Influence of OSHV-1 oyster mortality episode on dissolved inorganic fluxes: An ex situ experiment at the individual scale

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An ex situ experiment at the individual scale

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Abstract

Ostreid herpesvirus 1 (OsHV-1 µvar) infection has caused significant mortalities in juvenile oysters (Crassostrea gigas). In contrast to the practices of other animal production industries, sick and dead oysters are not separated from live ones and are left to decay in the surrounding environment, with unknown consequences on fluxes of dissolved materials. A laboratory approach was used in this study to test the influence of oyster mortality episode on dissolved inorganic fluxes at the oyster interface, dissociating (i) the effect of viral infection on metabolism of juvenile oysters and (ii) the effect of flesh decomposition on oxygen consumption and nutrient releases at the individual scale. Nine batches of juvenile oysters (Individual Total wet weight 1 g) were infected via injection of OsHV-1 enriched inoculums at different viral loads (108 and 109 OsHV-1 DNA copies per oyster) to explore infection thresholds. Oysters injected with filtered seawater were used as controls (C). Oysters were maintained under standard conditions to avoid stress linked to hypoxia, starvation, or ammonia excess. Before, after the injection and during the mortality episode, i.e. at days 1, 3, 7, 10 and 14, nine oysters per treatment were incubated in individual metabolic chambers to quantify oxygen, ammonium and phosphate fluxes at the seawater-oyster interface. Nine empty chambers served as a reference. Injections of the two viral loads of OsHV-1 induced similar mortality rates (38%), beginning at day 3 and lasting until day 14. The observed mortality kinetics were slower than those reported in previous experimental pathology studies, but comparable to those observed in the field (Thau lagoon, France). This study highlights that oxygen and nutrient fluxes significantly varied during mortality episode. Indeed (i) OsHV-1 infection firstly modifies oyster metabolism, with significant decreases in oxygen consumption and ammonium excretion, and (ii) dead oysters lead to a strong increase of ammonium (6 fold) and phosphate (41 fold) fluxes and a decrease in the N/P ratio due to mineralisation of their flesh. The latter may modify the structure of the planktonic community in the field during mortality episode. This study is a first step of the MORTAFLUX program. The second step was to in situ confirm this abnormal nutrient loading during a mortality episode and show its impact on bacterio-, phyto- and protozoo-plankton.

Keywords

Crassostrea gigas ; Mortality ; Ostreid herpesvirus 1 ; Juvenile ; Spat ; Mineralisation ; Oxygen consumption ; Nutrient fluxes

1. Introduction

Mortality episodes in cultured bivalves have been reported worldwide and have been associated with infection by a range of viral and bacterial pathogens (Barbosa Solomieu et al., 2015). Thus, the history of oyster culture consists of a succession of developmental phases using different species, followed by collapses caused by diseases as described in France (Buestel et al., 2009), where the indigenous species Ostrea edulis was replaced first with Crassostrea angulata in the 1920's, then C. gigas in the 1970s. First mortalities of the Pacific oyster, Crassostrea gigas in France, were reported in the early 1990s in hatcheries and nurseries, for larval and juvenile stages, and later in the field (Nicolas et al., 1992; Renault et al., 1994). These events have been primarily attributed to a herpes-like virus, based on histological and electron microscopy examinations (Renault et al., 1994; Renault et al., 2000a, 2000b) and PCR procedures (Le Deuff and Renault, 1999; Renault et al., 2000b). This herpes-like virus was later described and named ostreid herpesvirus 1 (OsHV-1) (Minson et al., 2000; Davison et al., 2005). Since 2008, a new variant, OsHV-1 µvar, has emerged (Segarra et al., 2010) and has induced abnormal juvenile mortality rates ranging from 80 to 100% along the French coast (Garcia et al., 2011). Mortality of oysters coincided with infections involving the ostreid herpes virus and also bacteria of the group Vibrio splendidus (Pernet et al., 2012; Petton et al., 2015b). These oyster mortality episodes have caused important economic losses in France since 2008 (Girard and Pérez Agúndez, 2014). The development of molecular tools has subsequently allowed the detection of these viruses in many countries in Europe, USA, Australia, New Zealand and in East Asia (Mineur et al., 2014 for review). In 2016, mortalities are still a key topic since they continue to occur each year in France at rates ranging from 35 to 70% (RESCO networks: http://wwz.ifremer.fr/observatoire_conchylicole). Thus mortality of Pacific oyster remain a huge problem, both from economic and scientific points of view in France and worldwide.

The search for a better understanding of these phenomena and to address this crisis have led scientists to focus on the causes and consequences of viral infection on oyster juvenile. Some studies have described viral entry (Jouaux et al., 2013) and distribution in organs and tissues (Segarra et al., 2016); others have identified changes in immune (Green et al., 2015, 2016, He et al., 2015, Moreau et al., 2015,), physiological and biochemical responses (Corporeau et al., 2014; Tamayo et al., 2014) in Pacific oyster spat infected with OsHV1. These studies have provided a better understanding of the interactions between OsHV-1 and oysters at the organism scale. In parallel, some studies have envisaged solutions by highlighting the factors controlling these mortality episodes, such as size, genetics (Dégremont, 2013), energetic status (Pernet et al., 2010), husbandry practices (Paul-Pont et al., 2013; Pernet et al., 2014a; Whittington et al., 2015) and temperature (Pernet et al., 2012; Renault et al., 2014; Petton et al., 2015a). However, to date, no studies have investigated the consequences of these mortality events in the environment, and especially on matter cycle.

Unlike in most other animal production industries, sick and dead individuals are not separated from conspecifics in shellfish farms, a practice that can favour cross-contamination and spread of disease. Dead oysters are kept in the rearing environment until their flesh totally disappears. The consequences of this practice for (i) the transfer of pathogens and (ii) particulate and dissolved fluxes into the environment remain unknown.

Apart from a mortality context, shellfish farms are known to modify the ecosystem functions of coastal environments. Indeed, shellfish modify particulate and dissolved fluxes via their (i) respiration (Chapelle et al., 2000; Richard et al., 2006), excretion (Mazouni, 2004; Richard et al., 2007; Jansen et al., 2012; Lacoste and Gaertner-Mazouni, 2016), (ii) filtration (Dupuy et al., 2000; Trottet et al., 2008) and (iii) biodeposition (Callier et al., 2006, 2009; Robert et al., 2013). At high stocking densities in confined environments, shellfish can control seston biomass via filtration (Smaal et al., 2013; Filgueira et al., 2014a, 2014b), stimulate primary production via nitrogen excretion (Chapelle et al., 2000; Souchu et al., 2001; Mazouni, 2004) and modify the microbial plankton community structure (Froján et al., 2014; Mostajir et al., 2015). Many studies have

focused on this topic in adult organisms (see Cranford et al., 2003; McKindsey et al., 2006; Forrest et al., 2009; Filgueira et al., 2015 for reviews), but few data are available on the juvenile stage, either under laboratory treatments (Dame, 1972; Goulletquer, 1999) or in situ (Meseck et al., 2012; van Broekhoven et al., 2014). Filtration, respiration and excretion rates depend on organism size (see Gosling, 2015a, 2015b for reviews), so juveniles would not have the same influence on biogeochemical fluxes and planktonic communities as would be observed for adults.

In the context of viral infection, the shellfish/environment interactions could be modified. Diseases are known to decrease filtration (Newell, 1985; Flye-Sainte-Marie et al., 2007), excretion and respiration rates (Flye-Sainte-Marie et al., 2007) of marine bivalves. In cases of mortality, oyster flesh probably decomposes and mineralises, thereby causing an increase of nutrients, as observed for mussels (Lomstein et al., 2006) and jellyfish (West et al., 2009). Increases in nutrient loading may in turn induce changes in planktonic components, but this possibility remains to be investigated in cases of oyster mortality. The aim of the MORTAFLUX project is to determine the influence of mortality episode of juvenile oyster on fluxes in the benthic-pelagic coupling of the Thau lagoon that corresponds to the most important French oyster culture site on the Mediterranean Sea.

In this paper, we present the first part of this project. The aim was to evaluate the influence of OSHV-1 mortality episode of oyster juveniles on the dissolved inorganic fluxes at the individual level, i.e. at the water-oyster shell interface, dissociating (i) the effect of viral infection on metabolism of oyster and (ii) the effect of flesh decomposition on oxygen demand and ammonium and phosphate releases at the individual scale. This first study was carried out under laboratory conditions, with the aim of inducing OsHV-1 infection by intramuscular injection of viral inoculum, as performed previously (Schikorski et al., 2011b), maintaining oysters in standard conditions used in ecophysiology to avoid any stress linked to stocking conditions (hypoxia, starvation, ammonia excess). Finally, recorded data of this first study will be up scaled from individual to the rearing structure to be further compared with in situ data issued from the second study of Mortaflux that was carried out before within and after a mortality episode in the Thau lagoon (France).

