FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) – risk mitigation, epidemiology and OsHV-1 biology

R Whittington, P Hick, A Rubio, N Dhand, O Evans

December 2015

FRDC Project No 2012/032

THE UNIVERSITY OF SYDNEY

ISBN **: 978 -1-74210-368-6**

Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) – risk mitigation, epidemiology and OsHV-1 biology

FRDC 2012/032

December 2015

Ownership of Intellectual property rights

This publication (and any information sourced from it) should be attributed to Whittington, R.J., Hick, P., Rubio, A., Dhand, N. and Evans, O. The University of Sydney. 2015. *Pacific oyster mortality syndrome (POMS) – risk mitigation, epidemiology and OsHV-1 biology*, Sydney, July.

Creative Commons licence

Disclaimer

Researcher Contact Details

Name: Richard Whittington
Address: 425 Werombi Road
Camden NSW 2570
Phone: 02 9351 1619
Fax: 02 9351 1618
Email: richad.whittington@sydney.edu.au
Web: www.oysterhealthsydney.org

FRDC Contact Details

Address:	25 Geils Court
	Deakin ACT 2600
Phone:	02 6285 0400
Fax:	02 6285 0499
Email:	frdc@frdc.com.au
Web:	www.frdc.com.au

In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

Table of Contents

List of Tables

List of Figures

Acknowledgments

This project was funded by the Australian Government through Fisheries Research and Development Corporation, with additional funding from the University of Sydney, Sydney Catchment Management Authority, Tasmanian Oyster Research Committee and Oysters Australia via the Seafood CRC. Bruce Alford, Broken Bay Oysters, provided skilled technical assistance for all experiments in the Hawkesbury River, while Len Drake managed field work in the Georges River. John Stubbs, Bob Drake, Ted Drake, Robert Hill and Keith Duggan were instrumental in ensuring the successful conduct and completion of field trials and provided shore facilities and access to oyster leases. Thanks are due to Reg Richards (deceased) and Steve Jones who gave free access to their commercial oyster sheds at Mooney Mooney in which the infrastructure for the upweller trials was installed. Commercial triploid Pacific oyster spat were kindly provided by Scott Parkinson, Shellfish Culture Tasmania. Oysters were grown in a region free from OsHV-1 by Leon and Angela Riepsamen, Goodnight Oysters, Greenwell Point. Technical assistance was provided by Alison Tweedie, Vickie Patten, Craig Kristo, Natalie Schiller, Ann-Michele Whittington and Stuart Glover. Serge Corbeil is thanked for useful discussions on disinfection studies. We acknowledge Peter Coad, Hornsby Shire Council, for his assistance with some of the environmental data analysis and participation with the sampling in the Hawkesbury River. We also acknowledge Hornsby Shire Council and Sydney Metropolitan Catchment Management Authority for sharing historical water quality data. The research was facilitated by NSW DPI Fisheries staff who issued a scientific collection permit and provided advice on biosecurity throughout the project. Marion Saddington provided skilled administrative support throughout the project.

Executive Summary

What the report is about

POMS is a viral disease with the potential to devastate Pacific oyster aquaculture in Australia. This project was an attempt by researchers from the University of Sydney to intervene in the face of a disease outbreak to discover ways to continue oyster farming. It was conducted in partnership with oyster farmers whose businesses had been wiped out, by working in real-time on affected oyster leases in the Georges and Hawkesbury Rivers, and in state of the art laboratories at the University of Sydney. It is the first study in Australia, and one of the few worldwide, to involve very intensive long term field work to find a solution for an oyster disease. The project addressed nationally agreed priorities and complemented research to develop a genetically resistant oyster. The solution to POMS will require both genetic improvement and science-based modifications to husbandry.

Background

There has been a disturbing pattern of emerging disease in oysters in Australia. Examples include QX disease, winter mortality and bonamiosis. These diseases have spread, persisted or recurred. Too little was known about their cause, or their biology (epidemiology) to enable rational control measures, and research was either not undertaken, or commenced too late to inform policy and strategy. Consequently the opportunity was seized to commence research on POMS at the outset of its emergence in Australia, and to communicate research findings in real time to both government and industry, to enable both policy formulation and commercial decisions. The Pacific Oyster Health Management Working Group under the Subcommittee for Aquatic Animal Health, Department of Agriculture Canberra was established to coordinate the flow of information.

Aims/objectives

The aims of the project were to confirm the identity of the virus, determine the mechanism(s) of transmission of disease, determine the major factors that contribute to outbreaks thereby identifying potential risk-mitigation management practices, to identify the natural reservoir(s) for the virus, its stability in the environment and disinfection guidelines, to develop a laboratory infection model to study the disease and to address future shortages of technical expertise through training a PhD student.

Methodology

The project required DNA sequencing to identify the virus. The samples for this were obtained from oyster leases by the university researchers who worked closely with oyster farmers during the disease outbreaks. Long term field study sites were set up on oyster leases in both the Georges River and the Hawkesbury River at the start of the project. The research team conducted an intensive real time investigation of the first outbreak of POMS in the Hawkesbury River in 2013, and set up controlled experiments in the Georges River to measure key aspects of the disease. Oysters were placed at multiple locations in both rivers over 2 years and sampled every few weeks to accurately determine the seasonal window for the disease. Experimental upwellers were set up at the Hawkesbury River to test the idea that the virus could be removed from seawater to protect hatcheries. Wild molluscs were sampled in the Georges River to try to find an environmental reservoir for the virus. Laboratory experiments were conducted to develop a reliable way to induce POMS under controlled conditions, and then these methods were used to study how long the virus remained infective in seawater, how it could be inactivated by disinfectants, and how the disease was dependent on water temperature.

Results/key findings

The virus that causes POMS in Australia is *ostreid herpes virus 1* µVar (OsHV-1 µVar). It is similar to the virus that has devastated Pacific oyster aquaculture in France, other European countries and New Zealand. The disease outbreaks in the Georges and Hawkesbury Rivers were caused by the same virus.

Detailed investigation of the Hawkesbury River outbreak revealed novel features of the disease: the virus was first detected months before the disease began, but it is likely that this was due to several separate infection events, the last one being massive and leading to widespread mortalities; the source of virus was not the farming operation, and was most likely to have been from a distant environmental source; once introduced en masse from an external source, local spread of the disease from oyster to oyster and lease to lease was minor; large adult oysters were relatively resistant.

A consistent seasonal pattern of disease was observed in both rivers. POMS was seen between October and May each year and was not present in the other, cooler months. Water temperatures in NSW when POMS occurred were about 4 °C warmer (i.e. 20^{o}C) than those observed in France when the disease occurs there (i.e. 16^{o}C). This is a very significant difference in disease behaviour between Europe and Australia. It was confirmed in an experimental infection trial at the University of Sydney where mortality was minimal at temperatures less than 18^{o}C.

Analysis of long term weather and environmental records revealed that the outbreaks in the Georges and Hawkesbury Rivers were not associated with anomalies in air temperature, water temperature, salinity, or chlorophyll-a levels in water. Harmful algae were variably present and did not explain disease occurrence.

The major factors determining the extent of mortalities during an outbreak were found to be the age of oysters (spat are highly susceptible, adults relatively resistant); growing height/immersion time (raising growing height by 300 mm in the intertidal zone reduced mortalities of adults by 50%); and location (some sites within an infected river were not affected at all). The type of cultivation system and the presence of non-susceptible bivalve species on adjacent leases were not important factors. Host energy status (feeding) and cultivation density were not able to be investigated and could be important.

POMS disease expression can be variable and the replication of trials over sites and over time was critically important to reveal risk factors.

Wild oysters, both Pacific and Sydney rock, tested positive for the virus, as did other mollusc species and other marine organisms. However, the levels of virus in their tissues were low, and their potential role in storing virus and amplifying and releasing it to infect farmed oysters is debatable. Further study is required.

The virus appears to remain stable in seawater for less than 48 hours. This was confirmed in laboratory experiments and in field trails with spat in upwellers in the Hawkesbury River. Water treatments based on ageing water for 48 hours and filtration to 5 µm were successful and can be used to protect hatcheries. Several disinfectants were effective and will be useful for decontamination of equipment. Chlorine is commonly available but was not effective in the presence of organic matter.

Implications for relevant stakeholders

Industry can adopt some findings from this study immediately. Hatcheries can treat incoming seawater using simple procedures to prevent mortality of spat due to the virus. It is possible to safely farm oysters in affected estuaries except between late October and mid May. Farmers

who already have suitable and flexible infrastructure, or who will make investments in infrastructure, can provide elevated intertidal growing height to substantially reduce mortalities in adult oysters. Partnerships between farmers in different regions and strategic movement of stock would allow for two stage farming to take advantage of feed availability and to avoid the danger period in POMS affected estuaries.

Policy makers should consider the findings related to disinfection guidelines for equipment. In addition, the potential for further spread of the disease due to unknown sources, probably oceanic, should be considered.

Recommendations

Further research is required to identify strategies to enable spat to survive in infected estuaries during the danger period. Identifying the reason for their profound susceptibility will fill a major knowledge gap.

The role of wild molluscs in outbreaks in farmed oysters, and identification of the main environmental sources of OsHV-1 requires further research.

Exposure of oysters to OsHV-1 at any given place or time is highly variable. This has major implications for surveillance, for prediction of risk and for assessment of the benefits of husbandry modifications and genetically selected oysters. We recommend that all field assessments be conducted using adequate experimental design involving independent professional supervision, multiple sites, replication, frequent observations and laboratory confirmation.

Active surveillance is required in regional oyster farming locations in Australia as an early warning system, for example to enable an orderly emergency harvest and rational business decisions (such as whether or not to purchase spat). A reliable active surveillance system needs to be devised based on understanding the spatial dynamics of OsHV-1 discovered in this project.

All outbreaks of POMS in Australia have occurred in relatively warm water. Further study of the consistency of seasonal patterns of POMS, the periodicity of infection within season, inter-estuary temperature variation in Tasmania and South Australia, and integration of this information to predict POMS behaviour is warranted.

It is important to determine whether water treatments prevent OsHV-1 infection of spat or merely prevent mortality, and whether they can be applied for biosecurity of hatchery effluent. In order to produce OsHV-1–free spat in an endemic region, and have batches certified for movement to disease free regions, greater confidence than that provided in the current project is needed to assess whether the virus can be excluded from hatcheries.

There is a question about the potential for persistence of infective OsHV-1 in fresh-frozen commercial oysters, and whether this could be a means of translocation of virus. Oysters may be diverted from the human food chain to be used as bait, or shells may be discarded in coastal waters from pleasure boats. Studies are required in Australia to assess the infectivity of OsHV-1 in imported fresh-frozen oysters, including those from New Zealand.

Sequencing of additional samples of OsHV-1 and additional parts of the genome of samples from Australia is recommended to further understand infection and disease spread patterns.

Keywords

Crassostrea gigas, Ostreid herpes virus -1, Pacific oyster mortality syndrome, POMS, disease

Introduction

POMS is a viral disease of *C. gigas* which is caused by the virus OsHV-1 u-Var. It has the potential to devastate *C. gigas* culture in Australia, as it has done overseas. For this reason national priorities have been discussed since early 2011.

There has been a disturbing pattern of diseases in commercial molluscs nationally. They have required a succession of government/industry responses, with no clear solutions: QX disease in Sydney rock oysters in NSW and QLD; Pacific oyster mortality syndrome in NSW with a national response; Abalone viral ganglioneuritis in VIC. To this list can be added diseases of unknown cause: Oyster oedema disease in pearl oysters in WA; Winter mortality in Sydney rock oyster in NSW; SAMS in Pacific oysters in SA; Winter mortality in Sydney rock oysters in NSW and PSMS in Pacific oysters in Port Stephens. Economic impacts have been substantial or devastating. Wild fisheries and aquaculture have been impacted. In NSW, the impact of QX disease in the Georges and Hawkesbury Rivers led to replacement of Sydney rock oysters by triploid Pacific oysters to re-establish the industry in these estuaries, but this is now threatened by POMS. Apart from generic responses, in each case it has not been possible to devise a specific intervention strategy that would halt disease spread or ensure the recovery of the industry.

Investigating the behaviour of POMS during its recrudescence in summer 2011/2012 in FRDC project 2011-053 afforded a unique insight into the disease, and these observations need to be extended over time to identify factors which may be used to reduce the impact of the infection. This project seeks to concurrently investigate the effect of host, environment and husbandry factors on POMS prevalence and mortality rate in Pacific oysters with the objective of discovering aspects of epidemiology that can be manipulated by oyster growers. If POMS spreads beyond its current limited distribution in NSW, commercial scale production of Pacific oysters in the face of POMS will be essential for the viability of the industry pending development of solutions such as genetically resistant lines and species diversification.

The project addresses FRDC strategic R&D theme 1 - biosecurity and aquatic animal health, and Aquatic Animal Health Subprogram priority - Nature of disease and host-pathogen interaction. The application was first developed in 2011. The project addressed priorities which were discussed at the FRDC Cairns conference on POMS in 2011. The initial expression of interest (RW044) had 9 objectives: 1. To correlate biotic and abiotic environmental factors with QX, POMS and winter mortality occurrence in selected oyster populations; 2. To develop an experimental laboratory transmission model for POMS in Pacific oysters; 3. To confirm experimentally whether biotic and abiotic factors affect POMS transmission; 4. To determine whether there are measurable immune responses in POMS and other key diseases; 5. To determine whether Pacific oysters can be protected from POMS and other key diseases by manipulating environmental conditions; 6. To determine whether oysters can be protected from QX and POMS by prior artificial exposure to agent factors; 7. To determine whether natural selection is a key driver of population survival following QX, POMS and other disease outbreaks; 8. To promote oyster health through oyster information portals; 9. Provide training for three PhD students to serve future industry needs. FRDC responded to develop a proposal addressing objective 1 which was funded as FRDC project 2011/053 “Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) - understanding biotic and abiotic environmental and husbandry effects to reduce economic losses” with the following objective: 1. To correlate biotic and abiotic environmental factors with POMS occurrence in selected oyster populations.

This project was successfully completed. A national process then commenced to define current research priorities for POMS. This process involved both the Sub-Committee on Aquatic Animal Health (SCAAH) and the FRDC Aquatic Animal Health Subprogram. This project faithfully addresses each of the research priorities identified by FRDC through this complex process.

Objectives

1 To determine/confirm the identity of the one or more variant(s) of Ostreid herpesvirus associated with the recent outbreaks of POMS

2 To determine the mechanism(s) of transmission of disease

3 To determine the major risk factors that contribute to precipitation of disease outbreaks thereby identifying potential risk-mitigation management practices

4 To identify the natural reservoir(s) for the virus

5 To determine the stability of the virus in the environment

6 To identify physical and chemical means for viral inactivation

7 To develop an infectivity model for POMS suitable for selection of resistant oysters and pathogenesis/environmental research

8 To address future shortages of technical expertise through the training and supervision of at least 1 PhD student

Method

Sequencing of *Ostreid herpesvirus-1* associated with recent outbreaks of POMS

Representative tissue samples from oysters from outbreaks of POMS were selected. These were from both the Georges River and the Hawkesbury River estuaries, i.e. the only two recognized locations where POMS has occurred in Australia. The samples are described in Table 1 and locations are illustrated in Figure 1. They represent repeated collection events at different sites in the same estuaries. All oysters sampled in the Georges River were experimental oysters that had been deployed and monitored by the University of Sydney team; all oysters sampled in the Hawkesbury River were commercially farmed oysters collected *in situ.*

Table 1. Time and location of sampling of farmed and experimental oysters for OsHV-1 DNA sequencing. Locations are according to Figure 1.

SVC	*Sample ID*	*Oyster/ pool details*	*River*	*Location*	*Date of collection*	*Mortality*
11/245	3	A13	Georges	Site A	24-Nov-11	high
12/025	9	B14	Georges	Site B	10-Feb-12	high
11/234	14	C15	Georges	Site C	16-Nov-11	high
12/194	22	105	Georges	Site A	7-Nov-12	high
12/212	26	B19	Georges	Site B	28-Nov-12	high
12/206	27	C16	Georges	Site C	13-Nov-12	high
13/084	28	Pool 15	Georges	Site A	11-Apr-13	medium
13/068	29	Pool 39	Georges	Site B	15-Mar-13	medium
13/068	30	Pool 93	Georges	Site C	15-Mar-13	medium
13/022	31	Mullet1	Hawkesbury	Mullet Creek	21-Jan-13	high
13/049	40	Porto5	Hawkesbury	Porto Bay	15-Feb-13	high
13/064	41	180	Hawkesbury	Coba Bay	29-Jan-13	high

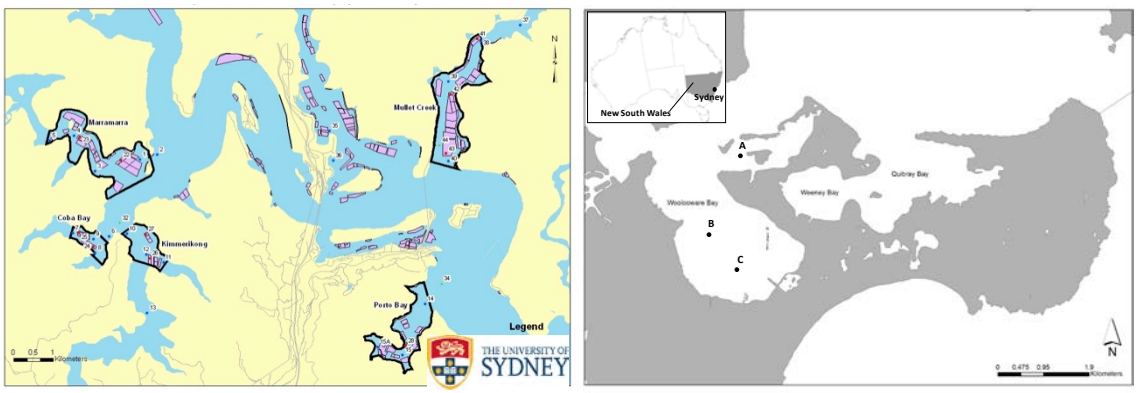


Figure 1. Locations for oyster sampling in the Hawkesbury River (left panel) and Georges River (right panel).

Collaboration with researchers at IFREMER, La Tremblade, France (Dr Tristan Renault) was established in 2012 to enable whole genome sequencing. Samples were provided to the French lab under a material transfer agreement and consistent with Australian guidelines for export of DNA from pathogens. The French lab was unable to provide any sequence data from the range of samples provided to them. Consequently multilocus sequence typing of OsHV-1 samples was completed at the University of Sydney using sub-samples of those provided to the French lab, and additional samples. Three genome regions were sequenced as recommended and described by Renault, et al. (2012), and the sequence data were analysed using Mega5 software.

Mechanisms of transmission of disease and determination of major risk factors that contribute to precipitation of disease outbreaks

Environmental data analysis

Retrospective analysis of environmental data was conducted in relation to OsHV-1 µVar disease occurrence.

Definitions and dates. The index cases and dates used for this investigation are defined in Table 2. The index case does not define the start of the season of infection as the virus might have been newly introduced during a favourable season rather than being detected at the start of it. The occurrence of POMS within an estuary has been reported to be seasonal (occurring during warmer months), discontinuous (mortality is not constant through time) and spatially variable (mortality varies by location within an affected estuary) (Paul-Pont, et al., 2013a; b). Hence while observed mortality events indicate that environmental conditions are favourable, the failure to observe a mortality event at a particular monitored location does not necessarily indicate that environmental conditions are unfavourable at that location at that time, and might instead indicate that exposure to OsHV-1 did not occur.

Table 2. Definitions of disease, infection and time period used in this study

Term	Definition	River	Value	Reference
Index case	The first OsHV-1 µVar associated mass mortalities reported by farmers	Georges River	16th November 2010	Jenkins, et al. (2013)
		Hawkesbury River	21st January 2013	Paul-Pont, et al. (2014).
First detected OsHV-1 µVar infection	Date of detection of OsHV-1 µVar in oyster tissues prior to the index case	Georges River	unknown as tests for the infection had not been used in Australia prior to the index case	
		Hawkesbury River	18th October 2012	Paul-Pont, et al. (2014).
Period prior to outbreak		Both	As early as 1st September prior to the respective index case	
Long term data		Both	5 to 10 year period prior to outbreak	

Sources of environmental data. Environmental parameters collected at high frequency were used in this study. They included both seawater and climatic variables. Real-time water quality data were obtained from two types of multi-sensor water quality probes. In the Hawkesbury River YSITM6820 sondes (John Morris Scientific) were used, while in the Georges River Hydrolab (Aqualab) sondes were used. Both types of probes were deployed 1.0 m below the water surface attached to a standard navigational buoy. These autonomous probes monitored temperature (°C), salinity (ppt) and chlorophyll-a (µg/L) at 15 minute intervals. Probes were calibrated and maintained to prevent marine fouling every three weeks and readings from the probes in the Hawkesbury were telemetered to a public website every 6 hours (http://www.hornsby.nsw.gov.au/environment/waterquality). Probes at both estuaries

were deployed as part of extensive water quality monitoring programs managed by Hornsby Shire Council in the Hawkesbury River and the Sydney Catchment Management Authority in the Georges River. Probes in the Hawkesbury River had been deployed for 7 years with the exception of the probe at Calabash which was deployed 11 years ago (2004). The monitoring stations at the Georges River were deployed for 4 years (2010-2014).

Samples to assess harmful phytoplankton in the Georges and Hawkesbury Rivers were collected fortnightly to monthly at a number of locations in the downstream oyster growing areas as part of the Shellfish Quality Assurance Program managed by the NSW Food Authority. Since 2012 phytoplankton was monitored more intensively every 3-4 weeks at the real-time monitoring stations in the Hawkesbury River as part of the Hornsby Shire Council monitoring program.

Water level data (15-min interval, 2004-2014) at the mouth of the Hawkesbury River were obtained from Manly Hydraulics Laboratory (NSW Public Works).

Daily air temperatures collected by the Bureau of Meteorology (BOM) were obtained for station 066037- Sydney Airport (period April 1939-May 2015 http://www.bom.gov.au/climate/data/). At one of the autonomous probe locations in the Hawkesbury, Calabash Point at Berowra Creek, the monitoring station recorded air temperature every 15min (period 2008-May 2015). Air Temperature datasets from the two locations were highly correlated (R^2= 0.87, Figure 4d); minimum air temperatures had a higher correlation (R^2= 0.96) compared with maximum air temperatures (R^2= 0.74) (data not shown).

Sea Surface Temperature (SST) daily data were obtained from NOAA data sets (http://www.esrl.noaa.gov/psd/data/gridded/data.noaa.oisst.v2.highres.html) for coordinates 33°34' / 151°21' (Hawkesbury River mouth) and 34°00'/151°14' (Georges River/Botany Bay mouth) for the period 1981 to 2014.

The monthly El Nino Southern Oscillation (ENSO) index Nino3.4 from 1870 to 2014 was obtained from a public website (http://www.esrl.noaa.gov/psd/gcos_wgsp/Timeseries/Nino34/).

Rainfall data were obtained from Berowra Heights Station ID#067052 and Sydney Airport Station 066037 (BOM).

Data analysis and statistics. Time-series and mean/median plots of 95%ile were used to represent environmental conditions during the period preceding the index case and at the onset of the mass mortalities at both estuaries. In order to identify data that departed from the long-term average, daily and monthly anomalies were calculated by removing the long-term monthly or daily climatology, depending on the resolution of the data set. Monthly climatology produced a mean value for each month while daily climatology produced a mean value for each day over a specified time period. Anomalies were calculated by subtracting climatological values from daily or monthly observations. This approach removes the seasonality inherent in the data. Anomalies were calculated only in data sets that were longer than 4 years and data collected at high-resolution, i.e. at least hourly. When calculating daily climatology the data were smoothed using a 7-day running average. The rate of change of a parameter was calculated as the change in the individual values relative to time. The data were first smoothed using a 7-day average. Statistical analyses were conducted using Statistica version 12, Statsoft and Matlab version 2012b, MathsWorks. A significance threshold of α=0.05 was adopted for all statistical tests.

Outbreak investigation Hawkesbury River January-February 2013

Case definition. The unit of interest was an oyster lease within a bay. The case definition 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.

Monitoring the spread and severity of the clinical disease.

Assessment of stock levels and trace forward investigation. Firstly, to identify oysters at risk we audited the stock present on farms with actively farmed leases 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.

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. 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. Sample sizes were calculated 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 was found to achieve the required target system sensitivity of 0.95. All leases were sampled, 36 oysters per lease. All calculations were made using online calculators: http://epitools.ausvet.com.au/content.php?page=2StageFreedom (least cost where herd size unknown). To account for clustering of infection within a lease, systematic sampling was undertaken. The stock audit 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.

Passive surveillance. Farmer observations of mortality in oysters were recorded.

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.

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.

Strategy for testing oysters for OsHV-1. Oysters that were collected as part of the surveys on 29th-30th January and 26th February 2013 were processed in batches according to the lease number and the bay of origin. A section of gill and mantle of approximately 100 mg was removed 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) for homogenization. For sentinel oysters that were sampled in 2011/2012 and all other oysters, 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 for bead beating. Each sample collected as part of the surveys on the 29th -30th January and 26th February 2013 was tested in qPCR in single wells. Each sample from sentinel oysters that were sampled in 2011/2012 and samples from all other oysters were tested in duplicate.

Estimation of prevalence. Individual animal prevalence was estimated from PCR results using a pooled prevalence calculator (www.ausvet.com.au).

Statistical analysis. The effects of age and size on mortality rate were evaluated using data pooled from a total of 21 leases (46 production units) using a restricted maximum likelihood linear mixed model (REML) (Genstat, VSN International). A production unit is defined as a part of a lease containing the same batch of oysters farmed using similar cultivation structure (i.e. tray, basket, tumbler). Mortality % was transformed to a logit and age, shell length and their interaction were included as fixed effects. The interaction was dropped from the final model as it was not significant. Lease nested within bay were included as random terms.

Determination of the window of infection in the summers of 2012-2013 and 2013-2014

The aim of this experiment was to determine when OsHV-1 was active and killing young spat i.e. POMS was present.

2012-2013. From February to June 2013 spat produced by Shellfish Culture Tasmania Batch SPL12FT were certified with respect to OsHV-1 and shipped every 2 weeks by air to Sydney airport. An average of 1500 spat per site was placed every two weeks in the Hawkesbury River and the Georges River in order to determine and compare the window of infection between the two estuaries. A total of 8 sites were monitored in the Hawkesbury River (Mullet Creek, Porto Bay, Marra Marra Creek, Kimmerikong, Patonga) and Georges River (Site A, Site B, Site C).

Spat were placed in socks inside floating baskets on floating longlines (500 spat per floating basket, 3 floating baskets per site located up to 50m apart from each other). Each basket was labelled and numbered and a temperature data logger was inserted inside basket (frequency of acquisition 30 min). A salinity probe was placed at each site to record data at 15 minute intervals until these probes failed in April 2013. Every fortnight the mortality was assessed in each basket by visual inspection and a random sample of 10 pools of spat was taken from each site (3 pools from basket 1, 4 from basket 2 and 3 from basket 3; pool size 3 oysters if >6 mm, otherwise 300 mg in total weight) and tested for OsHV-1 by qPCR. The surviving spat were placed in holding baskets nearby, but were not examined again. New spat from Shellfish Culture Tasmania were introduced and placed in the same baskets/location each time. The last deployment of spat was performed on 30th May.

2013-2014. A similar trial design was employed but the number of sites was increased to 14: A total of 8 sites were monitored in the Hawkesbury River (Patonga, Mullet Creek, Porto Bay, Marra Marra Creek, Kimmerikong and Mooney Mooney) and Georges River (Pelican Gut, Site A, Site B, Site C, The Shed, Sylvania Waters, Neverfail Bay and Limekiln Bar).

Two floating baskets each with 250 spat were placed at each site. Spat were sampled and replaced with new spat every 2 weeks between August 2013 and June 2014. All baskets were checked fortnightly, or weekly during November-December.

Georges River field Trial 2012-2013

To improve external validity the experiment described in the final report for FRDC project 2011/053 (Paul-Pont, et al., 2013a) in Woolooware Bay was repeated and enhanced during 2012-2013. A schematic diagram is provided to show the orientation and the organisation of the different cultivation systems at each site (Figure 1). The trial started on 19th October 2012 with the deployment of about 18,000 oysters of two ages (see below).

Oysters. Single seed triploid Pacific oysters obtained originally from a hatchery in Tasmania (Shellfish Culture Pty Ltd) as 2mm spat were grown in the Hawkesbury River for 8 to 13 months, then transferred to the Woolooware Bay site on 19th October 2012: Spat, batch SPL11G, 9.5 month old – 50.0 ± 5.2 mm; Adult, batch SPL11C, 17.5 month old – 92.2 ± 5.6 mm. The oysters were confirmed to be OsHV-1 free prior to shipment from the hatchery and by repeated sampling and testing using a TaqMan assay (Martenot, et al., 2010) performed fortnightly during 2012.

Experimental design. Oysters were allocated randomly to individually numbered 2m x 1m plastic trays (n=40 per segment, 8 segments/tray) and plastic baskets (n=92 per basket); commercial hanging baskets with a triangular cross-sectional profile (BST) and self-made pillow-shaped baskets were used. The design and treatment groups are shown in Table 3. The plastic trays were placed at two different heights in the inter-tidal range: High (H) and Low (L). The hanging baskets were placed on long-lines at two different heights: Very High (HH), High (H). Heights are defined in Table 1. The pillow baskets were floating (F) attached to a long-line that was extended between two floating rings that moved up and down with the tide on fixed poles; these baskets were thus immersed in surface water at all times; a foam block was attached to the top of each basket to maintain buoyancy and orientation. Individual numbered labels were attached to all the trays and baskets, and to minimize disturbance to putative sequential OsHV-1 transmission between units they were systematically returned to their original locations and orientations after each inspection of the oysters (Figure 2).

Table 3. Experimental design

Sites	Cultivation system	Cultivation zone	Treatment	Height differential	Age	No. replicates	No. oysters per replicate
A, B, C	Trays, rack and rail[1]	Intertidal	High (H)	L+300 mm	Adult	2	320
		Intertidal	High (H)	L+300 mm	Spat	2	320
		Intertidal	Low (L)	Standard	Adult	2	320
		Intertidal	Low (L)	Standard	Spat	2	320
	Baskets, long-line	Intertidal	Very high (HH)	L+600 mm	Spat	12	92
		Intertidal	High (H)	L+300 mm	Spat	12	92
		Subtidal	Floating (F)	0 mm	Spat	12	92

[1] these treatments match those in a previous experiment (Paul-Pont, et al., 2013a)

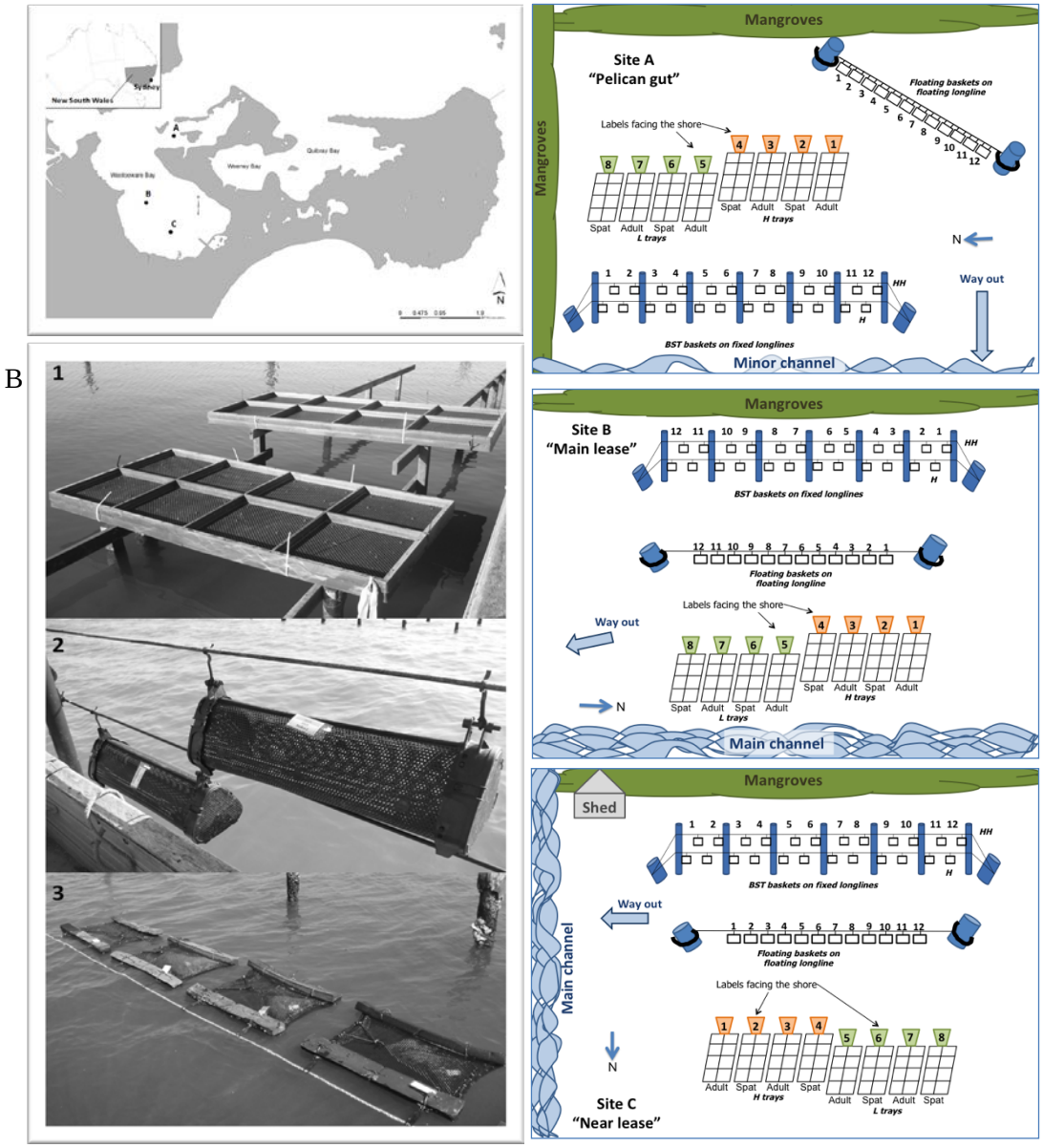


Figure 2. Location of sites A, B and C within Woolooware Bay, New South Wales, and cultivation systems used in the trial: intertidal trays on timber rack and rail (1), intertidal hanging baskets on a fixed longline system (2), and sub-tidal floating baskets on a floating longline system (3). Panel C, schematic showing the position and orientation of intertidal trays, hanging baskets and floating baskets at each site.

