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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.

Keywords : Aquaculture, bivalve, epidemiology, health, polymicrobial disease, OSHV-1, POMS

INTRODUCTION

Mortality outbreaks in Pacific oyster *Crassostrea gigas* associated with infection by viral and bacterial pathogens have increased during the last 10 years worldwide (AHAW 2015, Barbosa Solomieu et al. 2015, Pernet et al. 2016). The most striking example is the massive mortality syndrome which affects oyster juveniles (Pacific Oyster Mortality Syndrome, POMS) killing up to 100% of the farmed oysters. These mortalities coincide 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* are systematically associated to diseased oysters (Petton et al. 2015b, de Lorgeril et al. 2018a).

The economic costs associated with increased mortality has led to a major research effort to improve the knowledge on POMS. This research requires a method of pathogenesis which retains the whole complexity of the pathosystem and follows the natural route of infection. These last objectives are not fully reached 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 one or two pathogens of interest, generally a virus (OsHV-1), a bacteria (from the *Vibrio* genus) or both (i.e. Schikorski et al. 2011, Azema et al. 2016), and does reflect neither the complexity of the infectious process nor the natural route of the 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 recipient oysters (Le Roux et al. 2016) are 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 was 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), while the dynamics, relative weights and interactions of the different aetiological agents were not clearly established. Since then, this method was applied to demonstrate that POMS is a polymicrobial infection in which a OsHV-1 creates an immune-compromised state of oysters evolving towards subsequent bacteraemia by opportunistic bacterial pathogens leading to oyster death (de Lorgeril et al. 2018b).

Although this experimental pathosystem has been widely used for the last 5 years, 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 the seawater; and we tested the relationship between the cumulative exposure to these microorganisms and oyster mortality. As a corollary, we defined the required cohabitation time between

91 donors and recipients to exhibit significant mortality and we drew practical consequences
92 for both experimenters and growers.
93
94 **MATERIAL & METHODS**
95 **Animal production and maintenance**
96 Donors were progenies of 15 biparental oyster families that were produced to the
97 Ifremer facility at Argenton (Brittany, France) mid-March 2015 (de Lorgeril et al. 2018b).
98 The parents originated from wild stocks collected in farming and non-farming areas (2
99 areas) in the Mediterranean Thau lagoon and in the Bay of Brest (2 sites), and from a mass
100 selection programme to enhance their resistance to the mortality syndrome (2 areas × 2
101 sites + 1 selected = 5 origins, 3 families for each origin) (de Lorgeril et al. 2018b).
102 Recipients were progenies of wild oysters (n=90 individuals) collected in a farming-area
103 in Marennes-Oléron (France) produced on 28 April 2015 as previously described (Petton
104 et al. 2015a). These oysters were maintained free of specific pathogens (SPF) under
105 controlled bio-secured conditions until the onset of the experiment. The specific
106 pathogen-free status of donors and recipients was confirmed by the absence of OsHV-1
107 DNA detection by qPCR and a low level of *Vibrio* spp. (~1 cfu 100 mg^{-1} tissues, n=3 pools
108 of five individuals for donors and recipients) (Petton et al. 2015a, Le Roux et al. 2016).
109 This experiment was conducted at the Ifremer facility at Argenton in an area where
110 seawater temperature is lower than 14.5°C and Pacific oyster is absent. Therefore OsHV-
111 1 is locally unexpected. However, controlled bio-secured conditions were maintained by
112 filtrations and UV irradiation. The first treatment was applied after the pumping system
113 (filtrations on 50-µm lamellar filter, 10-µm pocket filter and 1-µm glass-fibber filter; UV-
114 device COMAP Water Treatment V3 Pehd model, 250 W). The second treatment was
115 applied in the experimental room (filtration on 1-µm glass-fibber filter, JBL ProCristal UV-

C 18W). The bio-secured conditions were confirmed by monitoring daily the levels of OsHV-1 DNA and *Vibrio* spp. in the seawater (see below for the protocol).

During the experiment, oysters were continuously supplied with treated seawater at 21°C under a 24h light cycle with a 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 one hour). During the cohabitation phase, seawater renewal was lowered at 5 to 10%. Oxygen saturation was always >95% and pH was between 7.9 and 8.2 which are suitable life conditions for *C. gigas*. Oysters were continuously fed a mixed diet of *Tisochryis lutea* and *Chaetoceros muelleri* (50:50, v/v) at 500 to 1000 $\mu m^3\ \mu L^{-1}$ at the outlet pipe of the tank. Temperature, salinity, pH, oxygen and phytoplankton concentration were measured each 12 hours with the WTW probes xi3101, cond340, pH3310 and FDO 925, and the Coulter particle counter (Multisizer 3) respectively. Seawater effluents were collected in a disposal tank and chlorinated for 2 hours (12 mg/L of free chlorine) before neutralization with sodium thiosulfate (20 mg/L).

Experimental cross-over design

On 3 September 2015, part of a mixed population containing an equivalent biomass of each oyster family (6.5-month-old, 1.4 g mean individual mass) was deployed in the Thau lagoon for 17 days and further used as pathogen donors (Petton et al. 2013). During this period, seawater temperature decreased from 23.9°C to 20.1°C, which is permissive for OsHV-1 infection (Pernet et al. 2012). Then they were brought back to the Ifremer facility (Argenton, France) within 24 hours and immediately placed in a flow through 350-L tank (200×70×60 cm), referred to as "pathogen-exposure tank". A subsample of the donor population (n=100 individuals) was placed in mesh-bags (25×30×1 cm) for regularly counting the dead. The remaining part of the mixed oyster population was left

141 undisturbed at the facility and placed in another 350-L tank to be used as uninfected
142 control. The biomass of oysters in both the pathogen-exposure and the control tanks was
143 1800 g, corresponding to *ca.* 1000 animals.

