Descriptive epidemiology of mass mortality due to *Ostreid herpesvirus-1* (OsHV-1) in commercially farmed Pacific oysters (*Crassostrea gigas*) in the Hawkesbury River estuary, Australia

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Abstract

Mortality of farmed triploid Pacific oysters (*Crassostrea gigas*) associated with *Ostreid herpesvirus-1* (OsHV-1) was first recorded in Australia in the Georges River/Botany Bay estuary (New South Wales) in late 2010. Two years later, the first sign of possible inter-estuarine spread was observed when commercial triploid Pacific oysters in the Hawkesbury River estuary, located 50 km north of Botany Bay, were affected by mass mortality. The aim of this study was to describe the epidemiological features of the Hawkesbury outbreak *via* a formal investigation which was conducted in real time and comprised: an assessment of stock levels, past oyster acquisitions and a trace forward investigation to identify stock at greatest risk due to transfers of oysters; passive surveillance of the spread of mortalities in the estuary; active surveillance using PCR to identify the distribution of OsHV-1 infection on farms in the estuary and mortality estimates to identify age and size classes affected; identification of the time of first infection using data from sentinel oysters; and assessment of environmental risk factors. Mortalities were recorded in all age classes but were greater in spat and juveniles than in adults. The incubation period for mass mortality was < 4 days, however subclinical OsHV-1 infection was detected three months prior to the first signs of mortality in the index case (first location affected), which suggests that low viral loads of OsHV-1 are insufficient to induce the disease. While inefficient oyster-to-oyster transmission occurred at two locations, a synchronous infection arising from a common environmental source was required to explain the

mass mortalities at the index case (Mullet Creek). Estuarine hydrodynamics then assisted rapid dispersal of viral particles throughout the estuary. Seawater temperatures were consistently above 24°C during the month preceding mass mortalities with variations (± 3°C) being observed over a few days during this period; however this did not necessarily lead to mortality events when the virus was present. There was no evidence of prior movement of potentially infected oysters or farming equipment into the Hawkesbury River estuary to explain the outbreak.

Key words: Disease outbreak investigation; Pacific oysters; Ostreid herpesvirus-1; disease transmission; incubation period; water temperature; epidemiology

1. INTRODUCTION

In late 2010, a few months after having decimated farms in bays on the North island of New Zealand, an acute disease caused by *Ostreid herpesvirus-1* (OsHV-1) occurred for the first time in Australia in a small population of commercially farmed triploid Pacific oyster (*Crassotrea gigas*) spat in Woolooware Bay within the Georges River / Botany Bay estuary, New South Wales (NSW) (Jenkins et al., 2013). A little over two years later, mass mortalities were observed in commercial *C. gigas* leases in the Hawkesbury River estuary, the mouth of which is located 50 km north of Botany Bay.

OsHV-1 is a DNA virus belonging to the family *Herpesviridae* that has been responsible for sporadic episodes of mortality of *C. gigas* during summer in France since 1992 (Dégremont et al., 2013). In 2008 a new strain was identified (OsHV-1 µvar) which has caused mortality of up to 100% in larvae, spat and juveniles in France, many other European countries and the USA (Renault et al., 1994; Friedman et al., 2005; Segarra et al., 2010; Lynch et al., 2012; Peeler et al., 2012). Sequencing of OsVH-1 recovered from oysters sampled in New Zealand in 2010 demonstrated a close relation to OsHV-1 µvar, however there were some significant differences in sequence (Renault et al., 2012). In Australia, partial genome sequencing work confirmed the role of OsHV-1 µvar in the mortalities observed in Woolooware Bay in 2010 (Jenkins et al., 2013).

Despite the worldwide impact that OsHV-1 has had on the Pacific oyster industry, the epidemiology of the disease in open marine environments is still poorly understood (Garcia et al. 2011). Many reports suggest the role of water temperature and the presence of some pathogenic *Vibrio* species in/prior to the onset of the disease (Burge et al. 2007, Sauvage et al. 2009, Saulnier et al. 2011). However, several field studies have reported an absence of mortality in areas where OsHV-1 and apparently favourable water temperatures were recorded (Dundon et al., 2011; Pernet et al., 2012; Paul-Pont et al., 2013a), suggesting the existence of other underlying environmental factors which may trigger the disease outbreaks. In Europe and New Zealand, movements of oysters or equipment from infected areas appeared to have played a major role in the spread of the disease between geographically distant areas (Bingham et al., 2013; Peeler et al. 2012). However, the spatial distribution of mortalities between and within bays, as well as the role of hydrodynamics and other environmental forces on OsHV-1 spread are still poorly understood and constitute major knowledge gaps that prevent implementation of efficient control mechanisms (Peeler et al. 2012, Pernet et al. 2012; Paul-Pont et al., 2013a).

The aim of this study was to describe the epidemiological features of the first massive outbreak of OsHV-1 disease in farmed *C. gigas* in the Hawkesbury River, Australia. A formal outbreak investigation was implemented in the Hawkesbury River estuary in January 2013 at the commencement of the outbreak and the disease event was monitored in real time (ie. daily from the day of the first mortalities observed). Critical features of the epidemiology of the disease were identified (latency, incubation period, transmission mechanisms, disease progression, risk factors) and the sequence of events that occurred during the outbreak is described.

2. MATERIALS AND METHODS

2.1. Overview

Mass mortality of *C. gigas* associated with the presence of OsHV-1 commenced on 21st January 2013 in the Hawkesbury River (Figure 1). All farmed oysters were triploid single seed oysters that originated from the same hatchery (Shellfish Culture, Tasmania). An outbreak investigation and response plan were initiated the same day and consisted of the following steps:

- Introduction of sanitary measures in an attempt to limit local spread by farmers;
- Audit of stock levels, past oyster acquisitions and a trace forward investigation to identify farms at greatest risk due to transfer of oysters from the site of the index case;
- Passive surveillance of the clinical signs of disease (mortality) to monitor local spread;
- Active surveillance using PCR to identify the extent of OsHV-1 infection among oyster leases in the estuary
- Examination of stock on farm leases which had been affected by mortality to identify age and size classes affected;
- Assessment of the time of first infection using data from sentinel oysters which had been placed in the estuary 15 months prior to the outbreak;
- Assessment of environmental risk factors.

The outbreak investigation started on the 21st January and stopped at the end of February when a large rainfall event forced oyster farmers to move most oysters located in the upper Hawkesbury River sites with low salinity (Marra Marra Creek, Cobay Bay, Kimmerikong) to downriver locations (Mooney Mooney Creek, Mullet Creek, Porto Bay) with higher salinity levels (Figure 1). Regardless mortalities occurred in adult oysters due to prolonged freshwater immersion and it became impossible to track the origin of the oysters (bay, lease, tray).

2.2. Case definition

The unit of interest was an oyster lease within a bay. The case definition used in this study for OsHV-1 associated mortality was the sudden onset of mortality with a cumulative total greater than 50% in juvenile oysters (< 1 year old, <60 mm length) over 1 week which could not be explained by other obvious causes (such as prolonged immersion in freshwater or heat wave conditions), and confirmation of the presence of OsHV-1 by PCR from a sample of *C. gigas* from the estuary. For adult oysters, the case definition was mortalities with a cumulative total greater than 20% with OsHV-1 associated mortalities in juvenile oysters in the same bay over 1 week, or a high load of OsHV-1 confirmed to be present in a sample of the affected adult oysters.

2.3. Estimation of prevalence

Individual animal prevalence was estimated from PCR results (see below) using a pooled prevalence calculator (www.ausvet.com.au) assuming 90% test sensitivity and perfect test specificity, 12 pools tested and a fixed pool size of 3 (survey oysters), 5 pools tested and a fixed pool size of 6 (sentinel oysters) or 6 pools tested and a fixed pool size of 5 (sentinel oysters). For leases/sites where none or most of the pools were positive, Bayesian methods were used to estimate prevalence. The Bayesian method requires specification of priors for prevalence, sensitivity and specificity. We specified a uniform beta prior for prevalence (alpha = 1 and beta = 1), an informative prior for sensitivity assuming a median of 0.9 with lower 5% interval of 0.7 (alpha = 15.03 and beta = 2.56), and an informative prior for specificity assuming a median of 0.995 with a lower 5% interval of 0.95 (alpha = 1137.5 and beta = 6.7).

2.4. Monitoring the spread and severity of the clinical disease

2.4.1. Assessment of stock levels and trace forward investigation

Firstly, to identify oysters at risk we requested an audit of the stock present on farms with actively farmed leases. This was conducted by the executive officers of Broken Bay Oyster Association (BBOA) on 23rd January. All oyster farmers were interviewed face-to-face or by telephone and data on stock levels, ages, sizes, type of cultivation and number of cultivation units were recorded in a spreadsheet. Secondly, we requested information on the destinations of movements of oysters from the index case (first case detected) in Mullet Creek to other bays since December 2012; these data were updated on 29th January to enable targeted active surveillance; all such trace forward records were based on the recollections of farmers. Thirdly, we evaluated farmer records of oyster movements and farming equipment into the estuary in the preceding year.

2.4.2. Survey of farm leases for OsHV-1 infection

We undertook a physical survey on 29th – 30th January 2013, at which time there was no evidence of disease spread from the index case (Mullet Creek) to leases in the upper river. The purpose of the survey was to detect OsHV-1 infection in Pacific oysters in the non-affected, actively farmed leases in the Hawkesbury River with a view to make recommendations about the risk of movement of oysters between bays to enable management for grow-out and harvest. It was assumed that, if present, OsHV-1 would infect more than 10% of oysters in a lease and more than 10% of leases in a bay; that infection may be clustered in some parts of a bay; that wild diploid Pacific oysters may be infected already but sampling of the far more numerous farmed oysters would be representative at bay level. Sample sizes were estimated using a two stage sampling approach for demonstration of disease freedom, with the leases to be selected in the first stage and oysters within leases in the second stage. All bays were considered to be independent populations as the aim was to demonstrate freedom of infection for each bay. Sample sizes were calculated for five bays to provide 95% confidence of detecting disease in a bay at 10% oyster and 10% lease-level design prevalences, assuming 90% test sensitivity, perfect test specificity and target system sensitivity of 0.95. A sample size of 32 oysters per lease for bays with 2 to 21 active leases with a maximum of 16 leases to be sampled per bay was found to achieve the required target system sensitivity of 0.95. Due to emergency harvest activities, there were no more than 16 active leases per bay on the day of sampling, hence all leases were sampled. A sample size of 36 oysters per lease was chosen to be conservative. All calculations were made using online calculators: http://epitools.ausvet.com.au/content.php?page=2StageFreedom (least cost where herd size unknown).

