Use of anionic polymer-coated magnetic beads to pre-concentrate *Ostreid Herpesvirus 1* from seawater: application to a UV disinfection treatment

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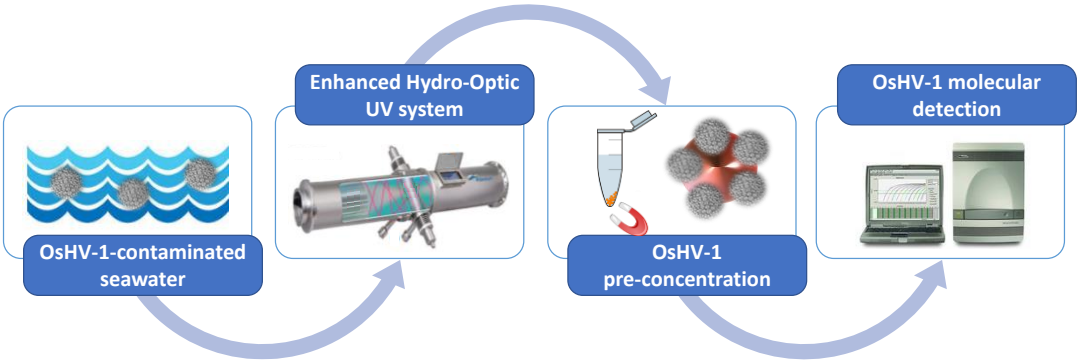
Abstract

Ostreid Herpesvirus 1 (OsHV-1) represents a serious threat to shellfish aquaculture worldwide. To minimise its impact, early warning systems able to detect the virus in seawater prior to infection of oysters are of utmost importance. However, monitoring OsHV-1 in seawater is challenging because of its presence at very low concentrations. Thus, a rapid and simple method to pre-concentrate the virus is needed to enable detection. Herein, magnetic beads (MBs) coated with an anionic polymer were used to pre-concentrate OsHV-1 from biological matrices including oyster homogenate and seawater samples. Following virus capture, OsHV-1 DNA detection was performed by quantitative PCR (qPCR). The MB-based approach combined with qPCR attained a limit of detection (LOD) as low as 0.1 viral copy/µL, which was 100 times lower than that of the qPCR alone. This approach was applied to the analysis of OsHV-1 in seawater from an Enhanced Hydro-Optic UV (HOD-UV) disinfection experiment operating at a UV dose of 1,360 J/m^2 in an open flow system. Our approach enabled detection of the virus in non-treated seawater and not in UV-treated seawater, discrimination that was not possible using qPCR alone. Moreover, the strategy provided data on the pattern of kinetics of the release of the virus in seawater. The approach could find applications in shellfish hatcheries and depuration plants to ensure biosecurity requirements.

Keywords: magnetic beads, *Ostreid Herpesvirus 1* (OsHV-1), virus pre-concentration, quantitative PCR (qPCR), Enhanced Hydro-Optic UV (HOD-UV) system.

Graphical abstract



Highlights

- Anionic magnetic beads enabled pre-concentration of OsHV-1 from seawater
- Magnetic beads enabled a reduction of the LOD of the subsequent molecular method
- A virus kinetics pattern during the disinfection experiment was observed

1. Introduction

Detection of microbiological contaminants in water is becoming increasingly important in environmental monitoring. Although much progress has been made in the development of new detection tools, other steps besides detection itself need to be improved to eventually implement such tools. In the field of water quality analysis, a sample pre-concentration step is particularly necessary because contaminants might be present in the sample at very low concentrations (Goodwin and Litaker, 2008). To achieve this, filtration or centrifugation are commonly used to pre-concentrate microorganisms such as bacteria, microalgae or protozoa prior to their detection (Karlson et al., 2010). Given their small size, pre-concentrating viruses is more complex. Even though conventional low-speed centrifugation has been used to pre-concentrate some viruses, which may be associated to larger particles (Evans et al., 2014; Liu et al., 2020), pre-concentrating viruses typically requires ultracentrifugation technology, which greatly increases costs and analysis time, thus being impractical for screening purposes. As a result, new methodologies to detect viruses in natural waters are highly desirable.

To tackle this challenge, other procedures have been proposed including polyethylene glycol (PEG)-mediated precipitation (Beyer et al., 2020), chromatography (Kutner et al., 2009), adsorption on membranes (Vincent-Hubert et al., 2017) and capture by magnetic beads (MBs) (Veyret et al., 2005). Because of its simplicity, rapidity, low cost and compatibility with subsequent analysis tools, the use of MBs as capture agents to pre-concentrate viruses has attracted particular interest. MBs coated with molecules that efficiently bind to the virus allow to separate and pre-concentrate virus particles from complex matrices by simply applying a magnetic field. In this sense, MBs coated with antibodies or, more recently, organic chemicals, have been successfully used to capture different types of viruses. Whilst the use of antibody-coated MBs intrinsically requires the availability of antibodies able to bind a specific type or family of virus (Myrmel et al., 2000), the use of organic polymers allows the capture of viruses in a more general manner. This is the case of polyethyleneimine (PEI) (Iwata et al., 2003; Uchida et al., 2007), poly (methyl vinyl ether-maleic anhydride) (poly (MVE-MA)) and their derivatives, whose molecular and physicochemical characteristics allow them to be used as bio-adhesives. MBs coated with anionic poly (MVE-MA) have been used to capture several types of viruses including non-enveloped viruses like adenoviruses (Sakudo et al., 2016), and enveloped viruses including human immunodeficiency virus (HIV) (Sakudo and Ikuta, 2012), respiratory syncytial virus (RSV) (Sakudo et al., 2009a), influenza virus (Sakudo et al., 2009b; Sakudo and Ikuta, 2008), borna disease virus (BDV) (Sakudo et al., 2011b) and dengue virus (Patramool et al., 2013). However, their universal applicability to all virus types has not been demonstrated. Although the exact mechanisms of interaction between the virus particle and the anionic MB remains to be fully elucidated, it has been hypothesised that electrostatic, hydrophilic and hydrophobic interactions are involved (Sakudo et al., 2016; Sakudo et al., 2011a). Recently,

MBs coated with anionic poly (MVE-MA) have been successfully used to capture *Ostreid Herpesvirus 1* (OsHV-1) from natural matrices (Toldrà et al., 2018).

