

Fine-scale temporal dynamics of herpes virus and vibrios in seawater during a polymicrobial infection in the Pacific oyster *Crassostrea gigas*

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ABSTRACT: The Pacific oyster *Crassostrea gigas* is currently being impacted by a polymicrobial disease that involves early viral infection by ostreid herpesvirus-1 (OsHV-1) followed by a secondary bacterial infection leading to death. A widely used method of inducing infection consists of placing specific pathogen-free oysters ('recipients') in cohabitation in the laboratory with diseased oysters that were naturally infected in the field ('donors'). With this method, we evaluated the temporal dynamics of pathogen release in seawater and the cohabitation time necessary for disease transmission and expression. We showed that OsHV-1 and *Vibrio* spp. in the seawater peaked concomitantly during the first 48 h and decreased thereafter. We found that 1.5 h of cohabitation with donors was enough time to transmit pathogens to recipients and to induce mortality later, reflecting the highly contagious nature of the disease. Finally, mortality of recipients was associated with increasing cohabitation time with donors until reaching a plateau at 20%. This reflects the cumulative effect of exposure to pathogens. The optimal cohabitation time was 5–6 d, the mortality of recipients occurring 1–2 d earlier.

KEY WORDS: Aquaculture · Bivalve · Epidemiology · Health · Polymicrobial disease · OSHV-1 · Pacific oyster mortality syndrome · POMS

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1. INTRODUCTION

Mortality outbreaks in Pacific oyster *Crassostrea gigas* associated with infection by viral and bacterial pathogens have increased worldwide during the last 10 yr (Barbosa Solomieu et al. 2015, EFSA AHAW Panel 2015, Pernet et al. 2016). The most striking example is the massive mortality syndrome, which can affect oyster juveniles (Pacific oyster mortality syndrome, POMS), killing up to 100% of farmed oysters. These mortalities have coincided with the recurrent detection of ostreid herpesvirus-1 (OsHV-1)

variants worldwide (Segarra et al. 2010, Lynch et al. 2012, Jenkins et al. 2013, Mortensen et al. 2016). However, other etiological agents, such as virulent bacterial strains assigned to the genus *Vibrio*, have been associated with diseased oysters (Petton et al. 2015b, de Lorgeril et al. 2018a).

The economic costs associated with high mortality have led to a major research effort to improve the knowledge on POMS. This research requires a method of pathogenesis that retains the whole complexity of the pathosystem and follows the natural route of infection. These objectives can not be fully reached

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using experimental systems in which the studied oysters are directly injected with a purified suspension of pathogens (OsHV-1 or vibrios) or placed in cohabitation with injected oysters. Direct injection relies on 1 or 2 pathogens of interest, generally a virus (OsHV-1), a bacteria (from the *Vibrio* genus) or both (i.e. Schikorski et al. 2011, Azéma et al. 2016), and reflects neither the complexity of the infectious process nor the natural route of infection. In addition, injection prevents the study of disease transmission. Cohabitation with injected oysters partly circumvents these problems. Recipient oysters placed in cohabitation with injected donors are 'naturally' infected, but the polymicrobial nature of the disease is not properly addressed. In addition, injection-based methods often require that the causative pathogen is well identified, isolated and cultivable or amplifiable, which may not be the case when diseases emerge.

To circumvent these issues, we used an experimental pathosystem in which specific pathogen-free (SPF) recipient oysters (Le Roux et al. 2016) were placed in cohabitation in controlled conditions with diseased oysters ('donors') that were naturally infected in the field when disease-induced mortalities were occurring (Petton et al. 2013). This method retains the whole complexity of pathogens (OsHV-1 and populations of virulent bacteria) and follows the natural route of infection.

This approach has been successfully used to investigate disease risk factors such as seawater temperature (Petton et al. 2013, Pernet et al. 2015), salinity (Fuhrmann et al. 2016), pH (Fuhrmann et al. 2019), water renewal or biomass of infected hosts (Petton et al. 2015a), bacterial populations (Lemire et al. 2015, Bruto et al. 2017), toxic algae (Lassudrie et al. 2015) and physiological condition of oysters (Tamayo et al. 2014, Fuhrmann et al. 2018), but the dynamics, relative weights and interactions of the different aetiological agents have not been clearly established. Recently, this method was applied to demonstrate that POMS is a polymicrobial infection in which OsHV-1 creates an immune-compromised state of oysters evolving towards subsequent bacteraemia by opportunistic bacterial pathogens, ultimately leading to oyster death (de Lorgeril et al. 2018b).

Although this experimental pathosystem has been widely used for the last 5 yr, the dynamics of disease transmission through the water column remains to be characterized. In the present study, we evaluated the effect of the cohabitation time between donors and recipients on the mortality of recipients, we followed the temporal dynamics of OsHV-1 and *Vibrio* spp.

during the cohabitation phase in seawater, and we tested the relationship between the cumulative exposure to these microorganisms and oyster mortality. As a corollary, we defined the cohabitation time between donors and recipients required to exhibit significant mortality, and we drew practical consequences for both experimenters and growers.