As OsHV-1 infection can be clustered (Paul-Pont et al., 2013a), it was important to ensure samples were collected from all parts of each lease. To do so would require sampling over a wide area which would be an extremely difficult and time consuming task, and true randomisation would be impossible. Therefore, within a lease, systematic sampling was undertaken. The audit of stock made by BBOA on 23rd January 2013 was used to determine the number of cultivation units to sample. For example, in a lease where there were 480 trays on 5 racks, every 480/36 = 14th tray was sampled; the first or second tray (determined by toss of a coin) was sampled on the first rack and then every 14th tray was sampled afterwards; one oyster was selected from each 14th tray. All oysters were tested in pools of 3 (12 pools of 3 oysters per lease tested by PCR).

In addition to systematic sampling from all leases, on one lease at Coba Bay (lease 20) where an unusual number of dead oysters were observed, 15 oysters (5 pools of 3) were non-randomly collected on 29th January from across several trays that had been moved there from Mullet Creek on 19th January.

The survey was repeated on selected leases in Coba Bay (leases 16 and 21) and Marra Marra Creek (leases 6 and 11) on the 26th February to assess the progression of OsHV-1 infection over time.

2.4.3. Passive surveillance

Farmer observations of mortality in oysters were recorded and reported to us by BBOA and a summary of the key observations in January and February 2013 was created in order to follow the progress of mortality.

2.4.4. Active surveillance

We performed a physical audit of mortality in all actively farmed oyster leases (OL, Fig 1 and 2) in Mullet Creek (on 04/02/13), Porto Bay (27/02/13), Mooney Mooney Creek (26/02/13) and Coba Bay (11/02/13 and 26/02/13) where mortality was observed to identify the range of age and size classes affected. Three widely separated locations in each OL were inspected for each age class identified. In addition wild *C. gigas* were inspected in Mullet Creek on 4/2/13. Data records comprised the OL identification and owner, the type of cultivation system and number of units, the number of oysters per unit, the range of size and age in each OL, and the range of percentage mortality that we observed.

2.4.5. Assessment of the time of first infection using sentinel oysters and opportunistic tests for OsHV-1

The time of first infection of oysters in the Hawkesbury River with OsHV-1 was assessed by retrospective testing of archived sentinel oysters and opportunistic samples that had been collected since September 2011. From September 2011 to August 2012 sentinel oysters comprised a batch of 4000 single seed triploid spat (age 6 weeks; size 9.1±3.4 mm in September 2012) produced by a Tasmanian hatchery (Shellfish Culture, Tasmania) and placed in floating baskets at up to four different sites (downstream: Mullet Creek, Porto Bay; upstream: Marra Marra Creek and Kimmerikong). Random sampling of 30 oysters per site was performed every fortnight (September 2011 - January 2012) and then once a month (January - August 2012). This sample size was calculated to provide 95% probability of detecting disease at a prevalence of 10% in the oyster population, assuming perfect test sensitivity and specificity. For these sentinels PCR testing was performed between September 2011 and September 2012 on pooled samples (5 pools of 6 oysters per site per time point).

From 3rd August to 20th September 2012 no samples were collected from sentinels.

Oysters from Porto Bay were opportunistically sampled on 20th September, 1st October and 12th October 2012 for unrelated laboratory experiments and 30 oysters per batch were tested for OsHV-1 by PCR between September and December 2012 to confirm the absence of the virus prior to each experiment (6 pools of 5 oysters per batch).

On the 18th October 2012 a small batch of dead spat was detected in a basket in Mullet Creek and some adjacent live spat were collected by farmers. These were stored frozen and were tested by PCR for OsHV-1 in February 2013.

On the 19th October 2012, a total of 18,000 oysters, comprising 14,000 juveniles (8 month old - 50 ± 6 mm) and 4,000 adult oysters (12 month old – 92 ± 6 mm), was collected from Porto Bay to be deployed in the Georges River as part of another field trial. Prior to deployment, 90 oysters (30 adults and 2 groups of 30 juveniles) were collected and these were tested for OsHV-1 by PCR in November 2012. PCR testing was performed on pools (6 pools of 5 oysters per group).

From the 26th November 2012 the presence/absence of OsHV-1 virus was monitored fortnightly by random sampling of spat collected from a series of randomly selected floating baskets that were part of normal production runs in Mullet Creek and Porto Bay (n=30 per site per time point). PCR testing was performed in February 2013 on pools (6 pools of 5 oysters).

2.5. Laboratory tests

Oysters that were collected as part of active surveillance on 29th and 30th January 2013 were placed in pre-labelled sealable plastic bags, identified with date, location, lease number, and placed in

eskies that were taken to the laboratory the same day. These oysters were kept at 4°C, opened and processed on 31st January, and tested by PCR on the 1st February, except for oysters from leases 9, 10, 15, 18, 20 and 29. In this case the oysters were removed from the shell and frozen in pools of 5 at -20 °C for 6 weeks prior to testing.

Oysters that were sampled as part of active surveillance in the second survey (26th February) were collected as described above and kept at -80°C for a week prior to dissection and PCR analysis.

Sentinel oysters that were sampled in 2011/2012 and oysters that were sampled as part of other research projects were collected as described above and kept at -80°C for a period of 2 weeks to 6 months prior to dissection and PCR analysis.

2.5.1. Dissection and tissue homogenization

Oysters that were collected as part of the surveys (29th-30th January and 26th February 2013) were processed in batches (fixed pool size of 3) according to the lease number and the bay of origin. Oyster knives and lab equipment were disinfected in 1 % Virkon® (oxidising disinfectant - Antec International Ltd) and rinsed in hot then cold water between each oyster. Each oyster was opened by removing the top shell then rinsed in tap water. A section of gill and mantle of approximately 100 mg was removed using a sterile scalpel blade with the lower shell used a firm base for cutting, and placed in a 1.5mL tube containing silica beads and 1.2 mL of distilled water. Tissues from 3 oysters were pooled into each tube (dilution rate of 1:4 W/V) and homogenized by bead-beating (Fastprep System, MP Biosciences, USA). Tissue homogenates were then clarified by centrifugation at 3000 × *g* for 10 min in a microcentrifuge, and the supernatants were stored at − 80 °C until DNA extraction and PCR analysis. The shells and remaining tissues were discarded.

Sentinel oysters that were sampled in 2011/2012 and oysters that were sampled as part of other research projects were opened as described above. Tissue homogenates were prepared on tissue pools of 5 to 6 oysters (gills and mantle; tissue weight: 3-50 g) at a dilution rate of one part tissue plus 4 parts distilled water (1:4 W/V) using a stomacher. An aliquot of 500 µL of each homogenate was placed in a 1.5mL tube containing silica beads and was disaggregated by bead-beating prior to supernatant collection as described above.

2.5.2. DNA extraction and Polymerase Chain Reaction (PCR)

DNA extraction and real-time PCR were performed following the protocols described in Paul-Pont et al. (2013b).

Each sample collected as part of the surveys on the 29th -30th January and 26th February 2013 was tested in single wells. Each sample from sentinel oysters that were sampled in 2011/2012 and oysters that were sampled as part of other research projects were tested in duplicate.

A valid run was defined as a run exhibiting no amplification of the negative control, amplification of the positive control (cycle threshold (Ct) within the range of the standard curve), a standard curve with $r^2 > 0.95$ and efficiency between 90-110%. The threshold setting for each run was manually locked in based on the standard curve series. When tested in a single well, a sample was defined as positive when it exhibitedan exponential accumulation of fluorescence and a valid cycle threshold. When tested in duplicate a sample was defined as positive when both replicates exhibited an exponential accumulation of fluorescence and a valid cycle threshold. When a sample exhibited one replicate positive and one replicate negative, it was considered to be “inconclusive”.

The detection limit was evaluated at 3 copies per mg of tissue and the quantification limit at 12 copies per mg of tissue based on guidelines previously published (Bustin et al., 2009; Martenot et al., 2010).

2.6. Environmental monitoring

Real-time water quality data were obtained from a multi-sensor water quality probe (YSITM 6820 sonde; John Morris Scientific, Sydney) deployed 1.0m below the water surface in the main river

channel outside Porto Bay (Figure 1). The probe is deployed as part of an extensive water quality monitoring program managed by Hornsby Shire Council. Information collected by this probe represents the environmental conditions of the main channel in the estuary. This autonomous buoy monitors salinity (ppt) and temperature (°C) at 15 minute intervals. The probe is calibrated and maintained to prevent fouling every three weeks and readings are telemetered to a public website every 6 hours (http://www.hornsby.nsw.gov.au/environment/water-catchments/hawkesbury-estuary/hawkesbury-temperature-and-salinity-monitoring).