OsHV-1 is an enveloped virus of double-stranded DNA with a diameter size of about 120 nm (Davison et al., 2005). This virus represents one of the major threats to shellfish aquaculture, particularly to the production of Pacific oysters (*Crassostrea gigas*) (EFSA, 2015). In recent years, periodic mass mortality episodes of *C. gigas* have been reported worldwide (Burge et al., 2006; Jenkins et al., 2013; Keeling et al., 2014; Renault et al., 1994; Roque et al., 2012; Segarra et al., 2010), causing important economic losses to the sector. Therefore, the availability of early warning detection tools is fundamental to minimise the impact of the OsHV-1 infection to the oyster industry. Surveillance of OsHV-1 commonly relies on its detection in bivalve tissues using quantitative PCR (qPCR) (Pepin et al., 2008; Whittington et al., 2019), though this approach is far from being considered an adequate early warning system. In contrast, monitoring the presence of OsHV-1 in water may allow the detection of the pathogen even before oysters become infected and/or ill, facilitating a rapid action and thus being closer to an early warning system. Although Paul-Pont and co-workers (Paul-Pont et al., 2013) observed that OsHV-1 was not uniformly distributed in seawater over time and space, detection of the virus in seawater is important, since it is through this medium that horizontal transmission (i.e. transmission from oyster to oyster) is suggested to occur (Sauvage et al., 2009). The virus could come from the discharges from depuration plants and/or from contaminated production areas, eventually reaching healthy shellfish. OsHV-1 has already been detected using qPCR without a pre-concentrating step in natural seawater during an experimental infection performed in a closed aquarium system (Schikorski et al., 2011a). Although the limit of detection (LOD) of the molecular technique was high (4 copies/µL), it was possible to detect the virus in the seawater due to its presence at high concentrations (>10 copies/µL). However, such high amount of OsHV-1 in seawater is not likely to be found in aquaculture facilities (e.g. hatcheries and open-water systems). Conventional centrifugation at relatively low-speed has been used to pre-concentrate OsHV-1 that was associated to larger particles (≥10 µm), such as plankton (Evans et al., 2014; Liu et al., 2020). However, OsHV-1 may also exist as free virus in seawater, making centrifugation unsuitable. Therefore, the combination of a pre-concentrating agent such as anionic MBs followed with qPCR is a promising approach to enhance sensitivity of OsHV-1 detection in real situations.

The implementation of water disinfection systems in shellfish depuration and hatchery plants able to properly inactivate OsHV-1 from the outgoing or incoming seawater becomes paramount to limit the potential spread of OsHV-1 to the ecosystem and/or to protect bivalve stocks. In this context, Whittington and colleagues (Whittington et al., 2015) demonstrated that the inactivation or removal of OsHV-1 particles from seawater using aging for 48 h or 5-µm filtration, respectively, prevented oyster mortality. However, such oysters still presented low amounts of OsHV-1 DNA. Little is known about the effectiveness of UV-disinfection systems in the inactivation OsHV-1. Some studies reported that standard UV treatment did not

inactivate all OsHV-1 in seawater of a recirculating aquaculture system (Evans et al., 2016). In contrast, the use of a high dose of UV radiation as a reliable disinfection system for OsHV-1 in seawater has been demonstrated (Hick et al., 2016). In such work, however, the effectiveness of the disinfection system was evaluated through OsHV-1 transmission, which is not always a guarantee of complete inactivation of viruses. In such a live assay, viral particles at an abundance below the minimum infective dose may pass through the system undetected.

The aim of the present study was therefore: 1) to investigate the use of anionic MBs to pre-concentrate OsHV-1 particles from biological matrices such as an oyster homogenate and seawater; and 2) to apply the MB-based pre-concentration strategy to seawater treated with an Enhanced Hydro-Optic UV (HOD-UV) system. Pre-concertation of OsHV-1 using MBs was assessed by qPCR analysis and the improvement of the LOD over the qPCR alone was calculated. The presence of OsHV-1 in seawater that had been treated and not treated with the HOD-UV system was evaluated. Moreover, mortality monitoring and qPCR analyses of shellfish samples were conducted.

2. Materials and Methods

2.1. Reagents and equipment

Anionic polymer-coated magnetic beads (300 nm in diameter) were purchased from Ademtech (Pessac, France). Qiagen DNeasy Blood and Tissue kit and SYBR Green dye were supplied by Thermo Fisher Scientific (Madrid, Spain). Custom oligonucleotide primers, potassium phosphate dibasic, potassium phosphate monobasic, sodium chloride, ethylenediaminetetraacetic acid (EDTA), Trizma base and Tween-20 were acquired from Merck KGaA (Madrid, Spain).