Observations and sampling of oysters. Oysters were examined and sampling was conducted approximately fortnightly before (October) and after (19th December – 14th February) the expected outbreak, at weekly intervals during the outbreak period (1st November – 19th December), then at monthly intervals from 14th February until 21st May 2013. Every oyster was examined individually at each observation time. Sampling was random: tray segments and oysters within segments that were demarcated by an overlay grid were selected using random number tables; baskets were selected by systematic random sampling (every 2nd basket after toss of coin to select the first or second basket) and a blind grab sample of the required number of spat was taken after mixing the contents. Cumulative mortality was calculated taking into account the number of oysters sampled at each time point (Paul-Pont, et al., 2013a). Growth was assessed by measuring the length, width, and depth of randomly selected oysters on 28th November 2012 and 21st May 2013 (days 40 and 174).

Estimation of prevalence of infection with OsHV-1. Detection of OsHV-1 was performed on live oysters randomly selected from the trays and baskets after removal of dead individuals. Five pools of five oysters per treatment per site were sampled and analysed (n = 25 oysters) and oyster level infection prevalence was estimated from the proportion of positive pools following a Bayesian approach (Cowling, et al., 1999).

Water temperature. Temperatures were recorded every 15 min using data loggers (1921G, Thermocron®, Thermodata Pty Ltd) each of which was enclosed in a thin-walled waterproof polypropylene tube. The tube containing the data logger was fixed to the bottom of a floating basket at each site: the probes were approximately 200 mm below the surface and were immersed at all times. Temperature data were downloaded to a computer using commercial software (Thermodata 3.2.23, Thermodata Pty Ltd).

Statistical analyses. Mortalities recorded for oysters over time were compared using survival analyses, taking synchronous interval censoring into account (Hosmer, Lemeshow, 1999; Radke, 2003). The effect of age and height on survival was evaluated using univariable and multivariable models. Similar general linear model analyses were conducted for growth of oysters over time (length, width and depth) and predicted means and s.e. were calculated.

Pool positive and negative status was compared between age and height groups using a logistic regression approach or Fisher's exact test, as appropriate. Infection prevalence was compared among observation times using a similar logistic regression approach or Fisher's exact test. All analyses were conducted using the SAS statistical program (release 9.3, copyright© 2002–2010 by SAS Institute Inc., Cary, NC, USA) unless otherwise stated.

Epidemic curve analysis. Detailed observation of mortality patterns within each tray and basket was performed in order to assess the mechanism of transmission of OsHV-1. At each of three sites there were 8 trays, each with 8 segments, while there were 12 baskets on each longline at each of 3 heights. Epidemic curves were prepared from the observational data, and graphed as cumulative mortality over time. This was done for individual tray segments to look for patterns of transmission within segments and spread from segment to segment within a tray. Similarly, graphs were prepared for individual baskets looking for patterns of spread between baskets.

Identification of natural reservoir for the virus

Wild oysters and other organisms (shellfish, crustacean, algae) were opportunistically sampled in Woolooware Bay before during and after the outbreak to check for the presence of OsHV-1 and identify a potential reservoir host. In addition several opportunistic collections were made in the Hawkesbury River after the outbreak of POMS which began in January 2013. All samples were tested using qPCR.

Stability of the virus in the environment
This was assessed using bioassays in two types of experiments: i) laboratory ii) on-farm.

i) Laboratory experiments.

Oysters. Triploid Pacific oysters supplied by Shellfish Culture (batch SPL13C) were grown in the Shoalhaven River to approximately 5 cm shell length. They were transported to the physical containment level 2 aquatic facility at the University of Sydney and acclimatised in artificial seawater (ASW) at 30 parts per thousand salinity (Red Sea Salt), at 20^{o}C +/- 1.5^{o}C and fed a commercial diet daily (Shellfish Diet 1800) for 4-5 days prior to use.

Amplification of fresh OsHV-1. Oysters were relaxed by immersion in 75 mg/L $MgCl_2$ in freshwater conditioned for aquarium use for 30 - 60 mins (n=8). They were challenged by injection of 100 µl of the inoculum into the adductor muscle with a 25 g needle. The inocula were: i) OsHV-1 working stock V171 (cryopreserved 1/10 w/v OsHV-1 infected tissue homogenate) diluted 1/10 v/v in 0.2 µm filtered ASW (n=6). Prepared from naturally infected oyster tissue sampled from the Georges River 2011; ii) Negative control V172 (cryopreserved 1/10 w/v OsHV-1 negative oyster tissue homogenate) diluted 1/10 v/v in ASW (n=2). These donor oysters were held in ASW without food at 20^{o}C for 3 days after challenge. There was a single total water exchange 24 h after challenge. The mantle and gill tissues from the donor oysters were pooled and homogenised by stomaching with 10 ml of 0.2 µm filtered ASW. The preparation was made up to a 1/10 w/v homogenate with ASW and stomaching was repeated. The suspension was clarified by filtration to 0.2 µm and held at 4^{o}C briefly before aliquots were subject to the disinfection treatments. The same procedure for preparation of a filtered tissue homogenate was followed for the negative control oysters.

OsHV-1 samples. The three preparations containing OsHV-1 indicated below were incubated in the dark at 20^{0}C in a refrigerated incubator (Thermoline). Aliquots were removed at intervals for up to 7 days and tested in a bioassay for infectious OsHV-1 and by PCR for OsHV-1 virus DNA (Table 2). The following sample preparations were examined:

> *OsHV-1 in seawater.* An aliquot of the water in which the donor oysters were housed was retained and filtered to 0.45 µm. The concentration of OsHV-1 was increased by adding 5% v/v of the 1/10 filtered tissue homogenate. The OsHV-1 in seawater preparation was used neat.
>
> *OsHV-1 in wet oyster tissue.* A concentrated tissue paste was retained before mantle and gill tissues of the donor oysters was diluted to 1/10 w/v. An aliquot of this stored tissue was homogenised further by stomaching to a final concentration of 1/10 w/v in ASW at each time the stability was tested. This homogenate was centrifuged at 1000 *g* for 10 min at 4^{0}C; the supernatant was filtered to 0.2 µm using syringe driven filters (Minisart, Sarturius) before use. These OsHV-1 in tissue preparations were diluted 1/100 v/v in ASW for immersion or 1/10 for injection.
>
> *OsHV-1 in dry oyster tissue.* Multiple 1 g portions of the concentrated oyster tissue paste were spread thinly on the surface of sterile petri dishes and allowed to dry overnight at room temperature. Immediately prior to testing for OsHV-1 in a bioassay, samples of the dried tissues were removed from the petri dishes and reconstituted in sterile ASW to prepare a 1/10 w/v tissue homogenate, based on the weight of tissue before drying. The reconstituted material was filtered to 0.2 µm. These OsHV-1 in tissue preparations were diluted 1/100 v/v in ASW for immersion or 1/10 for injection.

Negative controls. Negative control wet and dry tissue and seawater preparations were prepared using the same methods, but based on mantle and gill tissues of oysters injected with OsHV-1 free oyster tissue homogenate.

Bioassays. Oysters were relaxed by immersion in 50 g/L $MgCl_2$ in fresh aquarium water for up to 6 hours prior to challenge using one of two methods.

Immersion. Relaxed oysters were transferred to a plastic vessel with 100 ml of inoculum for spat or 250 ml of inoculum for juveniles and held there for 2 hours with intermittent gentle mixing. The oysters and inoculum were then transferred to the maintenance aquarium.

Injection. A 100 µl aliquot of the inoculum was administered into the adductor muscle of juvenile oysters using a tuberculin syringe with a 25 g needle.

Oysters were monitored for mortality each day by testing the response of the valve or mantle to touch. Dead oysters were removed and stored at -80°C. The trial was terminated 7 days after exposure to OsHV-1 when the remaining live oysters were removed and stored at -80°C.

Strategy for detection of OsHV-1 by qPCR. Spat were tested in pools of 300-500 mg; they were homogenised in 1.5 ml molecular biology grade water. Juveniles were tested individually; a 0.1 g pool of mantle and gill tissue was homogenised in 1 ml of water..

Bioassay test definitions. Positive: OsHV-1 was not detected by qPCR in any oysters from negative control bioassays; and at a least one individual or pool of spat contained $\geq 1.0 \times 10^4$ copies of OsHV-1 DNA per mg of tissue tested either at the time of death or 7 days after challenge; this indicated replication of OsHV-1 had occurred. Negative: OsHV-1 was not detected or $<10^4$ OsHV-1 genome equivalents per mg of tissue were detected in oysters at the time of death or 7 days after challenge; and replication of OsHV-1 occurred in positive control bioassays.

ii) On-farm experiments.

Stability of virus in naturally infected seawater was determined using as bioassay, with appropriate controls. This work was conducted at the Hawkesbury River estuary. The main effect examined was stability of virus over time, but the possibility of rendering water safe for oysters by filtration was evaluated at the same time.

Culture system. Land-based upweller tanks to hold/rear spat were designed and installed in a large enclosed shed at Mooney Mooney in March 2012 (Figure 3). Four main treatment tanks, each containing 4 upwellers were installed within a large fibreglass holding tank, which was connected to waste for discharge of all water to the river (Figure 4). All of the treatment tanks were in close proximity to one another and it was impossible to control aerosols from the tanks, or movement of personnel and other equipment into and within the shed during the experiments.

Figure 3. Layout of upwellers in treatment tanks. Top left, detail of upweller. Bottom left, treatment tanks for Trial 1. Right, treatment tanks for Trials 2 to 7; filters are shown in the foreground and at the rear of the main holding tank; the UV unit is wall-mounted.

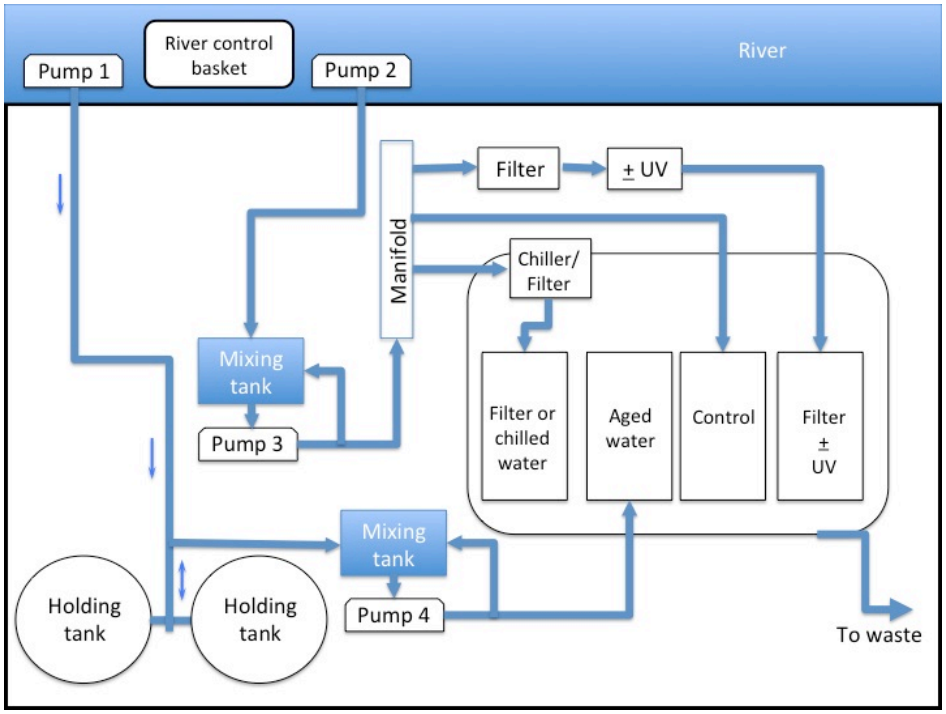


Figure 4. Physical design of the facility constructed to investigate treatment of water to inactivate OsHV-1. Treatments differed between experiments.

Control oysters were placed in a floating basket (BST, Australia) in the river. Upwellers were supplied with water from two sources in order to create different treatments: control, filtration, aged water, chiller (Figure 4). The water supply for the aged water treatment was independent of the others; it was drawn by gravity from twin 20,000 L (Trial 1) or 10,000 L (Trials 2 to 6) holding tanks that were filled intermittently using a pump (Davey). Its inlet point (foot valve) was located about 4 m from the submersible continuous flow pump (Dynapond, Davey, Australia) that supplied water to the other treatments. The reticulation for the others comprised the submersible pump in the river, pipework, a mixing tank and a manifold from which three individual treatment tanks were supplied. The control tank was supplied from the middle off-take on the manifold; prior to Trial 4, the filters on the first and third manifolds were swapped to avoid positional bias. The foot valve and the submersible pumps were 0.5 m below the water surface at all times, and could move up and down with the 2 m tidal movement as each was attached to a float mechanism on a wooden post. The posts were 4 m apart. The floating basket in the river containing control oysters was tethered to a rope located on a post between these two posts. It moved on a 1.5 m radius – thus at times it could be 0.5 m from the nearest post.

Each mixing tank (118 x 91 x 43 cm, L x W x D, 460 L) had a separate pump to regulate flow to the upwellers (Figure 4). Flow was controlled by partial recirculation of water from the off-take back into the mixing tank, and a ball valve on the outlet to the treatment tank containing the upwellers. This prevented excess flow and pressure through filters.

Upwellers were small scale 140 x 100 mm commercial units with fine mesh screen. Four were included per treatment tank (81 x 60 x 35 cm, L x W x D, 170 L) (Figure 3). Typical flow rates used in all trials except Trial 4 were 0.5 to 1L per min per upweller. For upweller controls and filter treatments in Trial 4a, flow rates of 1 to 4 L per min per upweller were used.

Water flow rates in filtration treatments were nominal; they were adjusted after cleaning filters, but as the filters became obstructed the flow rate reduced. Flow rates were adjusted so as not to exceed the maximum rated flow for the filters of 38 L/min. Filters were either cleaned or replaced each day (Figure 5). Cleaning was achieved using water under moderate pressure from a domestic water supply.

Figure 5. Examples of 5 µm pleated paper filter cartridges. Left, after washing; right, after 1 day in use.

Experimental treatments. Specific water treatments were tested. Treatments varied from trial to trial.

River control. Spat were deployed in a floating basket in the river, adjacent to the water intake points for the other treatments.

Upweller control. Water was pumped continuously from the river and supplied to the tank via a manifold to the tank, without any treatment.

Chiller. Water was pumped continuously from the river and supplied to the tank via a manifold (common to the control) and was then chilled by passing through a stainless steel heat exchange coil immersed in a cold water bath; the temperature reduction was 2-3°C in comparison with river temperature.

Filtration with or without UV. Water was pumped continuously from the river and supplied to the tank via a manifold (common to the upweller control) and filtered through a 5 µm pleated paper cartridge filter in a plastic flow through canister housing (Omnifilter BF7, USA) (Trials 1, 2, 3, 4, 5, 6) then if required exposed to UV light from a commercial aquaculture unit (VGX48VH, Atlantic-Ultraviolet) (Trials 1 and 2 only). From 25th April 2014, a 5 µm pleated polyester fabric cartridge filter (Puretec, Australia) in an Omnifilter housing was used (Trials 6, 7 only). In Trials 3 and 4 a 30 µm pleated paper cartridge filter (Omnifilter) was used as a separate treatment. Pre-filtration was used in all filtration treatments in all experiments, using either a 100 µm or a 55 µm stacked polypropylene disc filter (1" Short, Arkal, Israel). UV was used in Trials 1 and 2 only. Filter pore sizes are acknowledged to be nominal, and are listed here as specified by the manufacturers. All filters were cleaned or changed daily.

Aged water. Water was pumped from the river and held in tanks for 48 h before being supplied continuously via a mixing tank to the treatment tank. Two holding tanks were used in each experiment to enable the water to be held for 48 h prior to use.

Oysters. Pacific oyster spat (Shellfish Culture, Tasmania) were shipped by air and placed in each of the treatment tanks and the floating basket in the river at the start of each trial, unless otherwise stated. Spat were held out of water for <24 hours during transport and placement in the trial. There were 500 spat per upweller (2000 spat per treatment) unless otherwise stated. There were 2000 spat in the river control. No additional food was supplied to spat in upwellers during the time course of the experiments.

Variations. Due to high mortality by day 23, the river control in Trial 6 was replenished with new spat on day 28. The spat used for Trial 7 were received at the commencement of Trial 6 and were held in untreated river water in the mixing tank for the control and filtration upwellers, from 26th March, i.e. throughout Trial 6.

Random sampling and mortality. Oysters were examined daily (Trials 1, 2, 3, 5, 6, 7) or three times weekly (Trial 4) in each upweller and the river basket by visual inspection. Spat were tipped onto a tray or table for close inspection (Figure 6). Spat were considered to be dead if the valves were open or opened easily during handling, but not every freshly dead oyster was expected to be detected in this way, until the tissues had degraded. The mortality rate was assessed by manually counting dead spat in each treatment. At least 40 spat per treatment (10 per upweller) were sampled at these times by taking small numbers from each of multiple positions on the tray or table (approximated random sampling). As active sampling led to a reduction in the number of spat per treatment over time, this was taken into consideration when calculating the cumulative mortality as previously described (Paul-Pont, et al., 2013a). It was impossible to achieve aseptic conditions that would prevent the risk of cross-contamination between samples from different treatment groups in the oyster shed environment but the operator washed hands using soap and water, and rinsed the table or tray using tap water between the inspections of each treatment group.

Figure 6. Visual examination of spat from one of the treatments

Strategies for testing oysters for OsHV-1 by qPCR. Oysters were dissected if >6 mm length. For Trial 1, ten pools of oysters were tested per treatment; each pool contained 300 mg of whole spat if they were < 6 mm in length, or tissue removed from 3 oysters if they were >6 mm long. For Trials 2 to 7, five pools of oysters were tested per treatment; each pool contained 300-500 mg of whole spat if they were <6 mm in length, or 500 mg of tissues removed from the shell if they were >6 mm long; the number of spat per pool ranged from 6 to 12.

Water temperature monitoring. A temperature probe (Thermocron® temperature logger, Thermodata Pty Ltd) was placed into each upweller tank, as well as the floating basket in the river and each water storage tank, and recorded temperature at every hour.

Physical and chemical means for viral inactivation

The bioassay method, including the source of oysters preparation of a fresh OsHV-1 inoculum, challenge methods and detection and quantification of OsHV-1 by qPCR were as described above for the laboratory stability experiments. The following preparations of OsHV-1 were used:

> *OsHV-1 in seawater (OsHV-1 Preparation 1).* The seawater that held the oysters used to amplify OsHV-1 was held at 4°C and the quantity of OsHV-1 was increased by addition of 5% (by volume) of the tissue homogenate prepared from the donor oysters with OsHV-1 infection.
>
> *OsHV-1 in oyster tissue (OsHV-1 Preparation 2).* Clarified 1/10 w/v tissue homogenate was prepared from mantle and gill tissue of 6 donor oysters challenged by injection with OsHV-1. This was freshly prepared prior to the disinfection experiment.
>
> *Negative control oyster tissue (OsHV-1 Preparation 2b).* A clarified 1/10 tissue homogenate was prepared from mantle and gill tissue of 2 donor oysters challenged by injection with OsHV-1 negative control oyster tissue.

Disinfection treatments. Procedures were performed at 20°C in polypropylene laboratory tubes with minimal exposure to light, unless stated otherwise. Treatments for OsHV-1 in seawater were performed in a 10 ml volume and treatments on OsHV-1 in tissue were performed using 3 ml volume. Buffer exchange was performed for OsHV-1 preparations after chemical treatment to ensure that the disinfectant chemicals did not interfere with the bioassay. An Amicon Ultra-15 (Merck Millipore) Ultracel regenerated cellulose membrane with 30 000 molecular weight cut-off (MWCO) centrifuge device was prepared by pre-rinsing the membrane with milliQ water before loading up to 10 ml of the OsHV-1 preparation and centrifuging at 4000 x *g* for 5 minutes at 4°C. The filtrate was discarded and an equal volume

of ASW was added to the retentate before repeating the centrifugation. The retentate (containing OsHV-1) was recovered from the membrane and made up to the original volume in 0.2 µm filtered ASW. The potential for poor recovery of OsHV-1 or inactivation of the virus during this procedure was evaluated using a positive control. The potential for the procedure to be ineffective at reducing toxicity in the bioassay due to failure to remove the disinfectant was assessed using a buffer exchange procedure for each disinfectant treatment on the negative control oyster tissue preparation.

Treatments performed on seawater(OsHV-1 preparation 1)

Treatment 1. Positive control. Untreated OsHV-1 in seawater preparation held at 4°C.

Treatment 2. Positive control with buffer exchange: Untreated OsHV-1 in seawater preparation subject to buffer exchange to assess the effect of this procedure alone on the qPCR quantity and infectivity of OsHV-1.

Treatment 3. Heat: A 1 ml aliquot in a dry heat block at 42°C for 5 min.

Treatment 4. Ultraviolet light: The preparation was placed in a white plastic reagent reservoir (Vistalab Technologies, cat# 3054-1000) and exposed to UV irradiation for 10 min at a distance of 15 cm from 2 x Sankyo Denki G15T8 15W germicidal lamps rated at 4.9W output each, in the UVC range (>1000 mW/cm^2 at 254nm). The preparation was based on unfiltered ASW from the holding tanks, but some of the virus in the preparation was added from a 0.2 µm filtered tissue homogenate.

Treatment 5. Chlorine: Pool chlorine (Formula chemicals 125 g/L available chlorine as sodium hypochlorite) was added to a final concentration of 50 ppm available chlorine. The suspension was mixed thoroughly at the time of addition and the duration of the contact time was 15 min.

Treatment 6. Chlorine with high organic load: Pool Chlorine was added to a final concentration of 50 ppm according to the procedure described for Treatment 5 for an aliquot of OsHV-1 preparation to which 10% v/v foetal bovine serum had been added to increase the organic load.

Treatment 7. Virkon. The powdered product (Virkon-S DuPont) was freshly prepared in water according to directions of the manufacturer for a 10% w/v solution. This was added to the OsHV-1 preparation to a final concentration of 1%v/v. The preparation was gently mixed at the time of addition and the duration of contact time was 15 min**.**

Treatments performed on oyster tissue (OsHV-1 preparation 2)

Treatment 8. Positive control: untreated OsHV-1 in oyster tissue held at 4°C.

Treatment 9. Positive control: OsHV-1 in tissue subject to centrifugal buffer exchange procedure.

Treatment 10. Heat: a 1 ml aliquot was incubated at 50°C for 5 min in a hybridisation oven, (ThermoScientific).

Treatment 11. Chlorine 100 ppm: Pool chlorine (Sodium hypochlorite 125 g/L) was added for a final concentration of 100 ppm available chlorine with a 15 min contact time.

Treatment 12. Chlorine 200 ppm: Pool chlorine (Sodium hypochlorite 125 g/L) was added for a final concentration of 200 ppm available chlorine with a 15 min contact time.

Treatment 13. Virkon 1%: Addition of an appropriate volume of a 10% stock solution for a final concentration of 1% v/v, as described for Treatment 7 with a 15 min contact time.

Treatment 14. Iodine: Betadine antiseptic liquid (10% povidone iodine equivalent to 1% available iodine, FH Faulding & Co) was used for a final concentration of 0.1% available iodine. The preparation was gently mixed with a contact time of 5 min.

Treatment 15. Detergent product: Pyroneg (an alkaline detergent cleaning agent, JohnsonDiversey) was used at a final concentration equivalent to 2 ml per litre final concentration.

Treatment 16. Sodium hydroxide: A stock solution of 1 g NaOH in 10 ml water was used for a final concentration of 20 g/L.

Treatment 17. Formalin: A final concentration of 1/25 v/v 40% formalin solution was used with a contact time of 30 min.

Treatment 18. Quaternary ammonium compound (QAC): Livingston QAC used according to directions of the manufacturer for conditions with light soiling; a final concentration of 1 part to 25 parts OsHV-1 preparation for a contact time of 10 min.

Treatments performed on oyster tissue free from OsHV-1. Control procedures for each of the chemical disinfection treatments previously described were applied to 3 ml aliquots of the negative control OsHV-1 oyster tissue homogenates (Preparation 2b). The procedures were performed as described previously, before undertaking the buffer exchange procedure.

Development of an infectivity model for POMS suitable for selection of resistant oysters and pathogenesis/environmental research

Four experiments were performed sequentially in order to test (i) the infectivity of the Australian OsHV-1 µVar strain using infected material sampled from the Georges River during the summer of 2011/2012 (Experiments 1a and 1b), the dose-response relationship for OsHV-1 (Experiment 2) and the best storage conditions to keep purified viral inoculums (Experiment 3).

Oysters and husbandry. All naive oysters used in this study were hatchery single seed triploid oysters (Shellfish Culture, Tasmania) and came from the same batch (11 month old; size 40-50 mm length). Naive oysters were harvested from Porto Bay in the Hawkesbury River (Oyster Leases 68/178 and 85/023), which had been free of OsHV-1 until 2013. The oysters were harvested at different times depending on the experiment: July 2012 (Experiment 1a), August 2012 (Experiments 1b and 2) and September 2012 (Experiment 3). However as the sampling time of oysters was nested within experiment, there was no bias introduced in relation to the time of sampling.

The oysters were transferred to the acclimation tanks within 12h after sampling and water temperature was set to correspond to the conditions in the field (15-18°C) while the salinity was fixed at 30 ppt. The oysters were acclimatised for one week in 25 L tanks on a recirculation system connected to biofiltration and UV (Figure 7). The water temperature in the tanks was increased by 1°C per day until it reached 22°C used during the experiments. Oysters were fed daily with shellfish diet 1800 (Instant Algae®, Reed Mariculture, USA). No mortality was recorded prior to the experiments.

Figure 7. Experimental tanks within a PC2 animal laboratory (left) and placement of oysters within one tank (right).

Inoculum preparation. Oyster tissue homogenate from OsHV-1 µVar infected and non-infected Pacific oysters were used to prepare infected and control inoculums, respectively. OsHV-1 infected Pacific oysters (12 month old; length: 79.5 ± 4.6 mm, mean ± SD) were collected from the Georges River, NSW, Australia during a disease outbreak on the 24th November 2011. Control oysters (6 month old; length: 52.7 ± 8.8 mm, mean ± SD) were collected from the Hawkesbury River on the 19th December 2011. Both batches of oysters (i.e. infected and control) were previously tested by PCR to confirm their respective status (i.e. infected and control) and they were stored at -80 °C prior to use. For Experiments 1 and 2, five oysters from each batch were removed from storage at -80°C one day prior to the commencement of the experiment and were thawed at 4°C overnight. The following day, each batch of 5 oysters was processed separately with sterile materials, and working benches were carefully disinfected with 1 % Virkon® between batches to prevent cross contamination. Oysters were opened by removing the superior valve, and a small piece of mantle (0.08-0.12 g) was sampled from each oyster and placed at -80°C before DNA extraction and OsHV-1 detection by real-time PCR. The rest of the gills and mantle were dissected and pooled together (total weight: 10-12g) into a 50 mL sterile tube maintained in wet ice. These tissues were weighed and diluted by adding 10 volumes of 0.22 µm filtered synthetic seawater (FSSW) to the tube. Tissues were homogenised using a stomaching machine (MiniMix®, Crown Scientific, Australia) for one minute at maximum speed. After centrifugation (1000g, 5min, 4°C) supernatant was placed in a new sterile tube and diluted with 3 volumes of FSSW. Finally, the clarified tissue homogenate was filtered consecutively under aseptic conditions in a Class 2 Biosafety cabinet using syringe filters at 8 µm, 0.45 µm and 2 x 0.22 µm pore sizes. The purified homogenate, or inoculum, was maintained on ice prior to the injection.

For Experiment 1 the inoculum was freshly prepared. Syringes containing 100 µL of each purified homogenate (control and infected) were prepared in the Class 2 Biosafety cabinet and maintained on ice prior to the injection. The concentrations of the infected inoculums were 1.4×10^6 and 1.3×10^7 viral DNA copies per mL in experiments 1a and 1 b, respectively. No viral DNA was detected in the control inoculums.

For Experiment 2 the inoculum was freshly prepared and five concentrations were tested (neat at 5×10^6 viral DNA copies per mL, and 10-fold serial dilutions: 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) in parallel with a control group. Accordingly, the pure inoculum was diluted 4 times with FSSW before being filtered twice using 0.22 µm pore sizes syringe filters prior to injection. Syringes containing 100 µL of each purified homogenate were prepared in the Class 2 Biosafety cabinet and maintained on ice prior to the injection.

For Experiment 3, the inoculum that had been freshly prepared in July 2012 (experiment 1) and dispensed into four tubes (5 mL) separately to be kept at room temperature (RT), +4°C, -20°C, -80°C and liquid nitrogen was used. In early October 2012 (+ 3 months) the different inoculums were thawed overnight at + 4°C (if required) and filtered twice using 0.22 µm pore sizes syringe filters prior to injection. Syringes containing 100 µL of each purified homogenate were prepared in the Class 2 Biosafety cabinet and maintained on ice prior to the injection. The viral DNA concentrations of each inoculum are listed in Table 2.

Experimental infection. All experiments were conducted in a dedicated PC2 facility at the University of Sydney in accordance with the PC2 biosecurity regulations and protocols. Each intramuscular (IM) injection was carried out on the same day as the homogenate preparation, except for experiment 3 where stored inoculums were used. The IM injection was carried out using 8-10 oysters per treatment per 25L tank and was conducted in triplicate (n=3 replicate tanks containing a total n=30 oysters per treatment for experiments 1 and 3, and n=24 oysters per treatment for experiment 2). Oysters were placed out of the water (air temperature 22°C) for 24h prior to immersion in a magnesium chloride solution ($MgCl_2$; 50 $g.L^{-1}$) in seawater (1v)/distilled water (4v) for between 1 and 4 hours. Once relaxed, 100 µL of filtered tissue homogenate was injected into the adductor muscle of each oyster. The control and the infected oysters were injected on separated benches to prevent cross contamination and the inoculated oysters were put directly back in the water without any food supply or filtration system. This design ensured that (1) no water was shared among tanks due to recirculation; (2) the water temperature was uniform between tanks as it was controlled by equilibration with the air temperature (21.7 ± 1.2 °C, mean ± SD). The salinity was monitored daily and stayed stable (30 ppt). Water quality parameters (ammonia, nitrate, nitrite, pH) were monitored daily. In experiment 1b the water was occasionally changed to maintain water quality in acceptable ranges.

Sampling. A water sample was collected daily from each tank for PCR analysis (volume sampled=4mL, volume tested by PCR=200µL). Mortality was monitored daily for 10 days and no active sampling was performed on live oysters. Mean daily mortality ± SD was calculated among the three replicate tanks. Dead oysters were removed from the tanks during the experiment and a piece of gill and mantle (0.08-0.12 g) was sampled from each dead individual. Control and surviving oysters were all sampled and sacrificed at the end of experiment. All tissue and water samples were frozen at -80°C prior to DNA extraction and OsHV-1 quantification by real-time PCR.

Strategy for detection of OsHV-1 in oysters by qPCR. A piece of gill and mantle (0.08-0.12 g) was dissected from individual oysters using disposable sterile instruments that were changed between each oyster. After addition of 9 volumes of sterile distilled water, tissues were homogenised by bead-beating.

Statistical analyses. Kaplan Meier curves were created to compare survival between treatment groups for each experiment using the ggsurv function in R statistical package version 3.0.1 (© The R foundation for statistical computing). For the calculation of the mean viral copies/mg ± SD of tissues the authors firstly confirmed the absence of any significant tank effect (using an ANOVA test, $p > 0.05$; STATISTICA 10 software for Windows) before considering all replicate oysters, regardless of the tank number.