2. MATERIALS AND METHODS

2.1. Animal production and maintenance

Donors were progenies of 15 biparental oyster families that were produced at the Ifremer facility at Argenton (Brittany, France) in mid-March 2015 (de Lorgeril et al. 2018b). The parents originated from wild stocks collected in farming and non-farming areas (2 areas) in the Mediterranean Thau lagoon and in the Bay of Brest (2 sites), and also from a mass selection program to enhance their resistance to the mortality syndrome (2 areas × 2 sites + 1 selected = 5 origins, 3 families for each origin) (de Lorgeril et al. 2018b). Recipients were progenies of wild oysters (n = 90 parents) from a farming area in Marennes-Oléron (France); they were produced on 28 April 2015 as previously described (Petton et al. 2015a). These oysters were maintained SPF under controlled bio-secured conditions until the onset of the experiment. The SPF status of donors and recipients was confirmed by the absence of OsHV-1 DNA detection by qPCR and a low level of *Vibrio* spp. (~1 CFU 100 mg⁻¹ tissues, n = 3 pools of 5 ind. for donors and recipients) (Petton et al. 2015a, Le Roux et al. 2016).

The experiment was conducted at the Ifremer facility at Argenton in an area where seawater temperature is <14.5°C and Pacific oysters are absent. Thus, OsHV-1 is, locally, unexpected. Controlled bio-secured conditions were maintained by filtration and UV irradiation. The first treatment was applied after the pumping system (filtrations on 50 µm lamellar filter, 10 µm pocket filter and 1 µm glass-fiber filter; UV-device COMAP Water Treatment V3 Pehd model, 250 W). The second treatment was applied in the experimental room (filtration on 1 µm glass-fiber filter, JBL ProCristal UV-C 18 W). The bio-secured conditions were confirmed by daily monitoring of OsHV-1 DNA and *Vibrio* spp. levels in the seawater (see below for the protocol).

During the experiment, oysters were continuously supplied with treated seawater at 21°C under a 24 h light cycle with light aeration. Before the onset of the cohabitation phase, seawater renewal in the oyster

tanks was 50% (i.e. half of the tank volume was renewed within 1 h). During the cohabitation phase, seawater renewal was reduced to 5–10%. Oxygen saturation was always >95% and pH was between 7.9 and 8.2, which are suitable conditions for *Crassostrea gigas*. Oysters were continuously fed a mixed diet of *Tisochrysis lutea* and *Chaetoceros muelleri* (50:50, v/v) at 500–1000 $\mu\text{m}^3 \mu\text{l}^{-1}$ at the outlet pipe of the tank. Temperature, salinity, pH, oxygen and phytoplankton concentration were measured every 12 h with the WTW probes xi3101, cond340, pH3310 and FDO 925 and a Coulter particle counter (Multi-sizer 3), respectively. Seawater effluents were collected in a disposal tank and chlorinated for 2 h (12 mg l^{-1} of free chlorine) before neutralization with sodium thiosulfate (20 mg l^{-1}).

2.2. Experimental cross-over design

On 3 September 2015, part of a mixed population containing an equivalent biomass of each oyster family (6.5 mo old, 1.4 g mean ind. mass) was deployed in the Thau lagoon for 17 d and then used as pathogen donors (Petton et al. 2013). During this period, seawater temperature decreased from 23.9 to 20.1°C, which is suitable for OsHV-1 infection (Pernet et al. 2012). They were brought back to the Ifremer facility (Argenton, France) within 24 h and immediately placed in a flow-through 350 l tank (200 × 70 × 60 cm), referred to as the 'pathogen-exposure tank'. A subsample of the donor population (n = 100 ind.) was placed in mesh bags (25 × 30 × 1 cm) to regularly count the dead. The remaining part of the mixed oyster population was left undisturbed at the facility and placed in another 350 l tank to be used as the uninfected control. The biomass of oysters in both the pathogen-exposure and control tanks was 1800 g, corresponding to ca. 1000 animals.

In the meantime, the non-exposed batch of SPF animals was dispatched in 51 mesh bags (25 × 30 × 1 cm) at an identical density (100 ± 6 ind. bag^{-1} , 1.1 g mean ind. mass). Seventeen bags were directly placed in the pathogen-exposure tank in cohabitation with the donors and used as pathogen recipients (Petton et al. 2013). The biomass of donors equalled that of recipients. Seventeen other bags were placed in individual flow-through 50 l tanks (58 × 38 × 24 cm) referred to as 'safe tanks'. Then, each bag was either transferred from the pathogen-exposure to a safe tank (sequence 1) or from a safe tank to the pathogen-exposure tank (sequence 2; Fig. 1) according to a cross-over design (2 sequences × 2 treat-

ments; Jones & Kenward 2014). These permutations were conducted 1.5, 3, 6, 12, 24, 36, 48, 60, 72, 88, 110, 125, 150, 172, 196 or 220 h after the start of the cohabitation phase (n = 16 permutation times). The donors were removed from the pathogen-exposure tank after 220 h (9.2 d). Two oyster bags were left undisturbed in the pathogen-exposure tank and in one safe tank. Live and dead recipients were counted (1) at the permutation time in the relevant bags only and (2) at the end of the experiment in each bag 391 h (16 d) after the start of the cohabitation phase. Also, live and dead donors and uninfected controls were counted at each permutation time.