Magnetic separation was performed using a MagneSphere Technology Magnetic Separation rack (1.5-mL tubes x 12) from Promega Corporation (Madison, USA) and a Magnetic Separation rack (50-mL tubes x 6) from Eurofins Abraxis (Warminster, USA). The qPCR reactions were performed in a model 7300 real-time PCR system (Thermo Fisher Scientific, Madrid, Spain) and 7300 system 1.4.0 software was used to collect and evaluate data. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) was used to qualitatively check extracted DNA. Homogenisation of bivalve tissues for subsequent DNA extraction was carried out using a BeadBeater-8 from BioSpec (Bartlesville, USA).

2.2. Homogenate preparation

To optimise and characterise the MB-based pre-concentration protocol, a homogenate was first prepared. *C. gigas* oyster spats (~10 mm in length, ~8 months old) naturally infected with OsHV-1 were collected from Alfacs Bay (NW Mediterranean Sea) during a mortality event in April 2018. OsHV-1 prevalence in these

oysters was evaluated by qPCR following the protocol described in section 2.5. To prepare the homogenate, the flesh of 50 oysters was mixed with 20 mL of sterile seawater and homogenised using a stomacher for 1 min at maximum speed. After centrifugation (1,000 g, 10 min), the supernatant was collected. To evaluate the concentration of OsHV-1 in the homogenate, DNA was extracted from 100 µL of sample, checked for its quality using a NanoDrop 2000 spectrophotometer and quantified by qPCR as described in section 2.5. The homogenate was diluted with sterile seawater to a concentration of 2 OsHV-1 DNA copies/µL (200 OsHV-1 DNA copies in 100 µL) and stored at 4 °C until use.

2.3. Disinfection experiment

The experimental set up (figure 1) of the disinfection experiment was designed as follows. Decanted, filtered and UV-treated seawater (17 ± 1 °C) was fed into an upper tank (500 L) containing *C. gigas* oysters naturally infected with OsHV-1 (n=66, ~10 mm in length, ~8 months old) that had been collected in Alfacs and Fangar Bays (NW Mediterranean Sea) during a mortality episode in March 2019. Oysters were not fed after collection. A bifurcation in the outlet from the tank holding the infected oysters fed two bottom trays (80 L each). For one tray (HOD-UV tray), seawater passed (flow rate about 6-8 m^3/h) through a disinfection device based on Enhanced Hydro-Optic UV (HOD-UV) Light technology (RZ104-12 model) provided by Atlantium Technologies (Bet Shemesh, Israel). The HOD-UV system exploits the Total Internal Reflection (TIR) technology, which recycles UV light energy and ensures a uniform UV dose distribution. In this experiment, the two UV lamps of the system worked at dose of 1,360 J/m^2. For the other tray (non-HOD-UV tray, used as a control), seawater bypassed the HOD-UV system. Both trays contained naïve *C. gigas* oyster spats from the IRTA hatchery (n=60 per tray, ~20 mm in length, ~10-12 months old) and commercial *Mytilus galloprovincialis* mussels (n=30 per tray, ~60 mm in length, ~12-15 months old), which had been previously found to be negative for the presence of OsHV-1 DNA by qPCR following the protocol described in section 2.5. Mortality of shellfish was checked during the experiment. Seawater overflowing both trays was disinfected by ozonation, then discarded.

The experiment operated for 48 h non-stop, and included samplings at 10 different times: 0, 0.5, 1, 2, 3, 4, 5, 6, 24 and 48 h. At each time, 50 mL of seawater, 100 µL aliquots of seawater, 6 oysters and 6 mussels were collected. Seawater was collected at three sampling points: UP (seawater that left the upper tank containing infected oysters), HOD-UV (seawater that was exposed to the HOD-UV system and filled the HOD-UV tray) and non-HOD-UV (seawater that was not exposed to the HOD-UV system and filled the non-HOD-UV tray). The 50 mL of seawater were used to pre-concentrate viruses using MBs according to the protocol described in section 2.4. The MB-virus conjugates and 100 µL aliquots of seawater were stored at 4 °C until DNA extraction. Oysters and mussels were collected from the HOD-UV and non-HOD-UV trays and subsequently frozen at -80 °C until DNA extraction.

2.4. OsHV-1 pre-concentration using MBs

Pre-concentration of OsHV-1 by MBs was performed following the company's instructions with some adjustments: 1) 50 µL of MB suspension were transferred to a tube and washed three times with binding buffer (supplied with the MBs); for the washing steps, the tube was placed on the magnetic separation stand and the supernatant was removed; 2) the MB suspension was added to the 50 mL sample (homogenate or seawater from the depuration experiment) and it was incubated overnight (at least 8h) at room temperature with slow tilt-rotation; 3) the 50 mL tubes were placed on the magnetic separation stand for 1 h to enable capture of MB-virus conjugates; 4) the MB-virus conjugates were washed once with 1.5 mL of washing buffer (100 mM potassium phosphate, 0.05 % v/v Tween-20, pH 7.4) and resuspended in 100 µL of sterile seawater. MB-virus conjugates were then subjected to DNA extraction and subsequent qPCR analysis. The incubation time of the MBs/homogenate mixture was optimised by testing different times (from 30 min to 24h). The minimum OsHV-1 concentration able to be detected using MBs was calculated by performing serial dilutions of the homogenate (from pure to 1/4,700) and comparing the results of MBs + qPCR with qPCR alone (without MBs). All MB-virus conjugates were prepared in duplicate.

