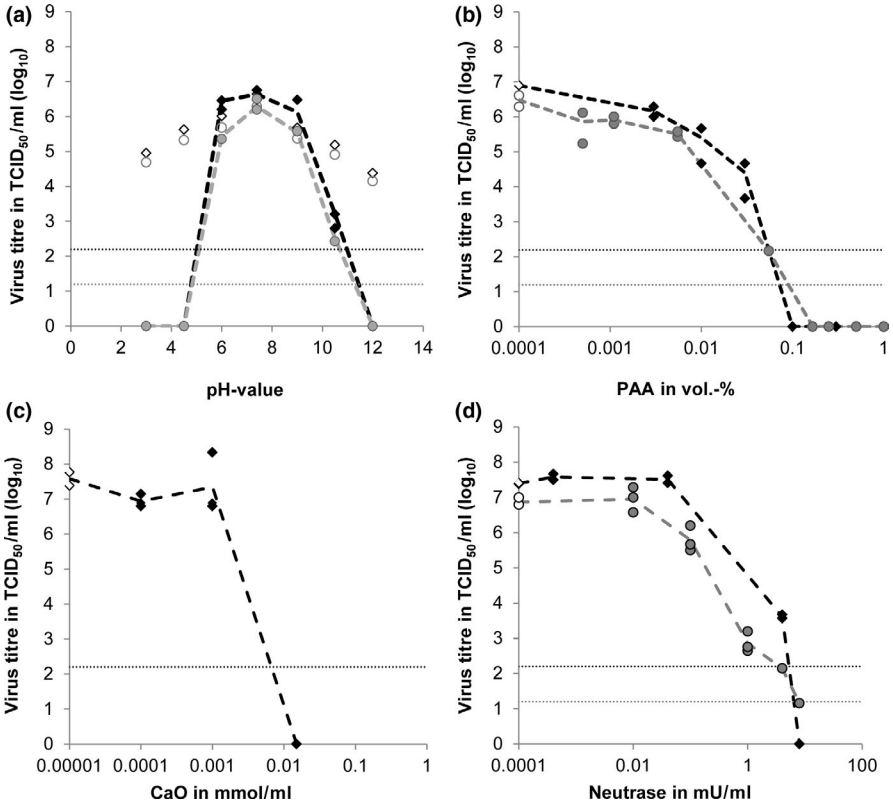

FIGURE 2 Efficacy of pH (a), PAA (b), CaO (c) and Neutrase® (d) against KHV and VHSV. Virus titre of KHV (◆, mean ---) or VHSV (●, mean ---), expressed as $\log_{10}$ ($TCID_{50}$/ml). Introduced virus load of KHV (◊) and VHSV (○) calculated for the respective sample based on the control and resulting dilution. Limit of the quantification for KHV (···) and VHSV (···) estimated by the $TCID_{50}$-assay and the respective dilution factor was 2.2 and 1.2 $\log_{10}$ ($TCID_{50}$/ml), respectively

Finally, the possibility of the application of proteolytic enzymes for the inactivation of KHV and VHSV was tested in this work. Virus suspensions were incubated at the same temperature and for the same period of time with various amounts of a commercially available protease from *Bacillus amylolioquefaciens*—Neutrase®—and the resulting titres were determined via $TCID_{50}$ (Figure 2d). The enzyme treatment of KHV reduced the virus titre up to more than three orders of magnitude at a nominal proteolytic activity of 4 mU/ml from an initial virus titre of 10^7/ml. The disinfection of VHSV (four or more powers of magnitude) was achieved already at nominal proteolytic activity of 1 mU/ml, whereas the incubation of both viruses with 8 mU/ml of Neutrase® resulted in titres under the detection limit of the assay.