2.3. Quantification of OsHV-1 DNA and *Vibrio* spp.

The levels of OsHV-1 DNA and *Vibrio* spp. were determined (1) in 3 pools of 5 donors and 3 pools of 5 recipients sampled at the onset of field exposure and in 5 dead recipients sampled in the undisturbed bags in the pathogen-exposure tank 136 h after the beginning of the cohabitation phase; and (2) in seawater samples collected with sterile 15 ml Falcon tubes at the outlet in the pathogen-exposure tank and in the control safe tanks 0, 1.5, 3, 6, 9, 12, 15, 21, 24, 28, 32, 36, 40, 44, 48, 54, 60, 72, 80, 88, 96, 110, 125, 150, 160, 172, 196, 220, 232 and 244 h after the onset of cohabitation (n = 30 sampling times). These analyses were conducted on aliquots of 200 μl taken from 2 samples of 10 ml seawater. All samples were stored at -20°C.

For oysters, whole tissues were removed from the shells and homogenized in sterile artificial seawater. Total DNA was extracted from oyster and seawater samples with a QIAamp tissue mini kit (Qiagen) according to the manufacturer's protocol. The extracts were stored at -20°C before detection and quantification according to a real-time PCR protocol based on SYBR Green chemistry (Pepin et al. 2008) with specific primers developed by Webb et al. (2007). The specificity and sensitivity of the detection test using these primers is similar to those reported by Pepin et al. (2008). The method used in our study was the recommended method for reasons of availability, utility and diagnostic specificity and sensitivity for OsHV-1 detection (<http://www.oie.int/en/standard-setting/aquatic-code/access-online/>). The results were expressed as the log of OsHV-1 DNA copies per ml of seawater or per mg of wet oyster tissue. Virus detection and quantification analyses were conducted by Laboceia, a French public diagnostic laboratory (Quimper, France), in compliance with approved quality

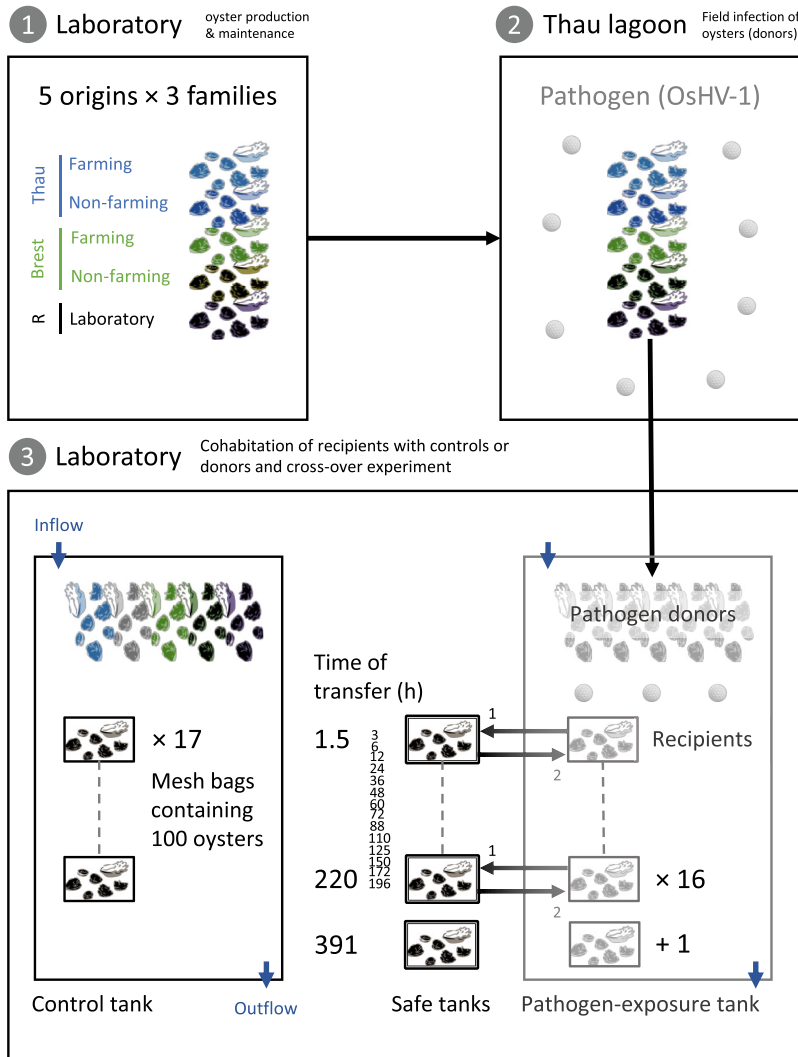


Fig. 1. (1) Families of Pacific oysters (15) from 5 origins were produced in the laboratory and (2) transferred in the Thau lagoon during the infectious period for 17 d and (3) used as pathogen donors in the laboratory. A batch of specific pathogen-free (SPF) oysters was kept under biosecured conditions. Seventeen bags were placed in the pathogen-exposure tank in cohabitation with the donors (pathogen recipients); 17 other bags were placed in individual 'safe' tanks. Each bag was either transferred from the pathogen-exposure to a safe tank (sequence 1) or vice versa (sequence 2). The remaining 17 bags of SPF oysters were displayed in the control tank. Live and dead recipients were counted in each bag at the end of the experiment. See Section 2.2 for further details