Experiments to apply the infectivity model. The infectivity model was adapted and applied for four specific purposes during this project.

i) Cohabitation and feeding. The aim was to investigate the role of feeding in OsHV-1 infection via co-habitation. Four hundred and twenty naive *C.gigas* oyster spat (50-60 mm length) were randomly allocated to groups: 1) High donor oyster stocking density with feeding (High+Feed), 2) High donor oyster stocking density no feeding (High-Feed), 3) Low donor oyster stocking density with feeding (Low+Feed), 4) Low donor oyster stocking

density no feeding (Low-Feed), 5) Naive oysters, no donor oysters, with feeding (Negative+Feed) and 6) Naive oysters, no donor oysters, no feeding (Negative-Feed). 90 oysters were used per treatment and 30 oysters were used per negative control. All tanks were supplied with artificial seawater. Oysters were collected during a POMS outbreak in Woolooware Bay, NSW in November 2012 and viral inoculum was prepared as described above on the day of injection and stored on ice until injection. Donor oysters were injected with 100 µL of inoculum in the adductor muscle and randomly placed into the tanks of one of the four treatment groups. Ten injected oysters were placed into each of the high IM oyster stocking density tanks and 2 injected oysters into each of the low IM oyster stocking density tanks. No donor oysters were placed into the negative control tanks. Feed rate per tank was calculated daily and feeding treatment oysters were fed twice daily with Instant Algae® Shellfish Diet 1800. The mortality was assessed daily in each tank and opportunistic, non-selective (approximated random) sampling of 6 live oysters per treatment and 2 live oysters per negative control was performed once daily prior to feeding. All samples and mortalities were analyzed by quantitative PCR (qPCR) for OsHV-1. As the active sampling led to a reduction in the number of spat per treatment over time, this was taken into consideration when calculating cumulative mortality. IM injected donors were removed when they died.

*ii) Influence of water temperature on infectivity of OsHV-1.*The aim of this study was to determine the effect of water temperature on the outcome of OsHV-1 µVar infection in Pacific oysters. A controlled laboratory environment was used so that temperature could be examined as an independent variable with a measured dose of OsHV-1 and standard infection challenge. Two batches of single seed hatchery produced triploid Pacific oysters from Tasmania were used that were 8 and 17 months of age, respectively. The oysters were grown at the Shoalhaven River under commercial conditions. Both of these regions are considered to be free of OsHV-1. The air temperature in the Physical Containment Level 2 Aquatic Animal Facility at the University of Sydney was 22°C. Oysters were maintained in artificial seawater at 30 ppt salinity. Tanks were 20L and were arranged as 4 groups of 6 tanks on a recirculation system that shared a common sump. The water in each recirculation system was passed through a fine sieve filter, an independent external canister bio-filtration unit, UV light and the desired temperature was maintained using a combination of thermostatically controlled aquarium heaters and an aquarium chiller unit. An additional 4 tanks were used for control oysters that were not exposed to OsHV-1 at each temperature. Each of these tanks had water that was independent from the recirculating systems and water quality was maintained with daily 50% water exchange pre-adjusted to the appropriate temperature. Temperature was monitored using aquarium thermometers and recorded every 15 min using data loggers in each system. Oysters were fed Shellfish diet 1800 (Instant Algae®, Reed mariculture).

The temperature in all systems was initially 20 C. The heater and/or chiller units were used to adjust the temperature by 1°C each day to the target temperature for each system: 14 C, 18 C, 22 C and or 26 C. Oysters were acclimated at the target temperature for 1 week prior to challenge with OsHV-1.

Inoculums were prepared as described above to contain $1x10^4$ and $1 x 10^6$ OsHV-1 genome copies per 100 µl in ASW immediately prior to challenge. The negative control homogenate was used at the same dilution factor as the most concentrated inoculum. Oysters were challenged by IM injection as described above then returned to ASW in the appropriate replicate tank.

Oysters were examined twice a day for 14 days after challenge. If an oyster was deemed to be dead it was removed and stored at -80°C until tested. Death was defined by a failure to close the valve on examination and no retraction of the mantle when stimulated by a 25 g needle.

A single control tank with 12 oysters was included for each temperature. There were 6 tanks on a common recirculation system for each of the 4 temperatures. Each tank contained 20 oysters (10 x SPL13B, 17 months old and 10 SPL14B, 8 months old). All oysters in 3 tanks

were challenged with a high dose (10^6 OsHV-1 genome equivalents per oyster) and all oysters in the other 3 tanks were injected with a low dose (10^4 OsHV-1 genome equivalents per oyster).

A sample was taken at day 7 to establish the prevalence of the OsHV-1 in the treatments without mortality (all 14°C and 18°C treatment groups). A number was assigned to each oyster based on position in the tank and a random selection of 5 oysters from each treatment group was taken using random numbers selected using the RAND function in Excel (Microsoft, 2010). All remaining oysters were sampled at the end of the trial after day 14 mortality counts.

iii) Stability of OsHV-1 in the environment. See methods described above

iv) Physical and chemical means for viral inactivation. See methods described above

Processing oyster tissues and detection of OsHV-1 by qPCR

Unless otherwise stated in sections above, all oysters were processed to detect OsHV-1 as follows. For dissections, oyster knives and lab equipment were disinfected in 1 % Virkon® (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 reverse osmosis purified water and tissues were dissected using a sterile scalpel blade with the lower shell used a firm base for cutting.

Tissue homogenisation. A piece of gill and mantle (0.08-0.12 g) was dissected from individual oysters using disposable sterile instruments that were changed between each oyster. After addition of 9 volumes, or a standard volume of 1 ml (individual oysters) or 1.5 ml (pooled oyster tissues) of sterile distilled water, tissues were ground by bead-beating (Speed 6.5 m/sec; Time: 15 sec using a Fastprep System, MP Biosciences, USA, or lysis for 2 min, rotate 180, 2 min lysis all at a frequency of 30 using a Qiagen Tissuelyser II machine). Tissue homogenates from adult oysters were clarified by centrifugation at 900 × g for 10 min while those from spat with shell were clarified by centrifuging at 1340 x g for 2 min in a microcentrifuge. Clarified tissue homogenates were stored at − 80 °C until required.

DNA extraction. Viral DNA extraction was performed via magnetic beads using a MagMax-96 Viral RNA Isolation Kit (Ambion, USA) following the manufacturer's recommended protocol, in a MagMax Express-96 magnetic particle processor with disposable tip combs and standard 96-well processing plates (Applied Biosystems, USA). Extraction was performed on a 50 µL aliquot of clarified tissue homogenate and a 200 µL aliquot for water samples.

Real-Time Polymerase Chain Reaction (PCR). The detection of OsHV-1 µVar DNA by real-time PCR was adapted from a previously published protocol (Martenot, et al., 2010) and fully detailed in (Paul-Pont, et al., 2013a). A valid run was defined as a run exhibiting no amplification of the negative control, amplification of the positive control (Ct within the range of the standard curve) and a standard curve with $r^2 > 0.95$ and efficiency between 90 and 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 exhibited an exponential accumulation of fluorescence and a cycle threshold within the range of the standard curve. When tested in duplicate a sample was defined as positive when both replicates exhibited an exponential accumulation of fluorescence and were assigned a 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).

Results

The identity of Ostreid herpesvirus associated with recent outbreaks of POMS

The aim of this part of the project was to identify the nature of the OsHV-1 strains responsible for mortality outbreaks at different times and different locations within the two affected estuaries in Australia, and in particular to determine whether OsHV-1 µVar was responsible for the outbreaks.

Twelve representative samples were subjected to sequencing. The samples were oyster tissues, from which DNA was extracted using standard approaches. The samples were field samples collected during disease outbreaks; consequently the quality (freshness) of the tissues varied. The sequencing approach used required PCR amplification of OsHV-1 DNA from these tissues, and it was not expected that all samples would be suitable for analysis in each of the reactions that was used.

Three regions of the genome were examined, consistent with recent recommendations for multiloccus sequence typing of OsHV-1 (Renault, et al., 2012).

C region. Sequence was obtained from all 12 samples. Analysis of a sequence alignment identified differences due to insertion/deletion and substitutions when compared with the OsHV-1 reference type. Samples showed the same repeat motifs as are accepted to be consistent with OsHV-1 µVar by the OIE (OIE, 2013). Sequence amplified from the C-region had 100% identity to published sequences from New Zealand and New South Wales (Genbank reference JN639858 and KC685525, respectively).

ORF42/-43 region. Sequence was obtained for 7 samples and identified a mutation represented by an A deletion at position 86. This was identical to the New Zealand 2010 OsHV-1 µVar isolate reported in (Renault, et al., 2012). The consensus sequence for 532 base pairs amplified from this region had 100% identity to a published sequence from a disease outbreak in New Zealand (JN800200).

ORF36-38 region. Sequence was obtained for 10 isolates. According to Renault et al (2012) variant OsHV-1 µVar is identified by a 605bp deletion covering ORF36 and ORF37 and part of ORF38. Three variants can be determined by size: the reference type gives a product of 989bp; OsHV-1 µVar gives a product of 384bp; the third variant gives no amplification. Only the µVar type was observed among Australian samples. The sequence had 100% identity in this region to those published from disease outbreaks in Ireland and New Zealand (Genbank reference JN800250 and JN800252, respectively).

Independent of this FRDC project, but using some samples from it, researchers coordinated by Dr Nick Moody (AAHL) completed partial sequences of 28 samples containing OsHV-1. DNA samples comprised the initial outbreak in 2010 in the Georges River, the 2011-2012 outbreak in the Georges River, and the 2013 Hawkesbury River outbreak. Both farmed and wild oysters were represented. All isolates examined were consistent with µVar with little variation in sequence among the regions examined (Moody, pers. comm. 2015).

In summary, the samples tested were consistent with OsHV-1 µVar according to the definitions of the OIE (OIE, 2013).

Mechanism of transmission of disease

Outbreak investigation Hawkesbury River 2013

A formal outbreak investigation was implemented in the Hawkesbury River estuary in January 2013 at the commencement of the first POMS outbreak in this estuary (Figure 8). It started on the 21st January when mortalities were first observed and stopped at the end of February when a large rainfall event killed oysters and/or forced oyster farmers to move their stock and it became impossible to track the origin and movements of the oysters.

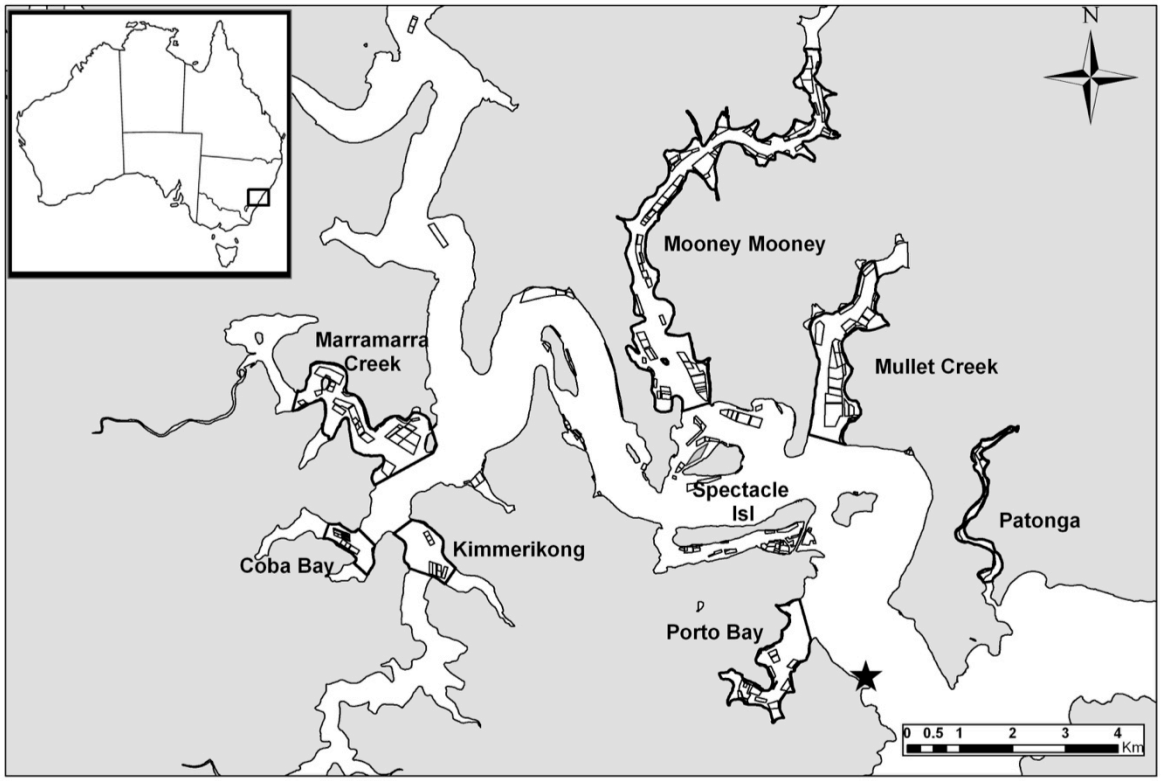


Figure 8. Pacific oyster farming areas in the Hawkesbury River located approximately 40 km north of Sydney, New South Wales. The thick black lines represent the harvest areas and the fine black lines represent the farming areas (oyster leases) in each Creek/Bay. The black star indicates the location of the multi-sensor water quality probe managed by Hornsby Shire Council at Gunyah point.

Detection of OsHV-1 associated mortality. 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 8). Four baskets of 10 - 20 mm oysters were inspected by one of us (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.

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 triploid single seed spat from a hatchery in Tasmania, which were acquired in two batches in April and October 2012, and had been certified by the regulatory authority as being negative for 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. The downstream sites (Mullet Creek, Porto Bay and Mooney Mooney Creek) represented the largest farming areas with 27 active oyster leases. More than 15 million oysters (spat / juveniles from 3 to 9 month old) were grown across these 3 nursery areas (Figure 8). 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 8). Most of the 2 million oysters spread across these 27 active 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 4). 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 4. 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

Passive surveillance

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 (Figure 8). 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.

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. 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. 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).

Active surveillance

Downstream sites. Dead wild diploid Pacific oysters were observed on the foreshore at Mullet Creek on 4th February. 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) in which the highest percentage mortality was observed (90-100%) (Table 5). Remaining oysters were older (11-30 month old) and farmed in trays (n=300). An apparent effect of age/size on mortality was observed at Mullet Creek as mortality was 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 there were young spat (3-9 month old) (Table 5). 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. In Mooney Mooney Creek, young spat (3-9 month) reared in hanging/floating baskets suffered from a high mortality (80-100%) while exhibiting a wide range of size 2-90 mm (Table 5). 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 60%.

Using data pooled from a total of 21 leases (46 production units) in Mullet Creek, Mooney Mooney Creek and Porto Bay in REML analysis, age was found to be a significant determinant of mortality rate (P=0.023) after allowing for variation in mortality due to size, but size was not significant after allowing for variation in age (Figure 9).

Table 5. 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 system (number of unit)	% mortality	Population at risk
Mullet Creek (D)	3-9 month	30-96 mm	baskets (28600)	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 (7560) + trays (50) + tumblers (50)	80-100	1 134 800
	11-14 month	70-100 mm	trays (503)	60	387 250
Porto Bay (D)	3-9 month	30-90 mm	baskets	90-100	514 640
	3-9 month	50-90 mm	baskets	40-70	
	3-9 month	50-90 mm	trays	60-90	623 520
	3-9 month	90-100 mm	trays	20-80	

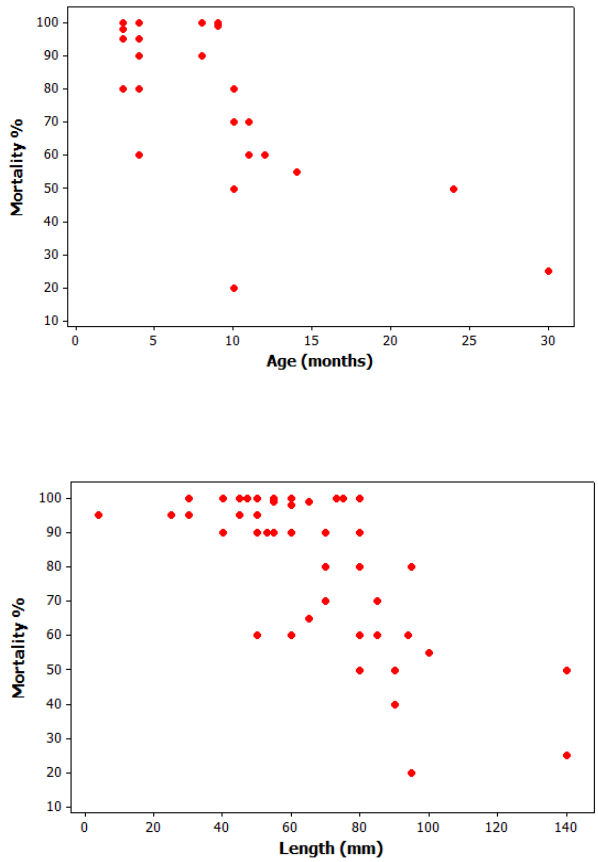


Figure 9. Scatter plot of mortality rate according to age and shell length for Mullet Creek (8 leases, 17 production units), Mooney Mooney Creek (5 leases, 11 production units), and Porto Bay (8 leases, 18 production units) in January-February 2013.

Upstream sites. Dead oysters were observed in some parts of Coba Bay (Figure 10). 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 3). No significant mortality (<5%) was observed in the other adjacent active leases on either occasion (Figure 3). No mortality was observed at Marra Marra Creek and Kimmerikong when inspected on the 29th January and the 26th February.

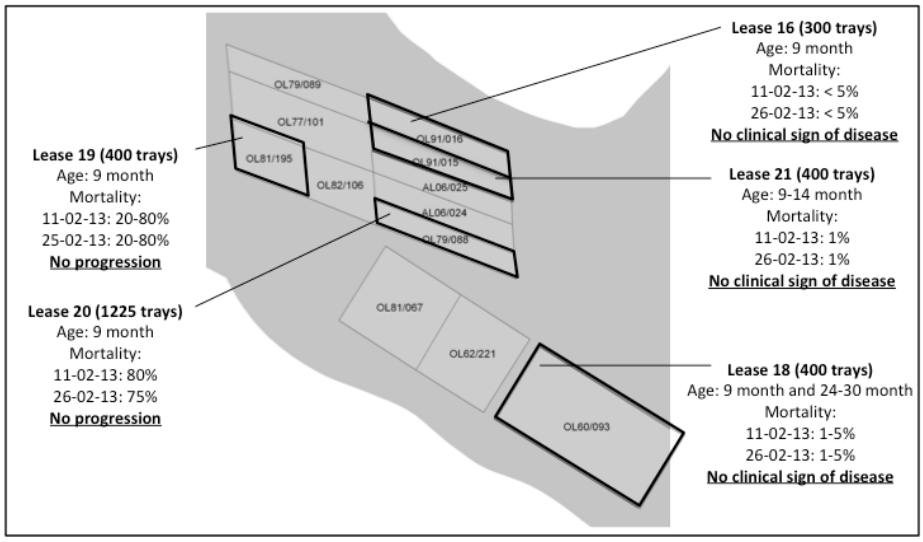


Figure 10. Mortality in active oyster leases examined in Coba Bay on the 11th and the 26th February 2013.

Sentinel oysters collected prior to and during the outbreak. OsHV-1 was not detected at any site between October 2011 and October 2012 (Table 6). The virus was first detected at very low level at Mullet Creek (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 while the virus was detected in one pool of oysters sampled from Mullet Creek on the 13th December (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 3). Prevalence > 40% and viral loads up to 9.4×10^4 copies per mg were observed in oysters sampled on the 21st January in Mullet Creek and Porto Bay and the 15th February at Porto Bay. The sentinel oysters sampled in Mullet Creek on the 21st January were apparently healthy and were collected two hours before the first dead oysters were observed in a lease on the other side of the bay. On this day 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.

Table 6. 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 x 10^1 - 9.4 x 10^4 (P) 5.6 x 10^1 - 1.1 x10^2	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 x 10^4 - 1.4 x 10^5	0.44 (0.12, 0.97)

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 8). Of the 432 pools of 3 oysters that were tested, 195 pools were positive for OsHV-1 DNA in PCR analysis. 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 5% to 31% among bays (Table 7).

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% CI)	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 x 10^1	No mortality
Kimmerikong (U)	2	24	3	0.05 (0.01 - 0.14)	BLOQ - 2.0 x 10^1	No mortality
Coba Bay (U)	6	72	28	0.17 (0.12 - 0.24)	BLOQ - 4.1 x 10^5	Mortality in lease 20
Porto Bay (D)	9	108	38	0.15 (0.11 - 0.21)	BLOQ - 3.7 x 10^1	No mortality
Patonga (D)	6	72	31	0.20 (0.13 - 0.27)	BLOQ - 2.0 x 10^1	No mortality

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. The prevalence declined over time in all oyster leases except for one lease 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 both occasions had low viral loads, either below or around the limit of quantification (1.2×10^1 copies/mg).

To determine the major risk factors that contribute to precipitation of disease outbreaks thereby identifying potential risk-mitigation management practices

Environmental data analysis

Geographical context. Both the Georges and Hawkesbury Rivers are classified as drowned river valley estuaries and are located approximately 20km south (-33.989011, 151.194878) and 50km north (-33.564377, 151.278477) of Sydney Harbour, respectively (Figure 11). Most oyster cultivation in the Georges River occurs at the mouth in Woolooware Bay and Quibray Bay, in the southern area of Botany Bay. The oyster leases are located in shallow areas among mudflats and surrounded by a foreshore dominated by mangroves and saltmarsh. Oyster growing areas in the Hawkesbury River are located in very similar ecological, bathymetrical and geomorphological estuarine conditions to the Georges River. The Hawkesbury River has 6 main oyster growing areas located in each of the main creeks that branch off the main channel of the river along the salinity gradient of 35 to 20ppt.

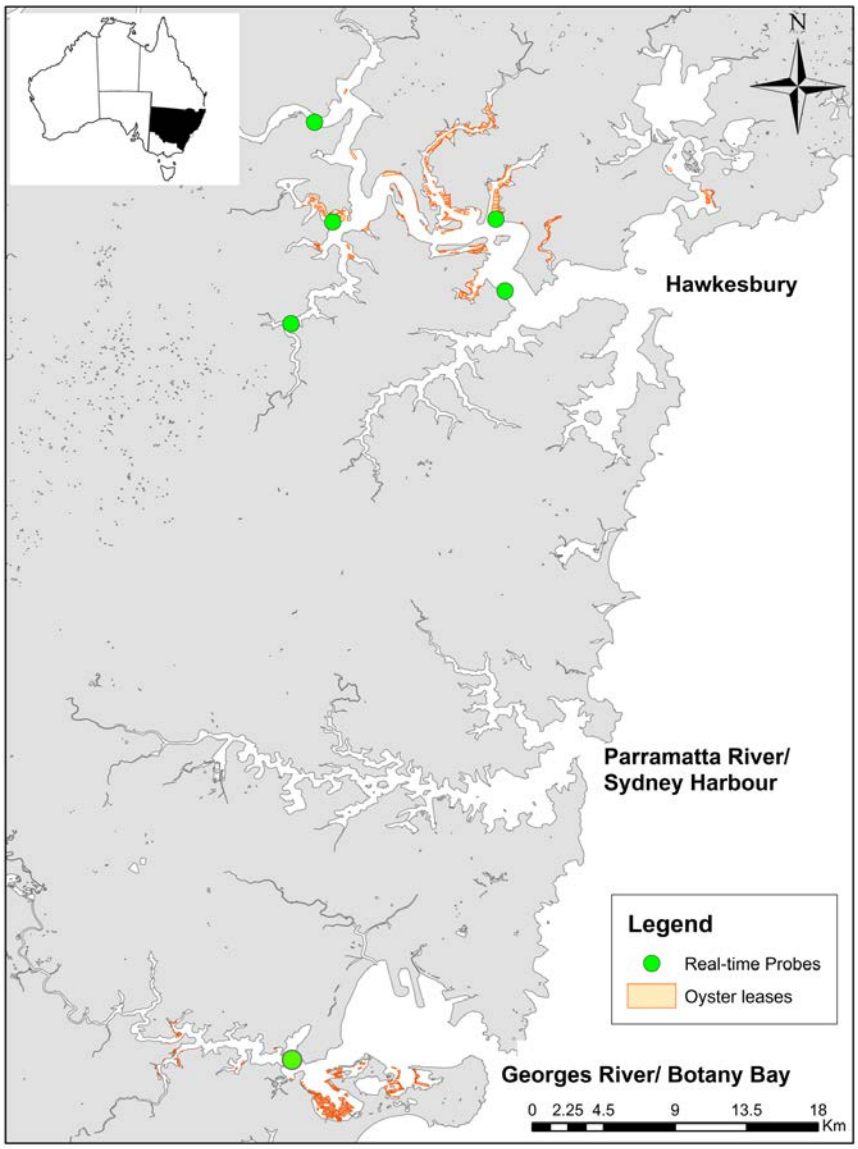


Figure 11. NSW estuaries impacted by OsHV-1 in Australia including the location of the oyster leases and real-time monitoring stations.

Conditions for the day of the index case and 24 hours prior. Environmental conditions averaged for the day prior to and including each index case are presented in Table 8. Average values for most parameters in the two rivers were quite similar taking into consideration that the outbreak in the Georges River occurred in mid-November compared with mid-January for the Hawkesbury River. However, the maximum air temperature in the Hawkesbury River reached 32.3°C while it reached only 21.7°C in the Georges River. Average air temperatures recorded were almost identical to the SST at the mouth of each estuary. Salinity levels were lower in the Georges River than in the Hawkesbury River, however this difference was persistent as the monitoring station in the Georges was located relatively more upstream than the Gunyah monitoring station in the Hawkesbury River. The average levels and range of chlorophyll-a and water level / tide were similar across both estuaries (Table 8).

Table 8: Summary of environmental variables for the day of the index case and 24 hours prior for the Georges and Hawkesbury River using data at15 minute acquisition frequency

Variable	Georges River (CMA Probe 5)			Hawkesbury River (HSC Gunyah Probe*)		
	Mean±stdev	Minimum	Maximum	Mean±stdev	Minimum	Maximum
Air temperature (°C)	20.52±2.0	17.5	21.2	23.67±3.4	19.26	32.3
Coastal Ocean SST (°C)	20.38±0.17	–	–	22.36±0.05	–	–
Estuary Water temperature (°C)	23.61±0.58	22.15	24.49	24.25±0.36	23.71	24.98
Salinity (ppt)	26.7±1.42	24.24	30.14	35.25±0.2	34.72	35.57
Chl-a (ug/L)	4.97±1.3	2.84	7.61	3.3±1.1	1.6	7.6
Water level/tides	Middle of neap tides (range -0.26m to 0.4m) 2 days after moon first quarter			Middle of neap tides (range -0.53m to 0.72m) 2 days after moon first quarter		

(*) Data for Air Temperature was obtained from the Hornsby Shire Council Calabash Point water monitoring station

National climatic index - Nino3.4. The National Climate Centre (NCC) uses the Nino3.4 index to classify ENSO conditions and identifies periods of warm and cool conditions when the index is more or less than 1 standard deviation (± 0.8°C), respectively.

Both OsHV-1 outbreaks occurred after a major shift in the ENSO index from warm conditions 12 months prior to each outbreak to cooler conditions at the time of each outbreak (Figure 12). This shift was more pronounced in the case of the Georges River outbreak than for the Hawkesbury River outbreak. However the range and scale of this shift in ENSO index have been recorded before, in particular during 1997-1998 (one of the 12 strongest El Nino events in history) but also between 2007-2008, with no association with OsHV-1 outbreaks. A year (Dec 2009) prior to the Georges River index case conditions were 2 standard deviations warmer than the historical mean. In the Hawkesbury River, the prior winter months were warmer by 1 standard deviation. A cooler period dominated between mid 2010 and mid 2012.

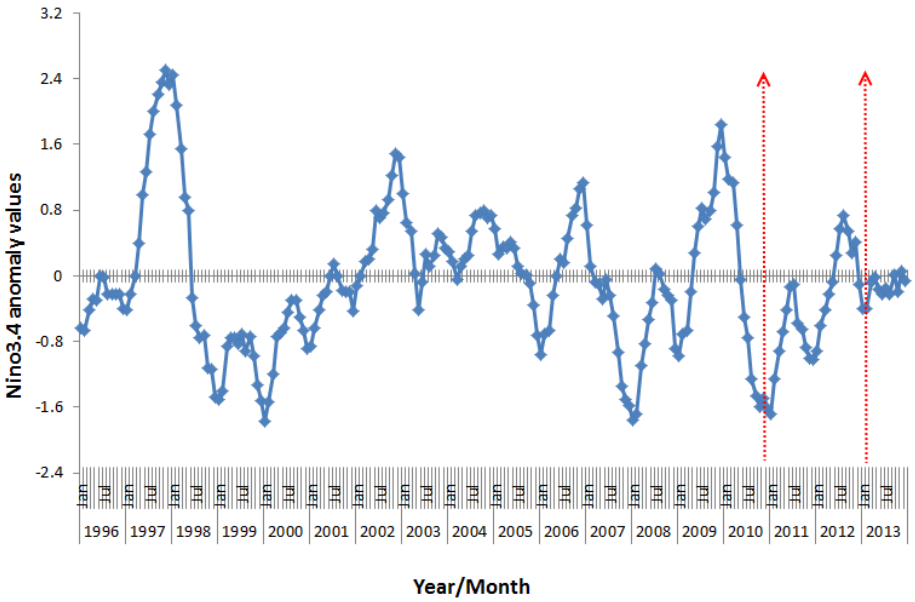


Figure 12. Nino3.4 index showing El Nino (>zero) periods and La Nina (<zero) periods. Arrows mark both OsHV-1 index case outbreaks in Australia

Oceanic input to estuaries. During both of the index case dates a strong warm southward coastal current was present as shown in Figure 13. The temperature of these currents was 23-24°C on the 16th November 2010 and 26-27°C on the 21st January 2013. The southward current travelled closer to the coast around 500 km north of the Hawkesbury and separated as it approached the Hawkesbury latitude. On both occasions satellite data showed the formation of an eddy off the coast slightly north of the Hawkesbury. Some of the warmer pools of water were close to the coast, potentially influencing the water temperatures at the mouth of the estuaries at the time of the index cases.

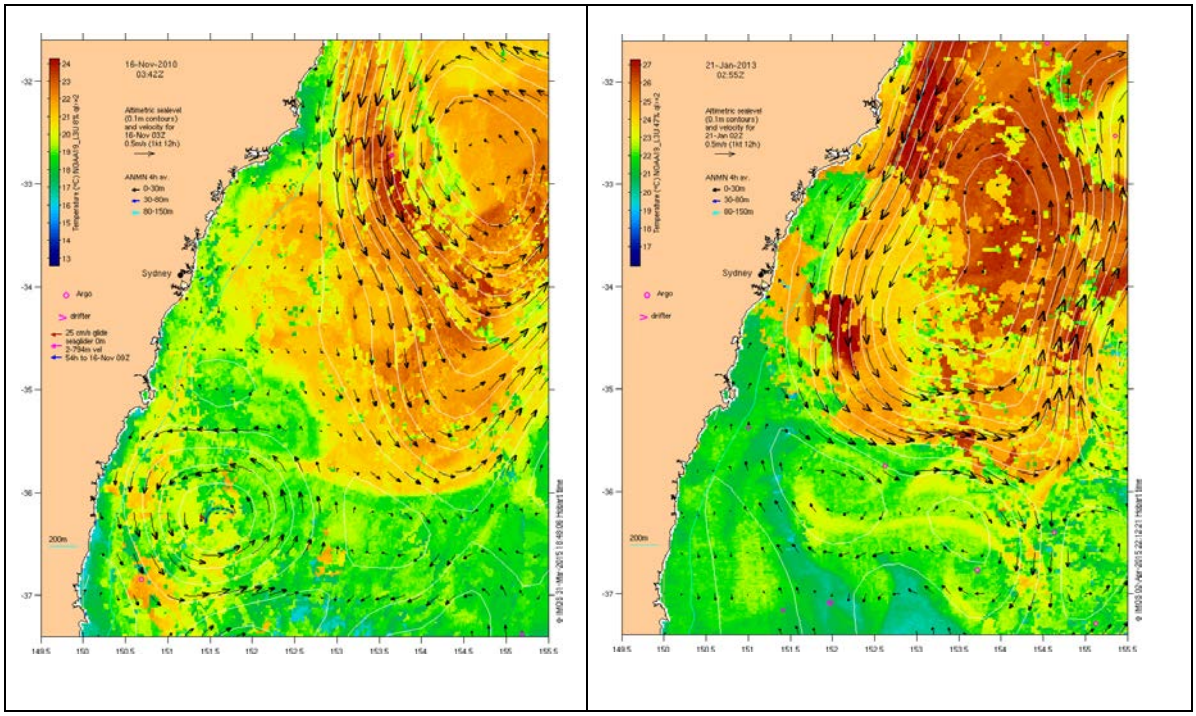


Figure 13. Thermal imagery of sea surface temperature for the south east coastline of NSW during the Georges River (Left) and the Hawkesbury River (Right) index case dates (Source: http://oceancurrent.aodn.org.au/sst.php#).