2. Materials and methods

2.1. Site and equipment

The experimental work was carried out at Ifremer's Aquaculture Research Facility at Palavas-les-Flots (France). Two rooms equipped with an effluent treatment system were dedicated to the pathology experiments. The first room contained a series of 24 similar aquaria (W × L × H: 35 × 60 × 30 cm); 12 were filled with 60 L of filtered seawater and equipped with an air bubbler, a thermostat (21 °C), a foam filter and a water circulation system driven by an airlift and were used for acclimation of the oysters. The second room included three aquaria (W × L × H: 35 × 60 × 30 cm), each equipped with a thermostat (21 °C) and an air bubbler, to be used for incubation experiments.

2.2. Viral inoculum

Viral inoculum was prepared as described by Schikorski et al. (2011b) using ten OsHV-1 laboratory-infected oyster juveniles. Briefly, the flesh was homogenised in 10 volumes of autoclaved seawater and this homogenate was clarified by centrifugation (1000 ×g for 10 min, 4 °C) before serial filtration (8.0, 0.45 and 0.22 µm). This inoculum contained 2 × 107 C9/C10 DNA copies µL−1 and was confirmed to be free of cultivable bacteria by plating 50 µL of inoculum on Zobell marine and TCBS agar plates. Preliminary tests showed that a 50-µL injection of this viral inoculum (i.e. 109 DNA copies oyster−1) induced in 4 days 67% mortality of oyster juvenile (1 g) that were confined at 22 °C in a low water volume (n = 15, 15 ind.5 L−1 :3 g L−1) without a filtration system. This viral inoculum was 10-fold diluted to obtain a second inoculum

containing 2 × 106 copies µL−1 to test the load effect on mortality kinetic and oyster metabolism. The two inoculums, called L1 and L2, were stored at 4 °C before injection during twelve days.

2.3. Oysters, acclimation and experimental procedure

A total of 370 juvenile diploid oysters were received on 3 March 2015 at Palavas. They had been produced and raised in the experimental hatchery of the Ifremer station in Argenton (Brittany, France), as described by Petton et al. (2015a, 2015b) and pre-grown in earthen ponds at the Ifremer station in Bouin (Vendée, France). Oyster spat production results from the reproduction of a large pool of broodstock sampled in the field. This material called "Naissain Standardized Ifremer" (NSI) is assumed to be "naïve", i.e. free of pathogens and not resistant to OSHV-1 pathogen. NSI are used as sentinel for the French shellfish observatory network (RESCO) and by several authors (Tamayo et al., 2014; Petton et al., 2015a; Green et al., 2015). Upon receipt, the oysters were immediately randomly divided into three batches and acclimated for 1 week in three acclimation aquaria. One week after receipt, each batch was sub-divided into three groups of 38 individuals, which were randomly assigned to an infection treatment (C: control, L1, L2: infected by viral inoculum at the 2 viral loads) in preparation for injection. The oysters were then distributed at low density among nine aquaria (38 1 g-ind 60 L−1 :0.6 g L−1) where they were kept until infection. During the acclimation and experiment periods, oysters were fed daily with Skeletonema sp. (supplied by SATMAR, Gatteville-Phare, France) with a ration equivalent to 5% of the oyster dry weight, to avoid potential starvation stress. Temperature and salinity were measured every day using a WTW-LF197S probe. The mean (±Standard Error: SE) temperature was 20.8 ± 0.96 °C and salinity was 38.78 ± 0.51. Nitrite, nitrate and ammonium concentrations were measured weekly with aquarium Red Sea kits to ensure the proper functioning of the foam filters and associated circulation system.

2.4. Experimental design

On 12 March 2015 (day −5), incubations and biometry were carried out on three individuals per aquarium to ensure that the aquaria or preassigned batches (C, L1, L2) had no effects on individual weight and metabolism of oysters before the viral inoculum injection (Table 1). On 16 March, all the oysters in each aquarium were anesthetised in a solution containing magnesium chloride (MgCl2: 50 g, 400 mL seawater + 600 mL freshwater) (Suquet et al., 2009; Schikorski et al., 2011b). Control oysters (C) received a 'sham' injection of filtered seawater (0.2 µm). Other oysters received a 50-µL injection of one of the two viral inoculums, corresponding to two different viral loads (L1: 108 or L2: 109 OsHV-1 DNA copies per oyster) (Fig. 1). After injection, the oysters were transferred into their corresponding experimental aquaria, giving a total of nine aquaria (3 acclimation aquaria × 3 treatments). During the experiment, the number of dead oysters was counted daily within a fixed batch of 20 oysters in each of the nine aquaria to determine mortality rates. The other 18 oysters were used in experimental incubations. Experiment duration was fixed to 14 days sincemortality episode occurred within two weeks in the Thau lagoon (Pernet et al., 2014b). Nevertheless, 10 oysters par aquarium were keep in "acclimation" in each aquaria until the 21 d to confirm the end of the mortality episode. Before (d −5) and after injection, at days 1, 3, 7, 10 and 14, three oysters per aquarium were randomly sampled for incubation in individual chambers for the measurement of dissolved inorganic fluxes. These oysters were alive or dead, according to infection treatment and time. At the beginning of each incubation series, three chambers were filled with water (i.e. without oysters) to serve as a reference for fluxes (W). Thus, 36 incubations were performed per date (3 series × 4 treatments (W, C, L1 and L2) × 3 replicates: Fig. 1).

2.5. Metabolic chambers and incubation procedure

Metabolic chambers, consisting of 125-mL glass bottles (H: 102 mm, Ø 51 mm) with rubber-sealed polypropylene caps (Fisher Brand 2911475), were equipped with a non-invasive optical oxygen sensor (PreSens) that adhered to the chamber wall. During the incubation procedure, 12 chambers (W, C, L1, L2 ×

3) plus three 125-mL bottles (W) were randomly deployed within an incubator, which was filled daily with filtered seawater (b0.2 µm), maintained at 21 °C and supersaturated with oxygen. Incubations were run for 2 h 30 min (i) in the dark in order to exclude photosynthesis and to ensure exclusive measurement of fluxes linked to respiration and mineralisation, (ii) in silence to avoid stressing the oysters and closing of the their valves. During the day, three series of incubations were performed using three incubators, located at three independent sites. Oysters were deprived of food 24 h prior to incubation.

At the beginning (T0) of each incubation procedure, three supplementary 125-mL bottles deployed in each incubator were used to sample seawater to estimate the average nutrient concentrations at T0. Oxygen concentration was measured in each chamber using the non-invasive optical oxygen spot (SP-PSt3-NAU-D5-YOP) and an immovable optical fibre linked to a Fibox 3 optical oxygen meter (PreSens) at T0 and Tf (final time) using OxyView-PST3-V6.02 software. Concentrations at Tf were estimated using the final sampling from each chamber. Water contained in the metabolic chambers was filtered using a syringe (50 mL) coupled with a fixed filter (0.2 µm cellulose acetate membrane). This water was next placed in two 60-mL plastic bottles and stored at −20 °C for NH4 and PO4 analysis. At the end of the incubation, oysters were individually collected for biometry measurements. Oyster shells were removed and the flesh was snap frozen in liquid nitrogen and stored at −80 °C for dry weight and qPCR analyses.

2.6. Mortality kinetic, oyster biometry and dissolved inorganic flux analysis

The cumulative mortality rates were calculated each day in each of the nine aquaria from the sums of dead oyster numbers observed from day 1 compared to the initial oyster number (n = 20). The lengths were determined with a calliper and total, flesh and shell weights were determined with a precision balance to 10−3 g. The AFNOR condition index (flesh weight/total weight) was calculated for each sampled oyster. The PO4 and NH4 concentrations were measured using Seal AA3 analytical autoanalysers following previously described methods (Aminot and Kérouel, 2007) with colorimetric (from SEAL Analytical, Germany) and fluorometric (from JASCO, FP-2020plus, France) detection, respectively. The Ifremer laboratory in Sète is accredited for these analyses in accordance with the recognised international standard ISO/IEC 17025, by the French Committee for Accreditation (COFRAC, Certificate n°1-1655). The O2 and nutrient fluxes were determined from changes in concentrations between Tf and T0, reported for incubation duration and multiplied by chamber volume. These data are expressed as µmol h−1 chamber−1 .

2.7. Nucleic acid extraction and PCR detection of OsHV-1

OsHV-1 DNA was detected and quantified. Genomic DNA was purified from oyster tissues using the Wizard® SV Genomic DNA Purification System (Promega). Whole animal samples previously stored at −80 °C were homogenised on ice in a 1.5-mL microtube using a pellet mixer in the digestion solution and incubated for 2 h at 55 °C. The remaining oyster tissues were pelleted (centrifugation at 2000 ×g for 2 min) and DNA was extracted from the supernatant according to the manufacturer's instructions. Samples were resuspended to a final concentration of 20 ng µL−1 . OsHV-1 genomic DNA was detected and quantified using quantitative PCR (qPCR) (Pepin et al., 2008). All amplification reactions were performed in duplicate using a Roche LightCycler 480 Real-Time thermocycler (qPHD-Montpellier GenomiX platform, Montpellier University). The PCR reaction volumes were 6 µL and contained LightCycler 480 SYBR Green I Master mix (Roche), 100 nM of pathogen-specific primers and 20 ng of DNA. Pathogen-specific primer pairs C9/C10 were obtained from the literature (Barbosa-Solomieu et al., 2004; Schikorski et al., 2011a) and their resulting amplification products were cloned into the pCR4-Topo vector and replicated in Escherichia coli DH5a (Invitrogen). Plasmids were extracted using the Wizard Plus SV miniprep DNA purification system (Promega) and standard curves of known concentration of plasmid were generated according to the Applied Biosystems manual of absolute real-time RT-PCR quantification. Absolute quantification of OsHV-1 genome copies in oyster samples was estimated by comparing the observed Cp values to known plasmid standards.