2.5. OsHV-1 DNA extraction and qPCR analysis

Total DNA was extracted using the Qiagen DNeasy Blood and Tissue kit as previously described (Toldrà et al., 2018). For samples that had not been treated with MBs (homogenate or seawater from the depuration experiment), 100 µL were mixed with 100 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), 20 µL of proteinase K and 200 µL of AL buffer. After 1 h at 56 °C, 200 µL of ethanol were added and the content was transferred into a spin column. After two washing steps with AW1 and AW2 buffers, elution was performed with 50 µL of AE buffer. For the MB-virus conjugates, the same protocol was used, the only exception being that MBs were removed after heating using a magnetic separation stand. For oyster and mussel tissue samples, a longitudinal slice of the whole body (including mantle, gill, digestive gland and adductor muscle) was mixed with 180 µL of ATL buffer and 20 µL of proteinase K. Zirconium glass beads were added and the tissue was disrupted using a BeadBeater-8 pulsed for 45 s at full speed. After digestion at 56° C overnight, 200 µL of AL buffer and 200 µL of ethanol were added. DNA was extracted using spin columns with a final elution of pure DNA in 100 µL of AE buffer. DNA quality and quantity were measured using a NanoDrop 2000 spectrophotometer. Extracted DNA was stored at -20 °C until qPCR analysis.

Detection and quantification of OsHV-1 DNA was performed by qPCR using the primer pair OsHVDPFor/OsHVDPRev (Webb et al., 2007) following the conditions described in our previous work (Toldrà et al., 2018). Briefly, each 20 µL reaction mixture contained 10 µL 2X SYBR Green dye, primers (final concentration 0.5 µM) and 1 µL of extracted DNA (DNA extracted from tissue samples was diluted to 50 ng/µL whilst DNA extracted from seawater and MB-virus conjugates was used directly). A negative control (no DNA)

and two positive controls (pure OsHV-1 genomic DNA, diluted at 10^6 and 10^3 copies/µL) were included, and each qPCR reaction was performed in triplicate. The qPCR conditions included 45 cycles of amplification following a three-step protocol (95 °C for 30 s, 60 °C for 1 min and 72 °C for 45 s) and a final step for melting temperature curve analysis at 60 °C for 1 min with a gradual increase of temperature (1 °C/15 s). Quantification of target copies of OsHV-1 genomic DNA was carried out using a standard curve based on 10-fold dilutions of OsHV-1 genomic DNA, obtained from an inter-laboratory exercise (Pepin, 2013), with an LOD of 10 copies/µL. Results were expressed as total OsHV-1 DNA copies per 100 µL of sample or MB-virus conjugate.

2.6. Statistical analysis

To evaluate differences among means of viral DNA amounts, analyses of repeated-measures ANOVA were carried out using SigmaStat software 3.1 (Systat Software Inc., California, USA). A *p*-value level of 0.05 was used to identify significant differences. Linear regression analysis comparing OsHV-1 quantifications obtained using MBs + qPCR and the amount of OsHV-1 present in the homogenate was performed using the same software.

3. Results and discussion

3.1. OsHV-1 pre-concentration using MBs

The ability of MBs to capture OsHV-1 from homogenate and seawater samples was previously demonstrated by our group (Toldrà et al., 2018). However, capture does not necessarily imply pre-concentration, and experimental conditions have to be carefully selected for that purpose. Herein, the use of MBs as pre-concentrating agents was assessed and characterised using the homogenate. To achieve this, some parameters were modified from our previous work (Toldrà et al., 2018): the ratio between the final elution volume and the initial sample volume was reduced (from 100 µL/100 µL to 100 µL/50 mL), whereas both the amount of MBs and the sample volume were increased (from 10 µL to 50 µL for the MBs, and from 100 µL to 50 mL for the sample volume). As demonstrated in our previous work, by increasing the amount of MBs, more OsHV-1 particles were captured, except when too many MBs were used, since steric hindrance decreased the efficiency. Although the use of 100 µL of MBs resulted in more OsHV-1 particles captured (data not shown), the use of 50 µL of MBs was selected for economic reasons. Similarly, 50 mL samples were chosen due to practical reasons (e.g. availability of magnetic separation stands for this volume).

The incubation time between MBs and non-diluted homogenate (200 total OsHV-1 DNA copies) was optimised in order to select the time leading to the highest virus capture. As shown in figure 2, the amount of OsHV-1 DNA copies detected by qPCR increased from 30 min to 8 h incubation. For longer incubation

times, the amount of OsHV-1 DNA detected was maintained, showing no significant differences among 8, 15 or 24-h incubation times. Consequently, an overnight incubation step of at least 8 h was established.

To demonstrate the ability of MBs to pre-concentrate OsHV-1 using the above-mentioned conditions, the non-diluted homogenate was analysed both with MBs + qPCR and qPCR alone (without MBs). As shown in figure 2a (i.e. non-diluted homogenate), the amount of OsHV-1 DNA copies detected with MBs + qPCR was higher (concentration factor ~50) than when only qPCR was used. Although the capture efficiency of MBs (calculated as the amount of DNA detected in the MB-virus conjugates divided by the total amount of DNA in the sample) from a natural matrix such as the homogenate was ~10%, the 500-fold reduction of the initial sample volume (from 50 mL to 100 µL) eventually facilitated the successful pre-concentration of OsHV-1.