144 In the meantime, the non-exposed batch of SPF animals was dispatched in 51 mesh-
145 bags (25×30×1 cm) at an identical density per bag (100 ±6 individuals per bag, 1.1 g mean
146 individual mass). Seventeen bags were directly placed in the pathogen-exposure tank in
147 cohabitation with the donors and they were used as pathogen "recipients" (Petton et al.
148 2013). The biomass of donors equalled that of recipients. Seventeen other bags were
149 placed in individual flow through 50-L tanks (58×38×24 cm) referred to as "safe tanks".
150 Then, each bag was either transferred from the pathogen-exposure towards safe tanks
151 (sequence 1) or from safe tanks towards the pathogen-exposure tank (sequence 2, Figure
152 1) according to a cross-over design (2 sequences × 2 treatments; Jones & Kenward 2014).
153 These permutations were conducted 1.5, 3, 6, 12, 24, 36, 48, 60, 72, 88, 110, 125, 150, 172,
154 196 or 220 hours after the start of the cohabitation phase (n=16 permutation times). The
155 donors were removed from the pathogen-exposure tank after 220 h (9.2 d). Two oyster
156 bags were left undisturbed in the pathogen-exposure tank and in one safe tank. Live and
157 dead recipients were counted *i*) at the permutation time in the relevant bags only and *ii*)
158 at the end of the experiment in each bag 391 h (16 d) after the start of the cohabitation
159 phase. Also, live and dead donors and uninfected controls were counted at each
160 permutation time.

161

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

163 The levels of OsHV-1 DNA and *Vibrio* spp. were determined *i*) in three pools of five
164 donors and three pools of five recipients sampled at the onset of field-exposure and in five
165 dead recipients sampled in the undisturbed bags in the pathogen-exposure tank 136 h

after the beginning of the cohabitation phase; and *ii*) 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, 244 hours after the onset of cohabitation (n=30 sampling times). These analyses were conducted on aliquots of 200 µL taken from two 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) (T. Renault pers. comm.). The method used in our study was the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity for OsHV-1 detection (OIE 2012, www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/2.4.09_INF_OSTREID _ HERPES.pdf). 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 Labocea, a French public diagnostic laboratory (Quimper, France), in compliance with approved quality management system ISO 17025 and COFRAC. In 2009 the OsHV-1 µVar 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 oyster immersed in the same tank during the cohabitation and reads were mapped on the OsHV-1 µVar. A genome

191 (KY242785) (Lucasson et al. in preparation). In order to determine if oyster were infected
192 by OsHV-1 or by OsHV-1 µVar we looked at the expression of ORF IN.1, .2, .3 and .4 which
193 are only present in OsHV-1 µVar (Burioli et al. 2017). We observed that reads mapping on
194 IN.1 to .4 genes represent 0.37% of the total reads mapped on the viral genome strongly
195 suggesting that oysters were infected by OsHV-1 µVar. Therefore, in the present study we
196 refer to 'OsHV-1' as 'OsHV-1 µVar'.
197 Homogenate of oyster tissue samples diluted in sterilized seawater (1:10) and
198 subsamples of seawater (100 µL) were spread on thiosulfate-citrate-bile salts-sucrose
199 (TCBS) agar in Petri dishes to quantify *Vibrio* spp. concentration. The plates were
200 incubated at 21.0°C for 48 h before counting the number of colonies forming units (cfu).
201 Results were expressed as cfu mg^{-1} of wet tissues in oysters and cfu mL^{-1} in seawater
202 samples. The study focused on total Vibrio load without knowing which species were
203 present including pathogenic strains as it is a reliable indicator of the bacteraemia typical
204 of POMS (Petton et al. 2015b, de Lorgeril et al. 2018b).
205
206 **Statistical analyses**
207 Regression models were used to examine the relationship between mortality of
208 recipients 391 h after the onset of cohabitation and time of transfer from pathogen-
209 exposure toward safe tanks (sequence 1) and from safe toward pathogen-exposure tanks
210 (sequence 2). Best fitted regression models were exponential growth and decay for
211 sequence 1 and 2 respectively:
212 $$y(t)_{seq1} = a_0(1 - e^{-a1t}), \quad a_0 > 0,\ a_1 > 0 \qquad \text{(eq. 1)}$$
213 $$y(t)_{seq2} = b_0 e^{-b1t}, \qquad b_0 > 0,\ b_1 > 0 \qquad \text{(eq. 2)}$$
214 Mortality reached a plateau at a_0 for sequence 1, and at 0 for sequence 2 since:
215 $$\lim_{t\to\infty} y_{(t)seq1} = a_0 \qquad \text{(eq. 3)}$$

$$\lim_{t\to\infty} y_{(t)seq2} = 0 \qquad \text{(eq. 4)}$$

The increase in mortality became not significant at the point where the confidence interval of the slope reached 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:

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

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

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

$$y'(t)_{seq1} \pm 1.96 se\left[y'(t)_{seq1}\right], \qquad \text{(eq. 7)}$$

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

$$y'(t)_{seq1} - 1.96 se\left[y'(t)_{seq1}\right] = 0 \qquad \text{(eq. 8)}$$

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

$$y'(t)_{seq2} + 1.96 se\left[y'(t)_{seq2}\right] = 0 \qquad \text{(eq. 9)}$$

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 in 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" which is a time-weighted summation of exposure intensities. In our study, the CEI were calculated for OsHV-1 and *Vibrio* spp. and corresponded to the areas under the polynomials between two time points (a, b) ϵ [0,244] as:

$$\int_{t_a}^{t_b} \left(\alpha_0 + \alpha_1 h + \cdots + \alpha_p h^p\right) dh$$

$$= \alpha_0 h + \frac{\alpha_1 h^2}{2} + \cdots + \frac{\alpha_p h^{(p+1)}}{p+1} \Bigg|_{t_a}^{t_b}$$

$$= \left[\alpha_0 t_b + \alpha_1 \frac{{t_b}^2}{2} + \ldots + \alpha_p \frac{{t_b}^{p+1}}{p+1}\right] - \left[\alpha_0 t_a + \alpha_1 \frac{{t_a}^2}{2} + \ldots + \alpha_p \frac{{t_a}^{p+1}}{p+1}\right] \qquad \text{(eq. 10)}$$

Where $t_{a,b} = \frac{a,b-\bar{t}}{24}$, $\bar{t} = 78.9$ and p was 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 as null.

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

$$logit\,(p) = \begin{cases} \delta_0 + \delta_1 x + \delta_2 x^2 \; if \; x < x_0 \\ \delta_0 + \delta_1 x_0 + \delta_2 x_0^2 \; if \; x \geq x_0 \end{cases} \qquad \text{(eq. 11)}$$

Finally, the model accuracy was measured by the area under the curve ROC (receiver operating characteristic). 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 (9.4. SAS Institute, NC). A significance threshold of 5% was adopted for all statistical tests.