2.8. Statistical analysis P

PERMANOVAs were used to test the effects of i) infection treatment, date and their interaction on mortality, biometry and flux results; and ii) oyster status (dead, infected and control oysters) on fluxes and nutrient ratios. First series of PERMANOVAs was done to test the influence of mortality episode on dissolved fluxes, considering alive and dead organisms, to describe mean changes at individual scale from a small number of organisms (n = 9). The idea was to extrapolate data from individual to lantern scale in order to simulate changes observed in the field before within and after a mortality episode. The second PERMANOVAs series was done to dissociate processes highlighting (i) the influence of infection on metabolism (respiration and excretion) of oyster, and (i) the effect of flesh decomposition on oxygen demand and ammonium and phosphate fluxes, in considering separately fluxes measured at the interface of infected (L) and dead oysters (D). A posteriori tests were then done to compare individual means with each other where there was significant variation. Analyses were done with JMP, PRIMER software and the PERMANOVA package (Plymouth Routines in Multivariate Ecological Research; Clarke and Warwick, 2001).

3. Results

3.1. Cumulative mortality rates

Cumulative mortality rates varied significantly with the interaction of date and treatment (N = 126, p-value b 0.0001). No mortality was observed during the first two days after viral injection within each infection treatment, but the first dead oysters were observed at day 3 in treatments L1 and L2. From day 4 until day 14, cumulative mortality rates were significantly higher in infected (L1, L2) than in control (C) treatments, with no significant differences between L1 and L2 (Fig. 2). At day 14, the cumulative mortality rates (± SE) were 0, 34.5 ± 2.7 and 41.6 ± 5.8 for C, L1 and L2 treatments, respectively. For information, no dead oyster was observed at day 21 whatever treatments.

3.2. Biometry

At d-5, the mean length and total weight (±SE) of the oyster juvenile were 17.7 ± 0.4 mm and 1.06 ± 0.01 g, respectively. From day 1 to day 14, the total weight of live oysters varied significantly with infection treatment (p = 0.004) and date (p = 0.002) (N = 135). The total weight was higher in the control than in the infected oysters, with no difference between viral loads (C: 1.16 ± 0.01 vs. L1 and L2: 1.12 ± 0.005). A slight increase was observed in the total weight and shell weight from d1 to d7, specifically for the C oysters. By contrast, neither flesh weight nor AFNOR condition index varied significantly among treatments during the experiment (d1 to d14). The means (± SE) were 0.158 ± 0.003 WWg and 14.04 ± 0.22, respectively, when the data were pooled within date (d1, d3, d7, d10 and d14) and infection treatment (C, L1 and L2).

3.3. Viral DNA

The number of viral DNA copies per ng total gDNA (NVDC), quantified for the flesh of live oysters varied significantly according to the interaction between date and treatment (p = 0.015) (N = 45). No significant variation was observed between C, L1 and L2 at d1 or d14, but higher NVDC values were observed in infected than in control treatments at d3, d7 and d10 (Fig. 3A). Intra-treatment variability was significantly higher in the L treatments, specifically for L2 at d3 and d7 (Fig. 3). The a posteriori tests showed that the NVDC observed for L1 increased at d3, followed by a decrease at d7 and a minimal value at d14. By contrast, the NVDC observed for L2 significantly increased from d3 until d10 and finally decreased at d14 (Fig. 3). The status of the oysters (C: control, L: infected, D: dead) was a significant source of variation for the NVDC (p = 0.0001), with the highest mean observed for dead organisms and the lowest for the control oysters (C: 1.81E + 01 b L: 1.13E + 04 b D: 1.54 + 05 C9C10 copies ng of total gDNA: Fig. 3B).

3.4. Dissolved fluxes

During the experiment, live and dead oysters were randomly incubated within individual chambers, with increase of numbers of incubated dead organisms from d3 to d14, in the infected treatments (Fig. 4). The O2, NH4 and PO4 fluxes varied significantly according to treatment (W, C, L1 and L2) and date, based on the fluxes at the interface of live and dead oysters (Table 2). More specifically, the presence of oysters was a significant source of variation for dissolved fluxes, with higher consumption of O2 and higher releases of NH4 and PO4 in the presence (C, L1 and L2) than in the absence of oysters (W) (cf. a posteriori tests in Table 2 and * Fig. 5). Consideration of the biogeochemical fluxes associated with live and dead oysters in the dataset (Table 2, Fig. 5) resulted in: i) a significant effect of infection treatments (L1, L2 vs. C) for O2 fluxes, with lower consumption observed for infected than for control oysters (Fig. 5A), ii) high NH4 and PO4 releases for L treatments at d7, d10 and d14 (Fig. 5B, C). The PERMANOVAs showed that the status of the oysters (dead, infected or control) had a significant effect on the fluxes of O2, NH4 and PO4, (i) with lower O2 consumption and NH4 release for the infected oysters than the controls (Fig. 6A, B, Table 3), (ii) with lower O2 consumption and higher NH4 and PO4 release at the interface of dead than of live oysters (i.e. C or L: Table 3, Fig. 6A, B, C). The oxygen demand observed at the interface of the dead organisms corresponded to 2/3 of the oxygen consumption of the control oysters. Dead oysters lead to very high ammonium and phosphate releases, especially at d7 when the mean release at the interface of dead organisms (0.58 µmol NH4 h−1 ind−1 ; 0.044 µmol PO4 h−1 ind−1) was 14 to 63 fold higher than that observed at the interface of live oysters (0.04 µmol NH4 h−1 ind−1 ; 0.0007 µmol PO4 h−1 ind−1), respectively. NH4 and PO4 releases at the dead oyster interface were on average 6 to 41 fold higher than ones observed at the interface of control oysters (Fig. 6B, C). Releases of NH4 and PO4 did not show the same proportions at the interface of control, infected or dead organisms. Thus, NH4/PO4 concentration ratios observed at final time of incubation (Tf) varied significantly according to oyster status (Table 3), with lower mean ratios for dead, followed by infected and control oysters (NH4/PO4: D: 11.3 b L: 22.4 b C: 35.4, Fig. 7C).

4 Discussion

In our study, we reproduced OsHV-1 infection by injection, as previously done by Schikorski et al. (2011b), maintaining oysters in standard conditions used in ecophysiology, with the objective to test the influence of oyster mortality episode on dissolved inorganic fluxes at the water-shell oyster interface, dissociating (i) the effect of a viral infection on metabolism (i.e. respiration and excretion rates) of oyster juveniles and (ii) the effect of flesh decomposition on oxygen consumption and nutrient release at the water-oyster interface at the individual scale.

4.1. Incidence of viral injection in juvenile oysters and kinetics of mortality

As expected, and as previously described by Schikorski et al. (2011b), intramuscular injection of OsHV-1 viral inoculum caused mortality in Crassostrea gigas, with the first dead oysters appearing three days after infection. The kinetics of mortality were slower in our study than typically observed in experimental pathology studies (Schikorski et al., 2011a; Schikorski et al., 2011b; Segarra et al., 2016) (Fig. 7), with cumulative mortality rates of 2.5, 15, and 40% at d3, d7 and d14, respectively, vs. 40–70 and 60–90% at d3 and d7, respectively (Schikorski et al., 2011a; Schikorski et al., 2011b). The variability in the mortality kinetics and magnitude could have been linked to differences in the injected viral loads, but it was not the case. We used higher loads than in other studies (108 and 109 vs. 2 × 104 to 1.5 × 107 viral DNA copies per oyster: Schikorski et al., 2011a, 2011b). We chose these doses after preliminary tests indicated that 108 viral DNA copies per oyster of our inoculum induced mortality. Our inoculum could possibly be less virulent than others, or else the quantification of DNA copies may not be a good indicator of virulence since real time quantitative PCR does not allow the quantification of infective virus particles, which are necessary to initiate the virus infection in host cells (Lyman and Enquist, 2009). PCR quantification does not give information about the