management system ISO 17025 and COFRAC. In 2009, the OsHV-1 μ Var had fully replaced the reference OsHV-1 genotype in oysters presenting mortality at all French oyster production sites (Segarra et al. 2010, Martenot et al. 2011, Renault et al. 2012), and this was confirmed in infected oysters collected in 2015 (de Lorgeril et al. 2018b). We also performed dual RNA-Seq on oysters immersed in the same tank during the cohabitation, and reads were mapped on the OsHV-1 μ Var A genome (KY242785) (J. de Lorgeril et al. unpubl. data). In order to determine if the oys-

ters were infected by OsHV-1 or by OsHV-1 μ Var, we looked at the expression of open reading frame insertions (ORF IN) IN.1–IN.4, which are only present in OsHV-1 μ Var (Burioli et al. 2017). We observed that reads mapping on IN.1–IN.4 genes represented 0.37% of the total reads mapped on the viral genome, strongly suggesting that oysters were infected by OsHV-1 μ Var. Therefore, OsHV-1 μ Var was hereafter referred to as OsHV-1.

Homogenate of oyster tissue samples diluted in sterilized seawater (1:10) and subsamples of seawater (100 μ l) were spread on thiosulfate-citrate-bile salts-sucrose (TCBS) agar in Petri dishes to quantify *Vibrio* spp. concentration. The plates were incubated at 21.0°C for 48 h before counting the number of CFUs. Results were expressed as CFU mg^{-1} of wet tissues in oysters and CFU ml^{-1} in seawater samples. The study focused on total *Vibrio* load without knowing which species were present, including pathogenic strains, as it is a reliable indicator of the bacteraemia typical of POMS (Petton et al. 2015b, de Lorgeril et al. 2018b).

2.4. Statistical analyses

Regression models were used to examine the relationship between mortality of recipients 391 h after the onset of cohabitation and time of transfer from pathogen-exposure to safe tanks (sequence 1) and from safe toward pathogen-exposure tanks (sequence 2). Best-fitted regression mod-

els were exponential growth and decay for sequence 1 and 2 respectively:

$$y(t)_{\text{seq1}} = a_0(1 - e^{-a_1 t}), \quad a_0 > 0, a_1 > 0 \quad (1)$$

$$y(t)_{\text{seq2}} = b_0 e^{-b_1 t}, \quad b_0 > 0, b_1 > 0 \quad (2)$$

Mortality reached a plateau at a_0 for sequence 1, and at 0 for sequence 2 since:

$$\lim_{t \rightarrow \infty} y(t)_{\text{seq1}} = a_0 \quad (3)$$

$$\lim_{t \rightarrow \infty} y(t)_{\text{seq2}} = 0 \quad (4)$$

The increase in mortality is no longer significant at the point where the confidence interval of the slope reaches 0. To estimate this time point for the sequence 1, the instantaneous increase in mortality (derived function) and its approximate standard error calculated using the Delta method (Oehlert 1992) may be written as:

$$y'(t)_{\text{seq1}} = a_0 a_1 e^{-a_1 t} \quad (5)$$

$$\text{se}[y'(t)_{\text{seq1}}] = e^{-a_1 t} \{ a_1^2 \text{var}(a_0) + [a_0(1 - a_1 t)]^2 \text{var}(a_1) + 2a_0 a_1 (1 - a_1 t) \text{cov}(a_0 a_1) \}^{1/2} \quad (6)$$

Therefore, the 95% confidence interval of the slope was:

$$y'(t)_{\text{seq1}} \pm 1.96 \text{se}[y'(t)_{\text{seq1}}] \quad (7)$$

and the instantaneous rate of change of mortality is no longer significant when the lower limit of this interval is equal to 0:

$$y'(t)_{\text{seq1}} \pm 1.96 \text{se}[y'(t)_{\text{seq1}}] = 0 \quad (8)$$

The same procedure (Eqs. 5 to 8) was applied to the data of sequence 2 using the upper limit of the interval for that case:

$$y'(t)_{\text{seq2}} \pm 1.96 \text{se}[y'(t)_{\text{seq2}}] = 0 \quad (9)$$

Polynomial regression models were fitted to the temporal dynamics of OsHV-1 DNA and *Vibrio* spp. concentrations in seawater during the first 244 h after the onset of the cohabitation phase. The independent variable (time) was centred at its mean ($\bar{t} = 78.9$ h) to reduce structural multicollinearity and converted into days. Also, OsHV-1 DNA was $\log_{10}(x / 10^6 + 1)$ transformed and *Vibrio* spp. concentration was $\log_{10}(10x + 1)$ transformed to meet the normality assumption.