RESULTS

In our study, recipient oysters were transferred from a tank containing naturally infected oysters (pathogen-exposure tank) toward individual safe tanks (sequence 1) or from safe tanks toward the pathogen-exposure tank (sequence 2) at 16 time points from the beginning to the end of the experiment (220 h). Level of OsHV-1 DNA and concentrations of cultivable *Vibrio* spp. were monitored in the pathogen-exposure tank, and the cumulative exposure indexes 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 was observed on donors and recipients. First mortalities were observed after 12 h in donors (2.3% mortality) and after 96 h in recipients in the pathogen-exposure tank (Figure A1). At the end of the experiment (391 h), mortality in donors was 51.7% and 26.4% in recipients that were left undisturbed in the pathogen-exposure tank (Figures 2A and A1). No mortality was recorded in the control tank but trace level of OsHV-1 DNA and low concentration of *Vibrio* spp. were occasionally detected in the surrounding seawater (Figures A1 and A2).

In oysters from sequence 1, mortality of recipients increased with time spent in the pathogen-exposure tank following an exponential growth pattern up until reaching a plateau at 21.1 % (Figure 2A). After only 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 ± 5.2×10^6).

In oysters from sequence 2, mortality of recipients decreased with time spent in the safe tanks following an exponential decay pattern (Figure 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 hours in the pathogen-exposure tank exhibited as much mortality as those which spent the next 165 hours. No mortality was observed in the recipients transferred in 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 predicts 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 (Figure 2B). After only 1.5 h of cohabitation, high level of OsHV-1 DNA (4×10^6 cp mL^{-1}) was detected in the seawater and remained between 4 $\times 10^6$ to 4×10^7 cp mL^{-1} until 220 h. Virus DNA was not detected afterwards. A fourth-order polynomial was fitted level of OsHV-1 DNA and time (Figure 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 one hour of cohabitation, increased up until reaching a maximum after 44 h (1650 cfu mL^{-1}) and gradually decreased to 0 cfu mL^{-1} after 172 h (Figure 2C). A third-order polynomial was fitted *Vibrio* spp. concentration and time. According to this model, *Vibrio* spp. concentration peaked at 49.6 h.

The cumulative exposure indexes (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 (Figure 3).

Mortality of recipients was strongly associated with CEIs to OsHV-1 and *Vibrio* spp (Figure 4). For the recipients in sequence 1, the relationship between mortality and CEIs was quadratic, suggesting that 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 (Figure 3). In

312 this case, the odds of mortality increased by 34.4% and 11.3% for every increased of 1
313 unit in CEI of OsHV-1 and *Vibrio* spp., respectively. The area under the curve ROC were
314 respectively 0.681 for sequence 1 and 0.720 for sequence 2 for both CEIs, so that the
315 model accuracy was fair.
316
317 **DISCUSSION**
318 Although the method of infection used in the present study has been widely applied
319 during the last five years, the dynamics of disease transmission through the water column
320 remains to be finely characterized. We addressed this issue in the present work. We
321 provided temporal dynamics of OsHV-1 DNA and *Vibrio* spp. concentrations in the
322 seawater in relation with mortality of "recipient" oysters that were placed in cohabitation
323 with "donor" oysters which were naturally infected in the field. As it was recently
324 demonstrated that infection by OsHV-1 is the first event that occurs during infectious
325 process followed by a secondary infection by bacteria including vibrios (de Lorgeril et al.
326 2018b), we followed the concentrations of these both pathogens in water tanks. We found
327 that levels of OsHV-1 DNA and *Vibrio* spp. concentration in the seawater of the pathogen-
328 exposure tank peaked at almost the same time (48 h and 50 h respectively).
329 Mortality of recipients increased with cohabitation time with donors and was
330 associated with the cumulative exposure to both OsHV-1 and *Vibrio* spp. In line with this,
331 mortality risk of recipients cohabited with donors increases with the biomass of infected
332 oysters and decreases with seawater renewal, two parameters that probably influence
333 pathogen concentrations in the surrounding seawater (Petton et al. 2015a). Also,
334 mortality risk of oysters injected with a viral suspension increases with concentration of
335 viral particles (Paul-Pont et al. 2015, Segarra et al. 2016). Finally, non-lethal infection of
336 oysters in the field were associated with the dilution of viral particles below a threshold

337 value under which no mortality occurs (Pernet et al. 2018). All together, these results
338 showed a clear relationship between mortality of recipients and pathogens concentration
339 in the seawater.

340 It took only 1.5 h of cohabitation with donors to detect OsHV-1 DNA in the
341 surrounding seawater and to observe mortality in recipients at the end of the experiment
342 (16 d, sequence 1). This result suggests that the transmission is very rapid. This could
343 probably explain why the disease spread so fast when the infectious conditions are in
344 place in farms. From an experimental standpoint, studies dedicated to the investigation
345 of the host response to the disease should focus on early times after exposure. Until now,
346 the earliest samples that were analysed were collected 6 hours after the cohabitation
347 starts (de Lorgeril et al. 2018b).

348 Mortality of recipients plateaued after 135 hours of cohabitation (5.6 d) with
349 donors, *i.e.* 39 h after onset of the first dead recipient. In previous experiments, the
350 cohabitation phase of donors and recipients lasted until appearance of the first dead
351 recipient which generally occurred after 4 days (Petton et al. 2013, Lassudrie et al. 2015,
352 Pernet et al. 2015, Petton et al. 2015a, Petton et al. 2015b, Fuhrmann et al. 2016).
353 However, to reach maximum mortality in recipients, it is necessary to continue the
354 cohabitation 1 to 2 d more. Then, the donors can be removed instead of leaving them
355 dying, decomposing, mineralising and therefore, altering the biogeochemical conditions
356 in the experimental tanks (Richard et al. 2017). This would avoid some confounding
357 effects during experiments.