status of viral capsids and membranes, which may be damaged, but we are unable to test this hypothesis. Variability of resistance traits between oyster juveniles used in this study with the other ones (Schikorski et al., 2011a; Schikorski et al., 2011b) could also explain this result. Nevertheless, the used oyster ("Naissain Standard Ifremer") were produced from large pool of broodstock sampled in the field (Petton et al., 2015a) and they supposed to be free of pathogens and not resistant to OsHV-1. However, the oyster stocking methods used in our study were not the same as those used in experimental pathology studies. We maintained oysters in standard conditions used in ecophysiology. In contrast to other studies, oysters were at low density (our study: 38 ind 1 g/60 L: 0.6 g L−1 vs. Schikorski et al., 2011a: 140 ind 5 g/25 L: 28 g L−1 , Schikorski et al., 2011b: 10 ind 5 g/5 L: 10 g L−1). Oysters were fed daily with Skeletonema sp. and seawater was continuously filtered through a foam filter and aerated with an airlift. The differences in stocking methods between our study and previous ones could also explain differences in mortality kinetics and magnitude. These conditions were chosen to avoid potential stress linked to starvation, hypoxia and excess ammonium and nitrite that could favour infection, mortality and metabolic changes. Finally, the low oyster density and filtration of seawater used in our study might have limited horizontal transmission processes induced by oyster cohabitation, as previously described in (Schikorski et al., 2011a; Petton et al., 2015a), and caused by releases of viral particles in seawater on the first day after infection (Schikorski et al., 2011a; Paul-Pont et al., 2015). These slow kinetics of mortality and intermediate cumulative mortality were also observed with diluted viral inoculum (Paul-Pont et al., 2015), which is adequate for conducting research on changes in metabolism in response to infection. Finally, our observations were comparable to those reported in natural environments, notably in the Thau lagoon (France), where cumulative mortality rates were 2.5–5% at the beginning of a mortality episode and reached 12–30% and 40– 47% at 7 and 14 days after the first observations of moribund and dead organisms (Fig. 7). The mortality of oysters in our study was due to OsHV-1 infection, as no mortality was observed in control oysters injected with filtered seawater. Higher numbers of OsHV-1 DNA copies were observed in the flesh of live infected oysters from d1 to d3 suggesting that OsHV-1 is the causative agent of the mortalities. However, as mentioned by previous studies (Petton et al., 2015b), we cannot exclude the role of pathogenic bacteria, especially vibrios, in the pathogenesis as co-factors of mortality. As suggested by Sauvage et al. (2009), the d1-d3 period may correspond to a replication phase that leads to irreversible cell damage before resulting in the first instances of mortality. The highest copy number for OsHV-1 DNA was observed between d3 and d10, with high individual variability. This result suggested that active virus replication occurred during this period in the injected oysters, as described by Schikorski et al. (2011b). The highest amounts of viral DNA were observed in dead oysters, followed by infected and control oysters (D: 1.54 × 105 N L: 1.13 × 104 N C: 1.81 × 101 DNA copies ng−1 of total DNA extracted from tissues). The same range of viral DNA amounts was observed previously in dead and dying oyster juveniles (Schikorski et al., 2011a, 2011b; Segarra et al., 2016). The quantity of viral particles reaches a threshold in oyster tissues before death (Paul-Pont et al., 2015). Thus, infected oysters, dissected alive, showed significantly lower viral concentrations in their tissues than dead oysters (Paul-Pont et al., 2015). At the end of our experiment, from 1 to 2 viral DNA copies ng−1 of total DNA were recorded in the flesh of live oysters, regardless of their infection treatment. Surviving oysters at d14 were probably genetically more resistant to the disease than others, as previously observed (Segarra et al., 2014) in a family selected for low susceptibility.

After 21 days, no further mortality was observed in the infected oysters. This result indicated the end of active infection and effective control of viral replication in the surviving oysters, as shown previously for juvenile oysters (He et al., 2015). Arzul et al. (2002) reported the detection of low virus DNA amounts in healthy oysters and suggested that these organisms corresponded to healthy carriers. Finally, no significant difference was observed in terms of mortality magnitude and kinetics between the two viral doses used in this study. A dose-response relationship between the load of OsHV-1 viral inoculum and mortality rate was reported in C. gigas by Paul-Pont et al. (2015). A sufficient initial dose of viral particles is needed to trigger mortality. It is likely that this threshold was exceeded in our study

4.2. Impact of viral infection on juvenile metabolism

Our study showed, under laboratory conditions, that oysters (i) consume oxygen via respiration processes and (ii) release ammonium and phosphate via excretion processes. The mean observed respiration rate (21.94 µmolO2 gDW h−1) was of the same order of magnitude than data reported in the literature (Table 4), that ranged from 23.6 to 33.6 µmolO2 DWg−1 h−1 for C. gigas at 20 °C with lower rates observed for juveniles. Respiration rates vary according to temperature, size, food availability, salinity and stressors (Gosling, 2015a). Ammonia is the main product excreted by marine bivalves (Gosling, 2015a). The NH4 excretion rates observed in our study were consistent with those obtained previously by Buzin et al. (2015), varying from 0.2 to 0.8 µmol NH4 DWg−1 h−1 according to temperature (Table 4). To our knowledge, no study has yet quantified phosphate excretion of marine bivalves at the individual scale. More often, phosphate release was documented at the community scale, i.e. at the interface of bivalve beds (Dame et al., 1989; Dame and Libes, 1993; Asmus et al., 1995; Bartoli et al., 2001), rearing structures (Richard et al., 2007; Jansen et al., 2011; Lacoste and Gaertner-Mazouni, 2016) or pools (Richard et al., 2006). These releases were attributed to mineralisation processes and excretion of benthic organisms, mainly represented by engineering species (i.e. oysters or mussels) in terms of biomass. In our study, the mean ratio between ammonium and phosphate excretion rates was 46 for the control oyster juvenile. A ratio of 35 was obtained between NH4 and PO4 concentrations at the end of the incubation, which was higher than that reported previously for phytoplankton (N:P = 16:1; Redfield et al., 1963). This observation illustrates that oysters act as an important sink for phosphorus, as highlighted by Dame et al. (1989) for oyster reefs, which released only 8% of TP uptake as PO4.

Several authors have highlighted, in an infection context, that bivalve diseases induce changes in filtration activities (Table 5); however, few have highlighted the variations in respiration and excretion activities of these infected organisms. Most of the cited papers listed in Table 5 focus on parasites (i.e. Martelia, Perkinsus marinus, Haplosporidium nelsoni, etc.), whereas few focused on bacteria and none investigated viral infection. To our knowledge, our study highlights for the first time that OsHV-1 infection induces a decrease in oxygen consumption and ammonium excretion by oyster juvenile. These results could be linked to the alteration of gill and heart cells, which have been characterised as targets for OsHV-1 replication (Arzul et al., 2002; Pepin et al., 2008; Sauvage et al., 2009; Schikorski et al., 2011a; Segarra et al., 2016) and are actively involved in respiration and excretion processes (Gosling, 2015a, 2015b).

Soletchnik et al. (1998) showed a negative correlation between respiration and gill abnormalities induced by Chlamia in C. gigas and related it to gill malfunction. Ruditapes philippinarum showed a parallel decrease in respiration and clearance rate in cases of infection by Vibrio tapetis (Flye-Sainte-Marie et al., 2007). These authors interpreted this respiration decrease as a compensatory reduction in the metabolic rate depending on feeding activity. No measurements of clearance rates were made in the present study; nevertheless, we assume that the filtration activity of oyster juvenile may be reduced in cases of OsHV-1 infection. We also assume that mucus secretion, observed at the extremity of valves of some infected oysters, would inhibit gill ciliary activities and in turn perturb filtration activities, as suggested by Flye-Sainte-Marie et al. (2007) for R. philippinarum infected with V. tapetis.

The valve-activity behaviour of infected oysters was modified during infection prior to death. When they were still alive, the infected oysters seemed to show longer and wider valve opening, so that the oyster juvenile seemed to be less reactive to external stress. The same phenomenon was observed with oysters infected with Perkinsus marinus (Mackin, 1962) which could not maintain valve as long as uninfected oysters. In the present study, this behaviour did not appear to show a correlation with respiration or excretion rates. The adductor muscles are also targets for OsHV-1 replication (Schikorski et al., 2011a), so infection could also induce dysfunction in their valve-closing function. Future experiments should attempt to confirm this observation with a valvometer as done by (Tran et al., 2010; Comeau et al., 2017). Changes in valve-opening behaviour could be a good indicator for predicting OsHV-1 mortality in the field.

4.3. Impact of flesh decomposition on dissolved fluxes

Our study showed for the first time that oyster mortality induced large releases of NH4 (×6) and PO4 (×41) via decomposition of flesh. Similar results were shown with decomposed mussels (Lomstein et al., 2006) and jellyfish (West et al., 2009; Chelsky et al., 2016). Nitrogen and phosphorus releases may result from leaching, followed by microbial mineralisation of dead tissue (West et al., 2009). A succession of bacteria is involved in oyster spoilage, including mainly proteobacteria such as Vibrio sp. (Madigan et al., 2014).