3. RESULTS

3.1. Detection of OsHV-1 associated mortality and initial responses to reduce spread

On 21st January 2013 an oyster farmer observed dead spat in 3-mm mesh socks in 12mm mesh floating pillow baskets in Mullet Creek (Figure 1). Four baskets of 10 - 20 mm oysters were inspected by one of the authors (RW) who confirmed mortality of 50% and collected a representative sample of live and dead oysters for testing by the Department of Primary Industries (DPI). OsHV-1 infection was detected by DPI on 22nd January and confirmed at the Australian Animal Health Laboratory. After obtaining technical advice and following formal discussions the Hawkesbury oyster industry group, Broken Bay Oyster Association (BBOA), immediately imposed voluntary quarantine and local movement controls to limit spread of the infection from the index case site at Mullet Creek. A state disease response plan was implemented by DPI, which involved quarantine and prevention of oyster movements from the Hawkesbury River. On 23rd January BBOA members agreed that there would be no movement of any oysters from any bay within the Hawkesbury River except for emergency harvest until surveillance testing of the entire River had been completed, with an underlying objective to harvest and sell marketable oysters as soon as possible. Other actions were to: limit boat movements from the lower part of the river (below Mooney Mooney Creek) to the upper river (Figure 1); although movements from a bay to a shore-base with return to the same bay was allowed. When boat movements were needed, biofouling from hulls of boats was removed and bilge water was disinfected with chlorine. Usual husbandry such as grading and cleaning of oysters was discouraged and kept to a minimum as stress could potentially result in further mortalities.

3.2. Assessment of stock introductions, stock at risk and trace forward investigation

The only Pacific oysters introduced between January 2012 and January 2013 were commercially produced spat from a hatchery in Tasmania, which were acquired in two batches in April and October 2012, and had been certified by the competent/regulatory authority as being free of OsHV-1 based on PCR tests. Sydney rock oysters were introduced from a hatchery at Port Stephens NSW in September 2012. There was no acquisition of oyster farming equipment previously used elsewhere.

An audit of the oysters present in all active oyster leases in the Hawkesbury River estuary was completed on 23rd January 2013 (Table 1). The downstream sites (Mullet Creek, Porto Bay and Mooney Mooney Creek; Figure 1) represented the largest farming areas where a total of 27 active Pacific oyster leases were recorded prior to the OsHV-1 outbreak. More than 15 million oysters (spat / juveniles from 3 to 9 month old) were grown across these 3 nursery areas (Figure 1). The farming areas located upstream (Coba Bay, Marra Marra Creek and Kimmerikong) and at the entrance of the estuary (Patonga) were used to fatten bigger oysters prior to harvest (Figure 1). Most of the 2 million oysters spread across these 27 active Pacific oyster leases were older than 12 month and their size range was between 60 and 150 mm (shell length).

Oyster movements from the index case (Mullet Creek) to other locations in the River (trace forward) were analysed on 29th January to enable sampling (Table 2). Oyster trays and baskets were transferred from Mullet Creek to Porto and Coba Bays as well as Mooney Mooney and Marra Marra Creeks on various occasions between the 28th December and the 21st January 2013, prior to the first mortalities being observed. While the last transfer of oysters to Marra Marra Creek and Porto Bay occurred no later than the 17th January, some trays were transferred to Coba Bay on 19th January

and to Mooney Mooney Creek on the morning of the 21st January, before the mortalities associated with OsHV-1 were confirmed in Mullet Creek (Table 2).

3.3. Passive surveillance

3.3.1. Downstream sites (Mullet Creek, Mooney Mooney Creek, Spectacle Island, Goat Island, Porto Bay)

Widespread mass mortality was observed in Mullet Creek by the afternoon on 21st January and the Creek is defined as the index case (first occurrence of the disease). Mass mortalities were reported at the entrance of another nursery area nearby (Mooney Mooney Creek) on 24th January and nearby down river leases at Spectacle and Goat Islands on 25th January (Table 3). The leases in upper Mooney Mooney Creek were observed to be affected 5 days later (29th January). Leases at Porto Bay were not affected on 2nd February but mortality was noticed on the 13th February in leases at the entrance to the bay and was widespread in the bay by the 15th February.

3.3.2. Upstream sites (Marra Marra Creek, Coba Bay, Kimmerikong)

Trays of oysters which were moved from Mullet Creek to Coba Bay on 19th January were observed to contain dead oysters on 29th January (lease 20), and the proportion of dead oysters increased over the next few days (farmer's observations). Oysters in trays on an adjoining lease at Coba Bay were affected by 8th February (lease 19), but mortalities were not observed on four other nearby leases on 11th and 27th February (Table 3). None of the movements of oysters from Mullet Creek to other upper River leases appeared to be associated with mortalities (Kimmerikong and Marra Marra Creek).

3.4. Active surveillance

3.4.1. Downstream sites

Based on age, size and cultivation system, oysters farmed in Mullet Creek could be divided into 3 different groups, the most numerous being the young spat (3-9 month) farmed in floating and hanging baskets (n=27,100) that also exhibited the highest percentage mortality (90-100%) (Table 4). Remaining oysters were older (11-30 month old) and farmed in trays (n=300). An age/size effect on mortality was observed as the percentage mortality was significantly lower in older/bigger stock (50-70% for 11-14 month old and 25-50% for 24-30 month old oysters). The cultivation systems used in Porto Bay were evenly represented by trays and floating/hanging baskets (50.5% and 49.5%, respectively), and all oysters farmed in the Bay were young spat (3-9 month old) (Table 4). However, there was great variation in size for oysters of the same age, ranging from 30 to 100 mm (shell length) with variation in mortality, ranging from 20 to 100%, regardless of the cultivation system. No size-mortality effect was observed in this bay.

In Mooney Mooney Creek, young spat (3-9 month) in floating/hanging baskets represented 93% of the population and suffered from a high mortality (80-100%) while exhibiting a wide range of size 2-90 mm (Table 4). The mortality observed for this age class was similar in other cultivation systems (mortality of 90% observed in trays and tumblers). Older/bigger oysters farmed in trays suffered less and exhibited mortality of 50-70%.

Dead wild diploid Pacific oysters were observed at all sites.

3.4.2. Upstream sites

Dead oysters were observed in some parts of Coba Bay (Figure 2). As upper River sites constitute the fattening areas, only old/large oysters were farmed in Coba Bay (age > 9 month; size range: 40-150 mm), Marra Marra Creek and Kimmerikong. As oysters in Coba Bay demonstrated mortality on 29th January in trays that had been transferred from Mullet Creek on the 19th January, it was decided to examine the Bay on two occasions fifteen days apart to record any potential spread of the disease among the adjacent oyster leases. High rates of mortalities were observed in leases 19 and 20 but with no sign of progression between the 11th and the 26th February (Figure 2). No significant mortality (<5%) was observed in the other adjacent active leases on either occasion (Figure 2). No

mortality was observed at Marra Marra Creek and Kimmerikong when inspected on the 29th January and the 26th February.

3.5. Detection of OsHV-1 infection in oyster leases and bays in the Hawkesbury River estuary

3.5.1. Data from sentinel oysters collected prior to and during the outbreak

All samples of oysters which were tested by PCR for OsHV-1 had negative results at all sites between October 2011 and October 2012 (Table 5). The virus was first detected downstream at very low level (site: Mullet Creek; estimated prevalence: 6%; viral load below the quantification limit; inconclusive PCR result) in October 2012 (Table 5). Oysters sampled at the same time in an adjacent bay (Porto Bay) were negative for OsHV-1. On the 26th November and 26th December 2012 oysters sampled from these two locations were negative for OsHV-1 by PCR while the virus was detected in one pool of oysters sampled from Mullet Creek on the 13th December (estimated prevalence: 4%; viral load below the quantification limit; inconclusive PCR result). From the 7th January 2013 onwards, OsHV-1 was systematically detected at each sampling time in Mullet Creek and Porto Bay, and the prevalence and viral loads were variable over time (Table 5). Higher prevalence (> 40%) and higher viral loads (up to 9.4×10^4 copies per mg) were observed in oysters sampled on the 21st January (Mullet Creek and Porto Bay) and the 15th February (Porto Bay). The sentinel oysters sampled in Mullet Creek on the 21st January were apparently healthy and were collected at 8 am two hours before the first dead oysters were observed in a lease on the other side of the bay. On the 21st January the intensity of the infection was higher in Mullet Creek (viral load ranging from 9.3×10^1 to 9.4×10^4 viral copies per mg) than in Porto Bay (viral load < 1.1×10^2 viral copies per mg). High viral loads (1.3×10^4 – 1.4×10^5 copies per mg) were observed in live oysters sampled from Porto Bay on the 15th February.

3.5.2. Survey of farm leases

Samples were collected on 29-30th January 2013 from 36 actively farmed oyster leases across the five major production areas in the Hawkesbury River that were not yet clinically affected. These included Patonga and Porto Bay (downstream sites) and Coba Bay, Kimmerikong and Marra Marra Creek (upstream sites) (Figure 1). Of the 432 pools of 3 oysters that were tested, 195 pools were positive for OsHV-1 DNA in PCR analysis (Table 6). OsHV-1 was detected at all locations in at least one pooled sample from 32 of 36 leases where oysters were cultured. The prevalence of infection ranged from 10% to 80% among infected leases and locations (Table 6). Overall, prevalence of infection among oysters across all leases was lowest at Kimmerikong (5%) and highest at Marra Marra Creek (31%) (Table 7).

The levels of OsHV-1 in tissues of oysters were low and below the quantification limit (< 1.2×10^1 copies per mg). However, in two oyster leases (leases 19 and 20) at Coba Bay, high viral loads were observed in several pools, showing a maximum of 1.7×10^4 to 4.1×10^5 viral copies per mg (Table 6). Lease 20 was the only lease where dead oysters were observed during sample collection for the survey on 29/1/13, and lease 19 was a nearby lease where mortality was subsequently observed. The trays which contained dead oysters on 29/1/13 (lease 20) had been moved from Mullet Creek to Coba Bay on 19th January, two days before mortalities were observed at Mullet Creek (Table 2). An additional sample (non-random) of 15 oysters was taken from lease 20 due to the unusual number of dead oysters observed there. The viral load present in these oysters ranged from 1.9×10^3 to 1.0×10^5 viral copies per mg (in 4 of the 5 pools tested) while one pool had a relatively low viral load of 1.3×10^1 viral copies per mg.