To subsequently determine the minimum OsHV-1 concentration that the MBs + qPCR approach can detect, serial dilutions of the homogenate were prepared (11 dilutions, from pure to 1/4,700), and results were compared with qPCR alone. As shown in figure 2a, qPCR was able to detect OsHV-1 DNA only in the non-diluted and 1/4.7-diluted homogenate. In contrast, when qPCR was used in combination with MBs, OsHV-1 DNA was detected at a dilution of 1/470-diluted homogenate. After this dilution (i.e. 1/1,000, 1/2,200 and 1/4,700), viral DNA was not detected neither using MBs + qPCR, nor qPCR alone. These results indicate that the use of MBs prior to qPCR analysis reduces the LOD of the qPCR at least 100 times under these experimental conditions, with detection as low as 0.1 copies/µL. This LOD is lower than other LODs of qPCR assays for OsHV-1 detection reported in the literature: 10 copies/µL (Pepin, 2013), 5 copies/ µL (Martenot et al., 2010), 4 copies/µL (Pepin et al., 2008) and 3 copies/µL (Evans et al., 2014).

Figure 2b correlates the amount OsHV-1 DNA detected using MBs + qPCR with the amount of OsHV-1 DNA present at each homogenate dilution. In this case, the amount of viral DNA in each homogenate dilution is a theoretical value, which was calculated from the non-diluted homogenate containing 200 total OsHV-1 DNA copies. After logarithmic transformation, a linear correlation between the two sets was obtained. This relationship makes it possible to calculate the amount of OsHV-1 DNA in a sample from the results obtained using MBs. Moreover, this dependence between sets (potential dependence when no log transformation is applied) indicates that both the capture efficiency and concentration factor increase following a potential relationship as the amount of OsHV-1 DNA in the sample increases.

3.2. Disinfection experiment

Depuration of bivalves is mandatory in many countries to guarantee food safety. In a depuration process, shellfish is held in tanks of clean seawater for 24-48 h to allow their natural filtering activity, resulting in the removal or reduction of human pathogens, both bacterial and viral, to a save level before taking them to the market for consumption (Lee et al., 2008). For such purpose, the incoming seawater to depuration plants receives disinfection treatment/s to eliminate microbial pathogens harmful for humans**.** However, the

outflow water from those plants might not be disinfected before its release. Therefore, the untreated effluents may contain not only human pathogens but also shellfish pathogens, such as OsHV-1, which if released into shellfish production areas would pose a risk to bivalves (Ramón et al., 2005; Rodgers et al., 2019). Therefore, assessing the risk of OsHV-1 dispersal into the production areas, depending on the treatment of the outgoing water from shellfish holding facilities, becomes paramount to stablish biosecurity protocols. Similarly, the implementation of water disinfection systems is also important in shellfish hatcheries to protect their stocks from incoming unwanted pathogens. With the same logics than for depuration plants, the effluent from such facilities should also be treated. Thus, in this work, the effectiveness of HOD-UV radiation in the inactivation of OsHV-1 in seawater was investigated, using an experiment designed to mimic the water release from a depuration plant.

MBs were used to evaluate the presence of OsHV-1 in seawater samples from the disinfection experiment. Additionally, seawater samples were analysed using qPCR alone (without MBs). Whilst OsHV-1 DNA was not detected using qPCR alone, OsHV-1 was successfully detected when using MBs combined with qPCR, thus demonstrating again that MBs were able to pre-concentrate the virus. Quantifications of OsHV-1 DNA in seawater obtained using MBs + qPCR at different sampling points (UP, HOD-UV and non-HOD-UV) and times are presented in figure 4. First, while OsHV-1 DNA was detected in both UP (seawater that left the upper tank containing infected oysters) and non-HOD-UV (seawater that was not exposed to the HOD-UV system) sampling points, no viral DNA was detected in seawater samples that were exposed to the HOD-UV system (HOD-UV). These results certainly demonstrate the ability of the HOD-UV system to disinfect water and inactivate OsHV-1. Secondly, a clear time delay pattern was observed between the viral DNA present in UP seawater and non-HOD-UV seawater. As shown in figure 4, the highest viral load detected in UP seawater was at 6 h, while in non-HOD-UV seawater the maximum was detected at 24 h and at a lower intensity. Both peaks were significantly different ($p < 0.05$) from the other means of the amounts of OsHV-1 DNA detected within each treatment. Similarly, but less marked, a peak in the viral detection was obtained at 3 h in UP seawater, and at 4 h in non-HOD-UV seawater (not significantly different from the amount of OsHV-1 DNA detected at 2 h and 3 h, respectively). Finally, another peak at 0.5 h in UP seawater (significantly different from time 0 h) was detected, while in the non-HOD-UV seawater this peak, though lower in intensity, was more prolonged lasting from 0.5 to 1 h. These results suggest that oysters released the virus as soon as they were placed into the system, possibly due to handling and transport stress, and 6 h later, maybe due to the period of acclimation to the new habitat. However, samplings between 6 and 24 h were not conducted and, consequently, higher peaks might not have been detected. As a general trend, the peaks in detection in this experimental system are temporally delayed between the UP samples and the non-HOD-UV samples, likely due to the nature of the hydrodynamic configuration of the system and the kinetics of the virus dispersal. Besides demonstrating the ability of MBs to pre-concentrate OsHV-1 from seawater and, consequently, the

correct operation of the HOD-UV system, the observation of this pattern could provide useful information on the viral cycle.