358 The relationship between recipient mortality and cohabitation time with donors
359 from both sequences 1 and 2 suggests that infectivity in the pathogen-exposure tank
360 decreased with time. For instance, oysters that spent the first 55 h in the pathogen-
361 exposure tank exhibited as much mortality as those who spent the next 165 h, and almost

362 no mortality (0-4%) was observed in oysters transferred in the pathogen-exposure tank
363 after 172 h. This agrees with the fact that levels of OsHV-1 DNA and *Vibrio* spp.
364 concentration in the seawater peaked after *ca.* 48 h and decreased to zero after 172-220h.
365 Although laboratory experiments do not fully reproduce field conditions, the
366 temporal dynamics of oyster mortality in the laboratory were similar to those occurring
367 in the field (de Lorgeril et al. 2018b). Also, cumulative final mortality was only 26% in
368 undisturbed recipients, reflecting a low pathogen-exposure that is typical of the fall in the
369 Thau lagoon (Pernet et al. 2012). Finally, concentrations of OsHV-1 DNA in the seawater
370 were slightly higher than those reported in laboratory conditions by Schikorski et al.
371 (2011) and Paul-Pont et al. (2015). To our knowledge, there are no data available on the
372 temporal dynamics of OsHV-1 concentration in seawater in the field to which we can
373 compare our laboratory data.
374 To conclude, we found that *i*) OsHV-1 and *Vibrio* spp. concentrations in the seawater
375 showed clear temporal dynamics, *ii*) mortality of recipients was associated with
376 increasing cohabitation time with donors and reflect the cumulative exposure to OsHV-1
377 and *Vibrio* spp., *iii*) after only 1.5 h of cohabitation with donors, OsHV-1 DNA was detected
378 at high concentration in seawater and mortality in recipients occurred, *iv*) mortality of
379 recipients plateaued after 5-6 d of cohabitation with donors following by 1-2 d the first
380 dead donor and *v*) infectivity in the pathogen-exposure tank decreased with time up to
381 values close to zero after 172 h. Although this experiment had not been replicated in time,
382 the mortality dynamics of the recipients observed here was consistent with all the others
383 published in the similar conditions. Therefore, the fine-scale dynamics of mortality and
384 OsHV-1 and *Vibrio* spp. concentration in seawater reported here can be generalized to
385 other experiments conducted in similar conditions.
386

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REFERENCES

AHAW EPAHW (2015) Oyster mortality EFSA Panel on Animal Health and Welfare (AHAW). Efsa Journal 13

Azema P, Travers MA, Benabdelmouna A, Degremont L (2016) Single or dual experimental infections with *Vibrio aestuarianus* and OsHV-1 in diploid and triploid *Crassostrea gigas* at the spat, juvenile and adult stages. J Invertebr Pathol 139:92-101

Barbosa Solomieu V, Renault T, Travers M-A (2015) Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas*. J Invertebr Pathol 131:2-10

Bruto M, James A, Petton B, Labreuche Y, Chenivesse S, Alunno-Bruscia M, Polz MF, Le Roux F (2017) *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. ISME J 11:1043-1052

Burioli EAV, Prearo M, Houssin M (2017) Complete genome sequence of Ostreid herpesvirus type 1 µVar isolated during mortality events in the Pacific oyster Crassostrea gigas in France and Ireland. Virology 509:239-251

de Lorgeril J, Escoubas J-M, Loubiere V, Pernet F, Le Gall P, Vergnes A, Aujoulat F, Jeannot J-L, Jumas-Bilak E, Got P, Gueguen Y, Destoumieux-Garzón D, Bachère E (2018a) Inefficient immune response is associated with microbial permissiveness

415 in juvenile oysters affected by mass mortalities on field. Fish Shellfish Immunol
416 77:156-163
417 de Lorgeril J, Lucasson A, Petton B, Toulza E, Montagnani C, Clerissi C, Vidal-Dupiol J,
418 Chaparro C, Galinier R, Escoubas J-M, Haffner P, Dégremont L, Charrière GM, Lafont
419 M, Delort A, Vergnes A, Chiarello M, Faury N, Rubio T, Leroy MA, Pérignon A, Régler
420 D, Morga B, Alunno-Bruscia M, Boudry P, Le Roux F, Destoumieux-Garzón D,
421 Gueguen Y, Mitta G (2018b) Immune-suppression by OsHV-1 viral infection causes
422 fatal bacteraemia in Pacific oysters. Nat Commun 9:4215
423 Fuhrmann M, Delisle L, Petton B, Corporeau C, Pernet F (2018) Metabolism of the Pacific
424 oyster, *Crassostrea gigas*, is influenced by salinity and modulates survival to the
425 Ostreid herpesvirus OsHV-1. Biol Open 7:10
426 Fuhrmann M, Petton B, Quillien V, Faury N, Morga B, Pernet F (2016) Salinity influences
427 disease-induced mortality of the oyster *Crassostrea gigas* and infectivity of the
428 ostreid herpesvirus 1 (OsHV-1). Aquaculture Env Interact 8:543-552
429 Fuhrmann M, Richard G, Quere C, Petton B, Pernet F (2019) Low pH reduced survival of
430 the oyster Crassostrea gigas exposed to the Ostreid herpesvirus 1 by altering the
431 metabolic response of the host. Aquaculture 503:167-174
432 Jenkins C, Hick P, Gabor M, Spiers Z, Fell SA, Gu X, Read A, Go J, Dove M, O'Connor W,
433 Kirkland PD, Frances J (2013) Identification and characterisation of an ostreid
434 herpesvirus-1 microvariant (OsHV-1 mu-var) in *Crassostrea gigas* (Pacific oysters)
435 in Australia. Dis Aquat Org 105:109-126
436 Jones B, Kenward MG (2014) Design and analysis of cross-over trials, Vol. Chapman and
437 Hall/CRC
438 Lassudrie M, Soudant P, Nicolas J-L, Fabioux C, Lambert C, Miner P, Le Grand J, Petton B,
439 Hégaret H (2015) Interaction between toxic dinoflagellate *Alexandrium catenella*
440 exposure and disease associated with herpesvirus OsHV-1µVar in Pacific oyster
441 spat *Crassostrea gigas*. Harmful Algae 45:53-61
442 Le Roux F, Wegner KM, Polz MF (2016) Oysters and Vibrios as a Model for Disease
443 Dynamics in Wild Animals. Trends Microbiol 24:568-580
444 Lemire A, Goudenege D, Versigny T, Petton B, Calteau A, Labreuche Y, Le Roux F (2015)
445 Populations, not clones, are the unit of vibrio pathogenesis in naturally infected
446 oysters. ISME J 9:1523-1531