PCR and prevalence results were provided to oyster farmers on 2nd February. On the basis of these results the voluntary local movement restrictions were relaxed to enable oysters to be moved from the downstream nursery areas to the upstream harvest areas for fattening. Biosecurity measures to clean boats and equipment were also suspended to reduce costs.

The survey was repeated a month later on 26-27th February 2013 in Coba Bay and Marra Marra Creek in order to assess the progression of infection over one month across oysters in selected leases (Table 8). The prevalence declined over time in all oyster leases except for lease 11 in Marra Marra Creek where the number of pools positive increased between January (n=3/12) and February (n=5/12). All pools that tested positive on 29th January and 26th-27th February exhibited low viral loads, either below or around the limit of quantification (1.2×10^1 copies/mg).

4. Identification of environmental risk factors

Prior to the Mullet Creek outbreak on the 21st January the estuarine conditions recorded by the YSI sonde were relatively stable (water temperature 22-26°C; salinity 33-35 ppt; CHL-a 1-5 µg/L) with consistent mean daily values and with the associated daily standard deviations being small for all recorded parameters (Figure 3). Greater variability in salinity was noted from the 30th January onwards due to a large rainfall event that occurred in late January / early February. Subsequent tidal flushing during the dry period following this rainfall event led to an a recovery in salinity levels to 28-34ppt before OsHV-1 associated mass mortalities occurred in Porto Bay on the 15th February (Figure 3).

The 1440 data points recorded by the YSI sonde in the main channel of the lower Hawkesbury River 15 days prior to the mass mortality events were pooled and compared between January (first period, Mullet Creek outbreak) and February (second period, Porto Bay outbreak) in order to determine (i) local environmental parameters, and (ii) whether a difference in one of these parameters could be associated with the spatial/temporal pattern of mortality observed in the Hawkesbury River (Figure 4). Substantial variations in water temperature (± 3°C over a few days) were recorded in January and February. The range of water temperatures were similar (23-26°C) between the first and the second outbreak periods even though the median values were 1-2°C cooler in February than in January. The range of salinity observed during the second period was lower and more variable than in January (Figure 4) as a result of the significant rain event.

5. DISCUSSION

5.1. First detection of OsHV-1 and associated mortalities

The first detection of OsHV-1 in Australia was in farmed Pacific oysters in Woolooware Bay/Botany Bay on the 24th November 2010 (Jenkins et al., 2013). A much larger Pacific oyster farming industry existed in the Hawkesbury River estuary, approximately 50 km to the north of Botany Bay. It remained free of signs of OsHV-1 disease until the January 2013 outbreak described in this paper. The first detection of OsHV-1 in the Hawkesbury River was in apparently healthy, farmed triploid Pacific oysters collected on the 18th October 2012 (archived and tested in February 2013; inconclusive PCR results), three months prior to mass mortalities. The virus was present at at low prevalence (2.7%-15%) and at a very low level (viral loads in individual oysters close to the detection limit of the PCR assay) between October 2012 and early January 2013. The first mortalities occurred on the 21st January 2013 at the index case site (Mullet Creek) associated with high prevalence (69%) and high viral loads (up to 9.4×10^4 copies per mg). The latter were consistent with the threshold value (10^4 copies per mg of fresh tissue) at which OsHV-1 is considered to be involved in mortality (Oden et al., 2011). Similarly, the virus was detected in Porto Bay as early as the 7th January but no mortality was observed prior to the 15th February. Whether several waves of infection (firstly in October, then higher exposures in January and February), several strains of the virus (one of low virulence in October *vs.* high virulence in January/February) or unidentified environmental/biological factors (*Vibrio* spp. were not investigated in this study) were responsible for the delay observed between the first detection of OsHV-1 and the first mass mortality event remains uncertain, but is discussed further below. These observations mean that from October 2012 until 21st January 2013, OsHV-1 virus was present in the Hawkesbury River while the environmental conditions were apparently favourable (water temperature above the limit of 16-17°C, data not shown) without leading to significant mortalities. This is in accordance with similar reports from the Netherlands, Spain (EFSA, 2010), Italy (Dundon et al., 2011), Republic of Ireland (Peeler et al., 2012), France

(Pernet et al., 2012) and Australia (Paul-Pont et al., 2013b) and confirms that the presence of OsHV-1 is necessary but not sufficient to induce the disease (i.e. mortality). In New Zealand, OsHV-1 was present in 2005, about 5 years before the first mortalities were detected in April 2010 (Webb et al., 2007; Bingham et al., 2013). From a practical standpoint this finding highlights the importance of active surveillance as an early detection tool / warning system to mitigate the economic losses of oyster mortality by emergency harvest.

5.2. Window of infection

The observations made in Woolooware Bay/Georges River in 2010 suggested that OsHV-1 infection caused mortalities over a period of months starting in November 2010 (Jenkins et al 2013). This corresponds to the beginning of the Australian summer which is consistent with observations made in Europe about the virus being active in summer (May-August) when the water temperature is above 16-17°C (Bedier, 2010). A controlled experiment conducted in Woolooware Bay the following year (2011-2012) demonstrated that the disease started at the same time (November) and remained active until May (Paul-Pont et al., 2013a). This was also confirmed in the same bay in 2012-2013 (unpublished data). In the Hawkesbury River mortalities associated with OsHV-1 were observed from 21st January 2013 to late April 2013 (in sentinel spat deployed at five different sites, data not shown). Now that OsHV-1 is endemic in this estuary it will be important to assess whether the period of activity for the virus is similar to that observed in Woolooware Bay, which is 50km south from the Hawkesbury).

5.3. Incubation period

Observations made in the Hawkesbury River provide knowledge about the incubation period for OsHV-1. Experimentally, the incubation period was shown to be as short as 2 days when the virus was injected into the adductor muscle of oysters, but when the transmission was performed by cohabitation of naïve oysters with infected individuals in a relatively small volume of water, up to 8 days was required to reach 50% mortality (Shikorski et al., 2011). Two field studies reported a peak in mortality between 5 and 14 days after deployment/exposure (Pernet et al., 2012; Dégremont et al., 2013) but overall very few data are available about the incubation period under natural conditions, probably because the frequent monitoring that is required to address this question could not be conducted. In the Hawkesbury River the history of oyster transfers from Mullet Creek (index case) to other locations can inform this question. Transfers of oysters were performed on the 19th January from Mullet Creek (downstream) to Coba Bay (upstream). Mortalities were observed at Coba Bay 10 days later localised to the transferred trays, and the role of OsHV-1 was confirmed by the high viral load detected in the dead individuals that were opportunistically sampled (10^4 - 10^6 copies per mg). Similarly, oysters were transferred to the upper part of Mooney Mooney Creek (downstream) on the morning on the 21st January and mortalities were subsequently detected in the transferred trays (and to a lesser extent in the surrounding trays) on the 24th January, 5 days prior to the observation of mass mortality at this site. This suggests that the oysters in Mullet Creek were already infected when moved to other locations from the 19th January onwards. Transfers of oysters from Mullet Creek to other sites (Porto Bay and Marra Marra Creek) were performed on the 17th January and earlier and no mortality was observed in these trays (Marra Marra Creek) or during the following month (Porto Bay), suggesting that these oysters were not infected. Overall, these results suggest that mass infection of the oysters farmed at the index case site occurred sometime between the 17th and the 19th January, leading to an estimated maximum incubation period of 4 days for the mass mortality event.

5.4. Transmission/spread of OsHV-1 in affected bays

Prior to 21st January 2013 OsHV-1 virus was detected in the Hawkesbury River at low levels (downstream sites; Table 5). As no other locations were monitored it is not possible to assess the extent of distribution of OsHV-1 prior to the first episode of mass mortality (21st January) where 12 million spat died within few days in Mullet Creek. Four days later (25th January) mass mortality was observed in an adjacent nursery area (Mooney Mooney Creek), and was confined to the entrance of

the creek as no mortality was observed in the upper part of the creek except in trays transferred from Mullet Creek (see above). Farmers' observations made on leases at the entrance to Mooney Mooney Creek were that the front of the leases close to the main channel were affected first, prior to the back of the leases close to the shore. Another farmer inspected his lease twice on the 25th January: he firstly reported that all spat were alive (12 pm), before reporting a mass mortality (>90%) in all baskets later in the day (5 pm). As millions of oysters died in Mullet Creek the release of billions of viral particles from the dead individuals into the water must have occurred over a few days. The hydrodynamic connectivity and the biomass of infected animals constitute major drivers for disease epidemics in aquaculture (Gustafson et al., 2007; Kristoffersen et al., 2009; Salama and Murray 2011: Petton et al., 2013), therefore the exposure of oysters *via* tidal movements of water is likely to explain the mass mortality observed in Mooney Mooney Creek and nearby farm leases at Spectacle and Goat islands (Figure 1). Similarly, mortality and detection of OsHV-1 DNA in oysters placed in Thau lagoon France, outside of a farming area, coincided with currents coming from the farming area where OsHV-1 associated mortalities were occurring. Therefore the authors concluded that mortality spread between healthy and infected oysters occurred as a result of hydrodynamic connectivity (Pernet et al., 2012). The survey performed on the 29th January revealed that OsHV-1 was already widespread in the estuary 8 days after the first observed mortality event (all sites infected, low viral loads, prevalence ranging from 10 to 80%, Table 6). An existing hydrodynamic model based on a simplified version of the water exchange and tidal flushing for the Hawkesbury River demonstrated that the influence of a single tide can be detected as far as 16 km upstream of the Mullet Creek entrance in the Hawkesbury River (data not shown), suggesting the important role of hydrodynamics in the rapid dispersion of viral particles at large scale. As an effective infection depends on the quantity of viral particles in contact with oysters (Schikorksi et al., 2011; Petton et al., 2013), it is likely that the quantity of the virus in the water in the upstream sites (Marra Marra Creek, Coba Bay and Kimmerikong) was too low to lead to significant mortality.