In addition to assessing the presence of the virus in seawater, OsHV-1 transmission in shellfish was evaluated by means of mortality monitoring and qPCR analyses of shellfish samples. Evaluation of virus transmission was previously used to investigate the effectiveness of disinfection using a HOD-UV treatment in closed systems (Hick et al., 2016; Schikorski et al., 2011b). In our work, naïve oysters and mussels were placed in contact with seawater exposed (HOD-UV) and not exposed (non-HOD-UV, control) to the disinfection system. Even though mussels are not an OsHV-1 host, they were included in the experiment given their capacity to both act as reservoir for the virus and transmit it to oysters (O'Reilly et al., 2018). No mortality of shellfish was detected during the 48-h experiment. Additionally, all shellfish samples collected at different sampling times were negative for the presence of OsHV-1 DNA in both HOD-UV and non-HOD-UV trays. These results suggest that, although a 48-h experiment was sufficient to detect the virus in water samples, the duration of the experiment was too short to infect naïve shellfish samples. Although in a static system, OsHV-1 DNA was detected in oysters after only 6 h and mortality at 48 h (Schikorski et al., 2011a), other works report OsHV-1 DNA presence at 11 days and mortality at 13 days (Whittington et al., 2015) and OsHV-1 DNA presence at 2 days and mortality at 4 days in a flow-through systems (Petton et al., 2015). Other factors that may have played a role could be: 1) the low viral load of the infected material used in the upper tank (oysters presented only ~50% prevalence and low viral load, i.e. 1,000 viral copies/µL); and 2) the poor susceptibility of the naïve animals used in the bottom trays as a consequence of age, genetic background, life-history traits, or physiological status (Pernet et al., 2016). Further efforts should be focused on setting experimental trials with higher viral loads and longer duration.

OsHV-1 DNA has been previously detected in seawater by qPCR in a closed system after cohabitation with oysters experimentally infected with OsHV-1, reaching concentrations of 10-1,000 DNA copies/µL (Schikorski et al., 2011a). However, in a recirculating system, viral quantities detected in seawater were always below the limit of quantification of the qPCR assay (Evans et al., 2016). The low LOD of our MBs + qPCR approach has for the first time enabled study of the presence and distribution of OsHV-1 in seawater in a flow-through system. In this study, a full-scale industrial HOD-UV disinfection system was implemented at a dose of 1,360 J/m^2. This dose is lower than that used in previous works using a seawater medium (6,000 J/m^2 (Hick et al., 2016)), or oyster homogenate (9,720 J/m^2 (Schikorski et al., 2011b)), where OsHV-1 inactivation was demonstrated by means of transmission studies. In contrast, other studies reported that a dose of 300 J/m^2 was not able to inactivate OsHV-1 in seawater (Evans et al., 2016). Given that our previous study (Toldrà et al. 2018) demonstrated that MBs were able to capture infectious viral particles, the OsHV-1 DNA detected using this novel pre-concentration approach could be used as a proxy of transmission studies. Although further experiments including longer time frames and different viral loads (certainly higher) of infected

material should be conducted to properly verify the effectiveness of the HOD-UV system, our results demonstrate that the use of MBs + qPCR enabled detection of OsHV-1 DNA in non-treated seawater and not in UV-treated seawater, discrimination that was not possible using qPCR alone. Thus, this detection method has the potential to be used as an early warning system for future outbreaks.

4. Conclusions

The use of anionic MBs to pre-concentrate OsHV-1 particles from natural matrices such as oyster homogenate and seawater samples was successfully demonstrated, attaining an LOD 100 times lower compared to the qPCR alone (without prior treatment with MBs). Additionally, the MB-based strategy was applied to a disinfection experiment based on HOD-UV system, where the presence of OsHV-1 in seawater that had been treated and not treated with the system was evaluated. The use of MBs in conjunction with qPCR enabled to detect OsHV-1 DNA in the non-treated seawater, which was not possible using only qPCR. Moreover, the use of MBs + qPCR provided data on the pattern of kinetics of the release of the virus in seawater in a flow-through system. Future works including longer times and higher viral loads should be performed to better understand the infection cycle of OsHV-1, to properly assess the effectiveness of the UV-disinfection treatment and also for risk management. So far, our results open the possibility to assess the efficiency of water disinfection systems in shellfish depuration plants and hatchery/nursery facilities to eliminate pathogens, specifically OsHV-1, from the incoming or outgoing water. This work may also contribute to limiting the potential spread of OsHV-1 to the ecosystem through effluent water from such facilities. Finally, the detection of the virus in seawater using the MBs combined with qPCR demonstrates the possibility of its implementation as an early warning system. This detection is particularly important because seawater is the vehicle of transmission of the virus.

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Authors' contributions

AT designed the study, performed the experimental work, analysed all data and wrote the manuscript draft. **KBA** and **AR** designed the disinfection experiment and performed experimental work. **AL** and **YR** provided the Enhanced Hydro-Optic UV (HOD-UV) system. **MDF** funded the project and provided coordination and supervision throughout. **MC** conceptualized and designed the study, performed the experimental work and discussed the results. All authors supervised the experimental work, participated in the discussion of the results and reviewed the manuscript.

Figure 1. Application of MBs to seawater samples from a HOD-UV disinfection experiment: (a) schematic representation of the disinfection experimental set up, showing the sample points for seawater (●) and shellfish (○); (b) protocol to pre-concentrate and detect OsHV-1 from seawater using MBs.