Bacteria are bio-accumulated mainly in the gills and digestive glands (Dapeng et al., 2014). In the present study, decomposition of oyster tissues was first observed in the mantle and gills and finally the digestive gland. Kasper et al. (2012) made a detailed description of the decomposition and mineralisation of decaying cadavers. The decay of a dead body typically starts under the influence of aerobic bacteria that deplete the body of oxygen. Oxygen consumption observed at the dead oyster interface could therefore be related to the biological oxygen demand exerted by these kinds of aerobic bacteria. Subsequently, decomposition continues with putrefaction by anaerobic bacteria. Putrefaction occurs predominantly by reductive processes and is accompanied by the production of putrefaction compounds, such as ammonia and hydrogen sulfide (Kasper et al., 2012).

Oxidation of these reduced compounds could also explain the increased oxygen demand observed at the dead oyster interface. Biological and chemical oxygen demands corresponded to half the oxygen consumption of uninfected oysters. Kasper et al. (2012) also noted that high molecular nutrients break down to their constituents. Thus, proteins degrade via peptides and amino acids to smaller sulfur-, nitrogen- and phosphorus-containing compounds. Triacylglycerides are saponified into glycerine and free fatty acids. Polysaccharides are hydrolysed into glucose and monosaccharides, which are typically decomposed to organic acids and other oxygenated compounds. Microbial activity on these low-molecular precursor substances leads to volatile organic compounds, responsible for the intense smell of decaying organisms. The efflux of fatty acids and dissolved organic nitrogen and phosphorus (DON and DOP, respectively) were also observed during decomposition of mussels and jellyfish (Lomstein et al., 2006; West et al., 2009). The volume of our metabolic chambers was too small to carry out both inorganic and organic N and P analyses. Nevertheless, we supposed that the increased decomposition of oysters also increased DON and DOP fluxes.

Releases from decaying organisms are called necronomes (Stroud et al., 2013). These necronomes can act as semiochemical signals, which are involved in animal communication. Certain semiochemicals, such as certain unsaturated fatty acids (oleic and linoleic acid), trigger strong necrophoric behaviour in isopod crustaceans (Yao et al., 2009 in Stroud et al., 2013). These kinds of organisms were observed in oyster lantern nets in the Thau lagoon during oyster mortality events. In our study under laboratory conditions, we did not take into consideration the role of scavengers in oyster degradation. Without predation, high ammonium releases at the interface of dead oysters may reduce the probability or amplitude of N deficiency in adjacent water, as found for healthy oysters in the adult stage (Souchu et al., 2011) or with mussels (Richard et al., 2006). Ammonia release at the interface of dead organisms may stimulate primary production and specifically favour blooms of small-sized phytoplankton, i.e. picophytoplankton, as observed for oysters at the adult stage (Chapelle et al., 2000; Souchu et al., 2001).

High inputs of PO4 related to oyster decomposition may increase the abundance of prokaryotes, pico-eukaryotes and cyanobacteria, as observed during a P enrichment experiment in mesocosms (Tsiola et al., 2016). Disequilibria in ammonia and phosphorus release kinetics were observed depending on oyster status, with lower N/P ratios observed at the dead oyster interface (NH4/PO4 at final time (Tf): D: 11.3, L: 22.4, C: 35.4). Justic et al. (1995) and Peñuelas et al. (2013) also reported that changes in the original nutrient ratios may induce changes in the specific composition of plankton communities. Considering that (i) growth of heterotrophic prokaryotic tend to be limited by P (Tsiola et al., 2016), and (ii) the reported molar C:N:P ratios of bacteria were near 45:9:1 (Goldman et al., 1987) and 50:10:1 (Fagerbakke et al., 1996), the disequilibria in ammonia and phosphorus releases observed during flesh decomposition may favour bacterial proliferation.

Thus, decomposition and mineralisation of oyster flesh may modify the structure of the planktonic community during mortality episodes.

5. Conclusions and perspectives

Our study highlighted for the first time, under laboratory conditions, that dissolved inorganic fluxes varied during a mortality episode since (i) OsHV-1 infection modifies oyster juvenile metabolism, with significant decreases in oxygen consumption and ammonium excretion; (ii) mortality of oysters leads to a profound increase in ammonium (×6) and phosphate (×41) releases and disequilibria in nutrient release kinetics at the water-dead oyster interface, due to mineralisation of flesh. Based on individual rates, fluxes were extrapolated to a lantern net scale (Table 6). Calculations showed that (i) a slight decrease (30– 40%) in oxygen consumption, (ii) a large increase in ammonia (×2–5) and phosphate (×8–19) releases and (iii) a decrease in the N/P ratio would be observed at the lantern net interface during mortality episodes, depending on the magnitude of these events (30–100% mortality). These estimated values will be compared with ones measured using pelagic chambers in the Thau lagoon as part of the second study of the Mortaflux program. This in situ experiments will help us to (i) confirm this abnormal nutrient loading during mortality episode, and (ii) demonstrate their influence in planktonic components (bacterio-, phyto and proto-plankton). Finally, this study will help us to elucidate pathogen transfers occurring during mortality episodes. This series of study is pioneer in its kind as it highlighted for the first time the consequences of the phenomena of oyster mortality on the cycle of matter.

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Fig. 1.

Diagram of the experimental design, composed of 3 infection treatments (C: control, L1, L2: Loads 1 and 2 corresponding to 0, 108 and 109 viral DNA copies ind−1), 6 dates (d −5, d1, d3, d7, d10, d14 after inoculum injection) and 4 incubation treatments, including a water treatment (W). All levels of treatments were replicated three times.

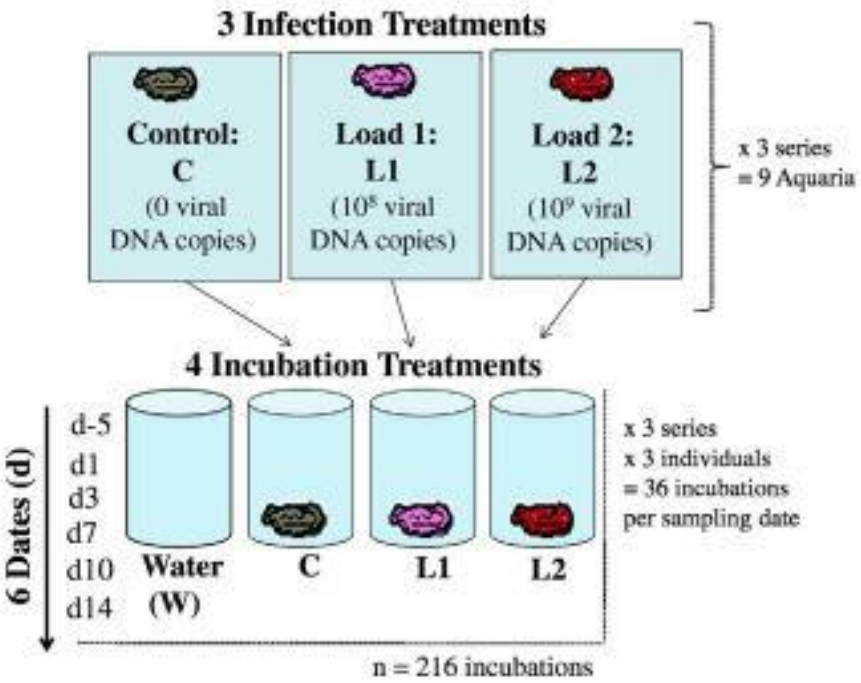

Fig. 2.

Mean (±SE) cumulative mortality (%) observed according to dates and infection treatments (C: control, L1, L2: Load 1 and 2). Stars indicate significant differences among treatments L1, L2 and C.

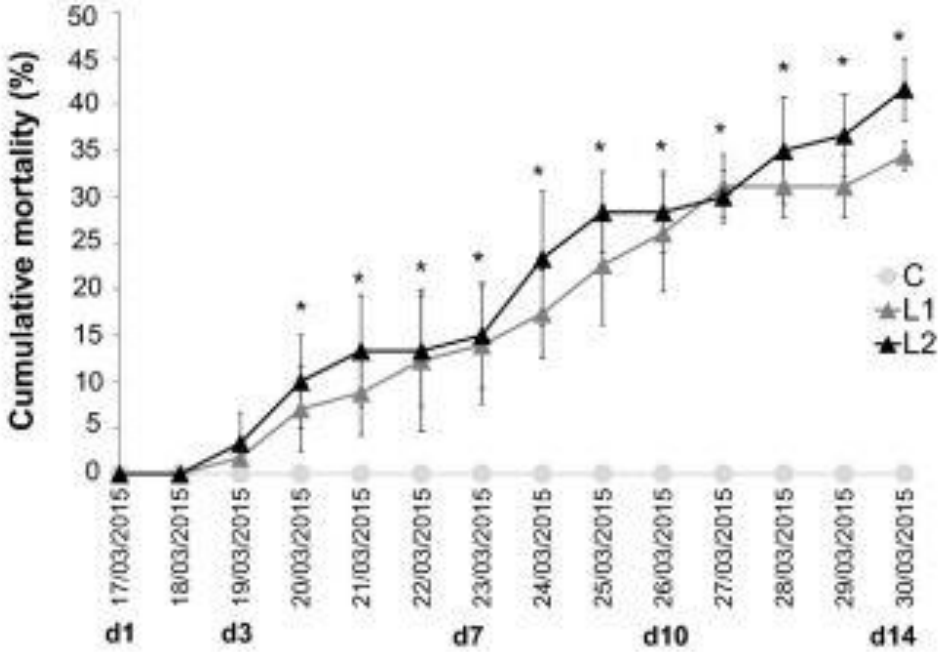

Fig. 3.