In Coba Bay mortalities were detected on the 29th January only in trays which had been transferred from Mullet Creek to lease 20, before being observed on the 8th February in an adjacent lease (lease19). Subsequent inspections were performed on February 11th and 26th but there had been no spread of mortality beyond leases 19 and 20. These results suggest that local spread of OsHV-1 from infected oysters to adjacent individuals occurred in the Hawkebsury River but was inefficient and did not lead to a mass mortality event in the upstream sites.

6. Relationship between oyster age/size and mortality

All spat supplied to oyster farmers in the Hawkesbury River were of same hatchery origin and supplied originally as 2.2 mm single seed in April or October 2012, but most oysters had grown considerably by January 2013. The most affected areas were the downstream nursery areas (Mullet Creek, Mooney Mooney Creek and Porto Bay) which contained millions of spat. This observation is consistent with the extreme mortality rates commonly observed after OsHV-1 µvar infection in young oysters (larvae, spat, juvenile) in Europe (EFSA, 2010). The mortality rate was age-dependent at most downstream sites (Table 4) but no clear relationship was observed between size and mortality rate as demonstrated by the homogenous percentage mortality observed in the 3-9 month old spat (80-100%) despite wide variation in shell length (2-96 mm). The variable mortality rate observed at Porto Bay for this age class (3-9 month old: 20-100% mortality) might be related to other confounding factors such as rearing structure and density, as previously reported in France (Pernet et al., 2012).

7. Role of sub-clinically infected adults as reservoirs for subsequent infection

Levels of OsHV-1 DNA in adult oysters sampled in the upstream growing areas (Marra Marra Creek, Kimmerikong, Coba Bay) and in Patonga on the 29th January were low and close to or below the limit of quantification of the assay (except for leases 19 and 20 in Coba Bay see above) (Table 6). As no mortality was observed at these sites this result confirms that OsHV-1 may persist in sub-clinically infected oysters (Arzul et al., 2002; Dégremont et al., 2013), which is also consistent with

herpesviruses infecting finfish such as cyprinid and pilchard herpesviruses (Whittington et al., 2008; Goodwin et al., 2009). A survey was performed twice in selected leases at Coba Bay and Marra Marra Creek in order to assess the progression of infection among oysters over time (Table 8). Infection prevalence and viral loads decreased over time for most leases confirming that surviving oysters are able to eliminate viral DNA from their tissues (Dégremont, 2011; Pernet et al., 2012; Petton et al., 2013; Paul-Pont et al., 2013b). However it is unclear how long those sub-clinically infected oysters constitute a reservoir for OsHV-1 and what their role is in subsequent infections. There is some evidence that sub-clinically infected oysters (demonstrating very low levels of viral DNA in their tissues) may either clear the infection (Petton et al., 2013) or transmit the disease after reactivation of the virus (Dégremont et al., 2013). Further work is required to determine the role of the sub-clinically infected population (including wild oysters) in the seasonal onset of the disease in the field.

8. Influence of environmental parameters

A body of European literature clearly identifies water temperature as the main risk factor for OsHV-1 disease expression (for review see EFSA, 2010). The threshold of 16°C below which no mortality is observed seems to be valid both experimentally (Petton et al., 2013) and in the field (Pernet et al., 2012). However the threshold above which no mortality is observed remains unclear: while some studies demonstrate an absence of mortality when the seawater temperature exceeds 24°C (Bouquet et al., 2011; Pernet et al., 2012), others show significant mortalities associated with OsHV-1 at higher temperatures (25-29°C) (Le Deuff et al., 1996; Sauvage et al., 2009; Petton et al., 2013). Based on the seawater temperatures recorded in the Hawkesbury River in January / February 2013 it is clear that OsHV-1 mortality outbreaks occur above the threshold of 24°C in Australian waters. Additionally, the detection of OsHV-1 associated mortalities in France and USA occurs after a marked increase in mean daily seawater temperature (+3°C over a couple of days) (Burge et al., 2006; Sauvage et al., 2009; Garcia et al., 2011). An intensive monitoring of the seawater temperature (frequency of acquisition every 15 minutes) performed during the second OsHV-1 outbreak (summer 2011/2012) in Woolooware Bay/ Georges River revealed that sudden increases in temperature of 2-3°C over a few days were commonly observed during the summer and did not necessarily lead to a mortality event (Paul-Pont et al., 2013b). Similarly, in the Hawkesbury River such sudden variations in water temperature were observed from August 2012 (winter, water temperature below 15°C) to December 2012 (summer, water temperature > 22°C) (data not shown) without leading to a significant mortality outbreak despite the virus being present since October 2012. In January, significant weekly increases and decreases in water temperature were recorded in the main river channel (Figure 1) but no significant mortality was reported at Porto Bay before 15th February. Overall these observations support the idea that sudden increases in seawater temperatures are a common phenomena in Woolooware Bay/ Georges River and the Hawkesbury River that do not necessarily trigger a mortality event. Furthermore, an unprecedented heatwave was recorded in the Sydney area on the 19th January 2013 and also during the preceding week (air temperature ≥ 46°C). This heatwave was not reflected by significant changes in the water temperature and its effects on oyster physiology and the onset of the mortalities remains undetermined.

Pacific oysters grow in various environmental conditions, they can withstand salinity in the range of 10-42 ppt and mortality generally occurs when salinity exceeds 50 ppt (Mann et al., 1991). There is no prior evidence to suggest that salinity plays any important role in OSHV-1 expression (EFSA, 2010). However, variation in salinity as well as extreme salinity values can significantly influence the immune systems of oysters and other bivalve molluscs, making them more susceptible to infection (Hauton et al., 2000; Gagnaire et al., 2006). The variation in salinity in the lower Hawkesbury River (26-38 ppt) was higher in February than in January but was within the acceptable range for this species. Extreme salinities were observed in the upper Hawkesbury River (down to 6 ppt mid-February, data not shown) at sites where no significant mortalities were detected (Marra Marra Creek and Kimmerikong) despite the virus being present. Overall these findings suggest that salinity did not play a significant role in the onset of mortalities associated with OsHV-1.

9. Source of infection of the Hawkesbury River estuary

In Europe and New Zealand, the spread of OsHV-1 associated disease between geographically distant areas was enhanced by movements of oysters and/or equipment from infected areas (Bingham et al., 2013; Peeler et al., 2012). However, there were no known movements of *C. gigas* into the Hawkesbury River estuary except for certified disease free spat from a commercial hatchery. These were also supplied to many other growing areas in New South Wales and South Australia with no evidence of disease arising at those locations. Similarly there were no known introductions of potentially infected farming equipment to the Hawkesbury River. At the time of the outbreak the only other known infected farming area was the Georges River/Botany Bay estuary, but very few Pacific oysters were being grown and this estuary was subject to strict quarantine orders preventing translocation of oysters. The only other known location of oysters with OsHV-1 infection was Port Jackson but these were exclusively wild populations (Jenkins et al., 2013). All other *C. gigas* farming areas in Australia were the subject of a national survey for OsHV-1 in 2011 and were free of the infection (Animal Health Australia, 2011).

Another possible mechanism for introduction of OsHV-1 to the Hawkesbury system is shipping movements, associated with either biofouling of vessels or ballast water. There is very little commercial shipping in the Hawkesbury River estuary, but there are many pleasure boat movements between the estuary and other estuaries along the coast of New South Wales.

A free oceanic source of OsHV-1, transported by current and tide, may also explain the introduction of OsHV-1. The detection of a low prevalence of oysters with infection, and low viral loads prior to the mass mortality event suggests there might have been several infection events of increasing magnitude during the spring and summer of 2012-2013.

10. Genesis of mass mortality

Mass mortality in the index case of farmed *C. gigas* at Mullet Creek occurred three months after the first detection of OsHV-1 in the Hawkesbury River estuary. Similarly in Porto Bay mass mortality lagged behind the first “arrival” of the virus. There was also a substantial wild population of diploid *C. gigas* in the Hawkesbury River estuary (NSW Department of Primary Industry, 2012) that was also affected although it was not monitored or sampled intensively in the present study. There are two possible explanations for these mass mortality events, either progressive amplification of OsHV-1 in the resident population of oysters to the point where infectious doses released into water were sufficiently high to trigger an outbreak, or successive infection events from an external source providing a sufficient infectious dose. Experimental infection trials have confirmed that a threshold dose is necessary to cause mortality of individual oysters as is typical for most infectious diseases (unpublished data). Evidence from this study suggests that there was an external source for infection rather than a progressive amplification of OsHV-1 in the population. Firstly, active surveillance through monthly sampling of sentinel oysters in Mullet Creek and Porto Bay in 2012 revealed the absence of dead oysters until the 21st January and viral loads were so low that they were barely detectable until 2 weeks prior to the first mass mortality (Table 5). Also, from October 2012 (first detection) to January 2013 (first mortality event), no increase in prevalence of infection or viral loads in tissues was observed at these sites. Additionally, prevalence of sub-clinical infection decreased over time in the upstream sites (Coba Bay, Marra Marra Creek) during the outbreak period (Table 8). These findings suggest that progressive amplification of OsHV-1 from the initial low-intensity, low prevalence situation was not a feature of the present outbreak. Secondly, the oyster-to-oyster spread that occurred in Coba Bay (and to a lesser extent in Mooney Mooney Creek) did not lead to a mass mortality event and was in fact quite limited. Thirdly, the rapidity with which mass mortality occurred in Mullet Creek is not consistent with oyster to oyster spread. It can be shown by simple modelling that even with an initial prevalence as high as 10% in a population of 10 million oysters, an incubation period of 3 days and a net reproductive rate of 5 or more, about one week is required for the entire population to be killed (data not shown), whereas all spat in Mullet Creek were killed within three days. For these reasons the most likely explanation for the mass mortality event in

Mullet Creek was synchronous exposure to a high infectious dose from an external source, i.e. the outbreak was initiated from a common environmental point source which infected most individuals at the same time (Thrusfield, 2007). Exposure to sub-lethal doses appeared to begin in October 2012 with probable super-infection in January 2013.