Oceanic sea surface temperatures (SST) at the mouth of the estuary were slightly higher (0.2°C) in the Georges River, in particular during the peak of summer and winter compared with the Hawkesbury River. Oceanic SST at the time of the index cases were similar to the long-term average (20.4 °C ± 2.2) for each of these locations. At the Georges River mouth, oceanic SST were 20.38 °C during the index case in November 2010. These SST were similar to the previous week (20.65±0.26 °C). Six weeks prior to the outbreak, SST increased from about 17 °C in two set of incremental steps of 2 °C (Figure 14). The first increment was 18-20°C over 4 days and the second increment twenty days later was from 18.7 to 20.8°C over 6 days. The average SST at the mouth of the Hawkesbury River during the index case in January 2013 was 22.36 °C. SST increased continuously over about 4 months prior to the index case with a couple of 2°C quick increments during a period of 4-6 days and similar to the Georges River (Figure 14).

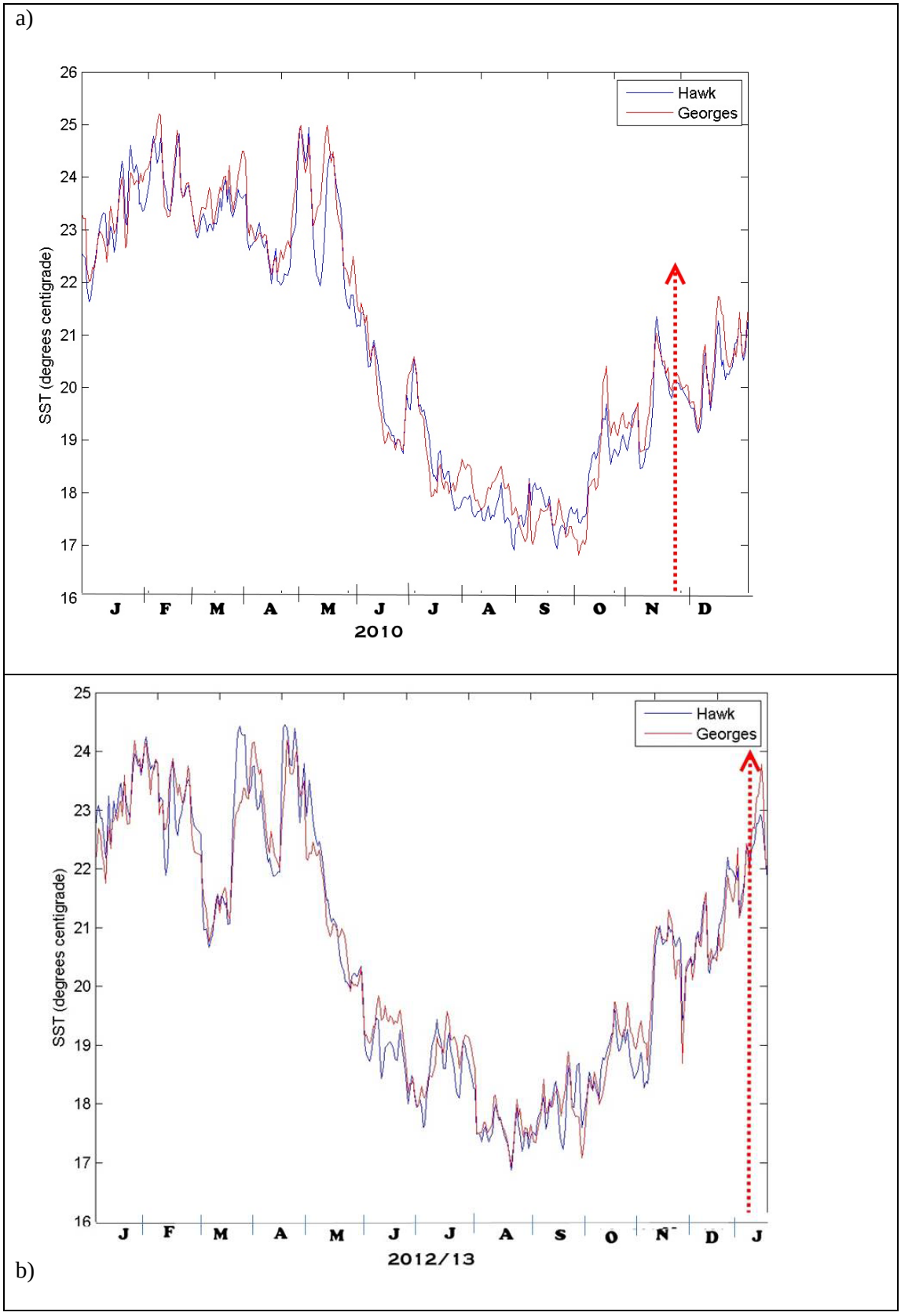


Figure 14. Daily sea surface temperature at the mouth of each river from a 25km x 25km grid. a) data for 1 year to early 2011; b) data for 1 year to early 2013. Index cases are shown by the arrows.

Estuarine environmental conditions

Georges River. The air temperature in the Georges River at the onset of mortalities was on average 20.5°C with a range of 3.7°C between maxima and minima. 7-9 days prior to the mass mortalities, air maximum temperatures ranged between 29.2 and 32.5°C, which were slightly higher than usual for this time of the year (Figure 15).

Water parameters in the Georges River were stable and within the levels expected for the time of the year when the first outbreak occurred. Water temperatures ranged between 22.15-24.49°C during the outbreak. In the 2 week prior to the outbreak, water temperatures increased from 18 to 23°C (a rate of 0.3°C/day). Salinity levels fluctuated around 32ppt a month prior to the outbreak. A drop in salinity of 5ppt was observed a week prior to the outbreak as a result of three rainfall events of 30mm. Rainfall run-off resulted in a slight increase in chlorophyll-a levels. A plume of discoloured water and algal blooms were noticed by farmers around the time of the initial mortalities (Jenkins, et al., 2013).

Environmental conditions during the 3 months prior to the outbreak did not reveal any unusual features that might have resulted in the mass mortalities. Water temperature increased from 15°C in early September to 23°C in mid November in a consistent way. Salinity levels were very high in September as a result of the dry conditions in mid 2010. Salinity then dropped in a continuous manner from mid September to early October as a result of a few rain events. Chlorophyll-a levels fluctuated between 2-5ug/L during the three months prior to the outbreak. Slightly higher levels 8-9ug/L were recorded during the week preceding the outbreak (Figure 16).

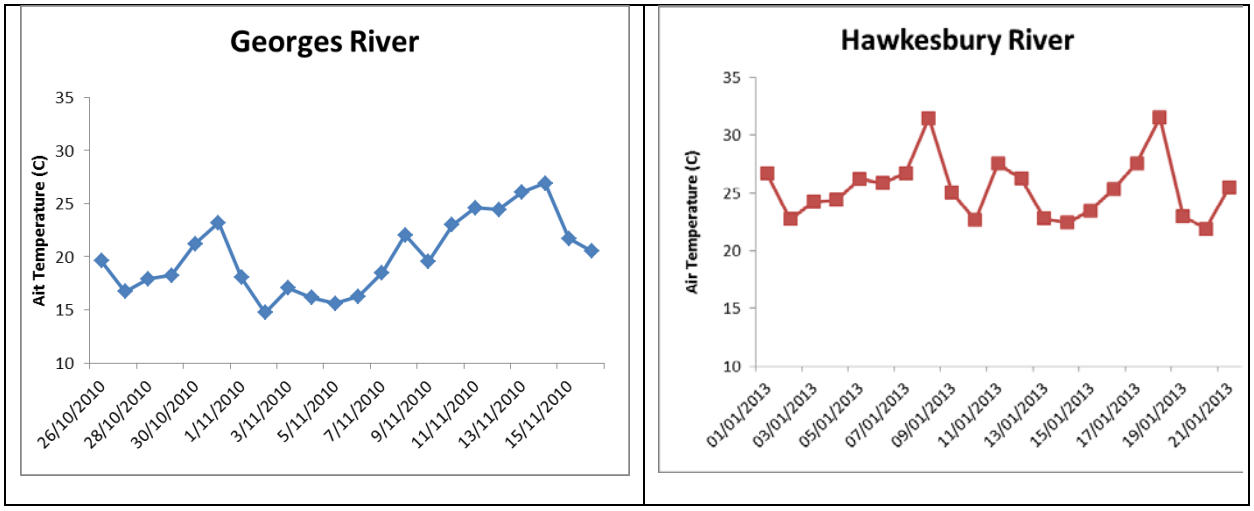


Figure 15. Daily average air temperature for three weeks prior to the index cases. Left panel, Georges River; right panel, Hawkesbury River.

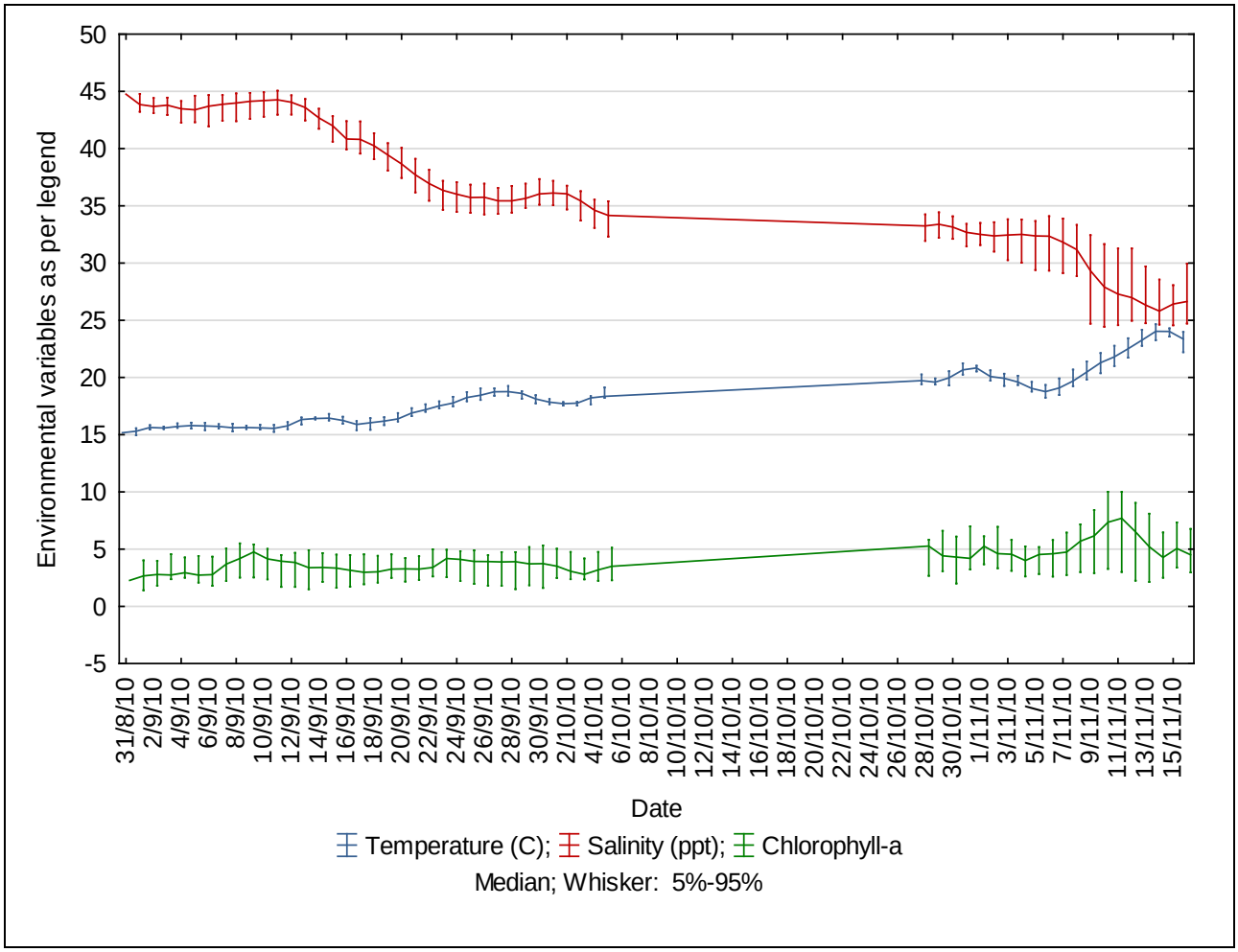


Figure 16. Environmental conditions in the Georges River during the period prior to the outbreak. There are missing data between 6th and 26th October 2010. Data with error bars represent the median and 95%ile for water temperature, salinity and chlorophyll-a

Levels of *Alexandrium catenella* were high in the Georges River during the index case, 1,100 to 22,000 cells/l between 12th-19th Nov 2010 (Table 9).

Hawkesbury River. The air temperature at the Hawkesbury River during the index case was 23.7°C (mean) with a maximum of 32.0°C and a minimum of 19.2°C (Figure 15). However three days prior to the mass mortalities, on 18th January, maximum air temperature reached 45°C. This was the highest temperature recorded in Sydney since 1939. The other noticeably hot day was 8th January. For these two hot days, 5% of daily recordings were above 40°C (Figures 17 and 18). Air temperature during the three months prior to the index case had large variability, with temperatures recorded above 32°C or below 11°C on 5% of occasions (Figure 18).

Water parameters showed no extreme values despite the significantly high air temperatures in the weeks preceding the outbreak (Figures 17 and 18). Water temperatures during the index case were 24.25 ±0.36°C, salinity levels were fully oceanic, chlorophyll-a levels were 3.3±1.1 ug/L which are typical for this season, and water levels were typical of a neap cycle (Figure 18).

From the 1st January, 3 weeks prior to the outbreak, water temperatures and salinity were relatively stable due to the absence of significant rainfall. Water levels from the 15th of January to the 21st of January decreased following a peak in the spring tidal cycle on the 14th January (Figure 18).

Conditions in the Hawkesbury River in the 3 months prior to the outbreak were relatively stable and as would be expected based on historical patterns (water temperature 16–26 °C; salinity 30–35 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 17). Salinity was greater than 32ppt approximately 95% of the time (Figure 17). These conditions indicate a lack of freshwater inflow to the estuary at this time. Substantial variations in water temperature (±3 °C over a few days) were recorded during January.

During the index case for the Hawkesbury no *Alexandrium catenella* species were detected (Table 9). However, *Alexandrium catenella* was present on 19/12/2012 (50 cells/L) and 24/10/2012 (300 cells/L) at the downstream sampling site close to the Gunyah water quality monitoring station and close to the harvest area that was severely impacted by OsHV-1 mass mortalities.

Table 9. Presence of *Alexandrium catenella* close to the sites of index cases.

River and date	*Alexandrium catenella* Presence and abundance (cells/L)
Georges River	
SEPT 2010	Present (trace levels)
OCT 2010	Close to main harvest areas (130)
NOV 2010	Close to main harvest areas (1100-22,000)
DEC 2010	Close to main harvest areas (350)
Hawkesbury	
SEPT 2012	Downstream sites (50)
OCT 2012	Downstream sites (300)
NOV 2012	Not detected
DEC 2012	Downstream sites (40)
JAN 2013	Not detected

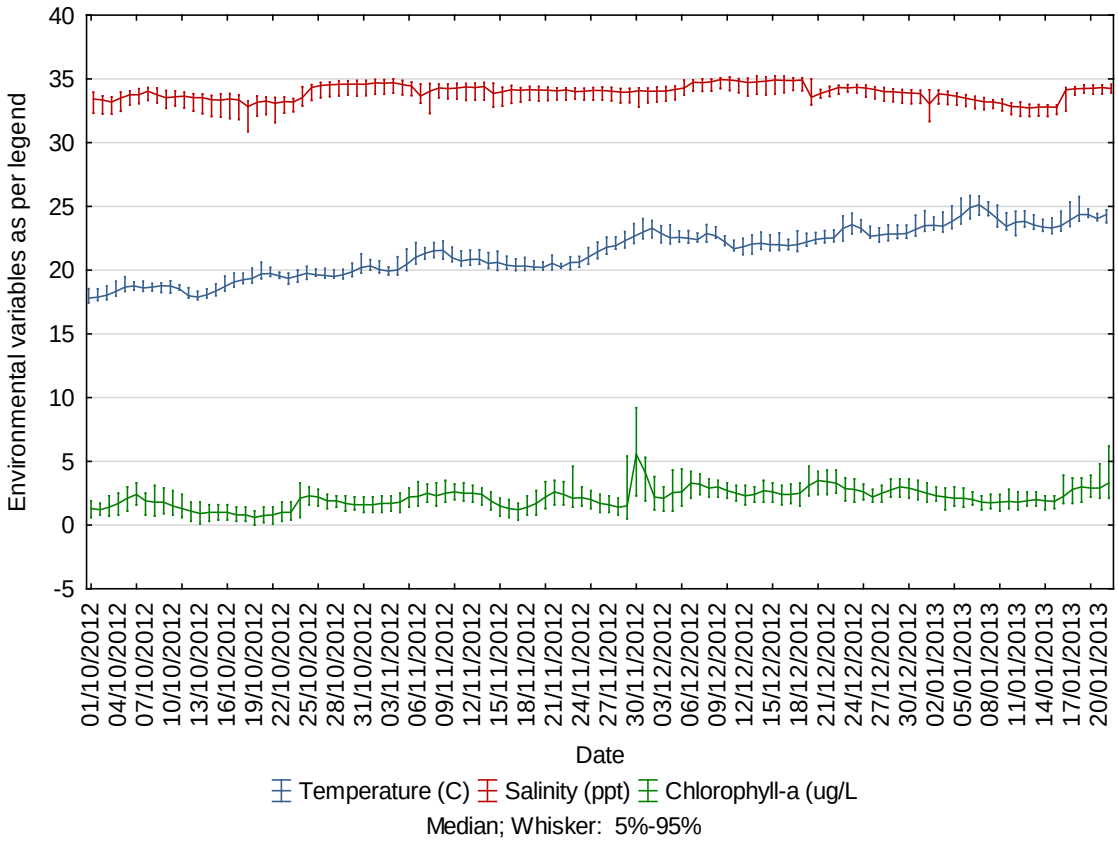


Figure 17. Environmental conditions in the Hawkesbury River prior and during the first POMS outbreak. Data with error bars represent the median and 95%ile

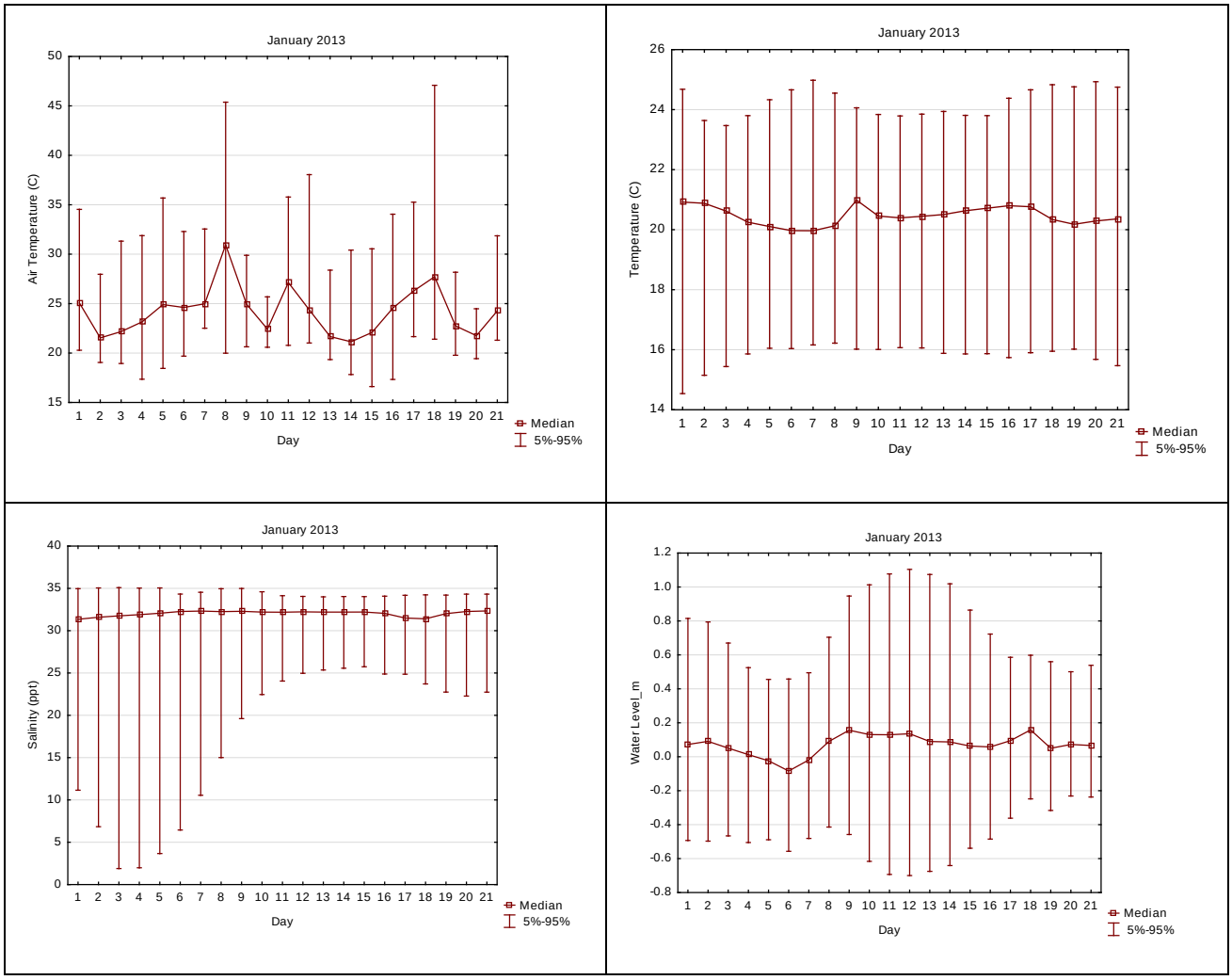


Figure 18. Environmental conditions for 21 days prior to the index case in the Hawkesbury River. Data with error bars represent the median and 95%ile for air temperature, water temperature, salinity and tide.

Anomalies in environmental conditions

Anomaly analysis was undertaken in order to identify deviations from long term average environmental conditions that occurred during 3-months prior or at the index cases. Five to ten-year time series of estuarine environmental data exist mainly for the Hawkesbury River. Historical information also exists for ocean climatic and air temperature data. Hence most of the anomaly analysis focuses on overall climatic ocean temperatures, air temperatures and water quality from the Hawkesbury.

Oceanic SST anomalies. Oceanic SST information was available from 1981. Historical annual SST ranged from 23°C in summer to 17.7°C in winter. The SST at the entrance to Botany Bay tend to be slightly warmer than at the entrance to the Hawkesbury River. In summer, generally it is 0.3°C warmer and in winter it is 0.1°C warmer at the entrance to Botany Bay. For the rest of the year SST at both estuaries are almost identical.

SST at the time of the first outbreak in the Georges River were not exceptional and were within the 95%ile. SST for 2010 were within this range except in April 2010 when they were significantly (3.5°C) warmer than the long-term mean. Oceanic SST at the time of the outbreak were 0.5°C cooler (Figure 19). The rate of change in SST did not appear to be

unusual at the time of the outbreak or in the months prior except for rapid changes during the warm period in April 2010.

SST temperatures were close to the historical average at the time of the Hawkesbury River index case, and during the previous 3 months. However SST anomalies for April and May 2012 were higher than the 95%ile. Exploring the data further, it was noticed that these months in most years had unusual warmer temperatures. The rate of change in SST did not appear to be unusual at the time of the outbreaks or in the months prior; rapid changes in SST were seen throughout the year, but for very short periods in each flux event (Figure 19).

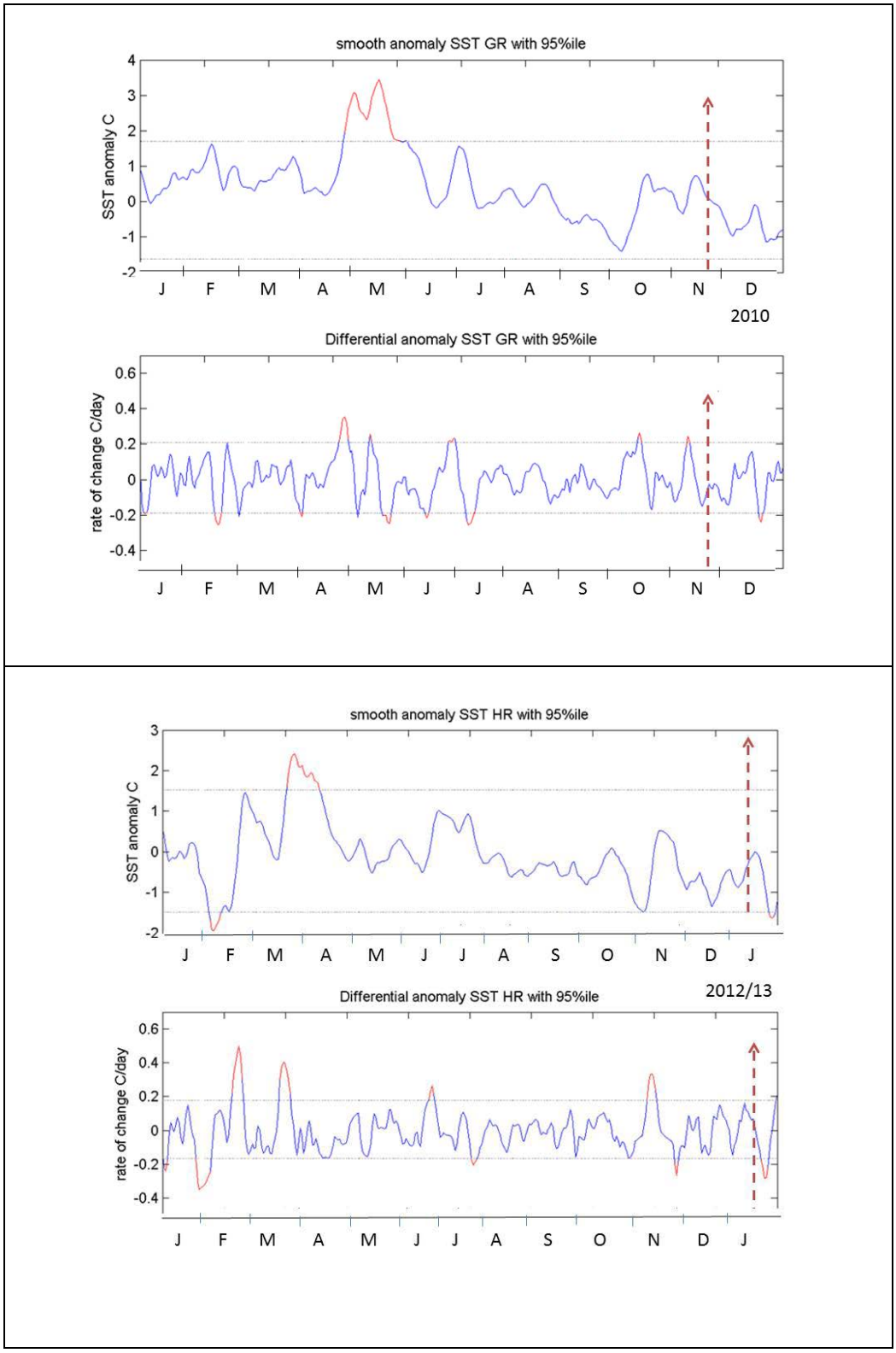


Figure 19. Sea surface temperature anomalies at the mouth of Botany Bay (upper panels) and the Hawkesbury River (lower panels) from a 25km x 25km grid. In each panel the upper plot shows the anomaly while the lower plot shows the rate of change. Dotted lines mark the 95%ile of the historical SST. Red lines on the time series represent the SST values that exceed the 95%ile. Vertical dotted red arrows show the time of the index cases.

Air Temperature anomalies. Air temperatures have increased significantly, in particular from the end of the 1970's. During the 2000s, maximum air temperature anomaly values were on average 1.31°C higher and minimum values were 0.83°C higher than the long-term average record from 1939 to 2015. Minimum air temperatures have increased more than the maximum air temperatures in recent years.

There was strong evidence that air temperatures for both index cases were warmer than historical averages. Although maximum air temperatures during November 2010 for the Georges River were not significantly different from the historical average, in November air temperature was 0.14°C warmer than the historical monthly mean. Minimum temperatures for November 2010 were 1.52°C higher than the historical mean (Figure 20).

Maximum air temperatures during January 2013 for the Hawkesbury River were 1.91°C warmer while minimum temperatures were 1.64°C warmer than their historical means (Figure 20). The maximum and minimum air temperatures for the prior September were 2°C warmer.

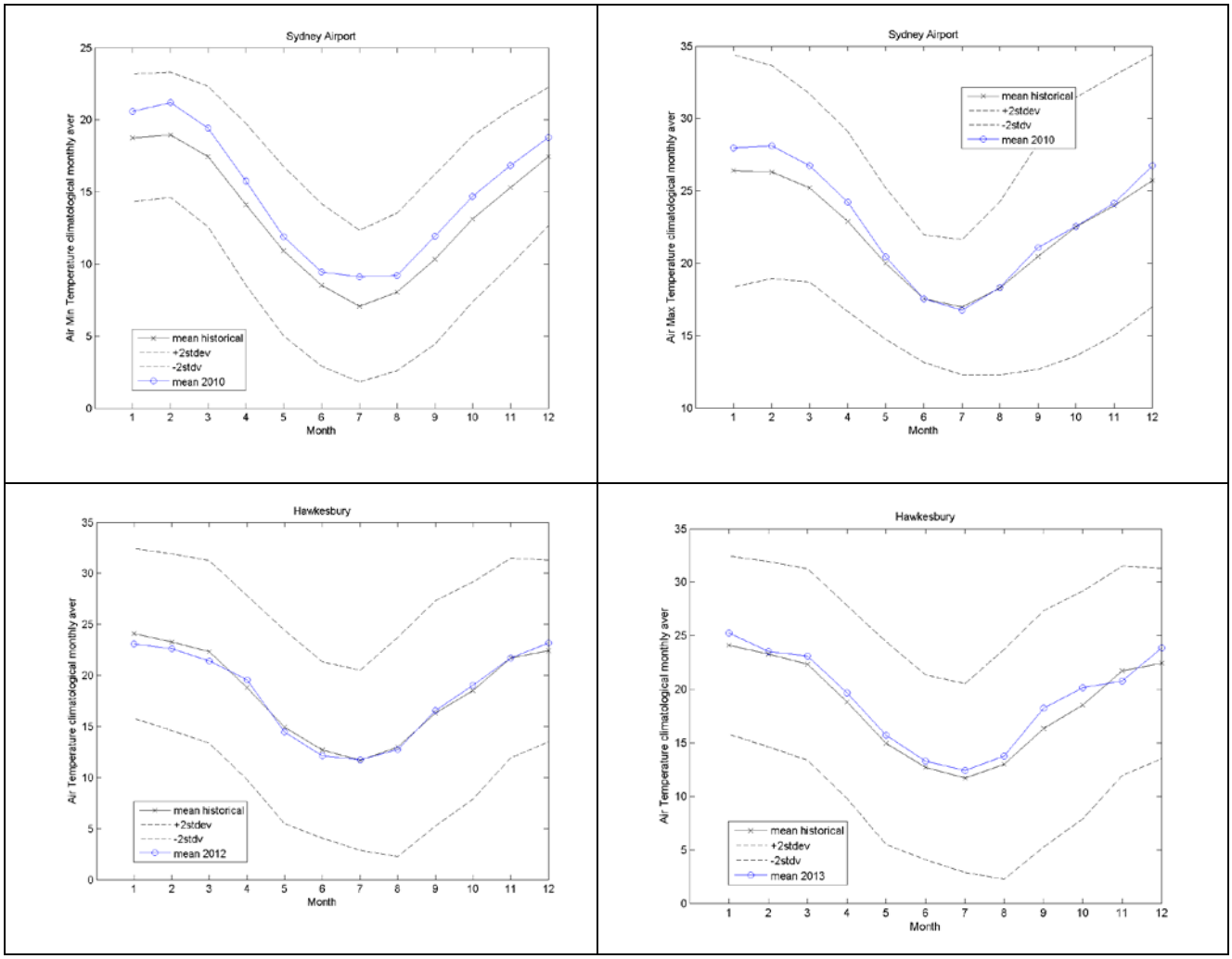


Figure 20. Monthly anomaly values for air temperature in the Georges River (upper panel) and the Hawkesbury River (lower panel). Blue lines represent the monthly averages for the year of the index case (i.e. 2010 for the Georges and 2012/13 for the Hawkesbury). Continuous black lines represent the historical monthly values and the discontinuous lines the ±2 standard deviations

Estuarine environmental anomalies. This section focuses on environmental data from the Hawkesbury as there were limited historical data for the Georges River. Monthly data during the index case and for months prior the first outbreak in the Hawkesbury showed no significant difference against the historical monthly mean for any of the variables considered (Figure 21). However, water temperature in January 2013 was 0.2°C higher than the average and salinity and chlorophyll-a levels were also marginally higher than their historical means (Figure 21).