3.4 | Inactivation of virus particles in the presence of quartz sand

Sand was reported to be one of the main components of the pond sediments in the Bavarian region with an average of 31% weight in mineral soil (Mletzko, Oberle, & Christian, 2014). So, calcined quartz sand and a defined standard soil (type Sp.2.44212) were used as model materials in this work to examine the possibility of KHV removal from the pond water via sorption on the sediment. The experiments were performed with five consecutive 10-fold virus dilutions that were incubated for 1 hr at 25°C with or without the solid material. Next, the remaining infective virus titre in the supernatant was determined via endpoint dilution assay, recalculated to undiluted virus to calculate virus sorption. Resulting titres were submitted to a paired *t* test to examine statistical significance. The mean KHV titre determined for samples incubated with quartz sand was 9.7×10^5 $TCID_{50}$/ml (*SD* of 9.3×10^5 $TCID_{50}$/ml) while the titre without sand was found to be 1.4×10^6 $TCID_{50}$/ml (*SD* of 3.6×10^5 $TCID_{50}$/ml). This suggests that ca. 30% of the viruses were adsorbed on the sand matrix. However, due to the high standard deviation related to the endpoint dilution method, no statistical significance was found for titres of samples incubated with or without sand (p_{value} = .429). After the incubation with the standard soil a significant removal of the virus (ca. 50%, p_{value} = .019) from the solution was detected. The mean titre calculated for the samples with the soil was determined to be 1.5×10^5 $TCID_{50}$/ml (*SD* of 5.1×10^4 $TCID_{50}$/ml), whereas the titre without soil was 3.3×10^5 $TCID_{50}$/ml (*SD* of 7.6×10^4 $TCID_{50}$/ml).

Furthermore, the inactivation of the virus using CaO, PAA and Neutrase® in the presence of quartz sand was investigated in this study. For that, KHV suspension was mixed with autoclaved sand and the disinfecting agent to reach the selected concentration of the latter. After a 24-hr incubation, the treated virus–sand mixture was placed on the cell culture in the T-flask and monitored up to 14 days for cytopathic effects typical to the KHV infection (Figure 3a,b,c). As positive control, virus–sand mixtures without any disinfectant and plain medium–sand samples were prepared and incubated with CCBs cells (Figure 3d,e). While no infection could be observed over 14 days for all examined agents, the cells incubated with KHV–sand samples untreated with disinfectant agent showed typical cytopathic effects already 4 d.p.i. Nevertheless, it has to be mentioned that the cell monolayer mixed with the protease treated virus