Mean (±SE) number of viral DNA copies (log10C9C10 totalDNA ng−1) quantified A) in flesh of live oysters according to dates (d1, d3, d7, d10, d14) and infection treatments (C: control, L1, L2: Load 1 and 2), B) in oyster flesh according to status (C: control, L: infected, D: dead). Stars indicate significant differences among treatments L1, L2 and C. Means linked with a horizontal line do not differ from one another within the same date. Different letters indicate significant differences among statuses.

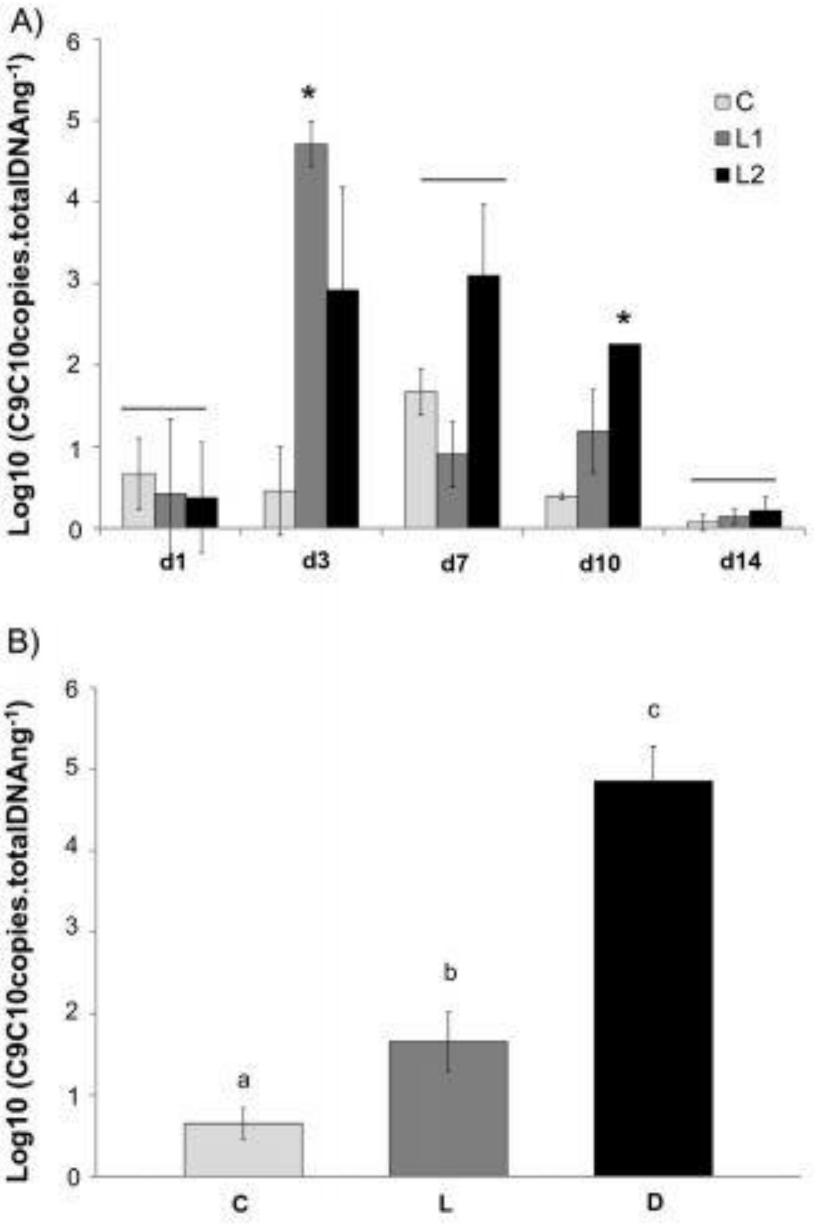

Fig. 4.

Number of dead oysters incubated in metabolic chambers according to dates (d1, d3, d7, d10, d14) and infection treatments (C: control, L1, L2: Load 1 and 2). A total of 9 individuals were incubated with the same incubation treatment for each same date.

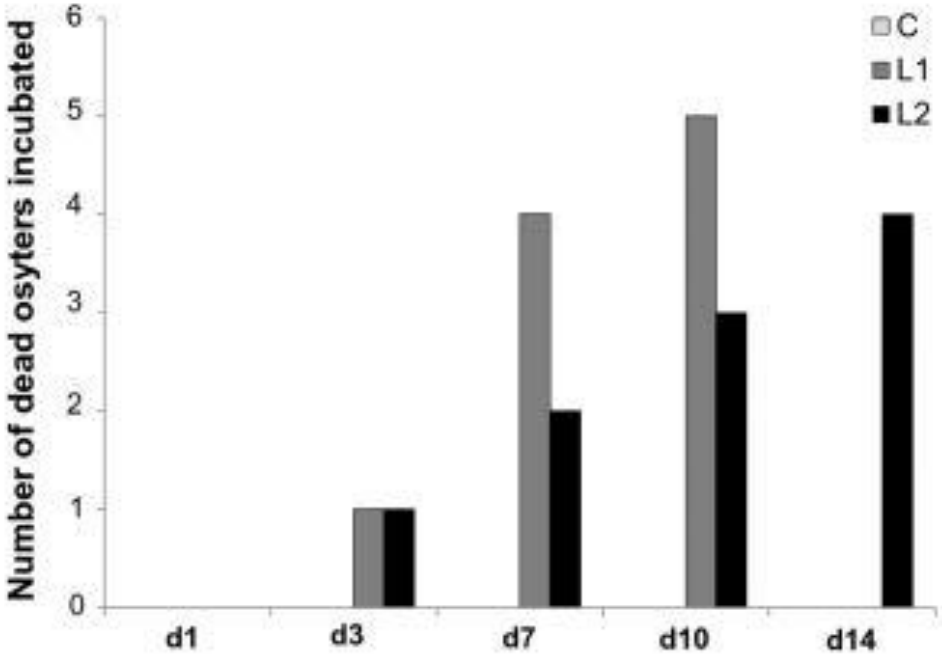

Fig. 5.

Mean (±SE) of A) O2 consumption, B) NH4, and C) PO4 fluxes, according to dates (d1, d3, d7, d10, d14) and incubation treatments (W: water, C: control oysters, L1, L2: infected oysters) considering fluxes at the interface of live and dead oysters. Stars indicate significant differences in absence of oysters (W vs. C, L1, L2). Means linked with a horizontal line do not differ from one another within the same date and were different from other means.