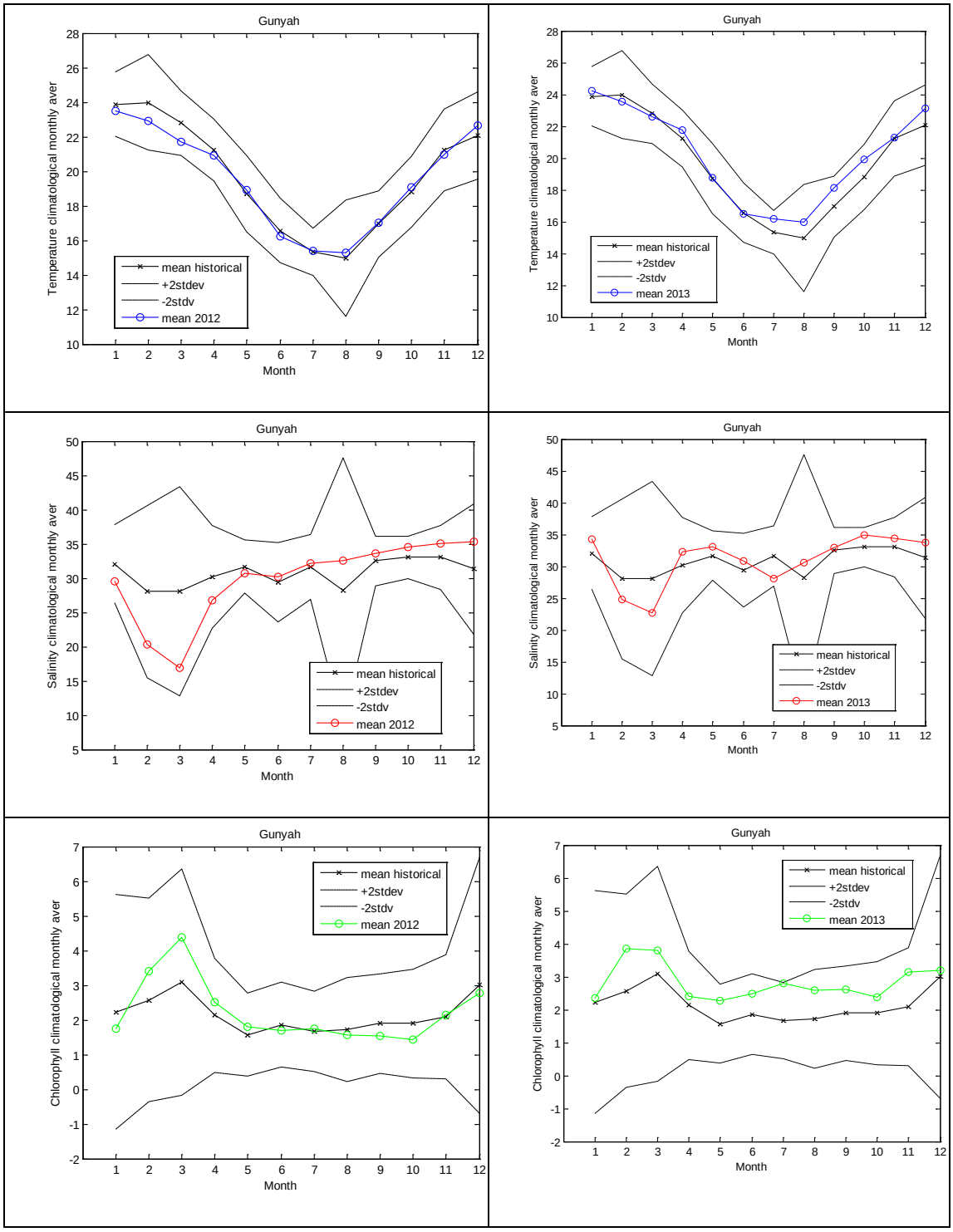


Figure 21. Monthly anomaly values for water temperature (upper panel), salinity (middle panel) and chlorophyll-a (lower panel) for the Hawkesbury River. The left column corresponds to 2012 monthly data and the right column to 2013 monthly data (coloured line). Data for historical averages each month ±2 standard deviations are shown in black lines.

The only parameter that showed a slight shift between the year of the first outbreak and previous years was salinity (Figure 21). Overall yearly median salinity concentrations were lowest in 2011 and 2012 compared with the 2009, 2010, 2013 and2014. This was a result of wet months in January 2011 and 2012 and dry conditions in January 2013. Air and water temperatures for January 2013 were slightly higher than in January 2012, the result of cooler and wetter climate in 2012. January 2013 had the highest air temperatures. There were no significant differences or notable observations in water temperature when comparing January data between years. There were no significant differences or notable observations in water levels when comparing January 2013 data with previous years.

Environmental data within the 2013-2014 POMS season. Upweller spat rearing experiments were run during the entire period of the 2013/14 season that runs from early November to end of May. During this period water temperature levels at the oyster shed where the experiments were run in the Hawkesbury River ranged from 18.7 to 25.9°C (Table 10).

Table 10. Summary of water temperature at two probe locations close to the oyster sheds in Mooney Mooney. Data correspond to dates when the virus was first detected in each of the upweller trials conducted in 2013 -2014. Virus was not detected in experiment 5

Spat Rearing Experiment	Temperature – Gunyah probe			Temperature – Mullet probe		
	Mean±SD	Min	Max	Mean±SD	Min	Max
1	20.8±0.16	20.4	21.1	N/A	N/A	N/A
2	21.1± 0.17	20.8	21.5	21.5±0.36	20.5	22.1
3	22.8±0.57	21.9	23.6	24.1±0.9	22.9	25.9
4	23.7±0.2	23.4	24.1	24.6±0.3	24.0	25.3
5	-	-	-	-	-	-
6	22.6±0.3	21.8	23.1	22.9±0.44	22.2	23.8
7	19.7±0.13	19.4	19.9	19.16±0.2	18.7	19.5

A wide range of environmental levels were recorded during the seven experiments. Environmental conditions were consistent throughout the experiments, independent of the day when the virus appeared and or when the oyster mortalities occurred (2 – 4 day incubation period) (Figure 22). Interestingly, OsHV-1 was not detected during Experiment 5. No major environmental difference was found between this experiment and the others in which the virus and mortality took place. Figure 23 shows the daily variations in environmental data for the 7 spat rearing experiments. Water temperatures fluctuated over 1-2°C prior to the virus appearing. In some cases this fluctuation was an increase, while in others it was a decrease. In half of the trials a drop in water temperature occurred prior to the virus appearing. The magnitude and the lag between the fluctuation and the presence of the virus was slightly variable depending on the trial.

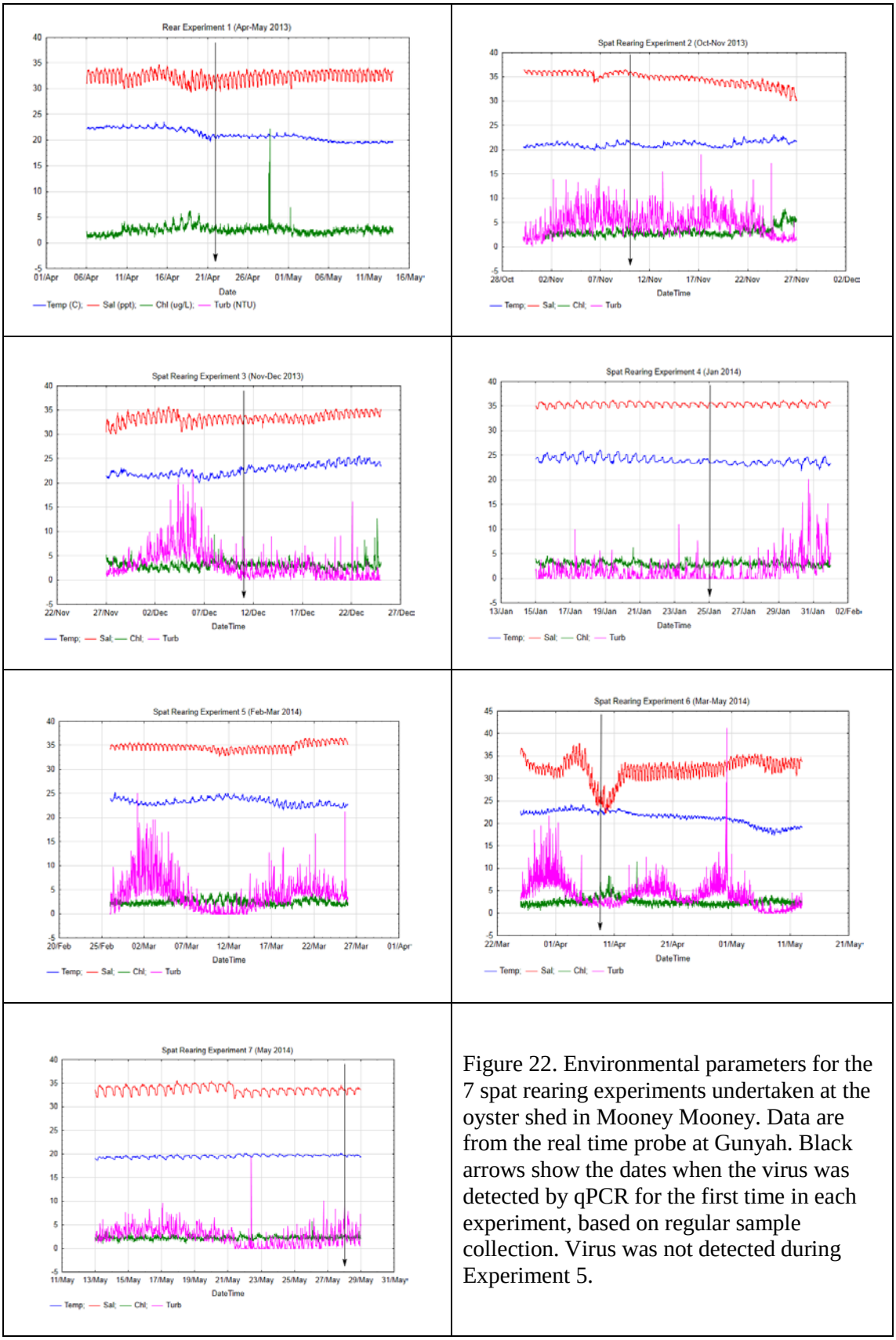


Figure 22. Environmental parameters for the 7 spat rearing experiments undertaken at the oyster shed in Mooney Mooney. Data are from the real time probe at Gunyah. Black arrows show the dates when the virus was detected by qPCR for the first time in each experiment, based on regular sample collection. Virus was not detected during Experiment 5.

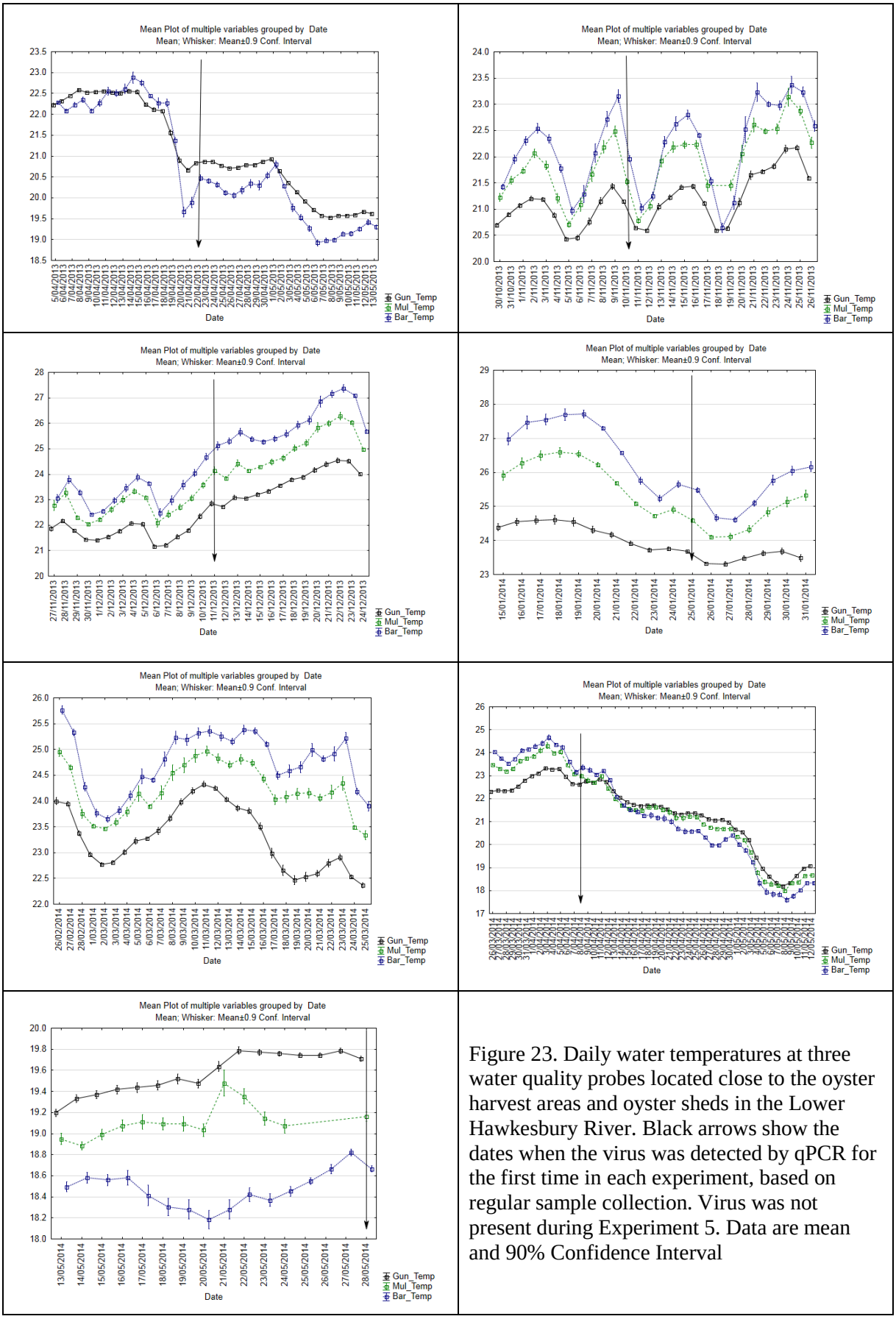


Figure 23. Daily water temperatures at three water quality probes located close to the oyster harvest areas and oyster sheds in the Lower Hawkesbury River. Black arrows show the dates when the virus was detected by qPCR for the first time in each experiment, based on regular sample collection. Virus was not present during Experiment 5. Data are mean and 90% Confidence Interval

Determination of the window of infection in 2012-2013 and 2013-2014

In both seasons and rivers there was great variability in expression of disease caused by OsHV-1 infection among replicate baskets at the same site, and between sites. In the first season (2012-2013) in the Hawkesbury River only Mullet Creek and Marra Marra Creek were affected out of the five sites studied. In the Georges River all three sites were affected over time. In the second season (2013-2014) only Kimmerikong and Marra Marra Creeks were affected in the Hawkesbury River but in the Georges River all sites except Pelican Gut and Site A were affected over time.

A summary of the seasonal occurrence of POMS is provided in Table 11. Spat deployed prior to or after the dates shown in Table 11 remained free of detectable OsHV-1 infection for at least two weeks after deployment. Observations to detect mortality events were fortnightly, so the resolution of the date when mortality occurred was within a 2 week period; therefore exact dates are not specified.

Table 11. The window of POMS disease based on fortnightly deployment and sampling of sentinel spat

Season	River	First case of POMS in sentinel spat	Last case of POMS in sentinel spat
2012-2013	Georges	na*	May 2013
	Hawkesbury	na*	May 2013
2013-2014	Georges	October 2013	April 2014
	Hawkesbury	October 2013	May 2014

*not applicable as sentinel spat were not deployed. However, POMS occurred in November 2012 at these sites in oysters in the Georges River field trail described below.

Georges River Field Trial 2012-2013

Mortality patterns. Oysters were deployed on 19th October 2012 and the trial ended on 21st May 2013 when the last samples were collected. Two main mortality events were detected at all sites; the first began in November 2012 and the second in March 2013. Site A was the first site affected with heavy mortalities observed on the 14th November, but by the end of November significant mortalities were apparent at Site B. Site C was the last to be affected. Mortality was slightly to markedly asynchronous across treatments (Figure 24).

Baskets. At each site there was sudden onset of mortalities for spat in floating baskets while they were slower to develop in oysters in hanging baskets, delayed by 2 weeks to 2 months, but the final mortality figures were similar or greater in the hanging baskets. There was also a delay in the onset of mortality for the highest baskets (+600 mm) (HH) compared to those at +300 mm (H) (see discussion). However, by the end of the summer final cumulative mortalities in HH baskets exceeded the mortalities observed in H baskets averaged over the three sites ($P<0.001$) (Table 12).

Trays. With respect to growing height in trays, at all sites, lower mortality was observed for oysters at high height (H, +300mm) compared to low height (L, standard growing height) ($P<0.001$) (Table 12). The absolute decrease was about 20% both for spat and adult oysters at Site A, 20% for adults and 10% for spat at Site B, and 20% for adults and 12% for spat at Site C (Figure 24; all P-values <0.001). The final cumulative mortality for H adults was between 30 and 40%. The results of survival analysis confirmed that oysters at L height died at a greater rate than those at H height, with hazard ratios between 1.4 and 2.5 depending on site.

With respect to age, the cumulative mortality for adults was significantly lower than for spat, at high height as well as at low height at all three sites (all P-values <0.001) (Figure 24).

Overall for all three sites, cumulative mortality at the end of the trial was 34% in H adults and 71% in H spat and 55% in L adults and 85% in L spat (both P-values <0.001) (Table 12). Results of survival analyses for oysters in trays suggested that spat had a 1.7 to 3.6 fold higher hazard of death compared to adults; the magnitude of the hazard varied between the three sites and tended to be greater at high growing height, due to the protective effect of height acting more on adults (Table 12).

With respect to cultivation system, a comparison was valid for spat reared at the same height in either high trays (H) or hanging baskets (H); in May 2013 the final mortalities in were similar at site level (Figure 24) and when averaged over all three sites, 71-73% (Table 12).

Table 12. Cumulative mortality by 21st May 2013 in oysters for all sites combined.

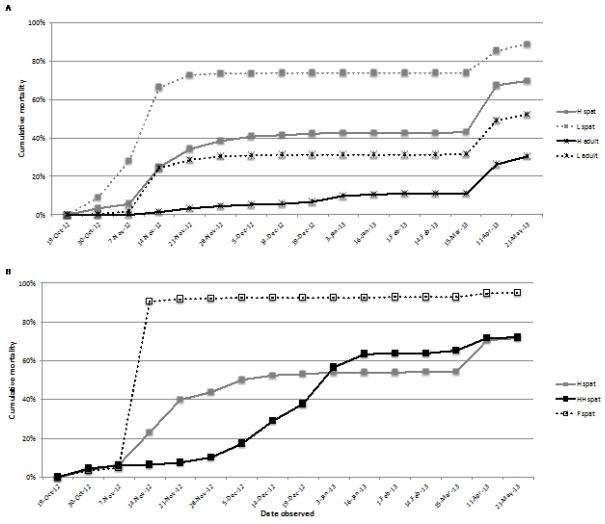
Age	Height	Total number	Adjusted cumulative mortality	P-value
Trays				
Adult				<0.001
	L	1923	55%	
	H	1918	34%	
Spat				<0.001
	L	2015	85%	
	H	1963	71%	
Baskets				
Spat				<0.001[1]
	F	3195	78%	
	H	3409	73%	
	HH	3174	84%	

[1] for the overall and all pairwise comparisons

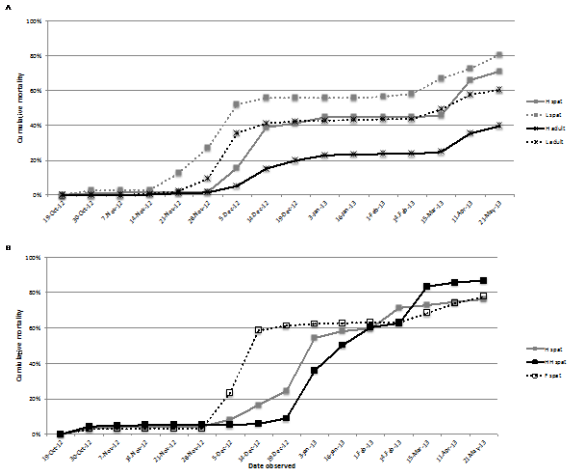
Prevalence of OsHV-1 infection in live oysters. OsHV-1 was first detected by PCR in inter-tidal oysters from trays. At Site A this was on 31st October, in 3 pools of spat, at Site B on 7th November in 1 pool of adults and at Site C on 13th November in 11 pools among spat and adults (Table 13). Based on sampling frequency, the actual time of detectable infection might have been up to 2 weeks earlier at Site A, but within 7 days of these dates at Sites B and C. The prevalence of OsHV-1 infection followed the pattern of mortality, with peaks in November and March, that is it declined after the first mortality event and increased again during the second.

In trays over all three sites and time points, the proportion of pools of spat that contained OsHV-1 were similar regardless of growing height, L (50.3%) and H (47.3%) (P =0.59). However, the proportion of positive pools of adults were lower at high height (23.0%) compared to low height (40.5%) (P<0.001). In baskets over all three sites and time points, the proportions of pools of spat that contained OsHV-1 were similar at each growing height: F 29.2%, H 31.7%, HH 22.7% (P=0.17).

Site A



Site B



Site C

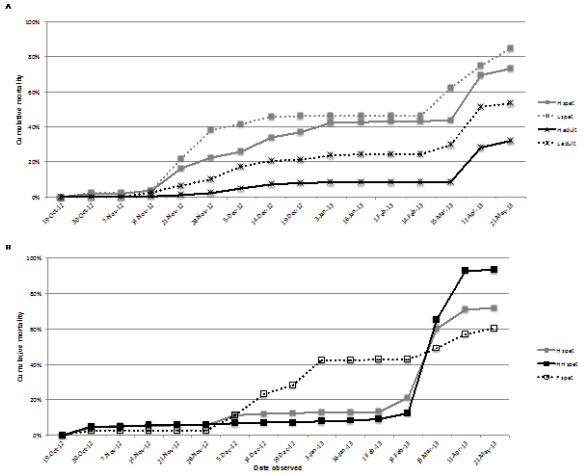


Figure 24. Mortality patterns in trays (upper panels) and baskets (lower panels) 2012-2013

Table 13. Prevalence of OsHV-1 infection in live oysters that were randomly sampled from the trays and baskets.

Data are the number of pools positive in a qPCR test for OsHV-1 DNA out of 5 pools tested. The degree of shading indicates the level of infection prevalence (white=0/5; black=5/5). Italicised numbers represent treatments where at least one pool had an inconclusive result, i.e. only one of two replicate DNA extracts from the sample contained detectable OsHV-1 DNA. NS: no sample, due to low numbers of oysters remaining, except for the final time point. Point estimates for prevalence based on 0, 1, 2, 3, 4 and 5 positive pools were 3%, 9%, 21%, 38%, 56% and 66% respectively based on Cowling, et al. (1999).

Age	Cultivation structure	Height	19-Oct-12	30-Oct-12	7-Nov-12	13-Nov-12	21-Nov-12	28-Nov-12	05-Dec-12	19-Dec-12	3-Jan-13	15-Mar-13	11-Apr-13	21-May-13
Site A														
Adult	Trays	H	0	0	0	1	3	2	2	0	1	1	***4***	4
		L	0	0	2	4	2	3	3	0	0	***3***	***3***	***3***
Spat		H	0	1	3	5	5	4	4	0	0	0	4	***5***
		L	0	2	4	5	5	5	3	0	NS	NS	NS	4
	Baskets	F	0	0	5	5	NS	NS	NS	NS	NS	NS	NS	***3***
		H	0	0	3	5	4	5	4	0	0	***2***	0	2
		HH	0	0	0	3	3	0	1	1	2	***3***	2	2
Site B														
Adult	Trays	H	0	0	1	1	1	1	4	0	0	0	1	4
		L	0	0	0	0	3	4	5	2	1	5	***1***	***3***
Spat		H	0	0	0	3	0	2	4	0	2	***2***	***3***	***2***
		L	0	0	0	3	4	5	5	***1***	0	4	0	0
	Baskets	F	0	0	0	0	0	0	5	2	0	3	1	0
		H	0	0	0	0	0	0	3	4	2	***2***	2	***1***
		HH	0	0	0	1	2	0	0	3	3	2	1	0
Site C														
Adult	Trays	H	0	0	0	2	1	0	1	1	0	1	1	0
		L	0	0	0	1	3	2	2	2	0	5	3	1
Spat		H	0	0	0	3	4	4	4	***4***	0	***3***	***4***	2
		L	0	0	0	5	5	4	3	1	0	5	***2***	0
	Baskets	F	0	0	0	0	4	0	5	***4***	***1***	0	0	0
		H	0	0	0	0	1	4	1	***1***	0	***5***	0	***1***
		HH	0	0	0	0	2	0	0	0	0	5	0	***1***

Growth of oysters in different cultivation systems and at different heights. In general oysters placed at high heights had less growth most likely due to reduced immersion time and opportunity to feed. Except for adults at site A and spat at site C in trays, at the end of the trial oysters kept at high height were significantly smaller than counterparts at a lower height. To illustrate this a comparison of the length of oysters at an intermediate time point (day 40) and at the end of the trial (day 174) is shown in Figure 25. Note that the oysters present at day 174 were the survivors of the outbreak. Differences in size appear more pronounced in spat kept in baskets because there was a more extreme range of growing heights compared to trays.

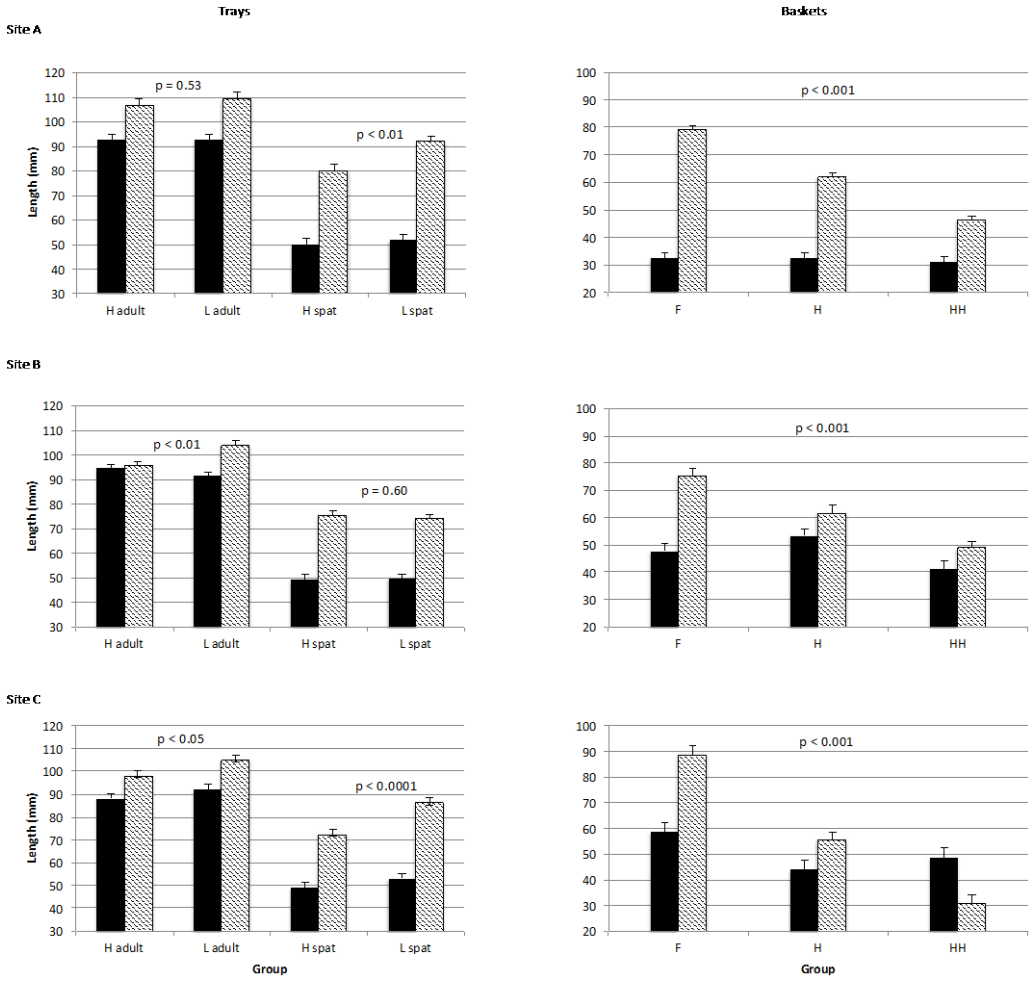


Figure 25. Lengths of oysters in trays and baskets at each site compared at two times about 4.5 months apart. Black bars, 28th November 2012 (day 40); stippled bars, 21st May 2013 (day 174). P values are shown for the difference between lengths at day 174 for oysters of the same age. For baskets, the differences were highly significant for all pairwise comparisons of F, H and HH.

Water temperature. The weekly mean temperature exceeded 19°C from the start of the trial until mid April 2013 when it commenced a steady decline to below 15°C.

Comparison of mortality patterns between 2011-2012 and 2012-2013. Compared to the previous summer mortality developed more slowly in 2012-2013 (Figure 26). Although statistical comparisons were not made between the two experiments as different batches (age, size, parentage) of oysters were used, lower final cumulative mortalities for spat were

observed in this study (70-90%) in comparison with 2011-2012 (100%) (Paul-Pont, et al., 2013a) (Figure 26). Spat used in the 2012-2013 experiment were older and larger (9.5 months old; 50 ± 5.2 mm length) than the spat used in the previous 2011-2012 experiment (5.5 months old; 28 ± 5.1 mm length). As the susceptibility to OsHV-1 infection is closely related to age, the early life stages (spat, juveniles) being the most susceptible (see below) the difference in age/size of oysters between the two studies could explain the lower mortalities observed in 2012-2013. The difference in final cumulative mortalities could also be related to differences in the quantity (dose) or quality (viability) of the virus in seawater, or to some environmental parameters.

Epidemic curve analysis. The outbreak was divided into major and minor episodes throughout the summer, with the peaks in mortality in November and April. Epidemic curves are illustrated in Figures 27 and 28. During the first period of mortality there were three epidemic curve patterns observed for the eight segments among the 24 trays:

i. synchronous onset. Mortality began synchronously in all 8 segments with cessation of new mortalities within two observation periods (14 days) (4 trays) (Site A low spat rep 2, Site A low adult rep 2, Site B high spat rep 1, Site B low adult rep 2)
ii. near-synchronous onset. Mortality began synchronously in 6 segments with cessation of new mortalities within three observation periods (21 days) (5 trays) (Site A low spat rep 1; site A low spat rep 2; Site A low adult rep 1;Site B high spat rep 2; Site C low spat rep 2)
iii. successive clustered onset with variable mortality rates. Mortality began successively in single segments or clusters of segments and progressed at a variable rate (15 trays).

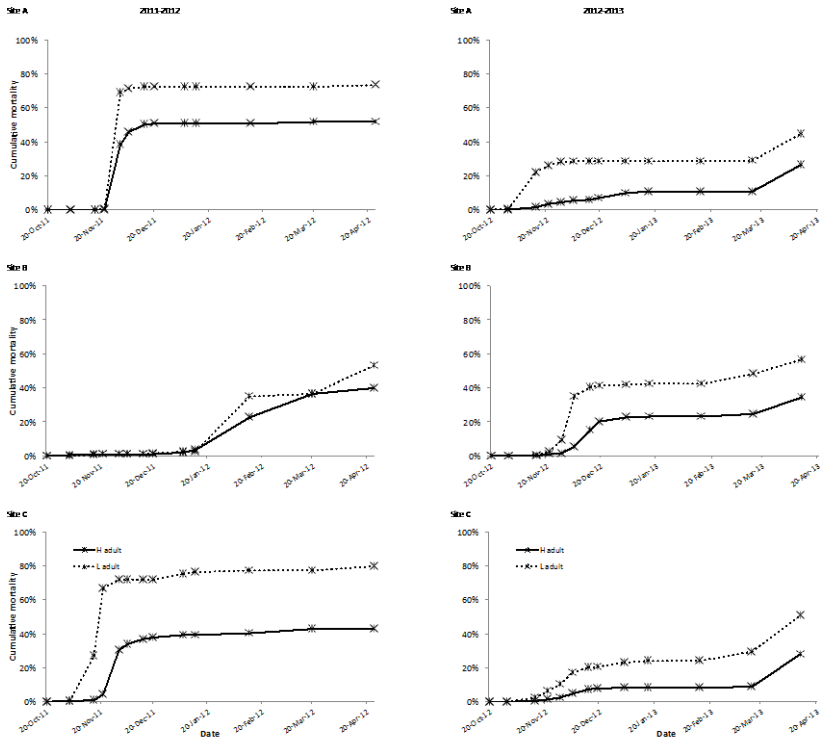
During the second period, mortality recommenced synchronously in most segments within a tray.