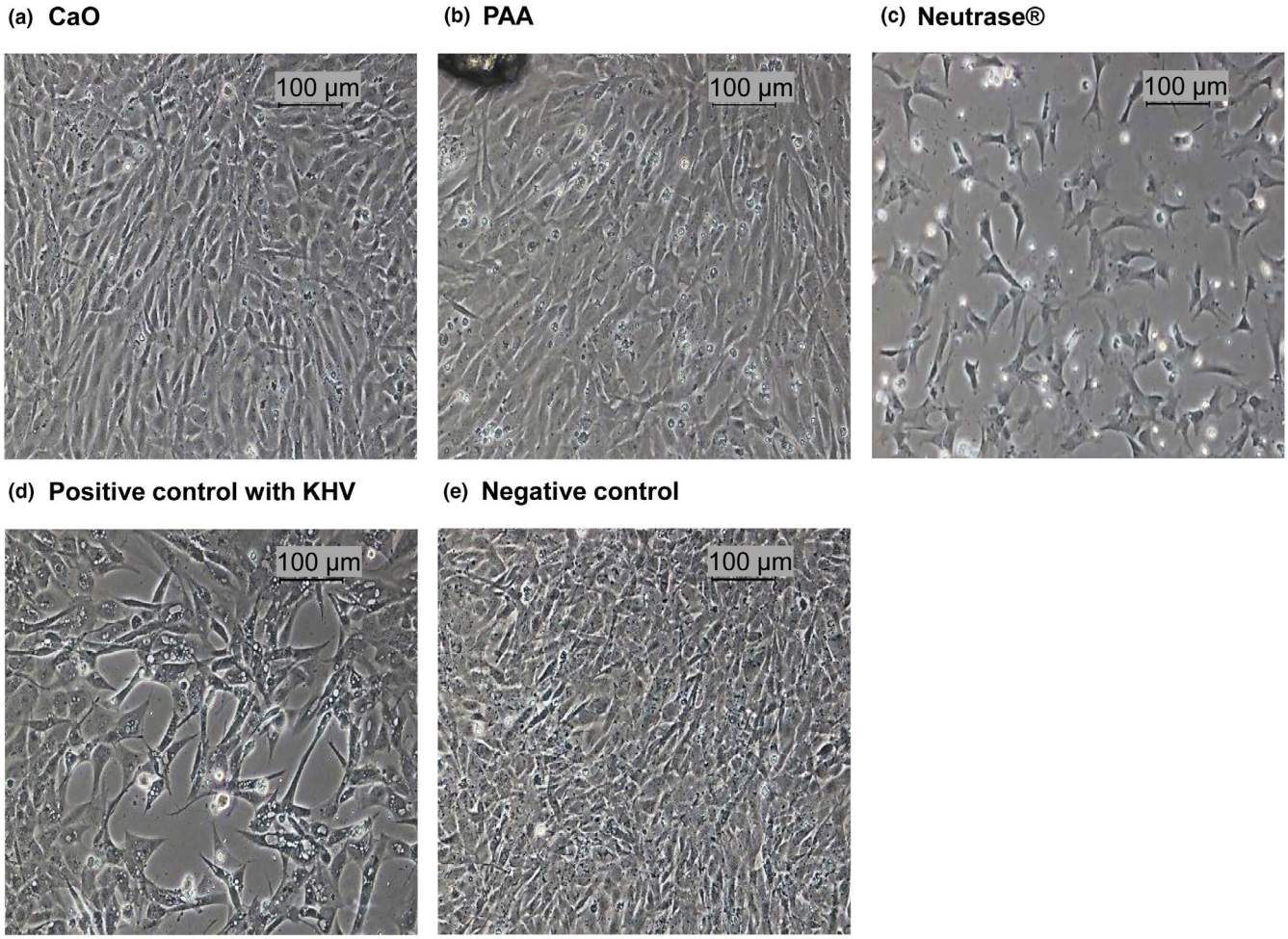

FIGURE 3 Disinfection of KHV in the presence of quartz sand with 0.01 M quicklime (a), 0.1 vol.-% PAA (b), or 53 mU/ml Neutrase® (c) for 24 hr at 8°C. Detection of possibly residual infective virus particles by incubation of suspension and quartz sand on CCB cells for 10 days at 25°C; positive control with cytopathic effect of KHV (d) and negative control without virus (e). Pictures taken on day four post-infection

suspension (Figure 3c) was apparently less dense and showed more stress symptoms in comparison to the samples with CaO and PAA treated virus. This is mainly due to the fact that while the cytotoxic effects of CaO and PAA were avoided by readjusting the pH to 7.4 of the virus suspension after inactivation, the protease remained active causing stress to the applied cell culture. No negative effects on the cell culture were observed for the negative control (sand mixed with cell culture medium).

4 | DISCUSSION

The stability of KHV and VHSV was influenced both by temperature and cell culture media used for incubation. At 8°C, the titre of both pathogens stayed more or less stable for up to 30 days, taking the standard deviation of the $TCID_{50}$ assay into account. In comparison, at higher temperature (20 or 25°C) a titre loss of one log step was observed already after 7 (KHV) or 14 days (VHSV), which can presumably be explained by a higher activity of enzymes in the virus preparations at elevated temperatures. These findings correlate well with the data published by Hawley and Garver (2008) or Parry and Dixon (1997) showing inverse correlation between survival of VHSV isolates and the temperature. While at 10°C VHSV incubated in filtered (0.45 µm) fresh and sea water kept active up to 300 and 6 days, respectively, at 20°C 99.9% loss of their infectivity were noticed accordingly around day 50 and 2. Shimizu et al. (2006) reported 2 or 3 decades reduction of KHV titre (Hanks' buffered salt solution or water) within 7 days at 15°C and 25°C, respectively. Perelberg et al. (2003) described that KHV added to water for immersion was still able to infect fish after 4 hr but not after 21 hr of incubation and finally Kasai et al. (2005) showed that a heat treatment (50°C) in cell culture medium (Leibovitz's L-15 medium with 10% FBS) led to effective inactivation of KHV already after 1 min. Furthermore, as mentioned above, the composition of the virus suspension or the cell culture media in which the virus is stored also seems to be relevant. In this study, the reduction of KHV titre during the incubation at 8°C was faster in MEM than in DMEM. As the experiment was performed without additional CO_2 in the atmosphere, and since the first medium requires 5% CO_2 to keep the pH value at 7.4, the quicker depletion of infectious viral particles can most probably be explained by the pH shift (pH ≥ 9) during the incubation. Even though no pH

TABLE 2 pH values of the cell culture medium at various peracetic acid concentrations

Concentration of PAA [vol.-%]	Measured pH
1.0000	3.2
0.5000	3.4
0.2500	3.6
0.1700	3.9
0.1000	4.2
0.0550	4.6
0.0300	5.4
0.0100	6.9
0.0055	7.1
0.0030	7.2
0.0011	7.3
0.0005	7.3
0.0000	7.3

measurement was performed in this experiment, this hypothesis is supported by the observed colour shift of the pH indicator present in the medium.