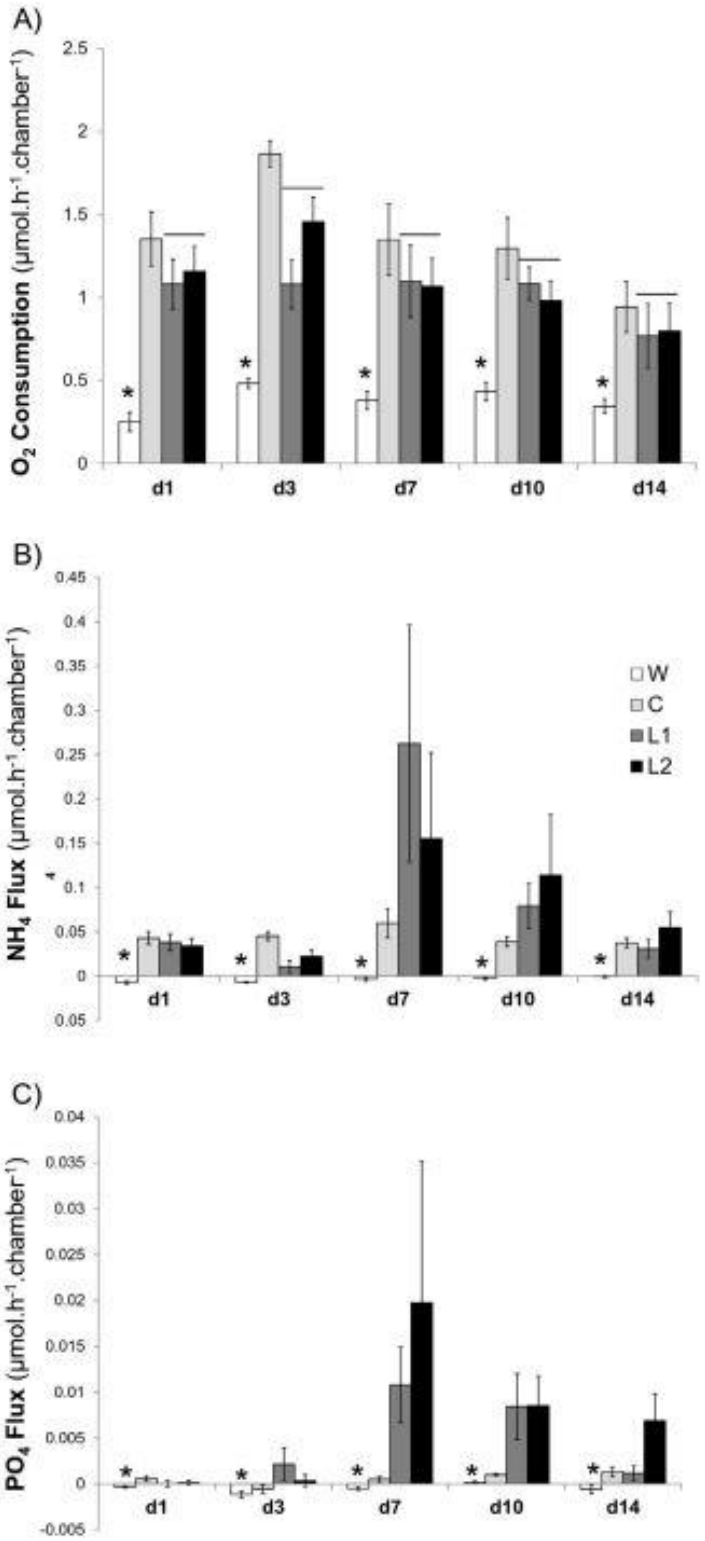

Fig. 6.

Mean (±SE) of A) O2 consumption, B) NH4 and C) PO4 fluxes, D) NH4/PO4 ratio at Final time of incubation (Tf), measured according to status (W: water, C: control oysters, L: pool of L1 and L2 infected oysters, D: dead oysters) regardless of date. Different letters indicate significant differences among statuses.

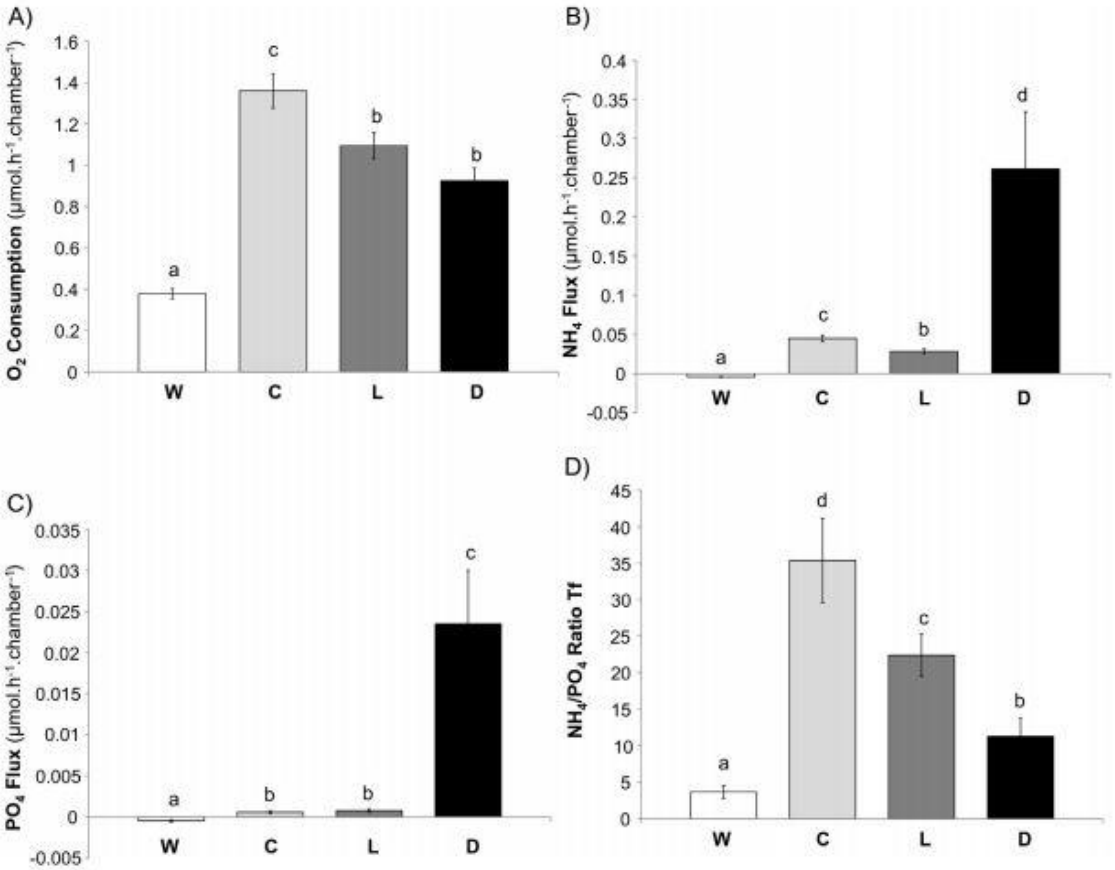

Fig. 7.

Comparison of mortality kinetics (%) of oyster juvenile observed in this study with those observed in classical pathology studies and in situ observations made in the Thau Lagoon.

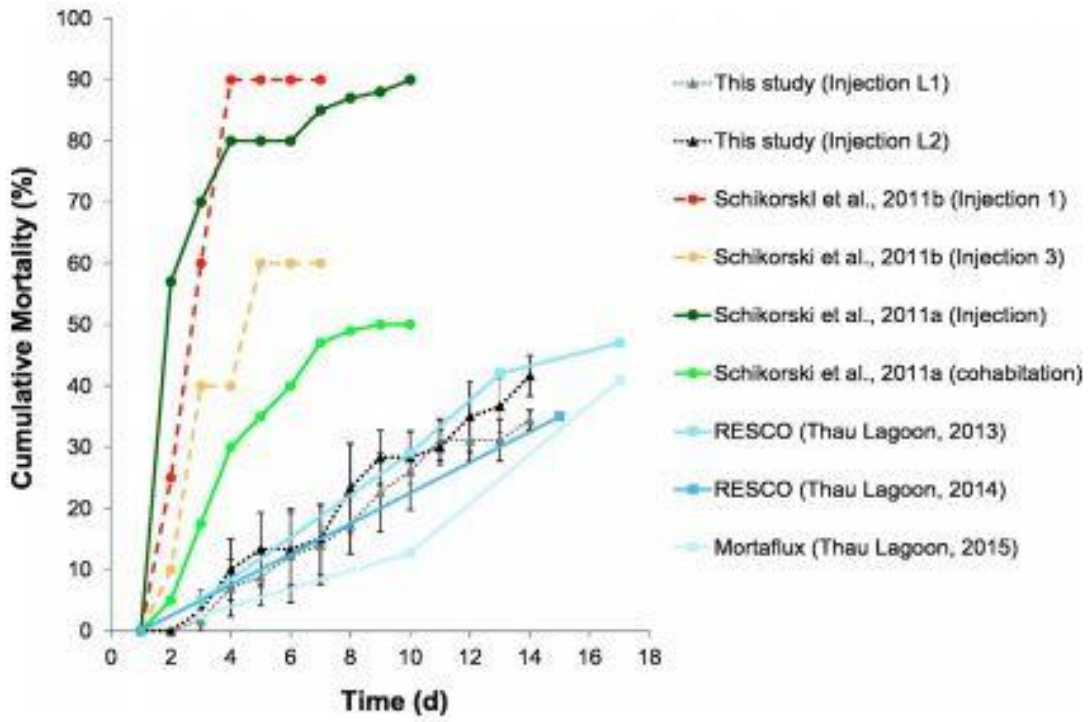

Table 1

Results of PERMANOVAs and a posteriori tests performed on length; total, shell and flesh weight; AFNOR; oxygen (O2) consumption; and ammonium (NH4) and phosphate (PO4) fluxes to test the effect of treatment (TR: W, C, L1, L2) at d −5 (i.e. 5 days before injection) to confirm that there was no initial effect of aquarium or pre-assigned batch (C, L1, L2) on oyster weight and metabolism before the viral inoculum injection. Df: degree of freedom, SS: sums of squares, Res: residual.

Variables	Source	df	SS	MS	Pseudo-F	p (Perm)	A posteriori test
Length	TR	2	0.26	0.13	0.09	0.92	
	Res	24	33.01	1.38			
	Total	26	33.27				
Total weight	TR	2	0.00	0.00142	1.13	0.344	
	Res	24	0.03	0.00126			
	Total	26	0.03				
Shell weight	TR	2	0.00	0.00069	0.72	0.484	
	Res	24	0.02	0.00095			
	Total	26	0.02				
Flesh weight	TR	2	0.00	7.70E−06	0.02	0.981	
	Res	24	0.01	5.08E−04			
	Total	26	0.01				
AFNOR	TR	2	0.21	0.10	0.02	0.98	
	Res	24	100.85	4.20			
	Total	26	101.06				
O_2 consumption	TR	3	14.57	4.86	52.05	0.001	W < C, L1, L2
	Res	29	2.71	0.09			
	Total	32	17.27				
NH_4 fluxes	TR	3	0.02	0.00536	9.17	0.001	W < C, L1, L2
	Res	30	0.02	0.00058			
	Total	33	0.03				
PO_4 fluxes	TR	3	0.00	3.29E−06	4.05	0.015	W < C, L1, L2
	Res	30	0.00	8.13E−07			
	Total	33	0.00				

Table 2

Results of PERMANOVAs and a posteriori tests performed on oxygen (O2) consumption, and ammonium (NH4) and phosphate (PO4) fluxes to test the effect of date (Da: d1, d3, d7, d10, d14), treatment (TR: W, C, L1, L2) and their interaction (Da × TR), considering fluxes observed at the interface of dead oysters in the dataset. df: degrees of freedom, SS: sums of squares, Res: residual.