For the baskets, the analysis was conducted by assessing the pattern of curves for all 12 baskets on each long line; there were 3 long lines at HH, 3 at H and 3 at F (total 9 long lines). The same epidemic curve patterns as described for trays were observed for baskets. Among the 9 long lines, synchronous onset and progression was observed for 2 long lines (Site A Floating, Site C HH), near-synchronous onset for 1 long line (Site B F) and successive clustered onset for 6 long-lines.

The staggered patterns of onset of mortality affecting different tray segments were consistent with successively acquired point source infections from an external source. The long time course for mortalities and presence of many survivors in most segments was not convincing for oyster to oyster spread within segments; if this had occurred there should have been rapid progression due to the short incubation period, and most oysters should have succumbed. The long time course of deaths within segments was better explained by newly acquired infections from outside the tray environment. Some trays had patterns consistent with "massive" point source exposure where all or most segments became affected concurrently. Oyster-to-oyster transmission within a tray, if it occurred, was incomplete or inefficient. PCR analysis of individual dead oysters throughout the trial confirmed that these oysters had high viral loads which clearly did not lead to rapid transmission from oyster to oyster within a tray.

In summary, the observational data suggest that a synchronous point source, and/or repeated point source exposure was required to explain the mortality patterns observed.

Adults



Spat

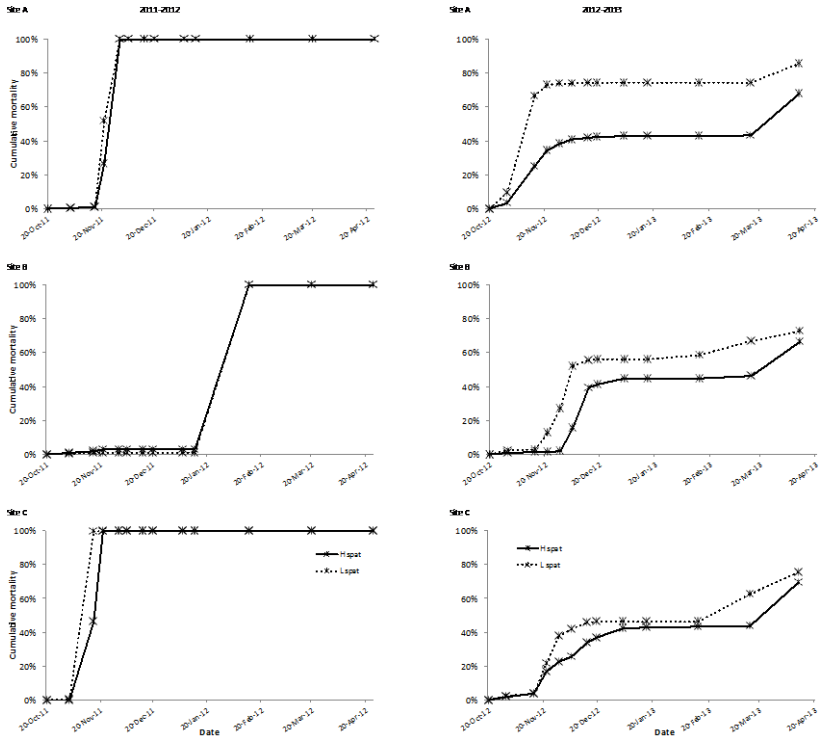
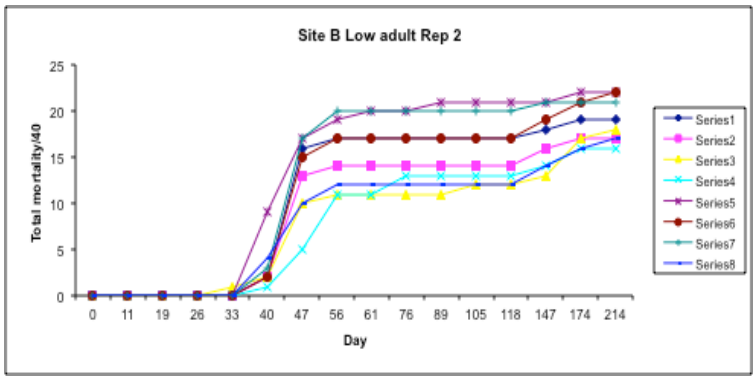
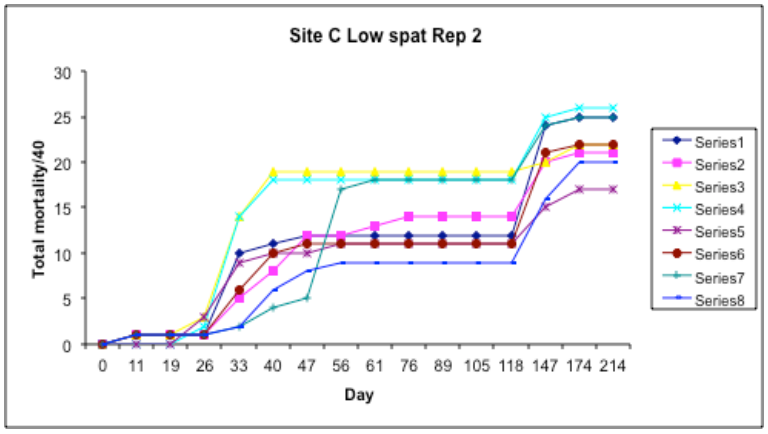


Figure 26. Comparison of cumulative mortality oysters cultivated in trays between experiments conducted in 2011-2012 (Paul-Pont, et al., 2013a) and 2012-2013 in Woolooware Bay.

A



B



C

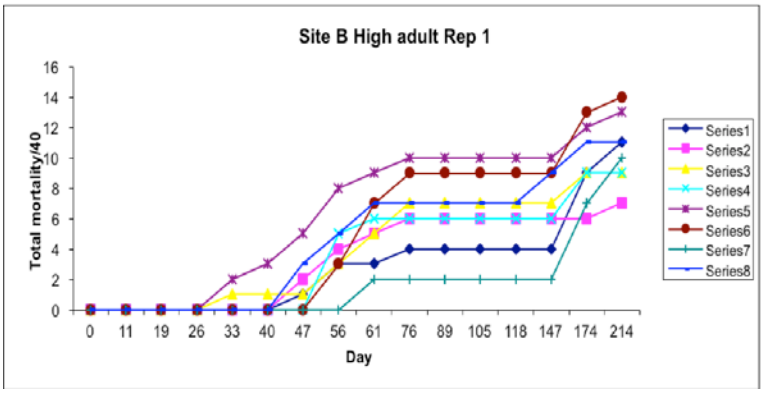
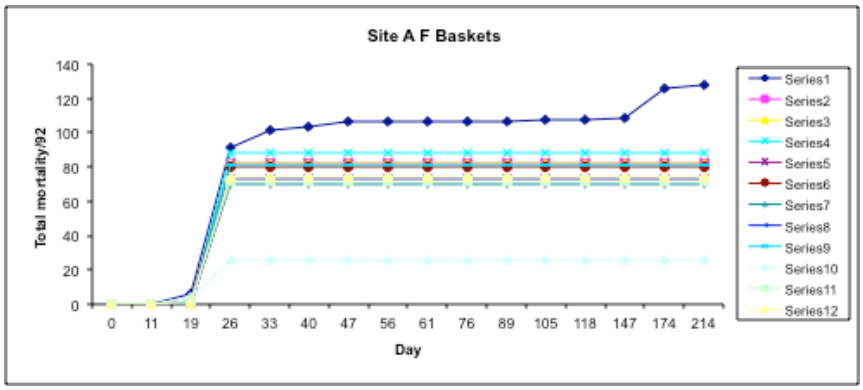
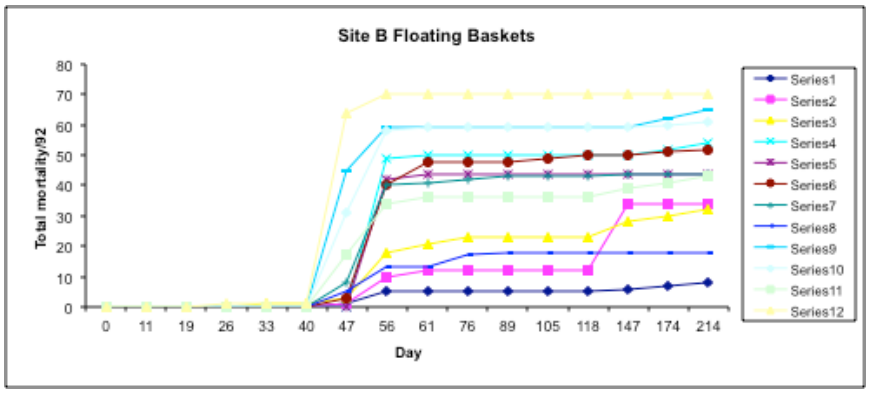


Figure 27. Representative examples of the main types of epidemic curve patterns observed among the 8 individual segments of 24 trays. Each segment contained 40 adult oysters. A. Pattern i) synchronous onset; B. pattern ii) near synchronous onset; C pattern iii), successive onset.

A



B



C

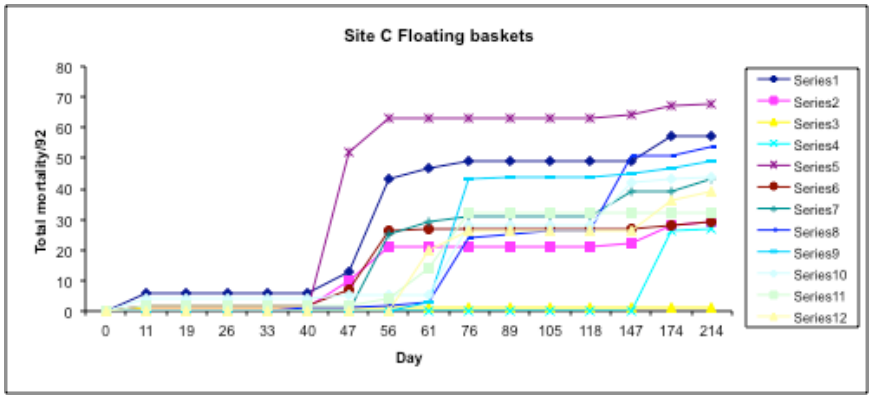


Figure 28. Representative examples of the main types of epidemic curve patterns observed among 9 long lines, each with 12 baskets. A. Pattern i) synchronous onset; B. pattern ii) near synchronous onset; C pattern iii), successive onset.

Identification of natural reservoirs for the virus

Opportunistic samples were collected from a wide range of locations in the Georges River between October 2012 and April 2013. This interval spanned the 2012-2013 summer season for POMS, during which time there were incidents of POMS at Sites A, B and C in Woolooware Bay. Approximate sampling locations for collections in the Hawkesbury River are shown in Figure 8 and for the Georges River in Figure 29.

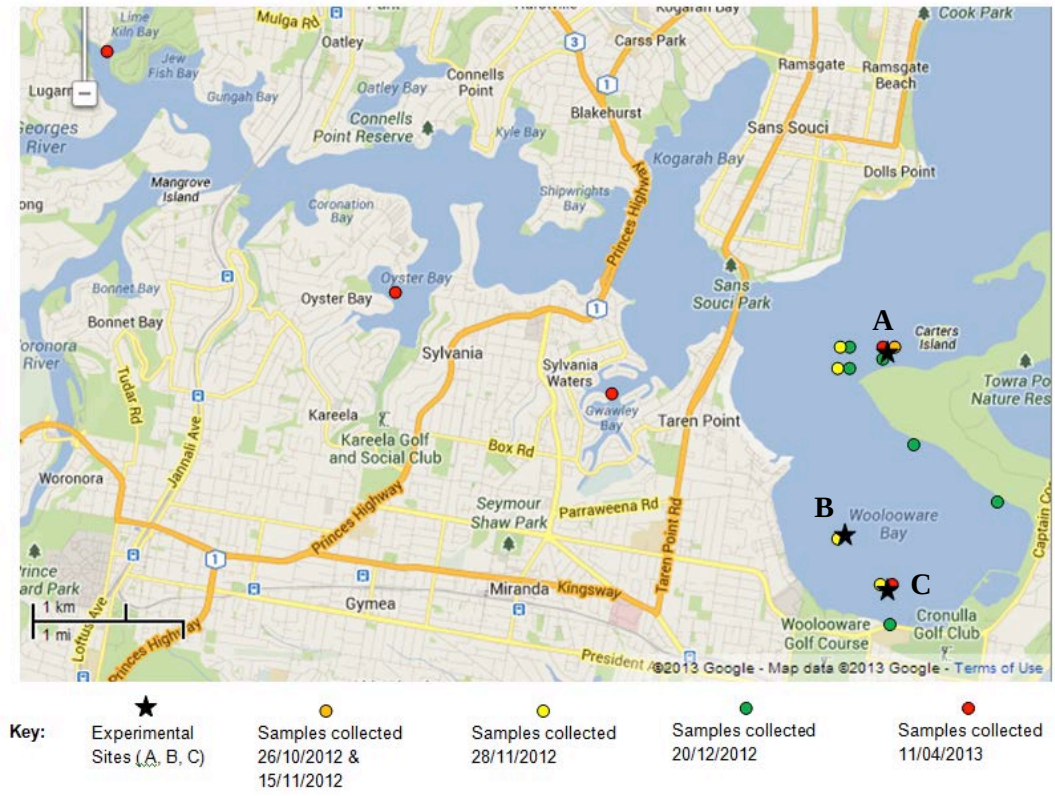


Figure 29. Location and date for wild species collection

Wild Pacific oysters and wild Sydney rock oysters contained detectable amounts of OsHV-1 (Tables 14 and 15) at several locations. Overall about 37% of the pooled samples of Pacific oyster were positive, compared to about 23% of pooled samples of Sydney rock oyster.

About 14% of pooled or individual samples of other mollusc species (Sydney cockle, blue mussel, hairy mussel, Australian mud whelk, periwinkles), barnacles cnidarians, and other types of samples such as seaweed and sediments also contained detectable OsHV-1. Species names where known are provided in Table 16.

Viral loads in positive samples were very low. Consequently the significance of these findings for the epidemiology of OsHV-1 in farmed oysters is unknown. It is possible that the virus detected in these samples was merely filtered from water (in the case of bivalves) or ingested by or attached to the other types of samples that were examined. It seems unlikely that the virus could actually infect such a wide range of species given the generally accepted high degree of host specificity displayed by herpesviruses. Furthermore, it would not be possible to test the viability of the virus detected because of the lack of a cell culture system, and because of the very low viral load in the samples, transmission trials using a bioassay could not be undertaken. It was beyond the scope of the project to analyse for viral transcripts or to compare quantities of immediate early and late viral genes in host cells as an indirect measure of viral replication.

Table 14. Results of qPCR tests for OsHV-1 in wild Pacific oysters. GR Georges River, HWY Hawkesbury River

Date	River	Location	No. individuals	No. samples per pool	No. of pools	No. positive
26.10.2012	GR	Site A Saggy Lease	3	3	1	0
			30	1	30	1
15.11.2012	GR	Site A Saggy Lease	20	5	4	4
28.11.2012	GR	Site A Location 1	2	2	1	0
	GR	Site B Location 3	2	2	1	0
	GR	Site C Location 4	1	1	1	0
	GR	Endeavour Oyster Shed (Site C)	5	5	1	1
20.12.2012	GR	Site A Location 1	5	5	1	1
	GR	Site A Location 3	25	5	4	1
			1	1	1	1
	GR	Shoal/shore SW Endeavour Oyster Shed (across bay)	30	5	6	0
			4	4	1	0
			2	2	1	0
	GR	Mangrove/Shoreline directly East of Shed	35	5	7	0
	GR	Mangrove SW Site B	15	5	3	0
04.02.2013	HWY	Mullet Creek Past Railway	30	5	6	6
14.02.2013	GR	Mangrove (collected by retired farmer)	3	3	1	0
11.04.2013	GR	Site C Main Channel	3	3	1	0
	GR	Site B	10	5	2	2
	GR	Site A Entrance	7	5	1	0
				2	1	0
	GR	Gwawley Bay	15	5	3	3
	GR	Oyster Bay	35	5	7	6
	GR	Lime Kiln Barr	40	5	8	8
			1	1	1	1
		Total	324		**94**	**35**

Table 15. Results of qPCR tests for OsHV-1 in wild Sydney rock oysters. GR Georges River, HWY Hawkesbury River

Date	River	Location	No. individuals	No. samples per pool	No. of pools	No. positive
26.10.2012	GR	Site A	24	4	6	2
15.11.2012	GR	Site A Saggy Lease	15	5	3	1
28.11.2012	GR	Site A Location 1	25	5	6	1
	GR	Site A Location 2	30	5	6	2
	GR	Site B Location 1	5	5	1	0
			4	4	1	0
	GR	Site B Location 2	10	5	2	1
	GR	Site B Location 3	10	5	2	1
	GR	Site B Location 4	5	5	1	0
			4	4	1	1
	GR	Site B Location 5	10	5	2	0
			3	3	1	0
	GR	Site C Location 1	2	2	1	0
	GR	Site C Location 2	7	5	1	0
			2	2	1	1
	GR	Site C Location 3	4	4	1	0
	GR	Site C Location 4	2	2	1	0
	GR	Site C Location 5	10	5	2	0
	GR	Endeavour Oyster Shed (Site C)	15	5	3	3
20.12.2012	GR	Site A Location 1	45	5	9	4
	GR	Site A Location 3	90	5	18	0
	GR	Shoal/shore SW Endeavour Oyster Shed (across bay)	35	5	7	0
			2	2	1	0
			4	4	1	0
	GR	Mangrove/Shoreline directly East of Shed	30	5	6	0
			12	4	3	0
			2	2	1	0
	GR	Mangrove SW Site B	35	5	7	0
14.02.2013	GR	Mangrove (collected by retired farmer)	3	3	1	0
11.04.2013	GR	Site C Main Channel	1	1	1	0
	GR	Site B	10	5	2	2
			4	4	1	0
	GR	Site A Entrance	10	5	2	0
			2	2	1	0
	GR	Site A Sand Bank/Oyster Reef (=Site A Location 2)	30	5	6	2
	GR	Gwawley Bay	15	5	3	3
			4	4	1	0
	GR	Oyster Bay	5	5	1	1
			2	2	1	1
	GR	Lime Kiln Barr	5	5	1	1
			4	4	1	0
		Total	537		**117**	**27**

Table 16. Results of qPCR tests for OsHV-1 in various wild molluscs and other types of samples. GR Georges River, HWY Hawkesbury River

Date	River	Location	No. individuals	No. samples per pool	No. of pools	No. positive
Sydney Cockle (Anadara trapezia)						
28.11.2012	GR	Site A Location 1	30	5	6	1
	GR	Site B Location 5	1	1	1	0
11.04.2013	GR	Site A Sand Bank/Oyster Reef (=Site A Location 2)	10	5	2	0
			4	4	1	0
		Total	45			
Blue Mussel (Mylitus galloprovincialis)						
28.11.2012	GR	Site A Location 1	1	1	1	1
	GR	Site C Location 5	4	4	1	0
04.02.2013	HWY	Mullet Creek past Railway	30	30	1	0
		Mullet Creek OL62/095	30	30	1	1
		Total	65			
Hairy Mussel (Trichomya hirsute)						
20.12.2012	GR	Site A Location 1	10	5	2	1
		Total	10			
Mud whelks (Batillaria australis)						
28.11.2012	GR	Site A Location 1	10	5	2	0
20.12.2012	GR	Site A Location 2	25	5	5	1
	GR	Site A Location 3	25	5	5	0
	GR	Mangrove SW Site B	35	5	7	0
		Total	95			
Small Gastropods (species unspecified)						
28.11.2012	GR	Site A Location 1	10	5	2	0
		Total	10			
Clam (species unspecified)						
28.11.2012	GR	Site A Location 1	1	1	1	0
	GR	Site A Location 2	1	1	1	0
11.04.2013	GR	Site B	2	2	1	0
	GR	Site A Sand Bank/Oyster Reef (=Site A Location 2)	1	1	1	0
		Total	5			
Barnacle species						
15.11.2012	GR	Saggy Lease	150	30	5	2
28.11.2012	GR	Site A Location 1	30	30	1	0
	GR	Site B Location 1	90	30	3	0
Cnidarians						
28.11.2012	GR	Site B Location 5	3 samples	*Unknown*	3	1
Blue periwinkles						
04.02.2013	HWY	Mullet Creek past Railway	30	30	1	1
Conch (species unidentified)						
11.04.2013	GR	Site A Sand Bank/Oyster Reef (=Site A Location 2)	1	1	1	0
Seaweed						
28.11.2012	GR	Endeavour Oyster Shed (Site C)	1 sample	*Unknown*	3	2
Unidentified Bird faeces + urates						
28.11.2012	GR	Site A Location 1	2 Samples		2	0
	GR	Site A Location 2	1 sample		1	0
Sediment samples						
20.12.2012	GR	Site A Location 1	4 samples		4	1
	GR	Site A Location 3	2 samples		2	0
	GR	Shoal/shore SW Endeavour Oyster Shed (across bay)	2 samples		2	0
	GR	Mangrove SW Site B	5 Samples		5	0
Flatworm (species unspecified)						
20.12.2012	GR	Site A Location 3	1	1	1	0
Total					**76**	**11**

Stability of the virus in the environment
This was assessed using bioassays in two types of experiments: i) laboratory trials; ii) on-farm upweller trials.

i) Laboratory trials. The relative sensitivity of the IM injection bioassay and the immersion bioassay were compared prior to the stability experiments. Injection of juvenile oysters was the most sensitive method of detecting OsHV-1; a 10^{-4} dilution of a preparation of OsHV-1 in seawater resulted in mortality in both replicates, in two separate titration experiments The limit of detection in the bioassay using immersion of spat was one to two 10-fold dilutions less sensitive.

In both the seawater and tissue preparation, the initial quantity of OsHV-1 was $>10^6$ genome copies per µl. Following storage at 20^0C for 24 hours, the amount of OsHV-1 genome detected in seawater had declined by 5 logs and it was undetectable by 5 days (Figure 30). The seawater preparation was tested by bioassay, using immersion of spat at each time point up to 7 days, and injection of juveniles at 7 days. At day 0 there was >50% mortality of spat (Table 17). Total cumulative mortality declined with increasing storage time of the seawater. The absence of infectious virus at 7 d was confirmed using both spat immersion and juvenile injection.

The wet oyster tissue homogenate initially contained 3.6×10^6 OsHV-1 genome copies per µl with a decline to 1.4×10^4 copies per µl after 7 d at 20^0C (Figure 30). The dry tissue contained $> 10^5$ genome copies per µl after reconstitution at each time up to 7 d at 20^0C (Figure 30). Using the bioassay the virus in these preparations was shown to be infectious at all time points over the 7 day period (Tables 18 and 19).

In all experiments dead oysters contained high quantities of OsHV-1, in excess of the amount of virus added to the tanks or injected, indicating viral infection and replication of virus.

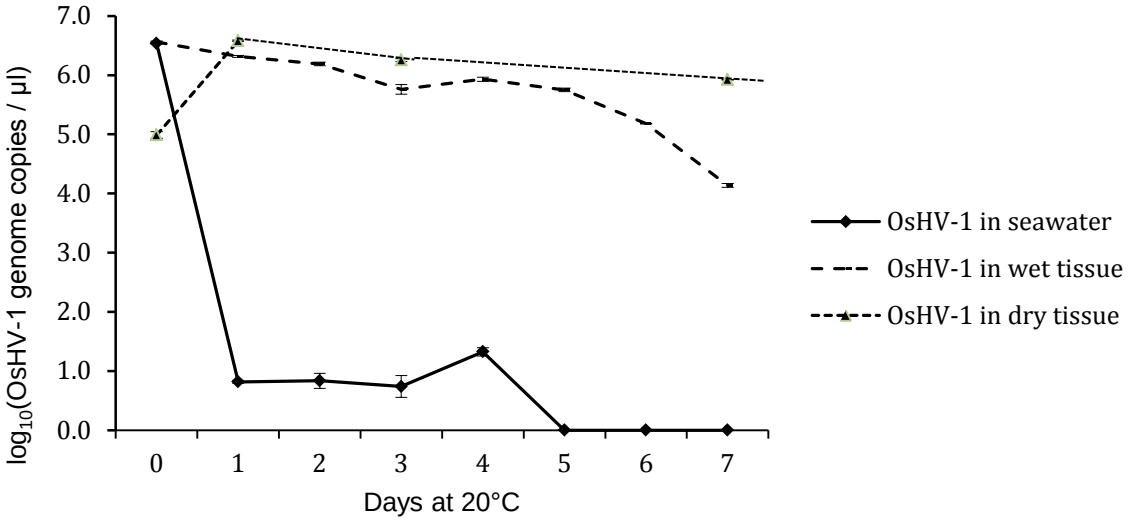


Figure 30. Stability of OsHV-1 in seawater, wet and dry oyster tissues during storage at 20^0C. Virus was quantified using qPCR.

Table 17. Stability of OsHV-1 in seawater at 20^0C

Inoculum	Bioassay method	Storage time (d)	Replicate	n	Cumulative mortality (%)	qPCR result		Bioassay outcome
						Dead oysters	Live oysters day 7	
Seawater containing OsHV-1	Immersion spat	0	1	23	57	Positive	Positive	Positive
			2	24	50	Positive	Positive	Positive
		1	1	20	0	No sample	Positive	Positive
			2	20	25	Positive	Positive	Positive
		2	1	20	5	Positive	Positive	Positive
			2	20	5	Positive	Negative	Positive
		3	1	24	4	Negative	Negative	Negative
			2	24	4	Inconclusive	Negative	Negative
		5	1	20	5	Not tested	Negative	Negative
			2	19	5	Not tested	Negative	Negative
		7	1	22	0	No sample	Negative	Negative
			2	21	0	No sample	Negative	Negative
	Injection juveniles	7	1	8	0	No sample	Negative	Negative
Negative control seawater	Immersion spat	0	1	24	0	No sample	Negative	Negative
			2	25	0	No sample	Negative	Negative
		7	1	20	0	No sample	Negative	Negative
			2	21	0	No sample	Negative	Negative
	Injection juveniles	7	1	8	0	No sample	Negative	Negative

Table 18. Stability of OsHV-1 in wet oyster tissue (1/10 tissue homogenate) at 20^0C.

Inoculum	Method	Storage time (d)	Replicate	n	Cumulative mortality (%)	qPCR		Bioassay outcome
						Dead oysters	Live oysters day 7	
OsHV-1 in oyster tissue	Immersion spat	0	1	25	8	Positive	Negative	Positive
			2	26	38	Positive	Positive	Positive
		1	1	20	10	Positive	Negative	Positive
			2	20	45	Positive	Positive	Positive
		3	1	32	9	Positive	Positive	Positive
			2	26	12	Positive	Negative	Positive
		7	1	20	15	Positive	Negative	Positive
			2	22	14	Positive	Negative	Positive
	Injection juveniles	7	1	8	88	Positive (n=7)	Positive (n=1)	Positive
Negative control (OsHV-1 free oyster tissue)	Immersion spat	0	1	25	0	No sample	Negative	Negative
		1	1	22	0	No sample	Negative	Negative
		3	1	25	0	No sample	Negative	Negative
		7	1	22	0	No sample	Negative	Negative
	Injection juveniles	7	1	8	0	No sample	Negative	Negative

Table 19. Stability of OsHV-1 in dry oyster tissue at 20^0C (reconstituted as a 1/10 tissue homogenate for testing).

Inoculum	Method	Storage time (d)	Replicate	n	Cumulative mortality (%)	qPCR		Bioassay outcome
						Dead oysters	Live at day 7	
OsHV-1 in oyster tissue (prepared from dried tissue)	Immersion spat	0	1	25	36	Positive	Positive	Positive
			2	24	21	Positive	Positive	Positive
		1	1	20	30	Positive	Positive	Positive
			2	20	25	Positive	Positive	Positive
		3	1	26	15	Positive	Positive	Positive
			2	24	13	Positive	Negative	Positive
		7	1	20	0	No sample	Negative	Negative
			2	21	0	No sample	Negative	Negative
	Injection juveniles	7	1	8	63	Positive (n=5)	Negative (n=3)	Positive
Negative control (dried oyster tissue)	Immersion spat	0	1	25	0	No sample	Negative	Negative
		1	1	19	0	No sample	Negative	Negative
		3	1	22	0	No sample	Negative	Negative
		7	1	20	0	No sample	Negative	Negative
	Injection juveniles	7	1	8	0	No sample	Negative (8/8)	Negative

ii) On farm upweller trials. Seven experiments were conducted between April 2013 and May 2014 inclusive. The treatments examined in each trial are shown in Table 4. Each trial was conducted during the window of infection for disease caused by OsHV-1 in the Hawkesbury River. As the pattern of infection at any specific site in the river is unpredictable, trials were repeated throughout the second season (summer 2013-2014) to obtain repeated test outcomes for treatments that appeared to be beneficial. Treatments were dropped from the design if results indicated that they did not prevent mortality of spat. Water aged for 48 hours was included as a treatment in 6 of 7 trials. UV irradiation treatment was included in Trials 1 and 2 only as it was shown not to be necessary in the second trial. The chiller treatment was used only once. The duration of each trial was determined by the appearance of mortality or by the reduction of the number of spat due to sampling.

Mortality patterns. In Trials 1, 2, 3 and 4 severe mortality occurred in both the river controls and the upweller controls. Although spat in the river control died in Trials 6 and 7, no mortality occurred in spat in control upwellers in trials in Trials 5, 6 and 7, therefore no conclusive data were obtained on the stability of the virus in trials 5, 6 and 7. The patterns of cumulative mortality in each trial are shown in Figure 31.

In trials 1, 2, 3 and 4 there were striking differences in mortality patterns of spat, and consistent results were obtained between trials (Table 20). There was high mortality in controls; it developed suddenly and peaked within a few days. No mortalities were observed in spat that were kept in water that had been aged for 48 hours. No mortalities were observed in spat that were kept in water filtered to 5 µm. In the third trial spat kept in water filtered to 30 µm survived but in the fourth trial 30 µm filtration failed to prevent mortality. Filtration to 55 µm did not prevent mortality in the third trial. UV irradiation of water did not appear to be necessary to prevent mortality after filtration of water to 5 µm, based on a comparison of filtration treatments with and without UV irradiation in the second trial.

Table 20. Outline of upweller trial design and the results of qPCR tests to detect OsHV-1 DNA. Data are the greatest viral load (copies per mg) detected and the highest number of pools with detectable DNA (in parentheses) on any day. Viral loads are the average of 4 pools (trial 1) or 5 pools (trials 2 – 7) of spat (pools with no DNA detected were excluded from the calculation of the average). Treatments in which mortalities were observed are shown in bold. The frequency of detection of OsHV-1 DNA (number of test days) in treatments without mortality is given in the results.