The variation of the pH value of the cell culture medium applied in this study resulted in the inactivation of both investigated viruses within 24 hr at pH ≤4.5 and ≥12 which correlates well with the published data (Dixon et al., 2012; Licek, 2011). For example, in the review of Licek (2011), KHV was reported to retain its infectivity only for up to 2 hr at pH of 3 or 11. In the same publication, it was described that VHSV resists inactivation only for 10 min at pH of 2.5 or for 2 hr a pH of 12. Additionally, Ahne (1982) described a successful disinfection of VHSV in a medium at pH of 3 within 3 hr. Furthermore, a similar behaviour was reported previously for other enveloped viruses. Chang, Chen, and Wang (1998) showed that white spot syndrome baculovirus was inactivated within minutes in highly acidic or alkaline solutions at room temperature.

The results described here as well as by Oberle et al. (2016) underline that composition, buffering capacity and pH of the matrix (e.g., soil, water), submitted to disinfecting actions, have to be considered carefully when selecting or calculating the amount of the agent that should be used. The higher the buffering capacity of the solution to be applied, the higher the amount of for example quicklime has to be considered, in order to obtain the same increase of the pH, thus leading in consequence to elevated costs. Therefore, both parameters should be examined prior to the use of a disinfection procedure, in addition to the necessary monitoring of the pH after the application of the agent to assure the desired disinfecting effect. On the other hand, it should always be considered that effective procedures against the pathogens involving particular high concentrations will also affect other species present in the pond and/or sediments. Such biological systems thus will require some time to recover from the treatment.

Due to its specific characteristics, the disinfecting properties of PAA consist of oxidation and acidification potential (Bützer, 2012). As the amounts of PAA that lead to inactivation of KHV and VHSV also resulted in pH shifts of the medium to ≤4.2, that lead to a disinfecting effect in its own right in addition to the oxidation, no conclusion about the individual contribution of acidification or oxidation can be made based on the data presented here.

Due to the predilution of CaO, the resulting temperature increase did not contribute to the inactivation of KHV in the experiment described here. Thus, the only effect that can be discussed is the influence of the pH shift due to the application of this agent. As CaO was prediluted in cell culture medium with a high buffering capacity (Table 1), only the highest used concentration resulted in pH value above 12 and led to complete inactivation of the virus. This observation corresponds well with the data regarding virus inactivation at various pH values (Figure 2c).

In the disinfection tests described here, the applied pH value seemed to play an important role regardless of the disinfectant used. This might be related to the fact that both investigated viruses possess lipid envelopes with integrated glycoproteins, which are crucial for attachment and penetration of the cell membrane (Bruss & Ganem, 1991; Cocquerel, Wychowski, Minner, Penin, & Dubuisson, 2000; Lecocq-Xhonneux et al., 1994; Mettenleiter, Klupp, & Granzow, 2009; Michel et al., 2010; Miyazaki et al., 2008). Strongly acidic or basic conditions not only catalyse protein hydrolysis (especially at increased temperature) but can also influence their structure and cause their irreversible denaturation. Furthermore, many proteins form aggregates at a pH value close to their isoelectric point resulting in precipitation. Any change in the structure of the viral envelope can hamper virus entry and therefore diminish or even completely obstruct virus replication. Thus, pH is an important antiviral parameter that has to be considered for pond disinfection. Furthermore, investigations by the Institute for Fisheries, Bavarian State Research Center for Agriculture showed that the pH values of water and sludge can vary strongly depending on season, environmental conditions or mineral composition of the investigated area (Oberle et al., 2016). Although the average pH of pond soil of all 86 examined ponds in Bavaria was 6.3, the values ranged from a minimum of pH 4.8 (around Schwandorf and Tirschenreuth, Germany) to a maximum of pH 7.8 (Aischgrund, Germany). Based on these results, a recommendation for a suitable disinfectant cannot be made without detailed knowledge of the characteristics of the matrix (water, sediment) that should be submitted to such a treatment.