447 Lucasson A, Petton B, Clerissi C, Toulza E, Escoubas J-M, Haffner H, Dégremont L,
448 Gueguen Y, de Lorgeril J, Mitta M (in prep) The mechanism of the polymicrobial
449 disease affecting Pacific oyster juveniles is conserved between oyster genotypes
450 and infectious environments
451 Lynch SA, Carlsson J, Reilly AO, Cotter E, Culloty SC (2012) A previously undescribed
452 ostreid herpes virus 1 (OsHV-1) genotype detected in the Pacific oyster,
453 *Crassostrea gigas*, in Ireland. Parasitology 139:1526-1532
454 Martenot C, Oden E, Travaille E, Malas J-P, Houssin M (2011) Detection of different
455 variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between
456 2008 and 2010. Virus Res 160:25-31
457 Mortensen S, Strand Å, Bodvin T, Alfjorden A, Skår CK, Jelmert A, Aspán A, Sælemyr L,
458 Naustvoll LJ, Albretsen J (2016) Summer mortalities and detection of ostreid
459 herpesvirus microvariant in Pacific oyster *Crassostrea gigas* in Sweden and
460 Norway. Dis Aquat Org 117:171-176
461 Oehlert GW (1992) A Note on the Delta Method. The American Statistician 46:27-29
462 Paul-Pont I, Evans O, Dhand NK, Whittington RJ (2015) Experimental infections of
463 Pacific oyster *Crassostrea gigas* using the Australian ostreid herpesvirus-1 (OsHV-
464 1) µVar strain. Dis Aquat Org 113:137-147
465 Pepin JF, Riou A, Renault T (2008) Rapid and sensitive detection of ostreid herpesvirus 1
466 in oyster samples by real-time PCR. J Virol Methods 149:269-276
467 Pernet F, Barret J, Gall PL, Corporeau C, Dégremont L, Lagarde F, Pépin J-F, Keck N
468 (2012) Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious
469 diseases and vary with farming practises in the Thau lagoon. Aquaculture Env
470 Interact 2:215–237
471 Pernet F, Fuhrmann M, Petton B, Mazurié J, Bouget J-F, Fleury E, Daigle G, Gernez P
472 (2018) Determination of risk factors for herpesvirus outbreak in oysters using a
473 broad-scale spatial epidemiology framework. Sci Rep 8
474 Pernet F, Lupo C, Bacher C, Whittington RJ (2016) Infectious diseases in oyster
475 aquaculture require a new integrated approach. Philos Trans R Soc Lond B Biol Sci
476 371:20150213
477 Pernet F, Tamayo D, Petton B (2015) Influence of low temperatures on the survival of
478 the Pacific oyster (*Crassostrea gigas*) infected with ostreid herpes virus type 1.
479 Aquaculture 445:57-62

480 Petton B, Boudry P, Alunno-Bruscia M, Pernet F (2015a) Factors influencing disease-
481 induced mortality of Pacific oysters *Crassostrea gigas*. Aquaculture Env Interact
482 6:205-222
483 Petton B, Bruto M, James A, Labreuche Y, Alunno Bruscia M, Le Roux F (2015b)
484 *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be
485 the killer in this polymicrobial opportunistic disease. Front Microbiol 6
486 Petton B, Pernet F, Robert R, Boudry P (2013) Temperature influence on pathogen
487 transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea*
488 *gigas*. Aquaculture Env Interact 3:257-273
489 Renault T, Moreau P, Faury N, Pepin J-F, Segarra A, Webb S (2012) Analysis of clinical
490 ostreid herpesvirus 1 (Malacoherpesviridae) specimens by sequencing amplified
491 fragments from three virus genome areas. J Virol 86(10): 5942–5947.
492 Richard M, Bourreau J, Montagnani C, Ouisse V, Le Gall P, Fortune M, Munaron D,
493 Messiaen G, Callier M, d'Orbcastel ER (2017) Influence of OSHV-1 oyster mortality
494 episode on dissolved inorganic fluxes: An *ex situ* experiment at the individual scale.
495 Aquaculture 475:40-51
496 Schikorski D, Faury N, Pepin JF, Saulnier D, Tourbiez D, Renault T (2011) Experimental
497 ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: Kinetics of
498 virus DNA detection by q-PCR in seawater and in oyster samples. Virus Res
499 155:28-34
500 Segarra A, Baillon L, Faury N, Tourbiez D, Renault T (2016) Detection and distribution of
501 ostreid herpesvirus 1 in experimentally infected Pacific oyster spat. J Invertebr
502 Pathol 133:59-65
503 Segarra A, Pepin JF, Arzul I, Morga B, Faury N, Renault T (2010) Detection and
504 description of a particular Ostreid herpesvirus 1 genotype associated with massive
505 mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus
506 Res 153:92-99
507 Tamayo D, Corporeau C, Petton B, Quere C, Pernet F (2014) Physiological changes in
508 Pacific oyster *Crassostrea gigas* exposed to the herpesvirus OsHV-1µVar.
509 Aquaculture 432:304-310
510 Vacek PM (1997) Assessing the effect of intensity when exposure varies over time. Stat
511 Med 16:505-513

512 Webb SC, Fidler A, Renault T (2007) Primers for PCR-based detection of ostreid herpes
513 virus-1 (OsHV-1): Application in a survey of New Zealand molluscs. Aquaculture
514 272:126-139
515

FIGURE LEGENDS

FIG. 1. (1) Families of oysters (15) from 5 origins were produced in the laboratory and (2) transferred in the Thau lagoon during the infectious period for 17 days and (3) used as pathogen donors in the laboratory. The remaining part of the mixed oyster population was left undisturbed at the facility to be used as uninfected control. A batch of specific-pathogen free oysters was displayed in 51 mesh-bags (100 individuals per bag) and kept under biosecured conditions. Seventeen bags were placed in the pathogen-exposure tank in cohabitation with the donors and they were used as pathogen recipients. Seventeen other bags were placed in individual flow through 50-L tanks referred to as safe tanks. Then, each bag was either transferred from the pathogen-exposure towards safe tanks (sequence 1) or from safe tanks towards the pathogen-exposure tank (sequence 2). These permutations were conducted 16 times in the time. Two oyster bags were left undisturbed in the pathogen-exposure tank and in one safe tank. 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.