11. Conclusion

The monitoring and analysis of this outbreak in real time provided important findings on the epidemiology of OsHV-1. The virus was detected months before the first mortalities were observed in the Hawkesbury River estuary despite favourable environmental conditions, suggesting the need for high infectious doses from an external source and/or other unidentified factors to trigger the disease. *Vibrio* species were not monitored therefore their role in this outbreak remains unclear and further investigations in the field are required. There was no evidence that introduction of oysters or farming equipment from another river initiated the outbreak. Instead, the distribution of OsHV-1 and associated mortalities over time and space in the Hawkesbury River suggested the existence of a common environmental point source that led to massive synchronous exposure of the oyster population at the index case site. Hydrodynamics then assisted the spread of OsHV-1 over a wide area. Spread of OsHV-1 with movement of infected oysters did not initiate the index case, or result in mass mortality events elsewhere in the estuary. The incubation period for mass mortality after putative synchronous environmental exposure was ≤ 4 days. From a practical standpoint these findings raise the importance of active surveillance in Australia to both monitor the spread of OsHV-1 to other locations, and to use as an early warning system for the oyster industry. There may be a substantial lag period to enable an orderly emergency harvest and rational business decisions (for example whether or not to purchase spat) prior to the onset of mass mortality. The identification of the main environmental reservoir for OsHV-1 as well as the existence of a potential vector (see Paul-Pont et al., 2013a) is of high priority in order to improve our understanding of the ecology of the disease and enable us to devise ways to mitigate the mortalities.

Acknowledgements

This study was funded by the Fisheries Research Development and Corporation (FRDC), The University of Sydney and the Sydney Metropolitan Catchment Management Authority. We thank Shellfish Culture Tasmania for supply of sentinel oysters andoyster farmers from BBOA, especially Bruce Alford, John Stubbs, Rob Moxham and Steve Jones for provision of oyster farm records. Thanks are also due to the following people who in addition to the authors volunteered their time and equipment on 29th January for sample collection: John Stubbs, Bruce Alford, Paul Hick, Shane Moxham, James Moxham, Rob Moxham, Steve McOrrie, Wayne O'Connor and Ian Lyall. Special thanks are due to laboratory staff at the University of Sydney: Anna Waldron, Alison Tweedie, Natalie Schiller, Vickie Patton, Ann-Michele Whittington and Craig Kristo. We are grateful to Mark Crane and Nick Moody from AAHL, and Paul Hick and Peter Kirkland from NSW DPI EMAI for control OsHV-1 samples and interlaboratory benchmarking of PCR protocols.

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Tables and Figures

Figure 1. Pacific oyster farming areas in the Hawkesbury River located approximately 40 km north of Sydney, New South Wales, Australia. The thick black lines represent the harvest areas and the fine black lines represent the farming areas (oyster leases) in each Creek/Bay. The black star indicates the location of the multi-sensor water quality probe (managed by Hornsby Shire Council).

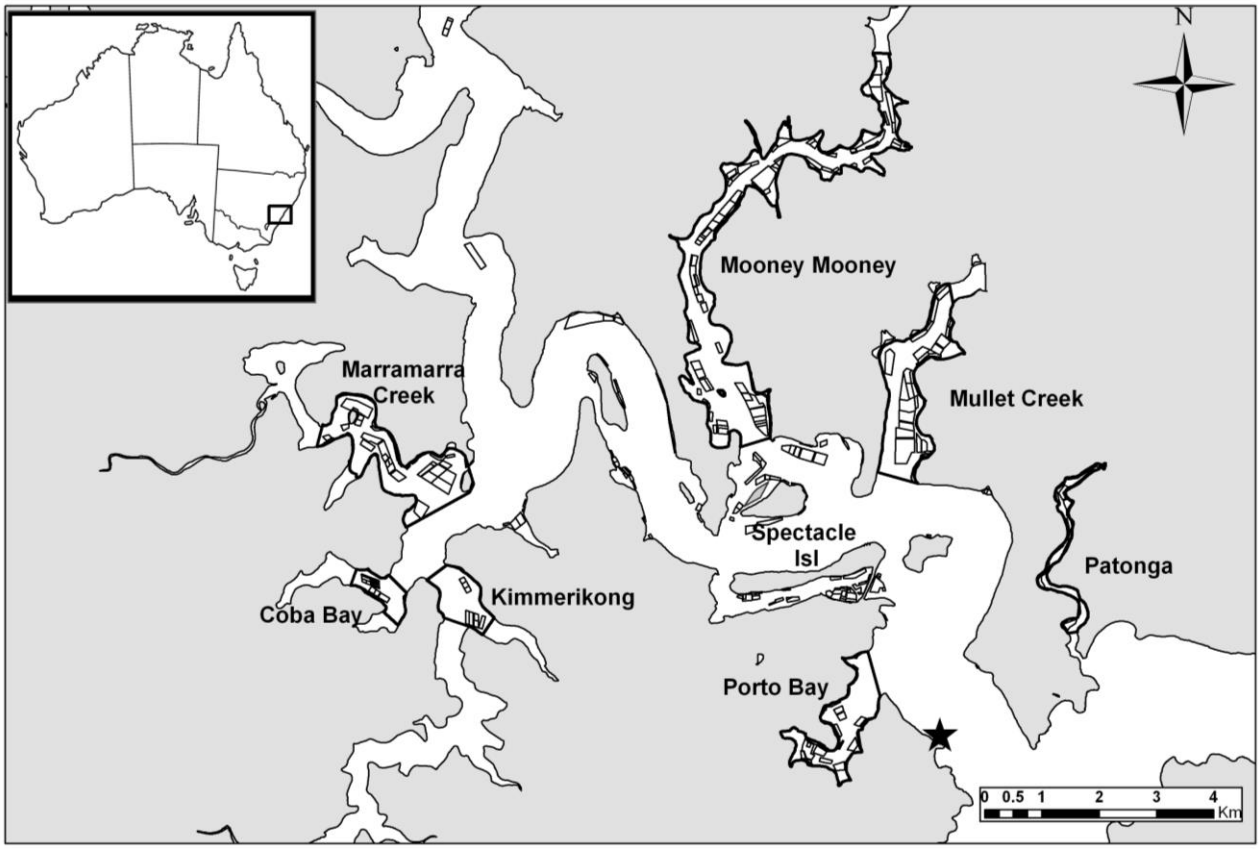


Figure 2. Mortality in active oyster leases examined in Coba Bay on the 11th and the 26th February 2013

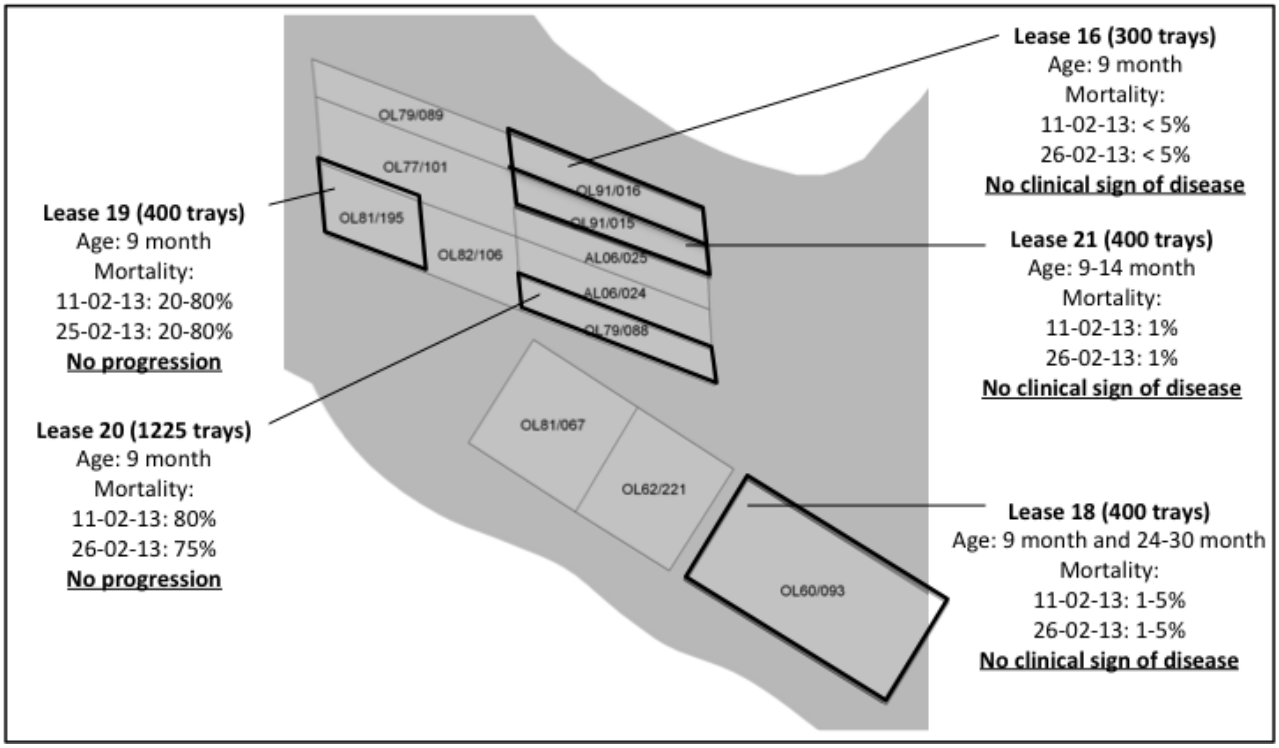


Figure 3. Water temperature and salinity recorded every 15 minutes by the multi-sensor water quality probe during 2013 (location shown with a star in Figure 1). Vertical dotted lines indicate the start of the mortality outbreaks observed in Mullet Creek (21st January) and Porto Bay (15th February).