Finally, an incubation of KHV and VHSV suspensions with a proteolytic enzyme—Neutrase®—produced with the help of *Bacillus amyloliquefaciens*, also led to reduction of titre of these two viruses above three powers of magnitude. The highest protease concentration applied for virus inactivation in this work (8 mU/ml nominal proteolytic activity) resulted in titres below the detection limit of the endpoint dilution assay for both investigated viruses. Thus, the application of the proteolytic enzyme can lead to an effective inactivation of the selected viruses and prevent their further spread. In principal, even more pronounced titre reductions at higher enzyme concentrations are most likely. However, since the residual activity of the enzyme (as visible at Figure 3c)

can have an influence on the cells used for the $TCID_{50}$ examination, it was not possible to test this correlation in this experimental set-up. Viral DNA can be quantified using real-time polymerase chain reaction (qPCR) thus giving an alternative assay for quantification. However, it cannot be regarded to be a valid substitute for virus titration using cell culture since the copies of viral DNA does not correlate with infective viral particles in such experimental set-up. For future studies however, a monitoring of the expression of viral genes in cell cultures after addition of enzyme-treated virus via reverse transcriptase qPCR could be included for a further evaluation of such experiments. Moreover, a possibility of enzyme inactivation or separation prior submission to $TCID_{50}$ assay can be considered.

However, the presence of 10% FCS in the cell culture medium used here seems not to affect the virus inactivation by Neutrase®, further investigations regarding the influence of organic matter on the protease treatment are needed. Nevertheless, as proteases are readily biodegradable they are a highly interesting alternative for the disinfection of fish virus-infected soil or water, particularly in organic fisheries. As the temperature applied in this study (8°C) was below the temperature optimum of this enzyme (40–50°C; product specification; Novozym) the treatment is expected to be significantly more effective at higher temperatures. The success of the proteolytic treatment can possibly be related again to the glycoproteins located in the viral envelopes that might be destroyed due to the enzymatic activity which in turn was responsible for the inactivation of the virus particles. This observation matches well the data reported by Yoshida et al. (2013) who described inactivation of KHV by incubation with various bacteria producing extracellular proteases, among others, *Bacillus amyloliquefaciens*. Similar findings were published by Shimizu et al. (2006), who found 12 bacterial strains (isolated from environmental waters) with anti-KHV properties. Although the disinfection with Neutrase® described here proved to be very promising in in vitro experiments, further investigations are necessary in order to evaluate the possibility of its use to combat viral disease in aquaculture at practical conditions. Again, as described above for other disinfectants, environmental conditions (such as pH, temperature, buffer capacity, water/sediment composition) have to be taken into account and compared with the optimum of enzymatic activity of the selected protease. Furthermore, although it was reported that Neutrase® is biodegradable and has no potential for accumulation (Safety data sheet, Novozym), further studies are necessary to evaluate in detail its impact on the aquatic environment in case of such an application. However, the low nominal proteolytic activity of Neutrase® used in this study (0.80 U/g) makes use of this particular enzyme preparation as disinfectant in practice meaningless from an economic point of view. Nevertheless, other enzymes with a higher specific activity, even as high as 500,000 U/g, are available on the market, so the amount of enzyme necessary for disinfection might be reduced substantially. Obviously, the inactivation of selected viral pathogens with such protease formulations has high potential, but requires further studies.

Finally, all agents tested here were able to inactivate KHV at selected concentrations even in the presence of quartz sand. While sorption on this matrix could not be measured directly in this study, KHV removal (~50%) from suspension due to the presence of a standard soil sample was observed, indicating a sorption, albeit a weak one. This observation is supported by literature, where sand is described as poor adsorbent for enteric viruses (Sobsey, Dean, Knuckles, & Wagner, 1980). However, the viruses investigated by Sobsey et al. (1980) were non-enveloped and thus might behave substantially different from KHV and VHSV.

5 | SUMMARY AND CONCLUSION

All investigated disinfection methods proved to have the potential to deactivate the two chosen representatives of notifiable viral pathogens of epizootic diseases, KHV and VHSV. The disinfectants that are already approved for application in organic aquaculture, such as peracetic acid and quicklime, as well as the newly introduced protease Neutrase®, were able to reduce the viral load of KHV and VHSV by at least four powers of magnitude within an exposure time of 24 hr. In summary, this is the first report of the application of this commercially available protease for inactivation of fish viruses, showing its principally high potential as an alternative disinfecting agent. Nevertheless, further studies regarding applicability of proteases at environmental conditions, as well as environmental and economic aspects are necessary.

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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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