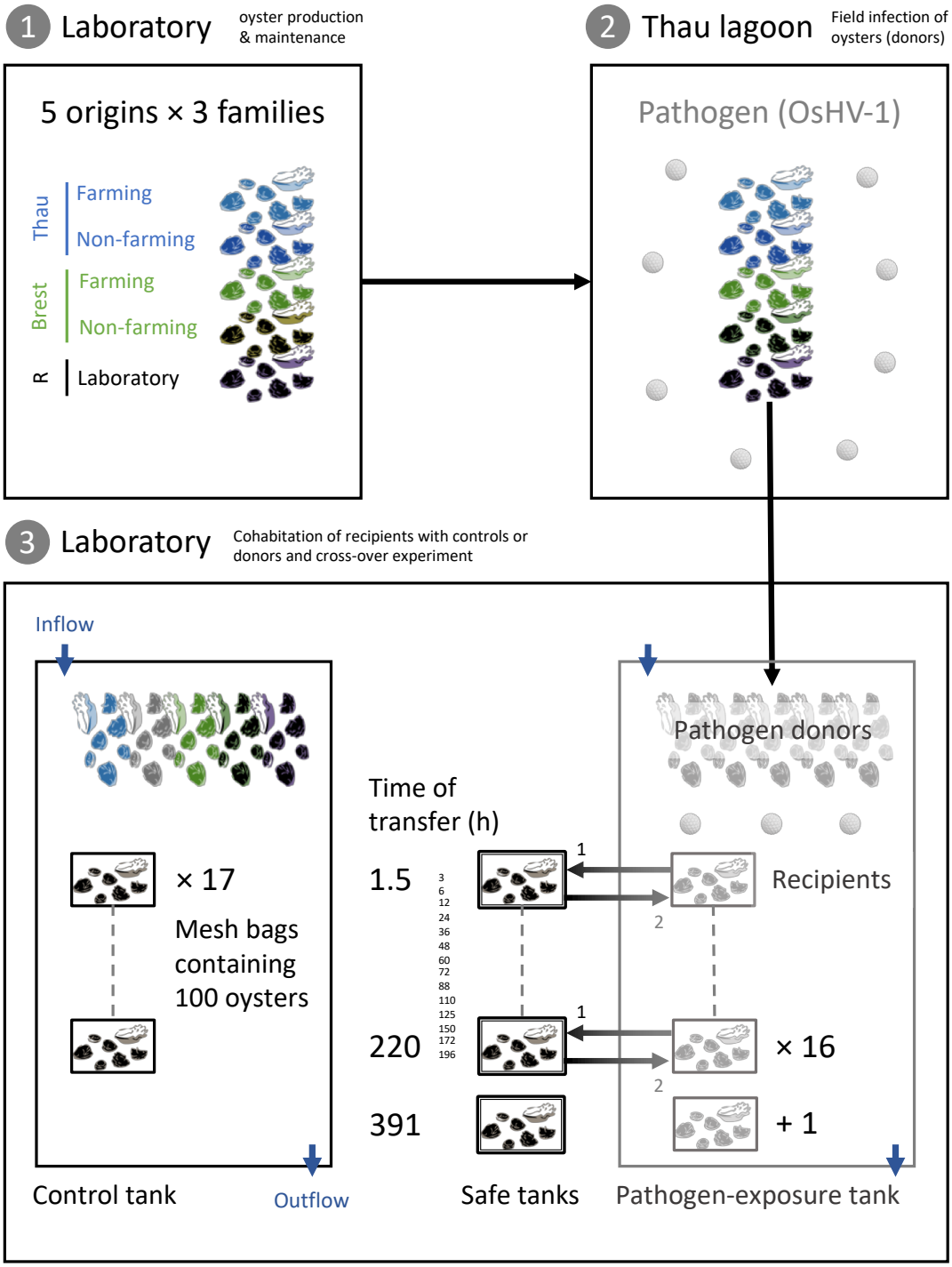
FIG 2. Mortality of recipient oysters at the end of the experiment (391 h) according to the time of transfer (A). Oysters were transferred from a tank containing infected oysters (pathogen-exposure tank) toward individual safe tanks (sequence 1) or from safe tanks toward the pathogen-exposure tank (sequence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

FIG. 3. Cumulative exposure index of oysters (see eq. 10) transferred from a tank containing infected oysters (pathogen-exposure tank) toward individual safe tanks

(sequence 1) or from safe tanks toward the pathogen-exposure tank (sequence 2) calculated for OsHV-1 DNA and *Vibrio* spp. concentrations.

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

547 **FIGURE 1.**



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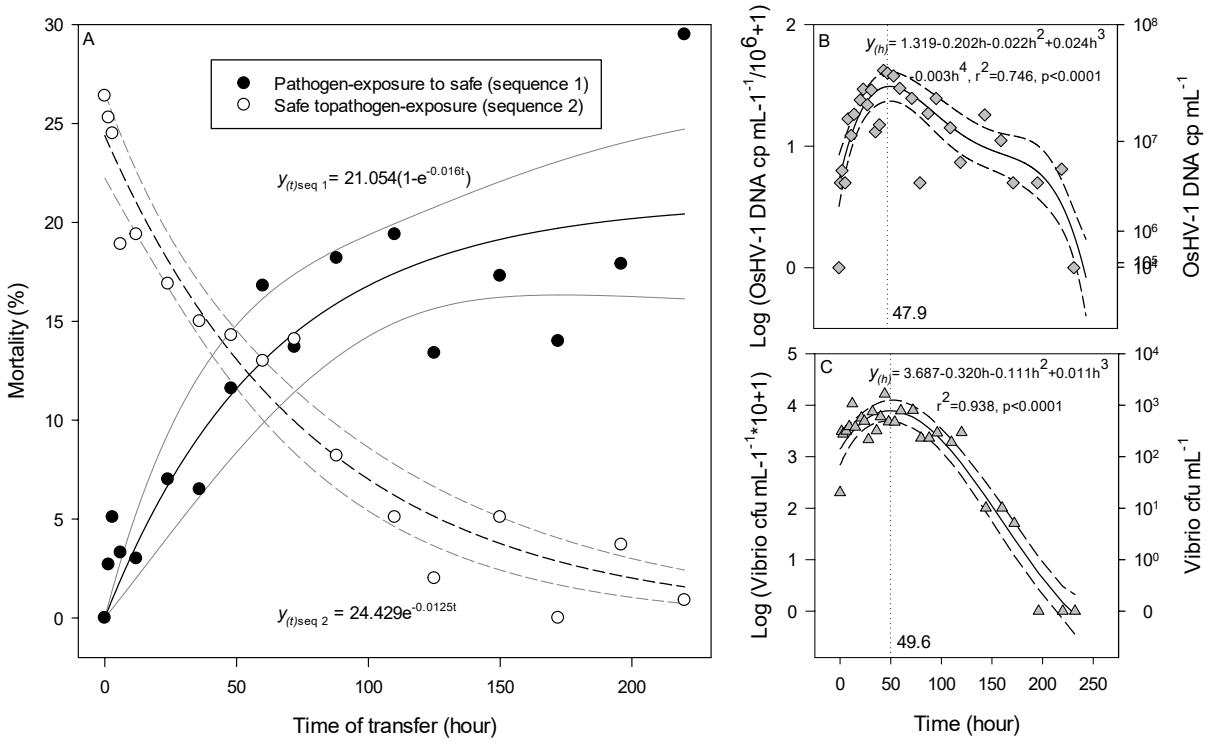
551 **FIGURE 2.**