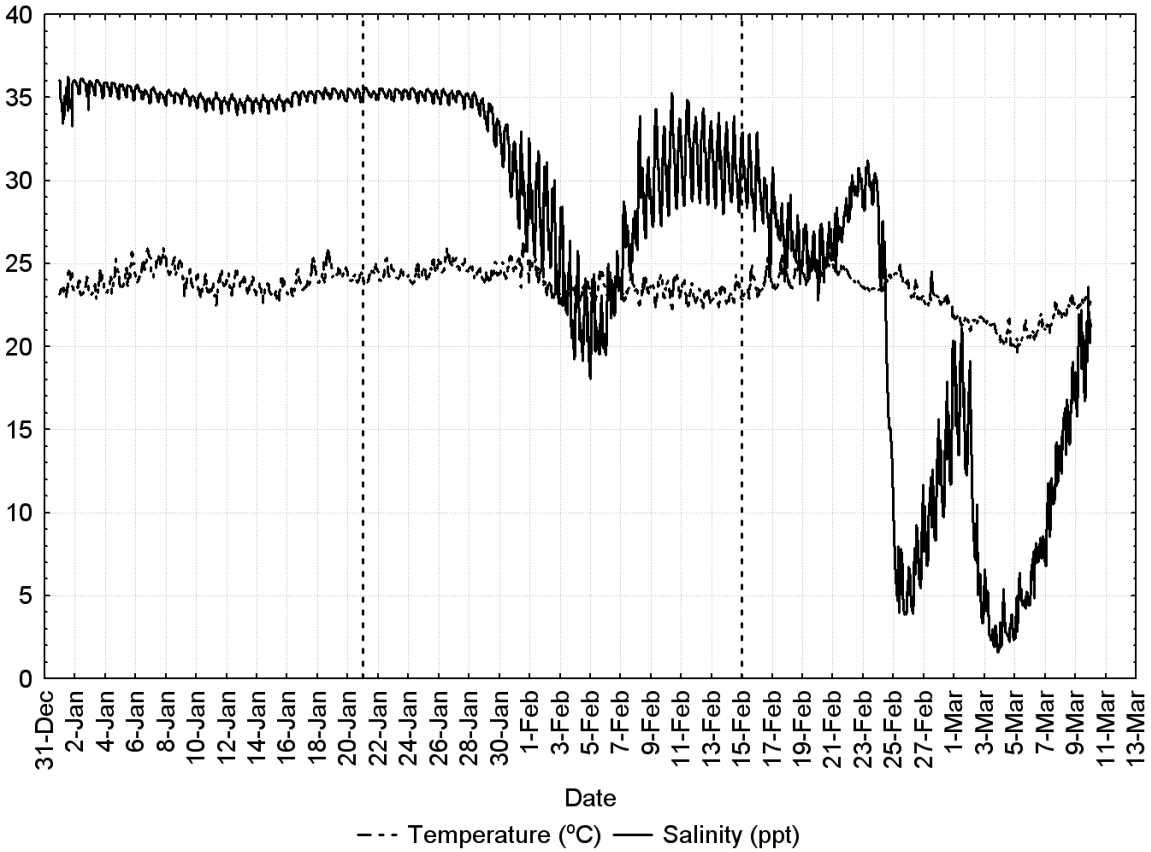
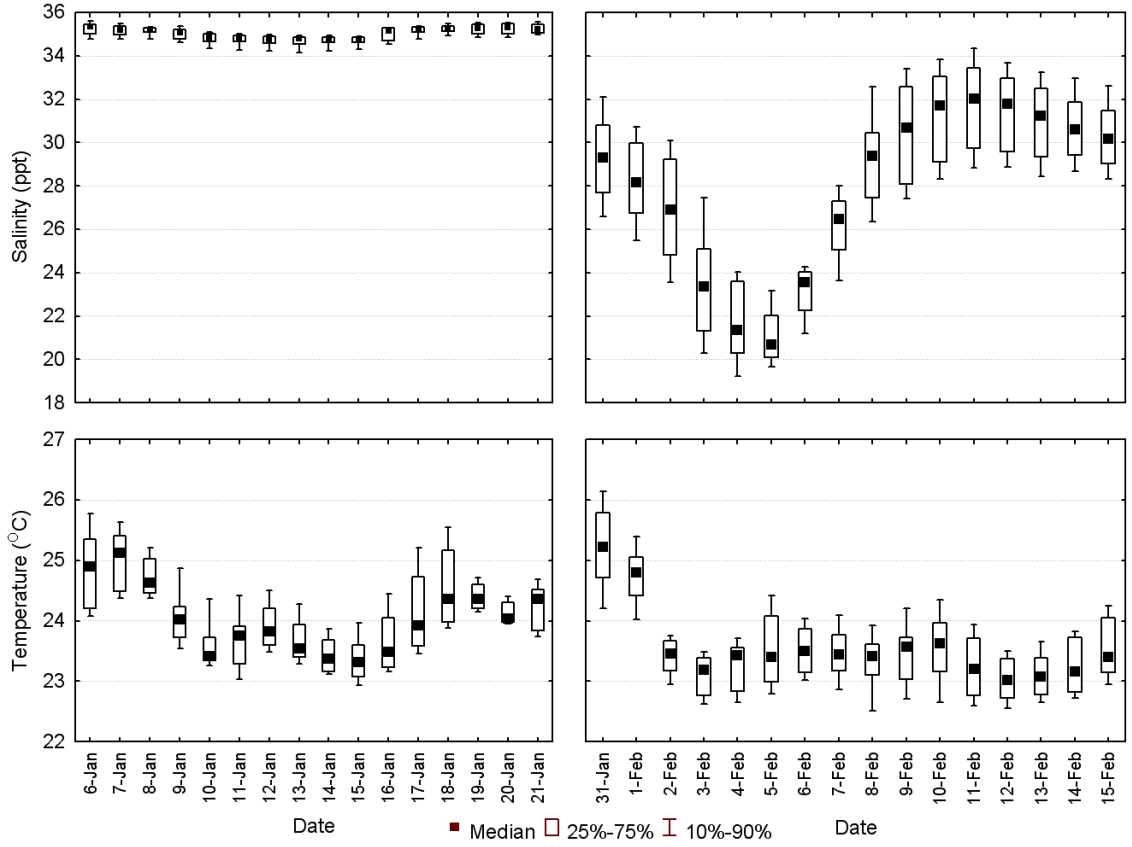


Figure 4. Box-and-whisker plots of the daily median water temperature and salinity data collected by the multi-sensor water quality probe during the 15-day incubation period prior to OsHV-1 outbreaks on 21st January and 15th February 2013 2013 (location shown with a star in Figure 1)**.**



Tables

Table 1. Characteristics of the main oyster farming areas in the Hawkesbury River. Information on surface area of harvest areas, farming areas and the number of registered oyster leases was calculated using ESRI GIS version 10 software from data managed by the Australian governmental agencies: NSW Food Authority and NSW Department of Primary Industries. The number of active Pacific oyster leases and farming businesses as well as the total number of Pacific oysters present in each farming area prior to the outbreak was estimated using data recorded by the executive officers of Broken Bay Oyster Association on 23rd January 2013 (audit of stock). (D): Downstream site; (U): Upstream site.

	Mullet Creek (D)	**Porto Bay (D)**	**Mooney Mooney Creek (D)**	**Coba Bay (U)**	**Marra Marra Creek (U)**	**Kimmerikong (U)**	**Patonga (D)**
Area of harvest areas (Ha)	216.1	137.1	498.4	43.1	230.6	75.0	53.5
Area of farming areas (Ha)	75.8	25.9	123.6	7.6	60.6	8.7	24.1
No. of registered oyster leases	29	31	81	12	32	8	15
No. of active Pacific oyster leases	10	10	7	6	13	2	6
No. of active Pacific oyster farming businesses	9	8	5	6	10	2	1
Approximated number of Pacific oysters prior to outbreak	12 000 000	1 138 160	2 117 050	720 400	942 488	391 000	298 800

Table 2. Oyster movements from the index case location in Mullet creek to other farming areas in the month preceding the onset of mortality in Mullet Creek (21st December – 21st January) and observed mortality at the destination sites. na: not applicable.

Destination sites	Dates of transfer	Most recent movement relative to index case	Mortality due to OsHV-1 detected at destination	Date mortality first observed
Mooney Mooney Creek (D)	21st January	Same day	Yes	25th January
Coba Bay (U)	28th December 9th and 19th January	2 days	Yes	29th January
Porto Bay (D)	7th, 8th, 9th, 10th, 14th, 16th and 17th January	4 days	Yes	15th February
Marra Marra Creek (U)	11th and 17th January	4 days	No	na
Kimmerikong (U)	na	na	No	na
Patonga (U)	na	na	No	na

Table 3. Mortalities observed and reported by BBOA members in oyster leases in the Hawkesbury River. Note – there were no reports of mortality in Patonga, Kimmerikong or Marra Marra Creek during January or February 2013. (D): Downstream site; (U): Upstream site.

Date	Location	Description
21st January	Mullet Creek (D)	Morning: Above average mortality noticed in young spat on one lease; other leases were not affected. Afternoon: mass mortality event
23rd January	Mullet Creek (D)	All leases were affected; most oysters were dead
24th January	Mooney Mooney Creek (D)	A few dead oysters were noticed in trays at the entrance to this bay.
25th January	Mooney Mooney Creek, Goat Island and Spectacle Island (D)	Dead oysters were noticed on leases at each site. At Mooney Mooney Creek mass mortality was confined to those leases at the entrance. Some dead oysters observed upstream confined to oyster trays that were transferred from Mullet Creek on the 21st January. No mortality observed other than in these trays.
29th January	Mooney Mooney Creek (D)	There were no boat movements or observations made in Mooney Mooney Creek between 25th and 29th January. Leases upstream were now massively affected.
	Coba Bay (U)	Dead oysters were observed on one lease (Lease 20); the affected trays had been moved from Mullet Creek on 19th January.
8th February	Coba Bay (U)	Dead oysters were observed on one lease upstream of the known-affected lease in Coba Bay (Lease 19).
11th & 26th February	Coba Bay (U)	All leases were inspected and there was no spread beyond the two known affected leases.
15th February	Porto Bay (D)	Widespread mortality was observed across leases.
28th February	Porto Bay (D)	All leases were inspected and were affected.