To relate microorganism exposure to risk of death at some specified time point, exposure history was summarized into a single value that represents the total amount of exposure experienced up to that time (Vacek 1997). The metric most frequently used is the cumulative exposure index (CEI), which is a time-weighted summation of exposure intensities. In our study, CEIs were calculated for OsHV-1 and *Vibrio* spp. and corresponded to the areas under the polynomials between 2 time points ($a, b \in [0, 244]$) as:

$$\begin{aligned} & \int_{t_a}^{t_b} (\alpha_0 + \alpha_1 h + \dots + \alpha_p h^p) dh \\ &= \alpha_0 h + \frac{\alpha_1 h^2}{2} + \dots + \frac{\alpha_p h^{p+1}}{p+1} \Big|_{t_a}^{t_b} \quad (10) \\ &= \left[\alpha_0 t_b + \alpha_1 \frac{t_b^2}{2} + \dots + \alpha_p \frac{t_b^{p+1}}{p+1} \right] - \left[\alpha_0 t_a + \alpha_1 \frac{t_a^2}{2} + \dots + \alpha_p \frac{t_a^{p+1}}{p+1} \right] \end{aligned}$$

where $t_{a,b} = \frac{a,b-\bar{t}}{24}$, $\bar{t} = 78.9$ and p is the polynomial order. For sequence 1, $a = 0$ and $b \in [1, 244]$, whereas

for sequence 2, $a \in [0, 220]$ and $b = 244$. After 244 h, levels of OsHV-1 DNA and *Vibrio* spp. concentration in the seawater were considered to be null.

Relationships between mortality and CEIs were tested using logistic (logit link) regression models. For sequence 1, the relationship between the logit of mortality and CEIs appeared quadratic-plateau, so that the model was:

$$\text{logit}(p) = + \begin{cases} \delta_0 + \delta_1 x + \delta_2 x^2 & \text{if } x < x_0 \\ \delta_0 \delta_1 x_0 + \delta_2 x_0^2 & \text{if } x \geq x_0 \end{cases} \quad (11)$$

Finally, model accuracy was measured by the area under the receiver operating characteristics (ROC) curve. An area of 1 represents a perfect test whereas an area of 0.5 represents a worthless test.

Statistical analyses were conducted using the SAS software package v.9.4. (SAS Institute). A significance threshold of $\alpha \leq 0.05$ was adopted for all statistical tests.

3. RESULTS

In our study, recipient oysters were transferred from a tank containing naturally infected oysters (pathogen-exposure tank) to individual safe tanks (sequence 1) or from safe tanks into the pathogen-exposure tank (sequence 2) at 16 time points from the beginning to the end of the cohabitation phase (220 h). The level of OsHV-1 DNA and concentrations of cultivable *Vibrio* spp. were monitored in the pathogen-exposure tank, and the CEIs of the recipient oysters to these microorganisms were estimated. Mortalities of the recipients were recorded at the end of the experiment (391 h) and the relationship between mortality and the exposure indexes were tested.

At the beginning of the cohabitation phase, no mortality of donors or recipients was observed. The first mortalities were observed after 12 h in donors (2.3% mortality) and after 96 h in recipients in the pathogen-exposure tank (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d135p097_supp.pdf). At the end of the experiment (391 h), mortality was 51.7% in donors and 26.4% in recipients that were left undisturbed in the pathogen-exposure tank (Figs. 2A & S1). No mortality was recorded in the control tank, but trace levels of OsHV-1 DNA and low concentration of *Vibrio* spp. were occasionally detected in the surrounding seawater (Figs. S1 & S2).

In oysters from sequence 1, mortality of recipients increased with time spent in the pathogen-exposure tank following an exponential growth pattern until reaching a plateau at 21.1% (Fig. 2A). After only