Trial	Start date	Duration of trial (days)	Mortality first observed (day)	Interval between PCR tests	OsHV-1 DNA first detected (day)	Maximum viral load (number of positive pools)								
						River control	Upweller control	Aged water	Filtration µm					Chilled water
									100/5+UV	100/5	55/5	100/30	55	
1	6 Apr 13	39	20	3-4 days	17	**$5.4x10^5$ (4)**	**$6.3x10^5$ (4)**	BLOQ (2^1)	BLOQ (1^1)	-	-	-	-	**$4.2x10^5$ (3)**
2	30 Oct 13	28	15	1 day	12	**$1.9x10^6$ (5)**	**$1.7x10^6$ (5)**	BLOQ (4^1)	BLOQ (3^2)	BLOQ (1^1)	-	-	-	-
3	27 Nov 13	28	20	1 day	15	**$2.8x10^5$ (5)**	**$2.1x10^5$ (5)**	BLOQ (2^1)	-	-	-	BLOQ (1^1)	**$8.3x10^4$ (5)**	-
4	15 Jan 14	15	14	1-4 days	11	**$2.0x10^5$ (5)**	**$4.5x10^5$ (5)**	BLOQ (1)	-	-	BLOQ (1^1)	**$6.0x10^4$ (5)**	-	-
5	26 Feb 14	28	Nil	3-4 days	28	Not detected	Not detected	Not detected	-	BLOQ (1^1)	Not detected	-	-	-
6	26 Mar 14	49	24	4-5 days	22	**$2.0x10^5$ (5)**	$5.5x10^3$ (1)	Not detected	-	Not detected	BLOQ (1^1)	-	-	-
7	14 May 14	15	15	4-5 days	15	**$6.5x10^4$ (5)**	Not detected	-	-	-	Not detected	-	-	-

-, not included in trial

BLOQ, DNA detected but level below limit of quantification

[1] all pools with detectable DNA were inconclusive – i.e only one of two replicate DNA extracts yielded a Ct

[2] 2 of 3 pools were inconclusive

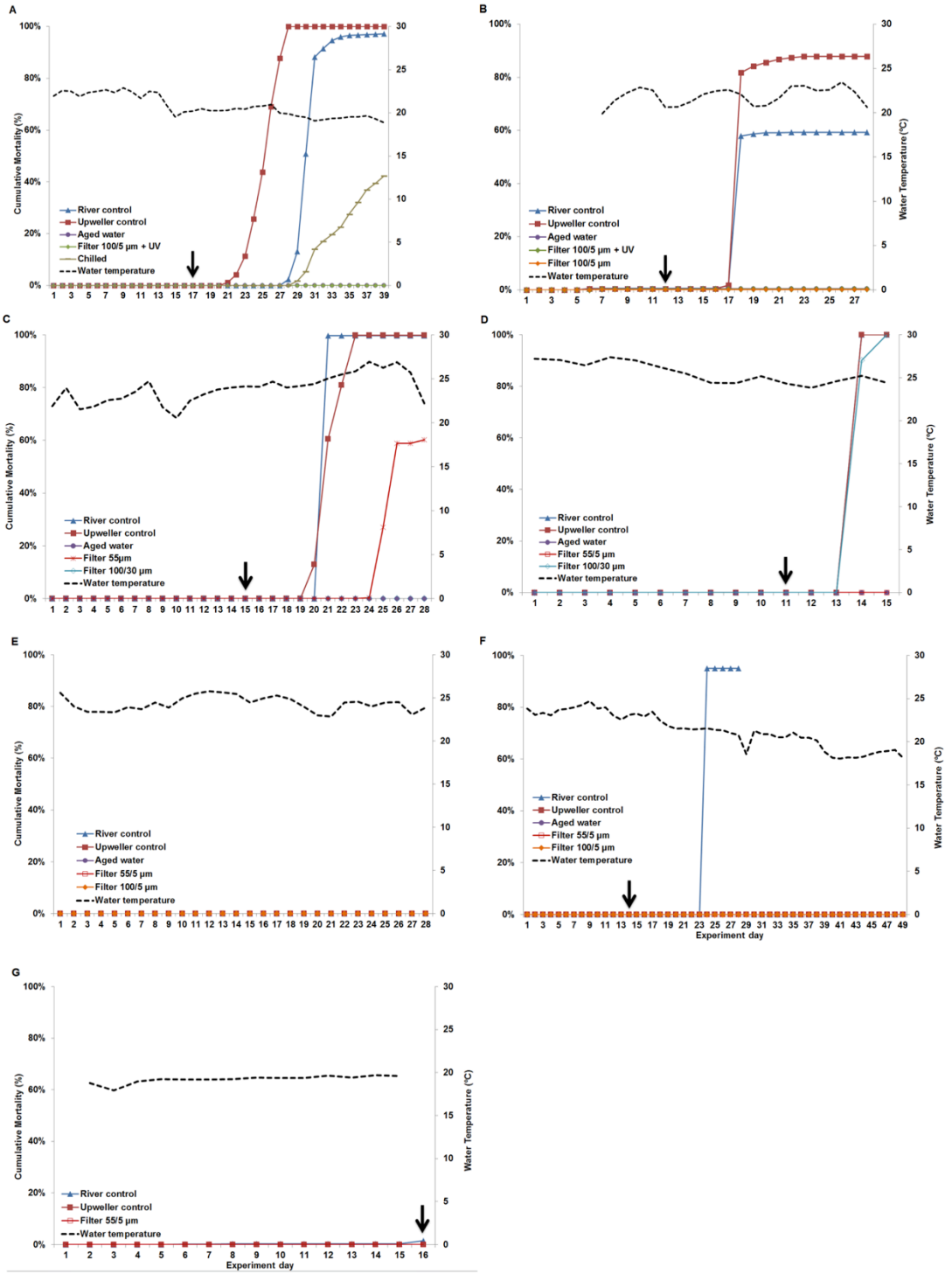


Figure 31. Mortality patterns of spat in each treatment group and average daily water temperature. Panel A, trial 1; panel B, trial 2; panel C, trial 3; panel D, trial 4; panel E, trial 5; panel F, trial 6; panel G, trial 7. The arrow indicates the day when OsHV-1 DNA was first detected in spat from the River Control and/or the Upweller Control. Temperatures are for probes in the control upweller tank.

Strategy for testing for OsHV-1. Tests to detect OsHV-1 were conducted on samples collected from each treatment each day in trials 2 and 3, but for trials 1, 4, 5, 6 and 7 the tests for OsHV-1 were done on samples from every 2nd to 4th day (Table 20). In treatment groups where there was high mortality ($\geq$ 60%), the viral load exceeded 10^5 copies per mg of spat tissue prior to or at the time of mortality, and all of the pools of spat that were tested contained detectable OsHV-1 DNA, suggesting a high prevalence of infection. This applied to the River Control in trials 1, 2, 3, 4 and 6, the Upweller Control in trials 1, 2, 3, 4, the 55 µm Filter treatment in trial 3 and the 100/30 µm Filter treatment in trial 4. The spat in the River Control had just begun to die on the last day of trial 7 and they contained 6.5 x 10^4 DNA copies per mg averaged over the 5 pools.

Among trials, OsHV-1 DNA was detected up to 5 days before the onset of mortality in the River Control and Upweller Control; this time was determined accurately in trials 2 and 3 where daily samples were collected and tested, and represents the incubation period for the disease. However, when the infection was first detected in these treatments the prevalence was low (1 of the pools positive) and viral load was also low, below limits of detection (BLOD) or below limits of quantification (BLOQ) to 10^2 copies per mg.

In the Chiller treatment in trial 1 there was a delay in the onset of mortality and a more progressive increase in mortality than in the control groups; cumulative mortality reached only 42% (Figure 31). OsHV-1 DNA was first detected on day 25 and mortality began on day 29 when viral load exceeded 10^5 copies per mg.

In treatments in which there was no mortality, OsHV-1 DNA was either not detected, or was detected intermittently, typically at low prevalence and low viral load (below the level of quantification of the qPCR assay) (Table 20). The only exception to this was in trial 6 where spat in the Upweller Control remained apparently healthy throughout the 49 day experiment but on day 14 one of 5 pools contained 5.5 x 10^3 copies per mg. Further evidence of low viral load was the inconsistent detection of OsHV-1 template DNA in the two replicate assays conducted on each DNA extract (Table 20) (Bustin, et al., 2009). Intermittent detection of OsHV-1 DNA was common: in trial 1 in the Aged Water and 100/5 µm Filter + UV treatments, OsHV-1 DNA was detected in spat on day 25 but not before that (i.e. on days 2, 5, 9, 13, 17 or 21), or after that (i.e. on days 29, 33 or 37). In trial 2, OsHV-1 DNA was detected in spat in the 100/5 µm Filter + UV treatment group on only 4 days out of the 28 for which samples were collected and tests conducted. Similarly for the 100/5 µm Filter treatment, it was for 3 test days out of 28, and for Aged Water 4 test days out of 28. Corresponding results for trial 3 were Aged Water 3 of 28 test days; 100/30 µm Filter 1 of 28 test days. In trial 5 the only detection of OsHV-1 DNA over 8 test days was from spat in the 100/5 µm Filter treatment on day 15. In trial 6 viral DNA was detected on 1 of 8 test days in the 55/5 µm Filter treatment group, and it trial 7 it was not detected in spat on any of 4 test days.

Growth of oysters. In the first trial there was growth retardation of spat in the aged water and filtered water treatments relative to the river controls and upweller controls, and those in chilled water. The size differential is illustrated in Figure 32. In subsequent experiments similar effects were observed although some growth was observed in all treatments.

Figure 32. Size comparison of spat in control and other treatments prior to the onset of mortality in Trial 1. Left panel, control upweller; middle panel, aged water; right panel filtration with UV.

Physical and chemical means for viral inactivation

The effectiveness of several common methods for disinfection of viruses was assessed. Disinfection treatments were applied to OsHV-1 in two different formats relevant to disease control: a relatively concentrated load of virus in seawater (3.5×10^2 OsHV-1 genome equivalents per µl) and concentrated virus in oyster tissue (2.1×10^5 OsHV-1 genome equivalents per µl). The disinfection treatments were applied according to recommendations of the manufacturer of the product, or commonly used protocols for other viruses of the order *Herpesvirales*. A bioassay was used to assess infectivity of the virus before and after treatment. In controls (prior to disinfection), the number of OsHV-1 genome equivalents injected per oyster was 3.5×10^4 for seawater, and 2.1×10^7 for tissue. These were high doses, sufficient to cause 100% mortality under the conditions of the bioassay.

Several of the chosen disinfection protocols adequately inactivated the high load of OsHV-1 in the test preparations (Table 21). Disinfection was achieved by several physical and chemical procedures: heating to 50^oC; exposure to a high dose of ultraviolet light; a quaternary ammonium compound (QAC, Livingston), Virkon-S (DuPont); sodium hydroxide; iodine; and formalin. Disinfection was not achieved by: heating seawater to 42°C or treating a tissue preparation with Pyroneg, an alkaline detergent. Importantly, the activity of chlorine was susceptible to dissolved organic load. Addition of chlorine to a final concentration of 50 ppm was sufficient to inactivate OsHV-1 in the seawater preparation. However, chlorine disinfection was ineffective in the same seawater preparation when the organic load was increased by addition of 10% foetal bovine serum. A final concentration of 200 ppm chlorine was ineffective for disinfection of OsHV-1 in the oyster tissue preparation.

Table 21. Disinfection of OsHV-1 in seawater and oyster tissues.

Quantities of OsHV-1 are genome equivalents per µl seawater or tissue homogenate, and per mg of pooled mantle and gill tissue of individual oysters in the bioassay.

Treatment ID	Disinfection method	Description	Buffer exchange	Inoculum after treatment (copies/ul)	Bioassay				Outcome
					n	Mortality (%)	qPCR oysters		
							No. positive	Quantity range (copies/mg)	
Disinfection of Preparation 1: OsHV-1 in seawater									
1	No treatment	Positive control	no	1.7×10^2	6	100	6	$1.4 \times 10^5 – 2.9 \times 10^6$	positive
2	No treatment	Positive control	yes	1.9×10^2	3	100	3	$3.0 \times 10^5 – 9.5 \times 10^5$	positive
3	heat	42°C 5 min	no	9.1×10^1	6	83	6 *	9.3×10^2 * – 1.2×10^6	positive
4	UV irradiation	10 min	no	1.4×10^1	5	0	0	0	negative
5	Sodium hypochlorite	50 ppm available chlorine 15 min	yes	1.8×10^2	5	0	0	0	negative
6	Sodium hypochlorite	50 ppm available chlorine 15 min	yes	2.2×10^2	5	100	5	$1.0 \times 10^4 – 1.7 \times 10^6$	positive
7	Virkon	1% w/v 15 min	yes	0	4	0	0	0	negative
Disinfection of OsHV-1 in oyster tissue									
8	No treatment	Positive control OsHV-1	no	9.5×10^4	6	100	6	$3.3 \times 10^4 – 1.1 \times 10^6$	positive
9	No treatment	Positive control, OsHV-1 after buffer exchange	yes	8.6×10^4	8	100	8	$5.7 \times 10^4 – 4.4 \times 10^6$	positive
10	Heat	50°C 5 min	no	6.3×10^4	8	0	0	0	negative
11	Sodium hypochlorite	100 ppm available chlorine 15 min	yes	4.5×10^4	8	100	8	$1.8 \times 10^5 – 1.4 \times 10^6$	positive
12	Sodium hypochlorite	200 ppm available chlorine 15 min	yes	3.5×10^4	8	100	8	$4.1 \times 10^3 – 1.1 \times 10^6$	positive
13	Virkon	Virkon-S (DuPont) 1% w/v, 10 min	yes	1.2×10^2	6	0	0	0	negative
14	Iodine	0.1% available iodine 5 min	yes	6.5×10^4	6	0	0	0	negative
15	Detergent product	Pyroneg alkaline detergent 0.2%, 10 min	yes	3.4×10^4	6	100	6	$1.0 \times 10^5 – 9.2 \times 10^5$	positive
16	Sodium hydroxide	NaOH 20 g/L, 10 min	yes	9.8×10^2	6	0	0	0	negative

Treatment ID	Disinfection method	Description	Buffer exchange	Inoculum after treatment (copies/ul)	Bioassay				Outcome
					n	Mortality (%)	qPCR oysters		
							No. positive	Quantity range (copies/mg)	
17	Formalin	40% formaldehyde solution diluted to 1/25 v/v 30 min	yes	2.2×10^4	6	0	0	0	negative
18	Quaternary ammonium compound (QAC)	Commercial preparation (Livingston) 1/25, 10 min	yes	3.1×10^4	6	0	0	0	negative
Oyster tissue without OsHV-1									
19	Chemical control	Chlorine 200 ppm for 15 min	yes	0	6	0	0	0	negative
20	Chemical control	QAC preparation 1/25 v/v	yes	0	3	0	0	0	negative
21	Chemical control	1/25 v/v 40% Formalin	yes	0	3	0	0	0	negative
22	Chemical control	Pyroneg 0.2 % final concentration	yes	0	3	0	0	0	negative
23	Chemical control	Iodine, 0.1% final concentration	yes	0	3	0	0	0	negative
24	Chemical control	Virkon 1% final concentration	yes	0	3	0	0	0	negative
25	Chemical control	Sodium hydroxide 20 g/L final concentration	yes	0	3	0	0	0	negative

* The lowest viral load was in the oyster that was alive at the completion of the bioassay; the dead oysters had viral loads $>5.3 \times 10^4$ copies/mg.

To develop an infectivity model for POMS suitable for selection of resistant oysters and pathogenesis/environmental research

A) Development of an infectivity model

These were the first experiments to set up a reproducible laboratory based experimental infection model using OsHV-1 µVar in Australia. Successful infection models using intramuscular injection had already been developed in France (Schikorski, et al., 2011a; Schikorski, et al., 2011b) and were used as a basis to develop a "local" infection model.

Experiments 1a and 1b. In experiment 1a oysters infected by intramuscular injection of OsHV-1 suspension had sudden onset of mortality reaching 70% by 48 hours post-injection (pi) (Figure 33). At 72h pi, the cumulative mortality was 90% and reached 97% at 96h pi. Only one infected oyster survived. None of the control oysters died. The viral loads in dead oysters were between 3.7×10^5 and 2.5×10^7 copies mg-1 of tissue (Figure 34). As each oyster from the OsHV-1 group was injected with 100 µL of an inoculum of 1.4×10^6 copies mL-1, the initial dose administered to these individuals was 1.4×10^5 copies. Viral loads measured in the dead individuals were much higher than the initial dose injected, and thus confirm replication of viral particles in oyster tissues (Figure 34). The one survivor had 1.1×10^3 copies mg-1 in its tissues. The viral concentrations in water increased from 9.0×10^4 to 1.8×10^6 copies mL-1 at 24 and 48h pi, respectively, before progressively decreasing to 2.5×10^2 copies mL-1 (Figure 34). A similar pattern of mortality was observed in experiment 1b (Figure 33); all OsHV-1 injected oysters were dead at 144h pi while no mortality was observed in the control group.

Experiment 2. There was a clear dose-response relationship between the quantity of viral particles injected and survival (Figure 33). One control oyster died at 72h pi. Mortality was low in the highest dilution group (10^{-4}) (two oysters died at 144 and 168h pi), while mortalities of 88-96% were observed in the groups injected with pure inoculum or the lowest dilutions (10^{-1} and 10^{-2}) (Figure 33). No virus was detected in the controls or in the oysters injected with the most diluted inoculum. In all the other conditions (P, 10^{-1}, 10^{-2}, 10^{-3}) the viral loads in dead oysters were between 1.1×10^5 and 1.2×10^7 copies mg-1 regardless of the concentration of the inoculum used for the injections, except for one individual injected with inoculum 10^{-2} (5.1×10^4 copies mL-1) sampled at 168h pi (viral load in tissue: 2.5×10^4 copies mg^{-1}) (Figure 35). The viral concentrations in seawater during the first 120h pi were between 5.1×10^3 and 9.6×10^4 copies mL^{-1}, regardless of the concentration of the initial inoculum. A decrease in the viral concentration in seawater was observed towards the end of the experiment (> 120h pi) as the tanks were progressively emptied by removing the dead individuals.

Experiment 3. Results are presented in Figure 33 and Table 22. The viral concentrations measured in the stored inoculums were lower than the concentration measured in the fresh inoculum in July 2012 (1.3×10^6 copies mL^{-1}), the lowest concentrations being found in the inoculums kept at room temperature and 4°C and the highest concentrations being observed in the frozen inoculums (-20 and -80°C) (Table 22). The inoculum kept at +4°C for 3 months induced significant mortality, similar to the fresh inoculum, with cumulative mortality of 94% after 96h (Figure 33) and the viral loads measured in the dead individuals varied between 6.7×10^5 and 3.0×10^7 copies mg^{-1} (Figure 36). Among the five oysters that died up to 72h after having been injected with the inoculum kept at RT, three were negative for viral DNA and two had $<10^2$ copies mg-1 (both were sampled at 48h pi). The -20°C and -80°C groups had mortality of 3% and 7% at 10d pi, respectively but no viral DNA was detected in the dead and live oysters. The injection of the inoculum kept in liquid nitrogen did not induce any mortality and viral DNA was not detected in those oysters. No mortality was observed in the control group (Figure 33). The virus was only detected in the seawater of the tanks corresponding to

the +4°C storage conditions and the concentrations were relatively stable over time, ranging from 2.2 x 10^3 to 7.7 x 10^4 copies mg-1 (Figure 36).

OsHV-1 was not detected in control oysters in any experiment, confirming the absence of cross-contamination during the experiments.

Table 22. Viral concentrations in a fresh inoculum (prepared and analyzed in July 2012) and some aliquots kept at different storage temperature for 3 months (analyzed in October 2012). Results are expressed in OsHV-1 DNA copies per mL.

Fresh inoculum	Room temperature	+4°C	-20°C	-80°C	Liquid nitrogen
1.4 x 10^6	6.4 x 10^2	7.1 x 10^3	3.1 x 10^5	3.1 x 10^5	6.9 x 10^3

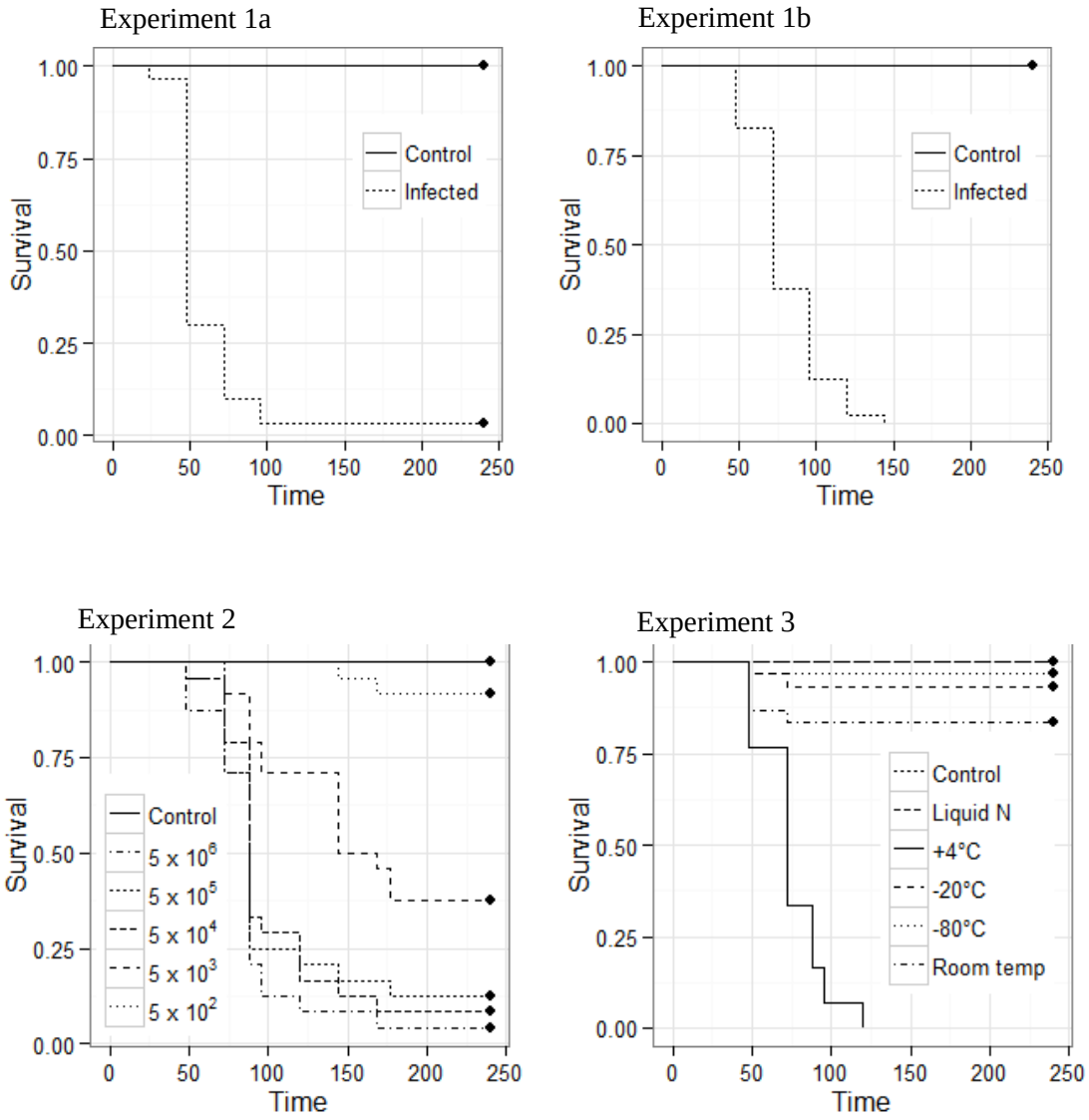


Figure 33. Survival analyses to compare mortality of Pacific oysters *Crassostrea gigas* exposed to different treatments in experiments 1a, 1b, 2 and 3.

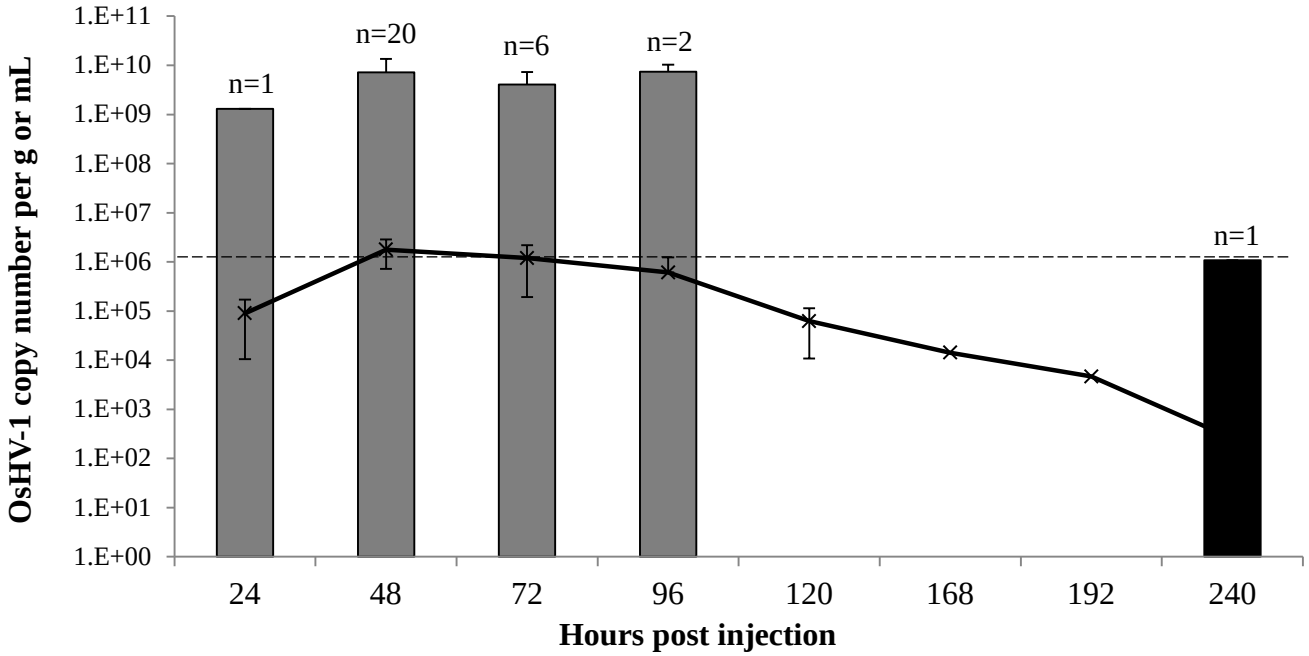


Figure 34. Viral load in oyster gills (bars), seawater (unbroken line) and inoculum (dashed line) in experiment 1a. All oysters were dead individuals, except one live individual sampled at 240h post injection (black bar). Data are copies per g for oyster tissues and copies per mL for seawater and inoculum (mean ± SD). The number of oysters analyzed is shown above each bar.

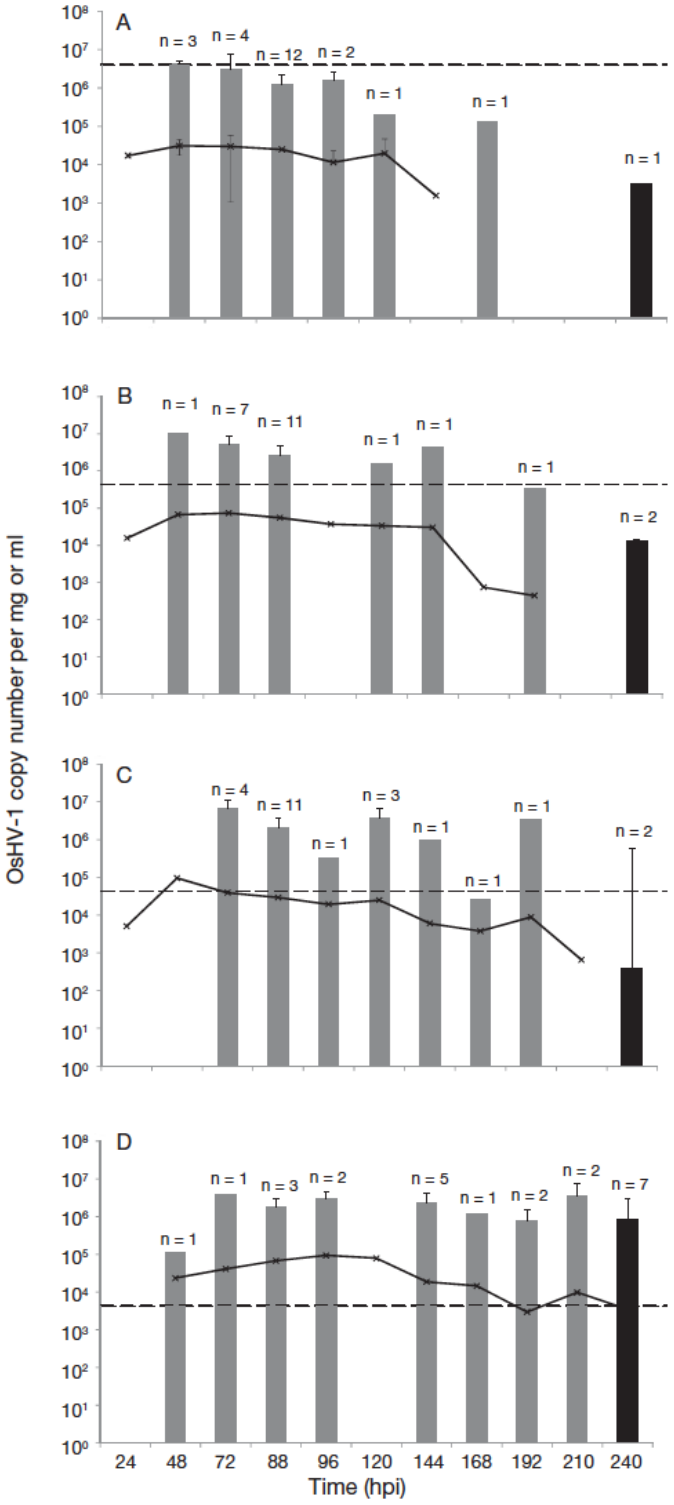


Figure 35. Viral load in oyster gills (bars), seawater (unbroken line) and inoculum (dashed line) in experiment 2. Different concentrations of viral inoculum were used: A) 5.1 x 10^6 B) 5.1 x 10^5, C) 5.1 x 10^4 and D) 5.1 x 10^3 copies mL^{-1}. All oysters were dead, except for the individuals sampled at 240h post injection (black bars). Data are copies per mg for oyster tissues and copies per mL for seawater and inoculum (mean ± SD). The number of oysters analysed is shown above each bar.

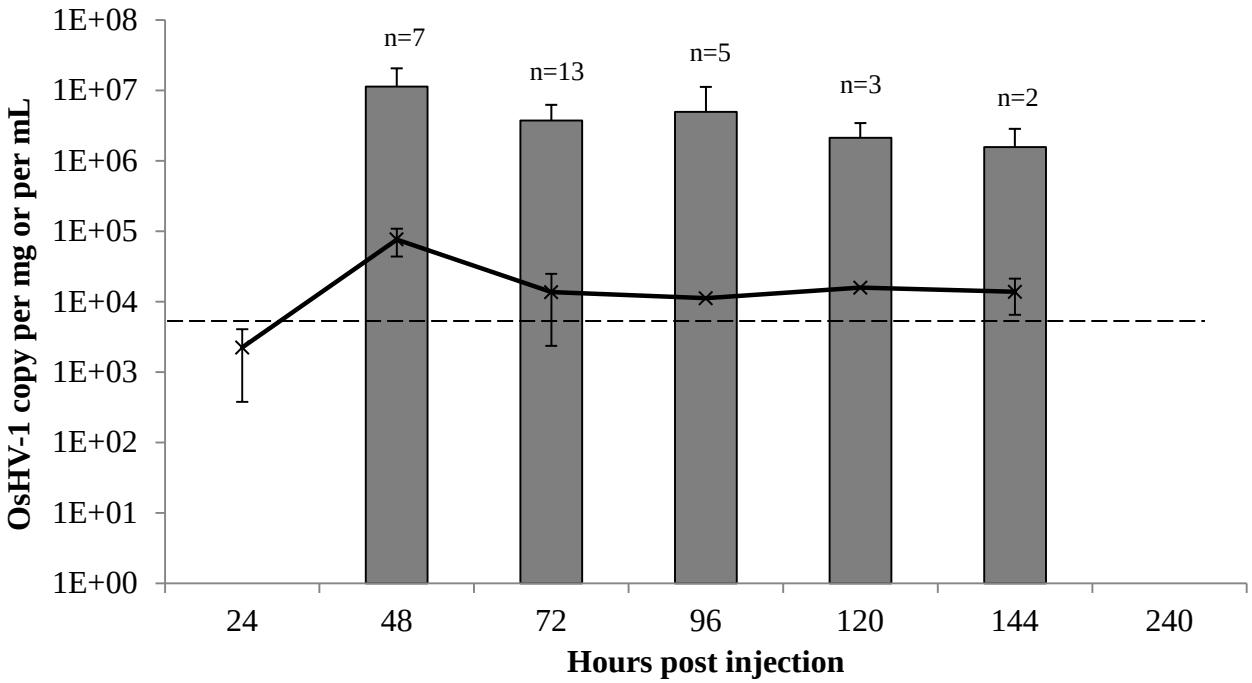


Figure 36. Viral load in oyster gills (bars), seawater (unbroken line) and inoculum (dashed line) in experiment 3. The inoculum was kept at +4°C for three months before use. All oysters were dead individuals. Data are copies per mg for oyster tissues and copies per mL for seawater and inoculum (mean ± SD). The number of oysters analyzed is shown above each bar.

B) Experiments to apply the infectivity model. The infectivity model was adapted and applied for four specific purposes during this project.

i) Cohabitation and feeding. Mortality of the cohabitated oysters began on day four in both the High+Feed and Low+Feed treatments (Figure 37). Cumulative mortality in the High+Feed treatment steeply increased from 1% on day 4 to 100% on day 10. In the Low+Feed treatment cumulative mortality started at 4% on day 4 and increased to 49% by day 10. Mortality of the cohabitated oysters in the High-Feed and Low-Feed treatments began on day 6. Cumulative mortality in the High-Feed treatment increased steeply from 5% on day 6 to 72% on day 10, while cumulative mortality in the Low-Feed treatment increased more gradually from 2% on day 6 to 22% on day 10. No mortality was seen in the Negative+Feed treatment. Mortality of 6% was seen in the Negative-Feed treatment on day 7 then did not change. The cause of mortality was confirmed to be OsHV-1 by qPCR. The pattern of mortality suggested that transmission of OsHV-1 occurred by cohabitation of infected oysters with naïve oysters in the experimental system and that both stocking density and feeding favoured disease expression.