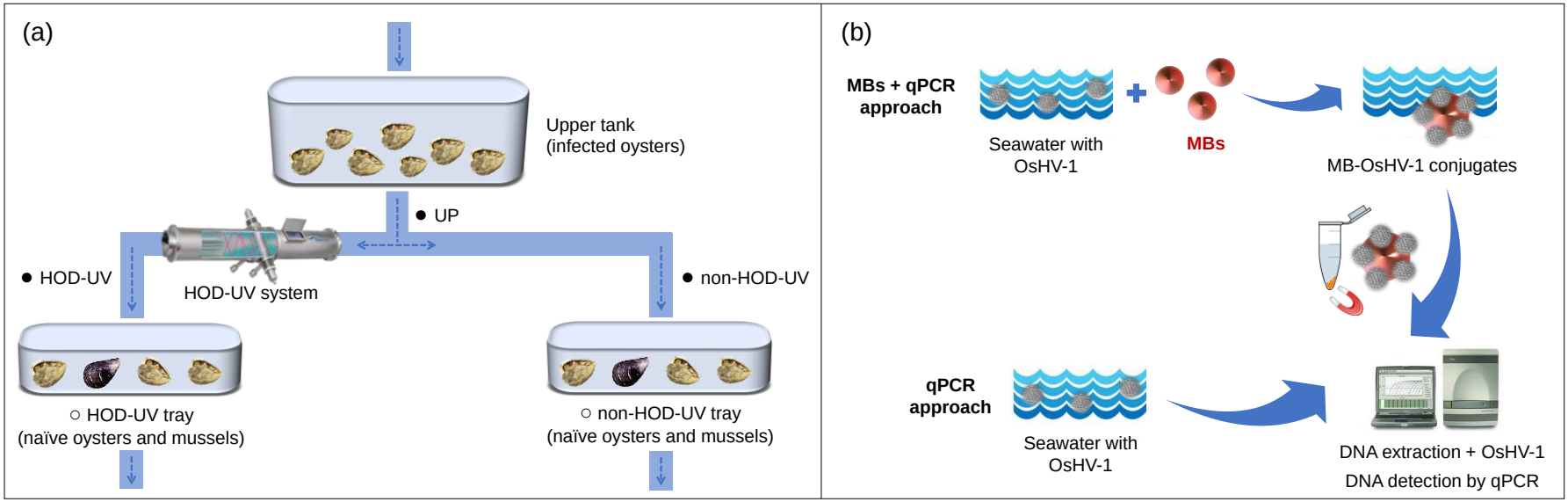


Figure 2. Effect of the incubation time between MBs (50 µL) and sample (50 mL) on the total amount of OsHV-1 in 100 µL of MB-virus conjugate detected by qPCR. MB-virus conjugates were prepared in duplicate and qPCR analyses were performed in triplicate. Error bars represent the standard deviation (n=6). * means significant at $p < 0.05$ (n = 6); n.s. means not significant at $p < 0.05$ (n = 6).

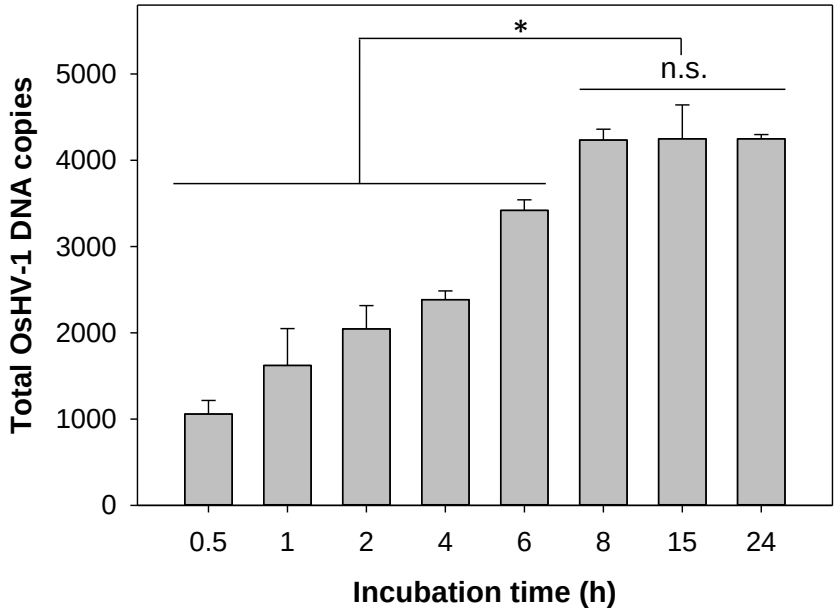


Figure 3. Pre-concentration capacity of MBs from serially diluted homogenate: (a) OsHV-1 DNA detected in serial dilutions of the homogenate using MBs + qPCR (black bars) and qPCR alone (grey bars); (b) linear regression between the OsHV-1 DNA detected using MBs + qPCR and the amount of OsHV-1 DNA in each homogenate dilution (theoretical values). MB-virus conjugates were prepared in duplicate and qPCR analyses were performed in triplicate. Error bars represent the standard deviation (n=6). Results were expressed as total OsHV-1 DNA copies in 100 µL of sample (qPCR alone) or MB-virus conjugate (MBs + qPCR).