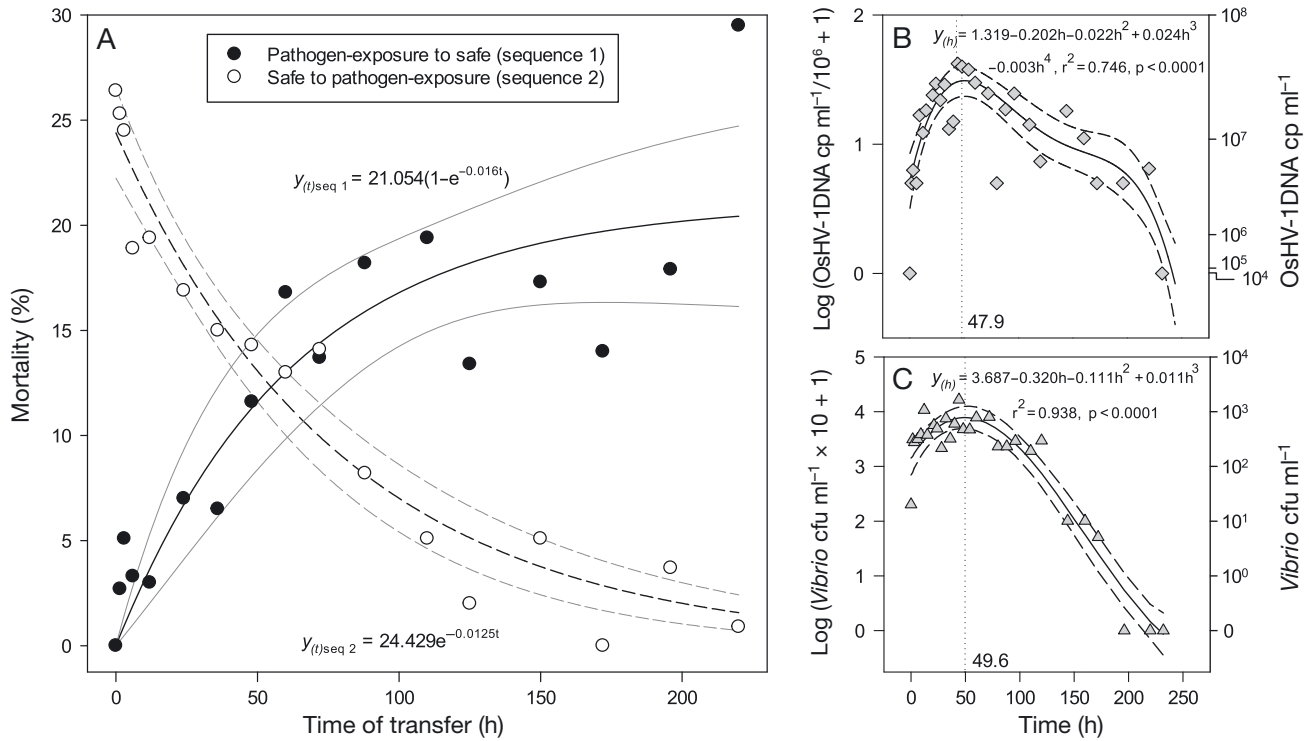


Fig. 2. (A) Mortality of recipient Pacific oysters at the end of the experiment (391 h) according to the time of transfer. Oysters were transferred from a tank containing infected oysters (pathogen-exposure tank) into individual safe tanks (sequence 1) or from safe tanks to the pathogen-exposure tank (sequence 2). Black lines: regression model fitted to the data; grey lines: 95% CI. (B,C) Dynamics of (B) OsHV-1 DNA and (C) *Vibrio* spp. concentration in the seawater of the pathogen-exposure tank as a function of time. Dotted lines indicate the time of peak concentration

1.5 h spent in the pathogen-exposure tank, recipients exhibited 2.7% final cumulative mortality in the safe tank. Mortality reached the plateau value after 135 h (5.6 d) of exposure in the pathogen-exposure tank. The level of OsHV-1 DNA in recently dead oysters collected after 136 h was 1.2×10^8 cp mg⁻¹ tissues (mean of 5 samples $\pm 5.2 \times 10^6$).

In oysters from sequence 2, the mortality of recipients decreased with time spent in the safe tanks following an exponential decay pattern (Fig. 2A). Mortality curves of recipients from sequences 1 and 2 crossed at $x = 54.9$ h and $y = 12.0\%$ mortality. This means that oysters that spent the first 55 h in the pathogen-exposure tank exhibited as much mortality as those which spent the next 165 h. No mortality was observed in the recipients transferred to the pathogen-exposure tank 172 h after the beginning of the experiment. However, few mortalities (1–4%) were reported in the subsequent transfers. The regression model predicted that mortality would plateau in recipients transferred 508 h (21 d) after the start of the cohabitation.

At the beginning of the experiment, OsHV-1 DNA was not detected in the seawater of the pathogen-

exposure tank (Fig. 2B). After only 1.5 h of cohabitation, a high level of OsHV-1 DNA (4×10^6 cp ml⁻¹) was detected in the seawater and remained between 4×10^6 and 4×10^7 cp ml⁻¹ until 220 h. Virus DNA was not detected afterwards. A fourth-order polynomial was fitted to the level of OsHV-1 DNA and time (Fig. 2B). This model indicates that OsHV-1 DNA in the seawater peaked at 47.9 h.

The *Vibrio* spp. concentration in the seawater of the pathogen-exposure tank increased from 20 to 310 CFU ml⁻¹ after only 1 h of cohabitation, increased until reaching a maximum after 44 h (1650 CFU ml⁻¹) and gradually decreased to 0 CFU ml⁻¹ after 172 h (Fig. 2C). A third-order polynomial fit *Vibrio* spp. concentration and time. According to this model, *Vibrio* spp. concentration peaked at 49.6 h.

The CEIs to OsHV-1 and *Vibrio* spp. that reflect both the concentration and the duration of exposure to these microorganisms increased with time spent in the pathogen-exposure tank (Fig. 3).