Variables	Source	df	SS	MS	Pseudo-F	p (Perm)	A posteriori tests
O_2 consumption	Da	4	4.3	1.1	5.7	0.001	d3 > d1, d7, d10 > d14
	TR	3	19.9	6.6	34.8	0.001	W < L1, L2 < C
	DaxTR	12	2.2	0.2	0.9	0.493	
	Res	151	28.7	0.2			
	Total	170	56.0				
NH_4 fluxes	Da	4	0.24	0.06	4.0	0.002	d7, d10, d14 > d10, d14, d1 > d1, d3
	TR	3	0.22	0.07	4.7	0.003	W < C, L1, L2
	DaxTR	12	0.26	0.02	1.4	0.161	
	Res	160	2.44	0.02			
	Total	179	3.15				
PO_4 fluxes	Da	4	0.0015	0.0004	2.8	0.005	d1, d3 < d14, d10, d7
	TR	3	0.0017	0.0006	4.3	0.002	W < C < L1, L2
	DaxTR	12	0.0017	0.0001	1.1	0.402	
	Res	160	0.0211	0.0001			
	Total	179	0.0259				

Table 3

Results of PERMANOVAs and a posteriori tests performed on oxygen (O2) consumption, and ammonium (NH4) phosphate (PO4) fluxes, and NH4/PO4 ratio at Tf (final sampling time) to test the effect of status (St: W: water, C: control oysters, L: pooled L1 and L2 infected oyster, D: dead oysters). df: degrees of freedom, SS: sums of squares, Res: residual.

Variables	Source	df	SS	MS	Pseudo-F	p (perm)	A posteriori tests
O_2 consumption	St	3	20.548	6.8495	32.3	0.001	W < D, L < C
	Res	167	35.407	0.21202			
	Total	170	55.955				
NH_4 fluxes	Status	3	1.0592	0.35306	29.7	0.001	W < L < C < D
	Res	176	2.0925	0.011889			
	Total	179	3.1517				
PO_4 fluxes	St	3	0.0095553	0.0031851	34.3	0.001	W < C, L < D
	Res	176	0.016331	9.28E−05			
	Total	179	0.025886				
NH_4/PO_4 Tf	St	3	21,971	7323.8	11.1	0.001	W < D < L < C
	Res	167	1.10E + 05	659.54			
	Total	170	1.32E + 05				

Table 4

Non-exhaustive review of oxygen consumption and ammonia excretion rates measured for several species of oysters. For conversion, we considered the formula of Bayne et al. (1987) Ys = (Ws / We)b × Ye, where Ys is the physiological rate for an individual of standard dry weight,Ws is the standard weight (1 g).We is the weight of the animal (g), Ye is the uncorrected measured physiological rate and b the weight exponent for the physiological rate function. b = 0.8 for respiration according Bougrier et al. (1995). Note that 1 mol O2 = 22.4 L = 32 g. Finally, our data were converted into DW assuming that mean weight of juvenile of this study was 158 WWmg corresponding to 20.54 DWmg considering the equation DW = 0.13 g WW (n = 30: this study, data not shown).

O_2 consumption	Conversion in ($\mu molO_2\ gDW^{-1}\ h^{-1}$)	NH4 excretion	NH4 excretion ($\mu mol\ NH_4\ gDW^{-1}\ h^{-1}$)	Species	Temp (°C)	Stage	References
$0.987\ mLO_2\ gDW^{-1}\ h^{-1}$	41.13	$5.542\ \mu gNH_4\ gDW^{-1}\ h^{-1}$	0.40	*Crassostrea virginica*	nd	Adult	Gale et al. (1991)
$0.33\ mLO_2\ gDW^{-1}\ h^{-1}$	13.75	–	–	*Crassostrea virginica*	20	Adult	Newell (1985)
$371.5\ \mu LO_2\ gDW^{-1}\ h^{-1}$	16.58	–	–	*Crassostrea virginica*	20	Spat, juvenile and adult	Dame (1972)
$0.51\ mLO_2\ gDW^{-1}\ h^{-1}$	22.77	–	–	*Crassostrea gigas*	Ambient		Bernard and Noakes (1990)
$1.02\ mLO_2\ gDW^{-1}\ h^{-1}$	45.54	–	–	*Crassostrea gigas*	22–23	Adult	Wan-Soo et al. (2002)
$0.7\ mLO_2\ gDW^{-1}\ h^{-1}$	31.25	–	–	*Crassostrea gigas*	22–23	Adult	Wan-Soo et al. (2002)
$1.022\ mgO_2\ gDW^{-1}\ h^{-1}$	31.94	–	–	*Crassostrea gigas*	20–22	Adult	Soletchnik et al. (1997)
$0.860\ mgO_2\ gDW^{-1}\ h^{-1}$	26.88	–	–	*Crassostrea gigas*	20	Juvenile and adult	Bougrier et al. (1995)
$0.851\ mgO_2\ gDW^{-1}\ h^{-1}$	26.59	–	–	*Crassostrea gigas*	20	Juvenile and adult	Bougrier et al. (1995)
$1.077\ mgO_2\ gDW^{-1}\ h^{-1}$	33.66	–	–	*Crassostrea gigas*	20	Juvenile and adult	Bougrier et al. (1995)
$0.71\ \mu LO_2\ gDW^{-1}\ h^{-1}$	31.70	–	–	*Crassostrea gigas*	20	Spat	Goulletquer et al. (1999)
$0{,}53\ \mu LO_2\ gDW^{-1}\ h^{-1}$	23.66	–	–	*Crassostrea gigas*	20	Spat	Goulletquer et al. (1999)
C: $0{,}98\ \mu molO_2\ gDW^{-1}\ h^{-1}$	21.94	C: $0.049\ \mu mol\ NH_4\ h^{-1}\ ind^{-1}$	0.50	*Crassostrea gigas*	20.8	Spat	This study
L: $0{,}71\ \mu molO_2\ gDW^{-1}\ h^{-1}$	15.89	L: $0.032\ \mu mol\ NH_4\ h^{-1}\ ind^{-1}$	0.33	*Crassostrea gigas*	20.8	Spat	This study
–	–	–	0.84	*Crassostrea gigas*	22	Adult	Buzin et al. (2015)
–	–	–	0.48	*Crassostrea gigas*	16	Adult	Buzin et al. (2015)
			0.23	*Crassostrea gigas*	13	Adult	Buzin et al. (2015)

Table 5

Non-exhaustive review of changes induced by pathogen infections on filtration, respiration and excretion rates in marine bivalves, nd: no data, NS: no significant variation, significant ↗ increase or ↘ decrease.

Host	Pathogen		Filtration/clearance rate	Respiration	Excretion	References
Crassostrea virginica	*Perkinsus marinus*	Protozoan	nd	NS	nd	Willson and Burnett (2000)
Crassostrea virginica	*Haplosporidium nelsoni*	Protozoan	↘	NS	nd	Newell (1985)
Crassostrea virginica	*Boonea impressa*	Gastropod	NS	NS	NS	Gale et al. (1991)
Mytillus galloprovincialis	*Martellia sp.*	Protozoan	↗	NS	NS	Anestis et al. (2010)
Crassostrea gigas	*Chlamydia*	Bacteria	↗	↘	nd	Soletchnik et al. (1998)
Ruditapes philippinarum	*Vibrio tapetis*	Bacteria	↘	↘	nd	Flye-Sainte-Marie et al. (2007)

Table 6

Individual fluxes (O2 consumption, NH4 and PO4 fluxes) extrapolated to lantern scale, considering measured individual rates, lantern stocking density (7 storeys × 350 ind storey−1 = 2450 individuals) and lantern density (1 m−2), before and during mortality events (if 30, 50 or 100% cumulative mortality rates were recorded). N/P indicates the ratio between NH4 and PO4 releases

	Before	Mortality (30%)	Mortality (50%)	Mortality (100%)
Oxygen consumption (μmol lantern^{-1} m^{-2})	2409.5	1619.0	1549.8	1342.2
NH_4 releases (μmol lantern^{-1} m^{-2})	119.9	269.9	365.0	650.4
PO_4 releases (μmol lantern^{-1} m^{-2})	2.6	21.6	30.9	58.7
N/P fluxes	46	12	12	11