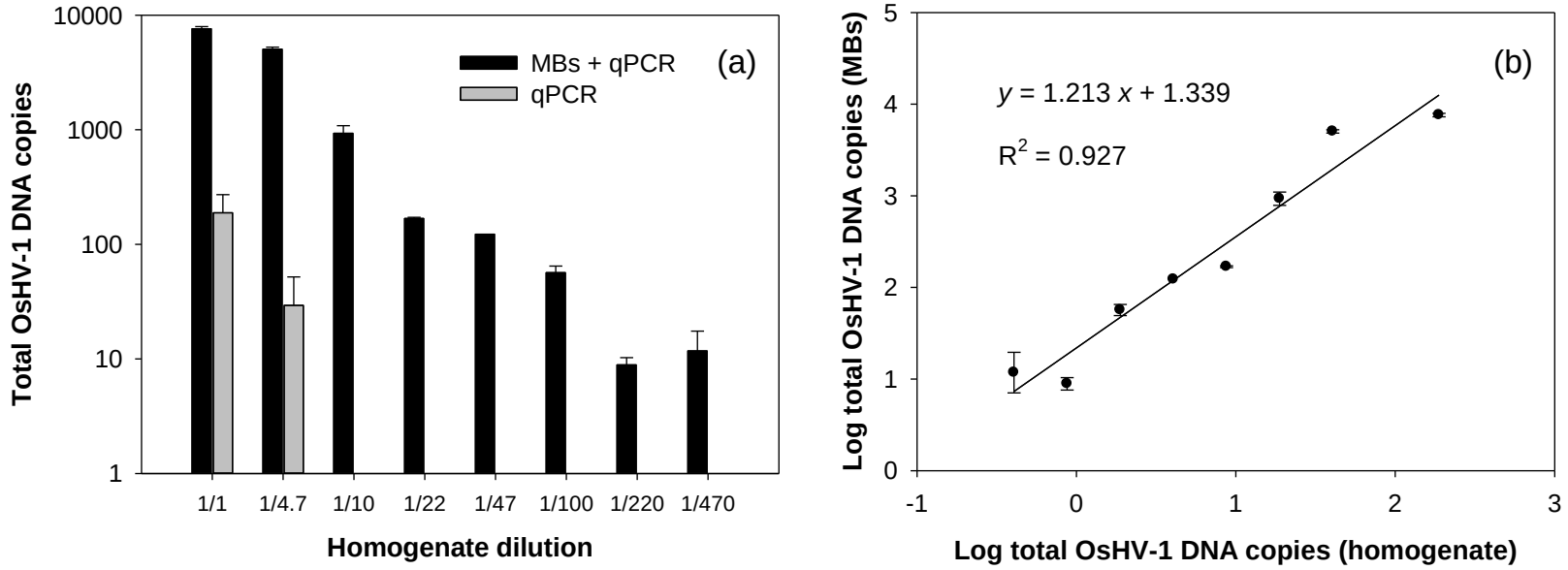
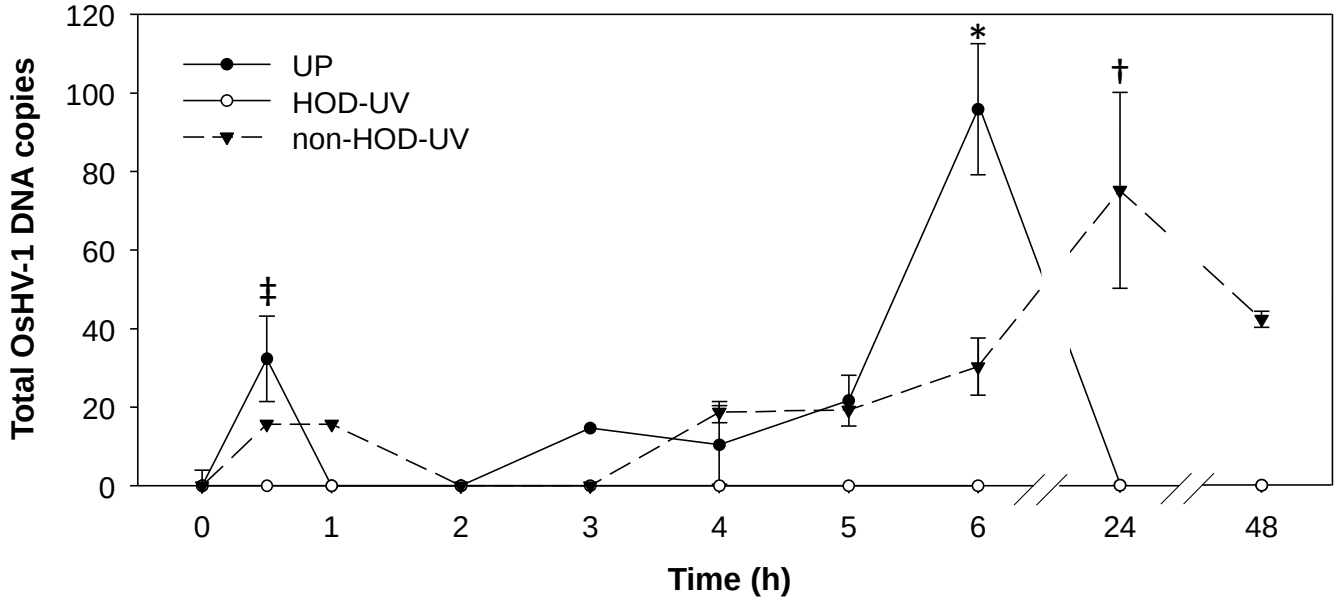


Figure 4. OsHV-1 DNA detected in the seawater using MBs + qPCR at different sampling points (UP, HOD-UV and non-HOD-UV) and times during the disinfection experiment. MB-virus conjugates were prepared in duplicate and qPCR analyses were performed in triplicate. Error bars represent the standard deviation (n=6). UP (seawater that left the upper tank containing infected oysters), HOD-UV (seawater that was exposed to the HOD-UV system and filled the HOD-UV tray) and non-HOD-UV (seawater that was not exposed to the HOD-UV system and filled the non-HOD-UV tray, used as a control). Results were expressed as total OsHV-1 DNA copies in 100 µL of MB-virus conjugate. ‡ means significant at $p < 0.05$ (n = 6) from the previous analysed time in UP treatment; * means significant at $p < 0.05$ (n = 6) from other OsHV-1 DNA amounts in UP treatment; † means significant at $p < 0.05$ (n = 6) from other OsHV-1 DNA amounts in non-HOD-UV treatment.



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