The mortality of recipients was strongly associated with CEIs to OsHV-1 and *Vibrio* spp. (Fig. 4). For the recipients in sequence 1, the relationship between mortality and CEIs was quadratic, suggesting that

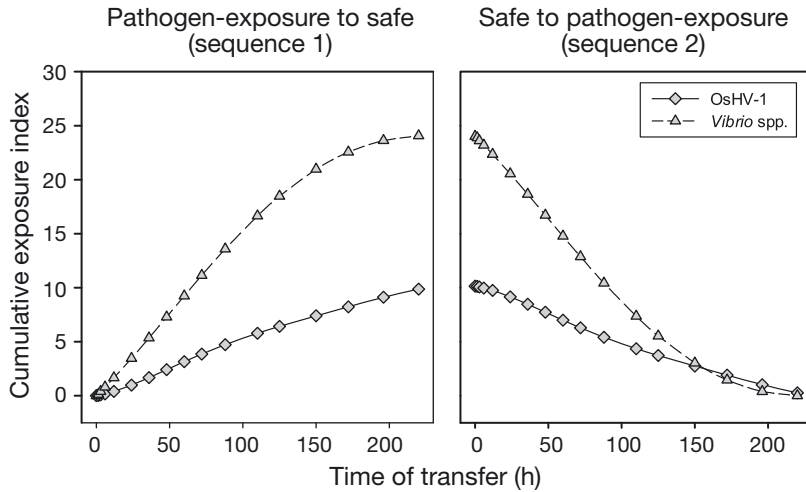


Fig. 3. Cumulative exposure index of Pacific oysters (Eq. 10) transferred from a tank containing infected oysters (pathogen-exposure tank) into individual safe tanks (sequence 1) or from safe tanks to the pathogen-exposure tank (sequence 2) calculated for OsHV-1 DNA and *Vibrio* spp. concentrations

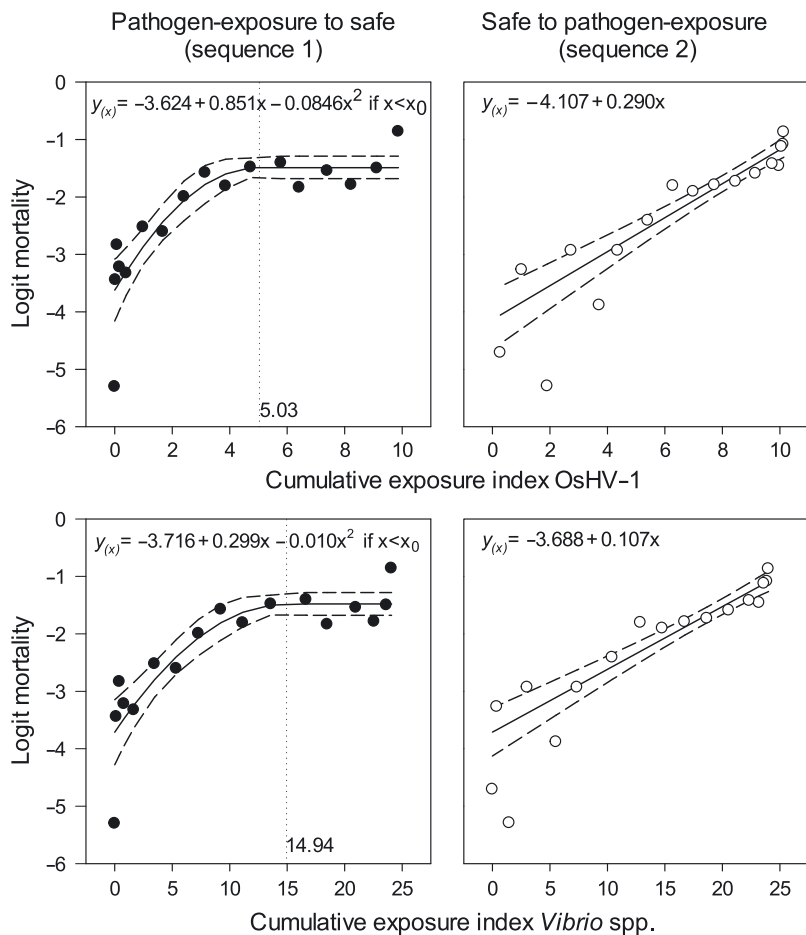


Fig. 4. Pacific oyster mortality versus cumulative exposure index to OsHV-1 and *Vibrio* spp. for oysters transferred from a tank containing infected oysters (pathogen-exposure tank) into individual safe tanks (sequence 1, left) or from safe tanks to the pathogen-exposure tank (sequence 2, right). Dotted lines: the value of x_0 beyond which the values of y are constant (plateau)

mortality plateaued and then reached a threshold CEI. However, for the recipients in sequence 2, the quadratic component was not significant, so that the relationship between mortality and CEIs was considered linear (Fig. 3). In this case, the odds of mortality increased by 34.4 and 11.3 % for every increase of 1 unit in CEI of OsHV-1 and *Vibrio* spp., respectively. The area under the ROC curves were respectively 0.681 for sequence 1 and 0.720 for sequence 2 for both CEIs, so that the model accuracy was fair.