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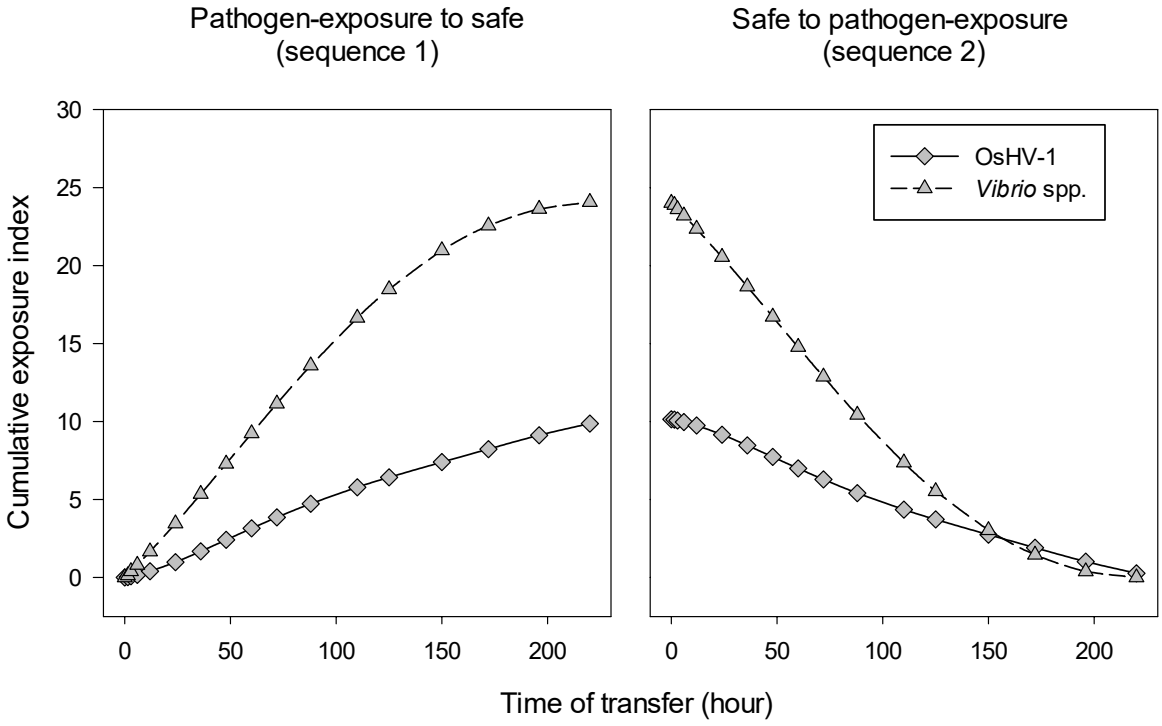
553 **FIGURE 3.**



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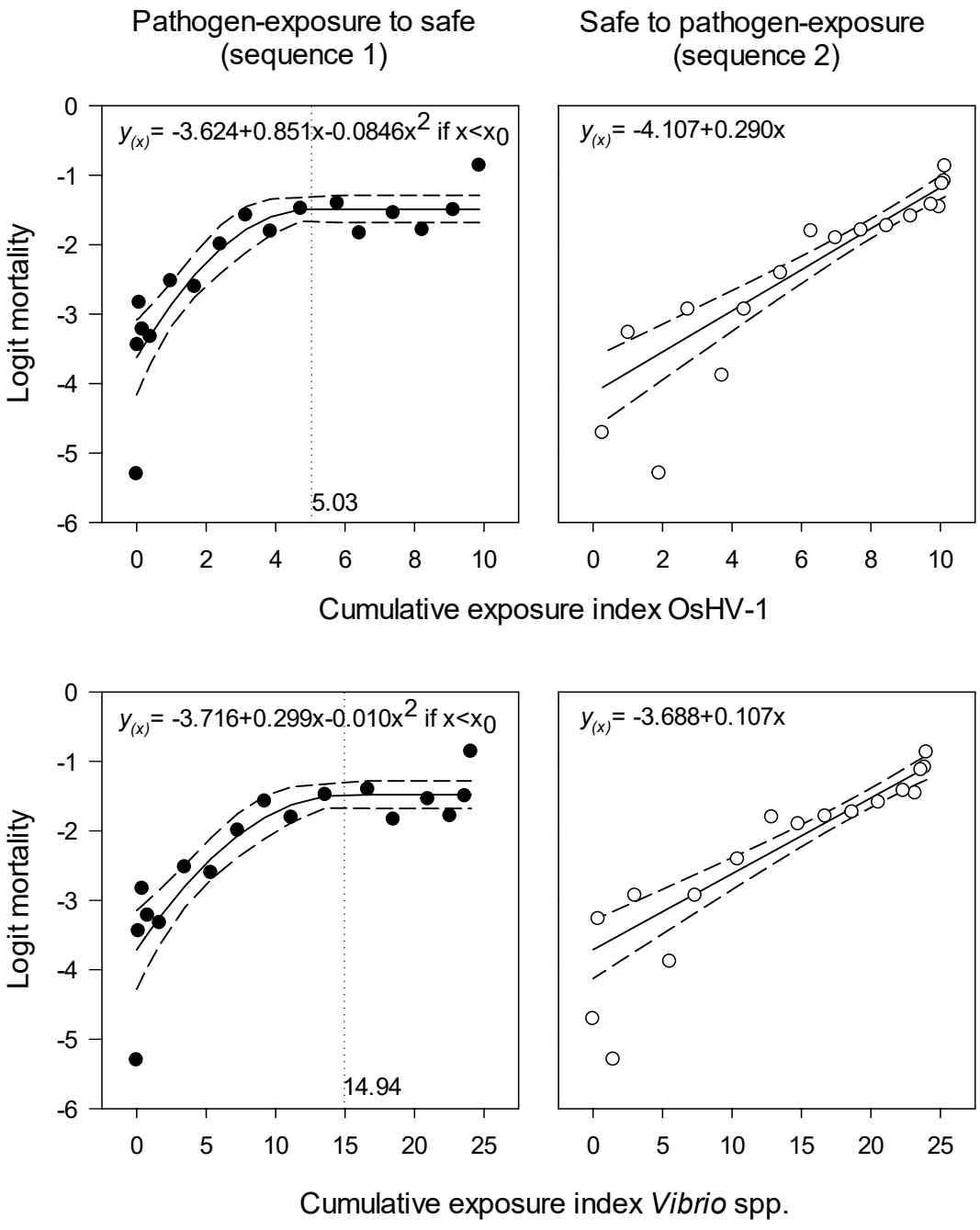
555 **FIGURE 4.**