Table 4. Mortality in active oyster leases examined in Mullet Creek (4th February 2013), Porto Bay and Mooney Mooney Creek (26th February 2013). (D): Downstream site.

Location	Age range	Size range	Cultivation types (number of units)	% mortality	Population at risk
Mullet Creek (D)	3-9 month	30-96 mm	baskets (27100)	90-100	11 928 000
	11-14 month	70-110 mm	trays (240)	50-70	57 600
	24-30 month	130-150 mm	trays (60)	25-50	14 400
Mooney Mooney Creek (D)	3-9 month	2-90 mm	baskets (27100); trays (50); tumblers (50)	80-100	1 144 800
	11-14 month	70-100 mm	trays (503)	60	377 250
Porto Bay (D)	3-9 month	30-90 mm	baskets	90-100	435 400
	3-9 month	50-90 mm	baskets	40-70	
	3-9 month	50-90 mm	trays	60-90	702 720
	3-9 month	90-100 mm	trays	20-80	

Table 5. Retrospective testing for OsHV-1 in sentinel oysters placed in different sites in the Hawkesbury River and from opportunistically sampled oysters from October 2011 to February 2013. Mullet Creek (M), Porto Bay (P), Marra Marra Creek (R) and Kimmerikong (K). The sample size was 30 oysters / site (except for Mullet Creek on 18th October 2012, n=21) and the pooling rate varied from 5 to 6. All oysters sampled were apparently healthy. *: inconclusive PCR result. BLOQ: positive/inconclusive PCR result but below the limit of quantification.

Date	Sites	Sample size	OsHV-1 qPCR	No. pools positive	OsHV-1 DNA copies per mg of tissue	Prevalence estimate (95% CI)
26-Oct-11	M,P	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
7-Dec-11	M,P	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
19-Dec-11	M,P	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
4-Jan-12	M,P	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
18-Jan-12	M,P,R,K	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
15-Feb-12	M,P,R,K	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
15-Mar-12	M,P	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
20-Apr-12	M,P	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
10-May-12	M,P	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
5-Jun-12	M,P,R,K	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
3-Aug-12	M,P,R,K	30	Negative	0/5 (all sites)	/	0.028 (0.001, 0.20)
20-Sep-12	P	30	Negative	0/6	/	0.027 (0.001, 0.15)
1-Oct-12	P	30	Negative	0/6	/	0.027 (0.001, 0.15)
12-Oct-12	P	30	Negative	0/6	/	0.027 (0.001, 0.15)
18-Oct-12	M	21	***Inconclusive***	1/5*	BLOQ	0.06 (0.001, 0.33)
19-Oct-12	P	30	Negative	0/6	/	0.027 (0.001, 0.15)
26-Nov-12	M,P	30	Negative	0/6 (all sites)	/	0.027 (0.001, 0.15)
13-Dec-12	M	30	***Inconclusive***	1/6*	BLOQ	0.04 (0.001, 0.22)
26-Dec-12	M,P	30	Negative	0/6	/	0.027 (0.001, 0.15)
7-Jan-13	M,P	30	***Inconclusive / Positive***	(M) 3/6** (P) 1/6*	BLOQ BLOQ	0.15 (0.028, 0.54) 0.04 (0.001, 0.22)
21-Jan-13	M,P	30	***Positive***	6/6 (all sites)	(M) 9.3×10^1 - 9.4×10^4 (P) 5.6×10^1 - 1.1×10^2	0.69 (0.28, 0.98)
2-Feb-13	P	30	***Inconclusive***	1/6*	BLOQ	0.04 (0.001, 0.22)
15-Feb-13	P	30	***Positive***	4/6	1.3×10^4 - 1.4×10^5	0.44 (0.12, 0.97)

Table 6. Prevalence of OsHV-1 in actively farmed oyster leases in the Hawkesbury River on 29th - 30th January 2013. There were 36 oysters tested per lease in pools of 3 (12 pools). The symbol # indicates that oysters were not processed as fresh samples, but removed from the shell and frozen at -20 °C for 6 weeks prior to testing. BLOQ: positive PCR result but below the limit of quantification. (D): Downstream site; (U): Upstream site.

Bay / Creek	Lease No.	No. pools positive	Range of OsHV-1 DNA copies per mg of tissue	Prevalence (95% CI)
Marra Marra Creek (U)	1	12	BLOQ - 1.5×10^1	0.8 (0.47 - 0.99)
	2	9	BLOQ - 5.6×10^1	0.45 (0.19 - 1)
	3	10	BLOQ - 5.4×10^1	0.58 (0.25 - 1)
	4	10	BLOQ - 9.1×10^1	0.58 (0.25 - 1)
	5	12	BLOQ	0.8 (0.47 - 0.99)
	6	9	BLOQ - 1.7×10^1	0.45 (0.19 - 1)
	7	12	BLOQ - 1.4×10^1	0.8 (0.47 - 0.99)
	8	10	BLOQ - 1.5×10^1	0.58 (0.25 - 1)
	9 #	0	0	0
	10 #	0	0	0
	11	3	BLOQ - 1.3×10^1	0.1 (0.02 - 0.29)
	12	3	BLOQ - 1.7×10^1	0.1 (0.02 - 0.29)
	13	5	BLOQ - 1.5×10^1	0.19 (0.06 - 0.42)
Kimmerikong (U)	14	3	BLOQ - 2.0×10^1	0.1 (0.02 - 0.029)
	15 #	0	0	0
Coba Bay (U)	16	8	BLOQ - 2.0×10^1	0.58 (0.25-1)
	17	4	BLOQ	0.14 (0.04 - 0.35)
	18 #	0	0	0
	19	6	BLOQ - 4.1×10^5	0.24 (0.09 - 0.5)
	20 #	4	4.3×10^1 - 1.7×10^4	0.14 (0.04 - 0.35)
	21	6	BLOQ - 1.2×10^1	0.24 (0.09 - 0.5)
Porto Bay (D)	22	3	BLOQ - 1.5×10^1	0.1 (0.02 - 0.29)
	23	5	BLOQ - 2.5×10^1	0.19 (0.06 - 0.42)
	24	4	BLOQ	0.14 (0.04 - 0.35)
	25	4	BLOQ	0.14 (0.04 - 0.35)
	26	4	BLOQ - 2.7×10^1	0.14 (0.04 - 0.35)
	27	3	BLOQ	0.1 (0.02 - 0.029)
	28	No stock to sample	/	/
	29 #	0	0	0
	30	10	BLOQ - 3.7×10^1	0.58 (0.25 - 1)
	31	5	BLOQ	0.19 (0.06 - 0.42)
Patonga (D)	32	3	BLOQ - 1.6×10^1	0.1 (0.02 - 0.29)
	33	3	BLOQ	0.1 (0.02 - 0.29)
	34	8	BLOQ - 2.0×10^1	0.36 (0.15 - 1)
	35	5	BLOQ - 1.2×10^1	0.19 (0.06 - 0.42)
	36	6	BLOQ - 1.6×10^1	0.24 (0.09 - 0.5)
	37	6	BLOQ - 1.7×10^1	0.24 (0.09 - 0.5)

Table 7. Prevalence of OsHV-1 in bays of the Hawkesbury River on 29th -30th January 2013. BLOQ: positive PCR result but below the limit of quantification. (D): Downstream site; (U): Upstream site.

Bay / Creek	No. leases tested	Total pools	Pools positive	Prevalence (95% Cl)	Range of OsHV-1 DNA copies per mg of tissue	Overall mortality observed at Bay level
Marra Marra Creek (U)	13	156	95	0.31 (0.25 - 0.38)	BLOQ - 9.1×10^1	No mortality
Kimmerikong (U)	2	24	3	0.05 (0.01 - 0.14)	BLOQ - 2.0×10^1	No mortality
Coba Bay (U)	6	72	28	0.17 (0.12 - 0.24)	BLOQ - 4.1×10^5	Mortality in lease 20
Porto Bay (D)	9	108	38	0.15 (0.11 - 0.21)	BLOQ - 3.7×10^1	No mortality
Patonga (D)	6	72	31	0.20 (0.13 - 0.27)	BLOQ - 2.0×10^1	No mortality

Table 8. Prevalence of OsHV-1 on two occasions one month apart in four selected oyster leases in the Hawkesbury River. There were 36 oysters tested per lease in pools of 3 (12 pools per lease). BLOQ: positive PCR result but below the limit of quantification. (U): Upstream site.

Bay / Creek	Lease No.	Date.	No. pools positive	Viral load (copies/mg)	Prevalence (95% CI)
Marra Marra Creek (U)	Lease 6	29.01.13	9	BLOQ - 1.7×10^1	0.45 (0.19 – 1)
		26.02.13	7	BLOQ	0.12 (0.29 – 0.61)
	Lease 11	29.01.13	3	BLOQ - 1.3×10^1	0.1 (0.02 – 0.29)
		26.02.13	5	BLOQ	0.19 (0.06 – 0.42)
Coba Bay (U)	Lease 16	29.01.13	8	BLOQ - 2.0×10^1	0.58 (0.25 – 1)
		26.02.13	1	BLOQ	0.03 (0 – 0.17)
	Lease 21	29.01.13	6	BLOQ - 1.2×10^1	0.24 (0.09 – 0.5)
		26.02.13	2	BLOQ	0.07 (0.01 – 0.23)