4. DISCUSSION

Although the method of infection used in the present study has been widely applied during the last 5 yr, the dynamics of disease transmission through the water column remains to be finely characterized. We addressed this issue in the present work. We provided temporal dynamics of OsHV-1 DNA and *Vibrio* spp. concentrations in seawater in relation to the mortality of recipient oysters that were placed in cohabitation with donor oysters that had been naturally infected in the field. As it was recently demonstrated that infection by OsHV-1 is the first event that occurs during the infectious process, followed by a secondary infection by bacteria including vibrios (de Lorgeril et al. 2018b), we monitored the concentrations of both these pathogens in the seawater tanks. We found that levels of OsHV-1 DNA and *Vibrio* spp. concentration in the seawater of the pathogen-exposure tank peaked at almost the same time (48 and 50 h respectively).

Mortality of recipients increased with cohabitation time with donors and was associated with cumulative exposure to both OsHV-1 and *Vibrio* spp. In line with this, previous studies have found that mortality risk of recipients cohabitating with donors increases with the biomass of infected oysters and decreases with seawater renewal—2 parameters that probably influence

pathogen concentrations in the surrounding seawater (Petton et al. 2015a). Also, mortality risk of oysters injected with a viral suspension increases with concentration of viral particles (Paul-Pont et al. 2015, Segarra et al. 2016). Finally, non-lethal infection of oysters in the field is associated with the dilution of viral particles below a threshold value under which no mortality occurs (Pernet et al. 2018). Taken together, these results show a clear relationship between mortality of recipients and pathogen concentration in seawater.

It took only 1.5 h of cohabitation with donors to detect OsHV-1 DNA in the surrounding seawater and to observe mortality of recipients at the end of the experiment (16 d, sequence 1). This result suggests that transmission is very rapid. This probably explains why the disease spreads so fast when infectious conditions are in place in aquaculture farms. From an experimental standpoint, studies dedicated to investigating the host response to the disease should begin sampling soon after exposure. Until now, the earliest samples that had been analysed were collected 6 h after cohabitation began (de Lorgé et al. 2018b).

Mortality of recipients plateaued after 135 h of cohabitation (5.6 d) with donors, i.e. 39 h after the death of the first recipient. In previous experiments, the cohabitation phase of donors and recipients lasted until the appearance of the first dead recipient, which generally occurred after 4 d (Petton et al. 2013, 2015a,b, Lassudrie et al. 2015, Pernet et al. 2015, Fuhrmann et al. 2016). However, to reach maximum mortality of recipients, it is necessary to continue cohabitation for 1–2 d more. At that point, the donors can be removed instead of leaving them dying, decomposing, mineralising and thus altering the biogeochemical conditions in the experimental tanks (Richard et al. 2017). Doing so would avoid some confounding effects during the experiments.

The relationship between recipient mortality and cohabitation time with donors from both sequences (1 and 2) suggests that infectivity in the pathogen-exposure tank decreased with time. For instance, oysters that spent the first 55 h in the pathogen-exposure tank exhibited as much mortality as those which spent the next 165 h in the tank, and almost no mortality (0–4%) was observed in oysters transferred to the pathogen-exposure tank after 172 h. This finding aligns with the fact that levels of OsHV-1 DNA and *Vibrio* spp. concentration in the seawater peaked after ca. 48 h and decreased to zero after 172–220 h.

Although laboratory experiments do not fully reproduce field conditions, the temporal dynamics of oyster mortality in the laboratory were similar to those occurring in the field (de Lorgé et al. 2018b). In addition, cumulative final mortality was only 26% in undisturbed recipients, reflecting a low pathogen-exposure that is typical of the fall period in the Thau lagoon (Pernet et al. 2012). Finally, concentrations of OsHV-1 DNA in the seawater were slightly higher than those reported in laboratory conditions by Schikorski et al. (2011) and Paul-Pont et al. (2015). To our knowledge, there are no data available on the temporal dynamics of OsHV-1 concentration in seawater in the field to which we can compare our laboratory data.

To conclude, we found that (1) OsHV-1 and *Vibrio* spp. concentrations in the seawater showed clear temporal dynamics, (2) mortality of recipients was associated with increasing cohabitation time with donors and reflects cumulative exposure to OsHV-1 and *Vibrio* spp., (3) after only 1.5 h of cohabitation with donors, OsHV-1 DNA was detected at a high concentration in the seawater and mortality of recipients occurred, (4) mortality of recipients plateaued after 5–6 d of cohabitation with donors 1–2 d after the appearance of the first dead donor, and (5) infectivity in the pathogen-exposure tank decreased with time, to values close to zero after 172 h. Although this experiment was not replicated over time, the mortality dynamics of the recipients observed here was consistent with other published studies using similar conditions. Therefore, the fine-scale dynamics of mortality and OsHV-1 and *Vibrio* spp. concentration in seawater reported here can be generalized for use in other experiments conducted under similar conditions.

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