556



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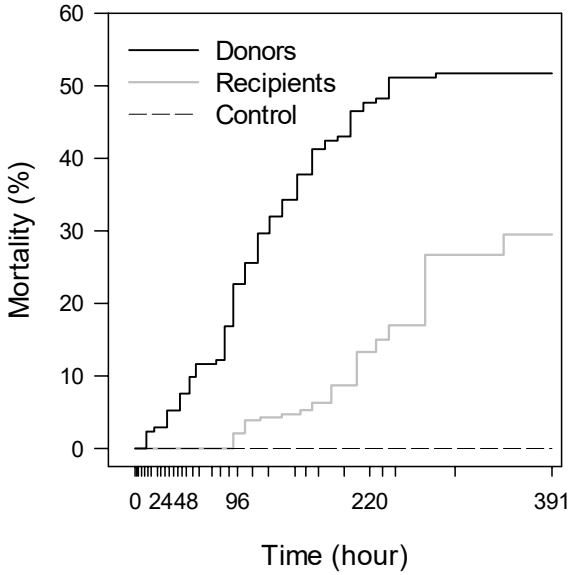
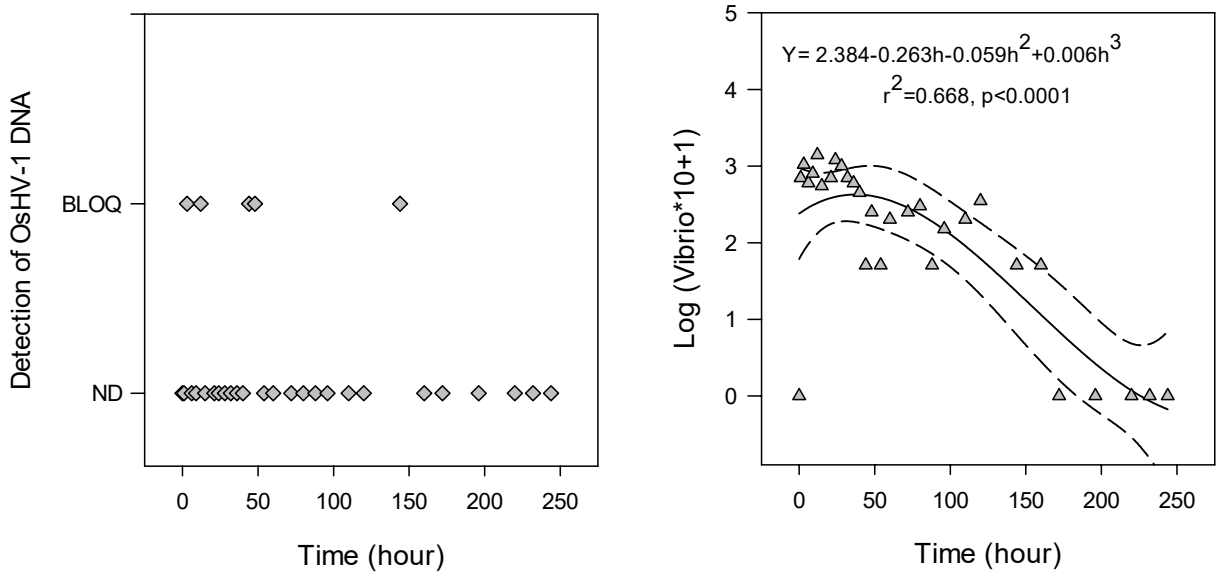


FIGURE A1. Dynamics of mortality in donors and recipients in the pathogen-exposure tank and in uninfected oysters (control). For each time between 1.5 and 220 h, recipient mortality was measured in the oyster bag that was transferred to the safe tanks. As a result, these mortality measures were independent of each other. After 220 hours, the donors were removed and there were no more transfers. Between 220 h and 391 h, recipient mortality was estimated on the oyster bag which remained in the pathogen-exposure tank.

Control oysters showed no mortality (Figure A1) but trace level of OsHV-1 DNA and low concentration of *Vibrio* spp. were occasionally detected in the surrounding seawater (Figure A2). Although these control oysters may not have been SPF during the entire duration of the experiment, absence of mortality suggest that they were healthy, and only the pathogen donors and recipients were considered hereafter.



39

40 **FIGURE A2.** Dynamics of OsHV-1 DNA detection and *Vibrio* spp. concentration in the
41 seawater of the control tank as a function of time. Abbreviations: ND, not detected, BLOQ,
42 detected but below the level of quantification (< 10 cp μL^{-1}).

43