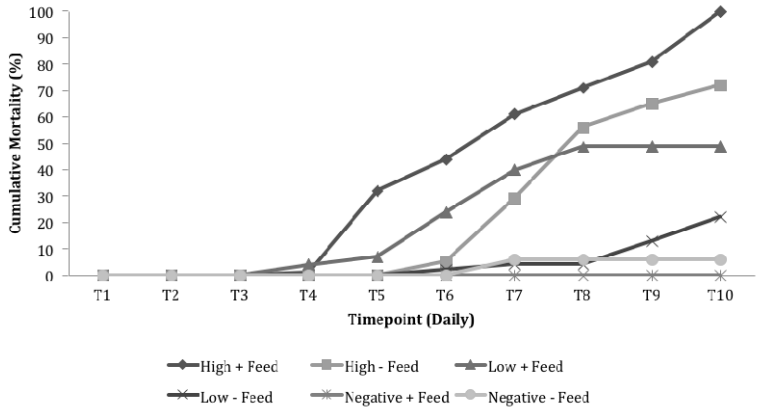


Figure 37. Cumulative mortality in naive oysters cohabitated with donor oysters at high or low stocking rates, with (+) or without (-) feed.

ii) Influence of water temperature on infectivity of OsHV-1. The desired water temperature was achieved in each system with no more than 1°C maximum deviation from the intended temperature at any time. There was no mortality in control oysters at any temperature. Mortality occurred at all temperatures except 14°C (Figure 38). The incubation period was lower and the mortality rate higher for oysters in water maintained at 22°C compared to 18°C. There was a further decrease in the incubation period and decrease in survival to just 6% for oysters maintained in water at 26°C. The viral load in the dead oysters ranged from OsHV-1 genome equivalents per mg of tissue. The viral load in 80% of dead oysters exceeded 1 x 10^4 OsHV-1 genome equivalents per mg of tissue (n=234), with a range of 6.2 x 10^1 – 1.6 x 10^7 for all dead oysters from all treatments. This, and the health of the control oysters injected with a matched tissue homogenate is consistent with OsHV-1 as the primary cause of mortality.

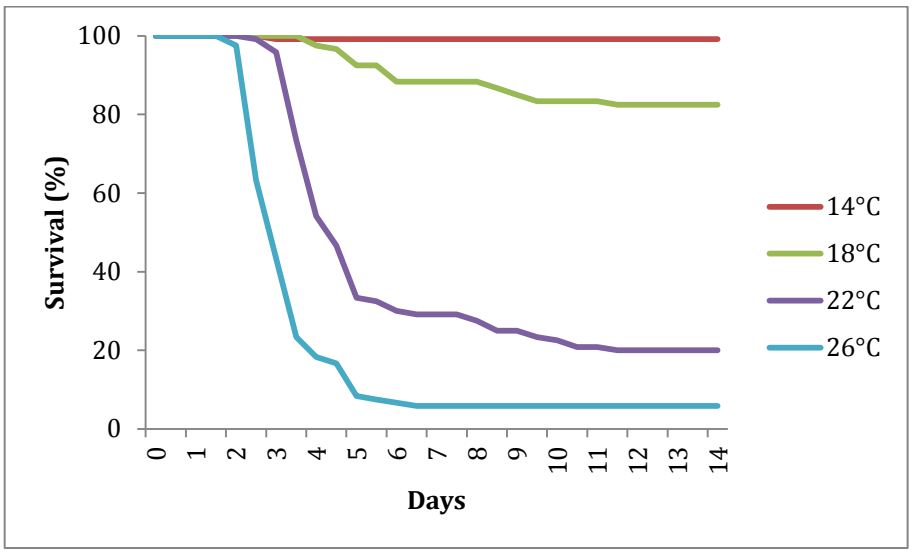


Figure 38. Survival curves for oysters challenged with OsHV-1 that were maintained at different temperatures (n=120). The results are pooled for the 8 and 17 month old oysters and those challenged with a high and low dose of OsHV-1. Each difference in survival at 14 days was significant ($p<0.05$). There was no mortality in control oysters at any of these temperatures (n=12).

iii) Stability of OsHV-1 in the environment. See bioassay results described above
iv) Physical and chemical means for viral inactivation. See bioassay results described above

Discussion

Discussion of results compared with the objectives

Objective 1. To determine/confirm the identity of the one or more variant(s) of Ostreid herpesvirus associated with the recent outbreaks of POMS

Three regions of the genome of OsHV-1 samples were examined in this study, consistent with recent recommendations for multiloccus sequence typing of OsHV-1 (Renault, et al., 2012). These regions, known as *C region, ORF42/-43 region, ORF36-38 region* provide a snapshot of the genome and appear to be useful for molecular epidemiological purposes. Analysis of a sequence alignment identified differences due to insertion/deletion and substitutions when compared with the OsHV-1 reference type and confirmed that the Australian samples were consistent with OsHV-1 µVar as defined by the OIE (OIE, 2013). There was no difference in the consensus sequence in these three regions of the genome between samples from outbreaks in the Georges and Hawkesbury Rivers. A large amount of sequencing work has been conducted recently by researchers in Europe, China and Japan (Martenot, et al., 2013; Shimahara, et al., 2012). The findings suggest that a diversity of OsHV-1 sequences are found in molluscs in endemically infected areas, and that variants of µVar are common. The limited sequence diversity among the isolates from NSW suggests that the virus may have been introduced very recently, i.e. there has been little time for mutation and diversity to arise. Sequencing of additional samples and additional parts of the genome is recommended to confirm this.

Objective 2. To determine the mechanism(s) of transmission of disease

This objective was addressed through field based intervention and observational studies, and through experimental transmission studies. The intervention study was a major controlled experiment in the Georges River to evaluate the uniformity of disease expression in different cultivation structures at three sites. The observational study was an outbreak investigation in the Hawkesbury River. Results from all of the studies will be presented in brief in this section and interpreted in relation to this objective.

Transmission is defined as the passage of infection from one individual to another, and is distinct from spread, which implies passage of infection from one population to another, usually over some distance. While the main objective of this project is to study transmission, some of the data are also relevant to understanding mechanisms of spread.

There are several possible mechanisms of transmission of OsHV-1: direct transmission from oyster to oyster by close contact, indirect transmission from oyster to oyster via water or an unknown vector, or indirect transmission from a remote source in the environment with or without a vector. The patterns of disease occurrence during an outbreak may reveal the mechanism of transmission, and studies in this project were designed with this in mind.

Previous studies by Schikorski, et al. (2011a) demonstrated that OsHV-1 can be transmitted in water by co-habitation of infected and healthy oysters. In FRDC project 2011/053 we provided evidence that OsHV-1 may be transmitted associated with plankton. This was based on the strong spatial clustering of oyster mortalities, at both the macro (bay level) and micro (individual rearing trays) scale, observed during POMS outbreaks in Woolooware Bay. This spatial clustering is similar to that seen in planktonic aggregations and communities, and provides support for the theory that OsHV-1 may be transmitted by a waterborne particulate source (Paul-Pont, et al., 2013b).

Epidemic curves were prepared from the Georges River 2012-2013 field trial data for fine-level assessment of transmission mechanisms. In summary, oyster trays contained 8 segments,

each with 40 oysters, while there were 12 baskets on each longline, each containing 92 oysters. Epidemic curves were made for individual tray segments to look for evidence of transmission within segments and spread from segment to segment. Similarly epidemic curves were prepared for individual baskets looking for evidence of spread between adjacent baskets. There was little evidence of segment to segment or basket to basket spread, and this provides evidence that passage of virus from one oyster to a closely located oyster is probably not important in the genesis of an outbreak. Instead the patterns observed were explained by synchronous point source, or repeated point source exposures. In other words oysters were exposed to virus coming from a more distant source.

The outbreak investigation that was conducted during the 2013 POMS incident in the Hawkesbury River revealed many facets of the mechanisms of transmission; the key findings from this study were that:

i. OsHV-1 was present in the Hawkesbury River for months without leading to significant mortalities. The virus was present from October 2012 at a very low level in some healthy oysters until early January 2013. The first mortalities occurred on the 21st January 2013 with high viral loads consistent considered to be involved in mortality (Oden, et al., 2011). OsHV-1 was detected in Porto Bay as early as the 7th January with no mortality seen prior to the 15th February. This pattern is in accordance with 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., 2013a) and confirms that OsHV-1 is necessary but not sufficient to induce mortality. In New Zealand, OsHV-1 was present in 2005; the first mortalities were detected in April 2010 (Bingham, et al., 2013; Webb, et al., 2007). In summary, when present at low level, the virus appears not to be readily transmitted.

ii. there was no obvious source to initiate the infection other than an environmental source. In both Europe and New Zealand, the spread of POMS between distant areas was enhanced by movements of oysters and/or equipment (Bingham, et al., 2013; Peeler, et al., 2012). However, there were no known movements of *C. gigas* into the Hawkesbury River except for certified spat from a commercial hatchery. These were supplied to many other growing areas in New South Wales and South Australia with no evidence of disease. There were no known introductions of potentially infected farming equipment to the Hawkesbury River. The only other known infected farming area was the Georges River/Botany Bay estuary, but very few Pacific oysters were being grown and there were quarantine orders preventing translocation of oysters. The only other known location of oysters with OsHV-1 infection was Port Jackson but these were 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 was shipping movements, associated with biofouling or ballast water. There is very little commercial shipping in the Hawkesbury River, but there are many pleasure boat movements between the estuary and both Botany Bay and Port Jackson. Therefore pleasure boats were a possible means of spread. A free oceanic source of OsHV-1, transported by current and tide, may also explain the introduction of OsHV-1 to the Hawkesbury River and its transmission.

iii. massive synchronous exposure from a point source was required to explain the onset of disease. 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 (this report). 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. 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. Therefore 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 transmission 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 local oyster to oyster transmission. 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 transmitted to most individuals at the same time (Thrusfield, 2007). Exposure to sub-lethal doses appeared to begin in October 2012 with probable massive re-exposure in January 2013.

iv. Once the outbreak was initiated, the virus was spread widely by tide and current. Millions of oysters died in Mullet Creek and released billions of viral particles into the water over a few days. 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. A hydrodynamic model based on water exchange and tidal flushing demonstrated that the influence of a single tide can be detected as far as 16 km upstream of Mullet Creek entrance, confirming hydrodynamics in the rapid dispersal of viral particles. As an effective infection depends on the dose (Petton, et al., 2013; Schikorski, et al., 2011a) it is likely that dilution led to the quantity of virus in the water in the upstream sites (Marra Marra Creek, Coba Bay and Kimmerikong) being too low to lead to significant mortality. The exposure of oysters *via* tidal movements is likely to explain the mass mortality observed in the lower river. Mortality 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 (Pernet, et al., 2012).

v. oyster to oyster transmission was inefficient and did not explain the pattern of disease. In Coba Bay mortalities were detected only in trays which had been transferred from Mullet Creek, with limited local spread to one adjacent lease. Inspections on two occasions revealed lack of further spread. These results suggest that local spread of OsHV-1 from infected oysters to adjacent individuals occurred in the Hawkesbury River but was inefficient and did not lead to a mass mortality event in the upstream sites.

vi. the incubation period for mass mortality was less than 4 days. The history of oyster transfers from Mullet Creek (index case) to other locations revealed this. Transfers on the 19th January to Coba Bay resulted in mortality localised to the transferred trays (observed 10 days later). Transfers to Mooney Mooney Creek on the morning on the 21st January led to mortalities in the transferred trays by the 24th January. Therefore oysters in Mullet Creek were already infected from the 19th January onwards. Transfers from Mullet Creek on the 17th January and earlier did not lead to mortality, suggesting that lethal infection was not present at that time. Overall, these results suggest that mass infection of the oysters farmed at Mullet Creek occurred between the 17th and the 19th January, with a maximum incubation period of 4 days for mass mortality. This is close to the value of 5 days observed in the upweller field trials in the Hawkesbury River in

2013-2014. This is also consistent with results from laboratory infectivity experiments, in which the incubation period has been shown to be as short as 2 days by IM injection, but up to 8 days by cohabitation (Schikorski, et al., 2011a). Two field studies reported a peak in mortality between 5 and 14 days after deployment/exposure (Degremont, et al., 2013; Pernet, et al., 2012) but overall very few data are available about the incubation period under natural conditions.

vii. Positive qPCR results were obtained from many apparently healthy individuals and lease-level oyster populations implying that subclinical infection was common. Low levels of OsHV-1 DNA were found in adult oysters sampled in the upstream growing areas and in Patonga Creek on the 29th January. No mortality was observed at these sites which confirms that OsHV-1 may persist in sub-clinically infected oysters (Arzul, et al., 2002; Degremont, et al., 2013). We observed that infection prevalence and viral loads decreased over time for most leases, confirming that surviving oysters can eliminate viral DNA from their tissues; this has been seen before (Dégremont, 2011; Paul-Pont, et al., 2013a; Pernet, et al., 2012; Petton, et al., 2013). However, it is unclear how long sub-clinically infected oysters would be a reservoir for OsHV-1 and what role they might play in subsequent infections. There is also some evidence that sub-clinically infected oysters may transmit the disease after reactivation of the virus (Degremont, et al., 2013). Further research 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, and whether persistent infection rather than repeated infection is the reason for positive tests for OsHV-1 in oyster populations over periods of time.

viii. The mortality rate was significantly age-dependent rather than size-dependent at the downstream sites. All spat supplied to oyster farmers in the Hawkesbury River were supplied as 2.2 mm single seed in April or October 2012, but most had grown considerably by January 2013. Extreme mortality rates occurred in downstream nursery areas (Mullet Creek, Mooney Mooney Creek and Porto Bay) which contained millions of spat. This observation is consistent with those Europe (EFSA, 2010).

The results of the infectivity model experiments demonstrate that disease can be induced by partially purified OsHV-1 from naturally infected oysters and did not require additional co-factors such as exogenous infectious agents to induce disease. The cohabitation experiment demonstrated that transmission of disease between oysters in a laboratory environment can occur where the water contains only the by-products of the oyster inhabitants and artificial feed. Both dose of OsHV-1 (donor oyster stocking density) and the availability of feed were positively correlated with the onset and rate of development of POMS. However, in the field, proximate oyster to oyster transmission was not responsible for evolution of an outbreak. Further investigation is required to determine the important factors in the field that modulate transmission of disease and result in the patterns of disease that are observed under commercial farming conditions.

Collectively these results suggest that OsHV-1 is transmitted to susceptible oysters via water, and that a factor other than the close presence of dead and dying oysters is necessary to initiate a field outbreak. The geographically and temporally clustered nature of disease occurrence is strongly suggestive of an environmental source (reservoir host in the environment rather than an on-farm source) of OsHV-1, and a transmission mechanism involving particulates in the water column. The findings from this work support observations made in a prior study in Woolooware Bay (for FRDC project 2011/053) and the hypothesis of particulate transmission has been published to encourage international studies (Paul-Pont, et al., 2013b).

Objective 3. To determine the major risk factors that contribute to precipitation of disease outbreaks thereby identifying potential risk-mitigation management practices

Based on accumulating evidence the major risk factors for POMS are clearly pathogen, environment, and host related. In the summary that follows the known or suspected risk factors and the evidence for them being risk factors are presented.

Pathogen risk factors

OsHV-1 µVar. OsHV-1 is a necessary factor for initiation of POMS. So far all examples of OsHV-1 that have been sequenced in Australia are consistent with OsHV-1 µVar. OsHV-1 is not sufficient to cause the disease syndrome known as POMS. OsHV-1 can occur in susceptible hosts for variable periods without transmitting to other oysters and without causing mortality. The evidence for this is:

- Data from FRDC project 2011-053 included an observation of subclinical OsHV-1 infection for several months at Site B in Woolooware Bay in 2011-2012.
- In the Georges River 2012-2013 field trial, high viral loads were occasionally recorded between November 2012 and March 2013 but no significant mortality occurred during that time, which suggests that other factors (physiology, environment) play a role in the onset of the mortality event.
- Sentinel oysters placed in the Hawkesbury River prior to the massive outbreak that occurred in January 2013 were exposed to OsHV-1 as early as October 2012. OsHV-1 was detected in trace amounts in October 2012, and then in more substantial quantities in the absence of mortality in early January 2013, with onset of the epizootic on 21 January 2013. The prevalence of subclinical infection did not increase prior to the epizootic.

Environment risk factors

Site/location of lease. In both 2011-2012 and 2012-2013 the occurrence of OsHV-1 associated mortality in Woolooware Bay was variable between the three sites that were studied intensively. Substantial mortalities were consistently detected first at sites A and C in November. Site B remained unaffected for months, until February 2012 in the first season and December 2012 in the second season. These observations suggest variations in exposure to the virus between sites and between years. They suggest that the source of exposure is external to any given site and that exposure is non-uniform within Woolooware Bay. The results of the window of infection experiments lead to similar conclusions that OsHV-1 exposure is non-uniform within an infected estuary. Some sites remained unaffected for >2 years, for example Patonga Creek. This has major implications for surveillance and for prediction of risk.

Reservoir hosts. Presence of OsHV-1 in reservoir hosts may be a risk factor. OsHV-1 has been detected in a proportion of wild Pacific oysters, Sydney rock oysters and several other mollusc species. These samples were collected from areas in which there have been severe natural outbreaks of POMS. However, it is unclear whether there is any link between wild reservoirs of OsHV-1 and disease outbreaks on farms. It is possible that the wild molluscs became infected due to exposure to virus released from infected cultivated oysters. It is also unclear whether the virus that has been detected in wild molluscs is viable and infectious.

Growing height/immersion time. A higher growing height was beneficial for survival of adult oysters as the hazard of death that was 2.1 to 2.5 fold greater at low height (L) compared to high height (H) (Table 4). Overall the final cumulative mortality over the summer risk period was reduced by about 50% when adult oysters were raised to a high height, and this confirmed the results obtained in 2011-2012. However the final mortality figures for spat remained extremely high (> 70%), as were the mortalities observed in all baskets (F, H, HH)

although reduced immersion time appeared to delay the onset of OsHV-1 associated mortality in spat (Figures 2A-2C). These results demonstrate that raising the growing height cannot be a practical option for increasing spat survival. The prevalence of OsHV-1 infection was significantly lower in adults kept at high height compared to low height suggesting that adult oysters kept at high height avoided infection, probably by reduced exposure due to the lower immersion time. However, regardless of cultivation height, spat had a similar prevalence of infection suggesting that spat were unable to avoid the infection even with reduced immersion time. Viral load in those oysters that became infected did not differ between heights. This probably means that once infected to a certain threshold dose, the replication of virus within the host was not affected by growing height. Growing height in the intertidal zone is a proxy for time spent in the water exposed to the risk of contact with the virus and for the time spent feeding. As OsHV-1 is a water-borne infection it is most likely that exposure cannot be avoided completely, but the probability of exposure to a lethal dose of virus might be reduced by reducing the time spent immersed in seawater. This was first noted in FRDC project 2011/053 and has been inferred in a risk factor study based on an oyster farmer questionnaire survey conducted in Ireland (Peeler, et al., 2012).

Cultivation structure. In the Hawkesbury River outbreak, oysters in all types of cultivation system succumbed to POMS. In the Georges River 2012-2013 field trial baskets did not appear to offer any advantage over trays, and the observation from the 2011-2012 field trial that spat in sub-tidal floating baskets were less affected by OsHV-1 than spat in inter-tidal trays (Paul-Pont, et al., 2013a) was not confirmed in 2012-2013. Spat of the same age reared at the same height showed similar cumulative mortality regardless of the cultivation system (trays *vs.* hanging baskets). However, the infection prevalence in pools of live oysters across all time points in the trial was significantly lower in baskets (27.8%) than in trays (40%), regardless of the height. This difference could be related to a difference in (i) oyster density, (ii) oyster movements (contact, collision) affecting the frequency of valve opening/closing activity, and (iii) small scale dynamics inside the cultivation structures. After a mortality event reductions in prevalence and viral loads were observed over time, suggesting clearance of the virus from infected oysters (as the reduction in prevalence of infection was not due to the death of infected oysters). Capacity to rapidly eliminate the viral DNA may be linked with virus resistance and survival in oysters (Pernet, et al., 2014), which is consistent with our observations in both 2011-2012 and 2012-2013.

Density of cultivation. This was not studied in this project. Conventional understanding is that hydrodynamic connectivity, density of infected animals and the size of the susceptible population (naive Pacific oysters) constitute drivers for epizootics in aquaculture (Gustafson, et al., 2007; Kristoffersen, et al., 2009; Petton, et al., 2013; Salama, Murray, 2013). Aquaculture practices, leading to high-density populations of hosts, create suitable conditions for epizootics. Close contact between animals facilitates infection and disease transmission, augmented by high quantities of viral particles released by the large number of infected hosts (Pernet, et al., 2012). In comparison with French farming sites, the density of cultured Pacific oysters is much lower in NSW coastal areas and especially in Woolooware Bay where there has been no commercial *C. gigas* farming activity since 2010 (Agreste, 2005; 2014; Honkoop, Bayne, 2002; Paul-Pont, et al., 2014). This could lead to the genesis of discontinuous disease epidemics in Woolooware Bay, even though the role of the wild population of Pacific oysters remains unknown.

Presence of non-susceptible bivalve populations. In FRDC project 2011/053 we noted that there were 900,000 adult Sydney Rock oysters (SRO) surrounding site B in Woolooware Bay 2011-2012; these were suspected to have acted as a "buffer" due to (i) their status as a species that is not susceptible to OsHV-1, (ii) their intense filtration activity leading to the potential removal of particle-attached virus from the water, and (iii) the coincidence between their removal from site B and the appearance of OsHV-1 related mortalities in adjacent Pacific oysters (Paul-Pont, et al., 2013a). New stocks of SRO spat were introduced to Woolooware Bay in 2012 as follows: 370,000 in March, 200,000 in September and 1,240,000 in late

October. They were located close to site B. These would mainly have been spat or small juvenile oysters in November-December 2012 and would not have had equivalent filtration capacity to the larger oysters present at Site B the previous season. Therefore the "buffer" effect of SRO remains unclear.

Season. In contrast to France where the disease is believed to remain active throughout the summer, with OsHV-1 associated mortalities continuously observed from May until September, the pattern of mortality in Woolooware Bay and the Hawkesbury River was discontinuous. In the Georges River mortality peaks were consistently observed in November and in March two seasons in a row, and little or no mortality associated with OsHV-1 was detected in between these peaks despite apparently favourable environmentals. In the Hawkesbury the pattern was highly variable within season by site. Two trials have been conducted to determine the seasonal window of infection in both the Georges River and the Hawkesbury River. The trial in 2012-2013 involved deployment of oysters in replicate baskets that were placed on floating long lines at 5 sites in the Hawkesbury River and 3 sites in the Georges river. New spat were placed at each site every 2 weeks, and samples were collected for PCR. Not all sites were impacted by POMS at the same time in the Georges River and in the Hawkesbury River only 2 of 5 sites were affected. The trial in the 2013-2014 season involved 5 sites in the Hawkesbury River and 8 sites in the Georges River and as in the previous season, the mortality was non-uniform: only 2 of 5 sites in the Hawkesbury were affected compared to 6 of 8 in the Georges River. The upweller trials conducted at Mooney Mooney in the Hawkesbury river in 2013-2014 revealed repeated (almost monthly) exposure events during the season. Based on data from these experiments pooled across years and rivers, the window of infection ran from October through to May inclusive. Susceptible oysters placed in these estuaries in these months are at risk of POMS.

Water temperature. A body of European literature clearly identifies water temperature as the main risk factor for OsHV-1 disease expression (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 (Pernet, et al., 2012) others show significant mortalities associated with OsHV-1 at higher temperatures (25-29°C) (Le Deuff, et al., 1996; Petton, et al., 2013; Sauvage, et al., 2009). 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; Garcia, et al., 2011; Sauvage, et al., 2009). This is not clear in the data from NSW. Sudden increases in temperature of 2-3°C over a few days were commonly observed but did not necessarily lead to a mortality event. In the Hawkesbury River sudden variations in water temperature were observed from August 2012 (winter, water temperature below 15°C) to December 2012 (summer, water temperature > 22°C) 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 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.

POMS commences when water temperature is above 19-20^{o}C in Australia. Figure 1 shows mean water temperature for three sites in the Georges River for the period July 2012 to May 2013. POMS occurs annually in this estuary between November and May. In NSW there is a period of nearly 2 months between September and November during which water temperature is above 16^{o}C and disease is absent (shaded area in Figure 39).

The significant role of water temperature in modulating mortality was demonstrated in a laboratory experiment. Oysters were exposed to OsHV-1 under conditions where water temperature was controlled. In these circumstances the mortality was very high at 26°C and 22°C, minimal at 18°C but nil at 14°C. This is consistent with the absence of disease in winter. The powerful effect of water temperature was demonstrated as all environmental factors that were not under the direct influence of temperature were standardised.

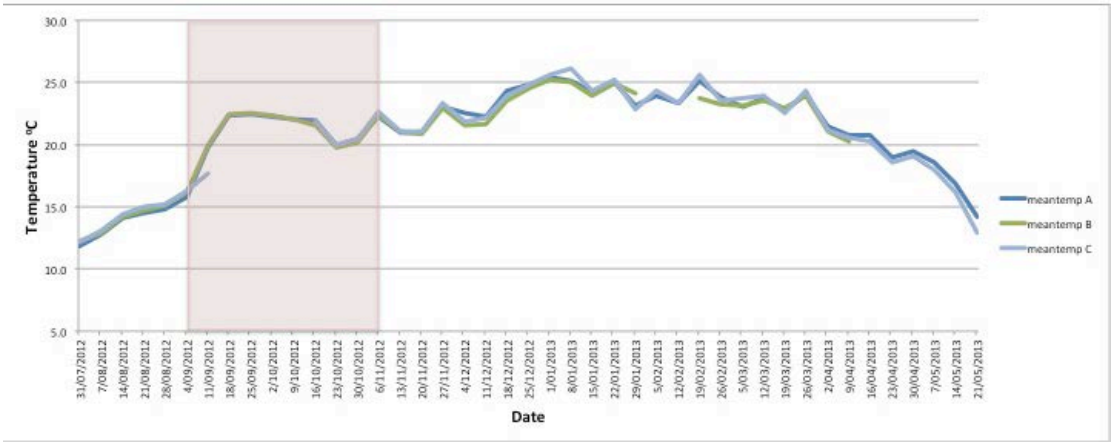


Figure 39. Mean water temperature for three sites in the Georges River for the period July 2012 to May 2013. The shaded area is the period when water temperature is above 16 C, which is the threshold for onset of disease in France, during which disease was not seen in the Georges River.

Other environmental factors.

A long term increase in air temperatures were observed in this study, but were not linked to the outbreaks. Extreme air temperatures were recorded just prior to Hawkesbury River outbreak in January 2013 – these were the highest temperatures on record for the Sydney area. However, extreme air temperatures did not precede the index case in the Georges River.

There were no major differences in the environmental variables water temperature, salinity, or chloropyll-a compared to long term historical data for the months of the index cases or the three previous months.

There is no prior evidence to suggest that salinity plays any important role in OSHV-1 disease expression in Europe (EFSA, 2010). The findings in this project also suggest that salinity did not play a significant role in the onset of mortalities associated with OsHV-1 in NSW. 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 (Gagnaire, et al., 2006).

The parameters chosen in this project do not imply they are the definitive factors, either singly or combined, that 'trigger' the occurrence POMS. They were chosen due to the availability of data and are the best 'surrogates' for the description of oceanic and estuarine processes and conditions.

There are several reports that toxic microalgae species, in particular *Alexandrium minutum* and *Alexandrium catenella*, tend to be present during OsHV-1 related mortalities (Jenkins, et al., 2013; Lassudrie, et al., 2015). However, *Alexandrium* species were not present at the time

of the outbreak in the Hawkesbury River. *Alexandrium* species in the downstream areas of the Hawkesbury tend to be present all year around at extremely low counts, below the threshold that requires oyster harvesting to cease. These levels tend to increase slightly (~600 cell/L) during April and October every year in the Hawkesbury River. *Alexandrium catenella* was present in the Hawkesbury River in October and December prior to the index case (50-300 cells/L). These levels were significantly lower than those reported during the Georges River index case (20,000cells/L) (Jenkins, et al., 2013) and during mortalities in France (in the order of $x10^5$ and $x10^6$ cells/L) (Belin, et al., 2015; Fleury, 2014).

Since 2005, there has been an increase in blooms of *Alexandrium* in south-eastern Australian coastal waters (Farrell, et al., 2013), which have resulted in over 50% of the algal-related shellfish harvest area closures. It is possible therefore, that phytoplankton blooms in the Georges River exacerbated virus spread or interacted in some way to induce the pathology observed. Indeed, the histological changes associated with the presence of PST-producing *Alexandrium* spp., which include haemocyte accumulation in the connective tissue surrounding the gut and digestive tubules (Haberkorn, et al., 2010) were also observed in the Woolooware Bay oysters (Jenkins, et al., 2013). Further analysis of biotic data is recommended.

Host risk factors

Age. Age was identified as an important determinant of mortality based on an intervention study conducted in FRDC project 2011/053 (Paul-Pont 2013b). In the present project we measured the increased susceptibility of spat compared to adults. Spat had 1.7 to 3.6 fold greater hazard of death compared to adult oysters depending on the site and the growing height. This is consistent with overseas studies showing a higher susceptibility of young stages (larvae, juveniles) in comparison with older oysters (Burge, et al., 2006; Burge, et al., 2007; Dégremont, 2011; Dégremont, et al., 2010; Miossec, et al., 2009; Renault, et al., 1994a). Age and size are important risk factors for mortality but are difficult to separate due to correlation between age and size. Within the classes of oysters termed spat and adults there are probably grades of susceptibility based on size/age differences. For example the final cumulative mortality for spat was different in the present trial compared to the 2011/2012 trial in project FRDC project 2011/053 in which 100% of the spat died. This might be related to the younger age (5.5 month old) of the spat in 2011/2012 compared to 2012/2013 (9.5 month old). In the Hawkesbury River epizootic age was a significant risk factor for mortality after allowing for variation due to size. Further controlled experiments are required to determine the interaction between age and size and whether practical applications exist for exploiting age/size based resistance.

Host energy status. It has been suggested that food quality/quantity, which influences host energy reserves, may have major implications for OsHV-1 epizootics (Pernet, et al., 2014). This was not investigated directly in the present study. However, the increased susceptibility of spat compared to adults may relate to the energetic status of young oysters. A decrease in carbohydrate concentration and lipid accumulation has been observed in association with OsHV-1 infection both in field (Pernet, et al., 2010; Pernet, et al., 2014) and laboratory studies (Tamayo, et al., 2014). It was suggested that this decrease was related to (i) the initiation of immune processes, (ii) the synthesis of macromolecules for virus assembly, or (iii) the reduced filtration/feeding activity that is characteristic of infections in bivalves (Flye-Sainte-Marie, et al., 2007). In addition, proteomic studies clearly indicated an increased rate of glycolysis and lipolysis in OsHV-1 challenged oysters (Corporeau, et al., 2014). Young oysters may have limited energy reserves in comparison to older oysters or energy reserves that are mainly mobilized for growth instead of immune defenses, in parallel with higher energy needs (Rico-Villa, et al., 2010). This could make spat more susceptible to the disease, especially at high height where they have less access to food due to shorter immersion times. There was an obvious growth penalty for oysters placed at high height (Figure 5), and this

was especially marked for spat placed at the highest height (HH, + 600mm); presumably these would have had the lowest energy reserves, and may have been the most vulnerable. Thus the worst mortality, which was observed in HH baskets, could be related to starvation or environmental exposure as well as to reduced resistance to OsHV-1 infection. It is noteworthy that regular checks confirmed that OsHV-1 was present at high viral load in dead oysters in all treatment groups, thus excluding/limiting other direct causes for mortality.

Genetic background. There is evidence that resistance to the effects of OsHV-1 µVar infection is heritable (Dégremont, 2011; Dégremont, 2013).

Identification of potential risk mitigation management practices

Based on these research findings it is possible to conceptualise an integrated production system leading to successful farming of *C. gigas* in the face of POMS. Mitigation strategies that can be recommended so far include:

1. Hatchery production of larvae to spat using seawater that has been treated to remove the risk of mass mortality

2. Rearing of susceptible spat in seawater free of OsHV-1 (in a disease-free region or through water treatment).

3. Holding spat in safe regions, restricting their growth, and shipping them for growout when they are older and likely to be more resistant to OsHV-1

4. Using knowledge of the window of infection to place susceptible stock in known-infected estuaries when it is known to be safe to do so

5. Using rapid growth strategies (including optimal management of stock placements according to feed availability and carrying capacity; trials of floating upweller systems) in infected estuaries during the safe period to enable spat to quickly reach a large size likely to be more resistant to OsHV-1

6. Use of a high cultivation height (+300mm) for adult oysters during the summer risk period to reduce mortality to less than 50%.

7. All of the above components are entirely compatible with cultivation of genetically selected POMS-resistant oysters. However these are unlikely to be commercially available before 2018. In the event that genetic resistance is partial rather than complete, the above strategies will be of enduring importance.

8. Use of two stage growth strategies involving cooperation between growers in different estuaries. For example, commercial trials have already commenced to import spat from Tasmania into estuaries in southern NSW where POMS does not occur, to grow them through one or two summers, then to move them for fattening to a POMS-affected estuary in the safe period. This takes advantage of opportunities and constraints that exist for growers in both locations.

   The success of this project is indicated by uptake of these options already by industry. Hatcheries are implementing risk management on incoming water (step 1) and growers are trialing steps 2 to 5. Step 6 is out of reach of growers who do not already have flexible infrastructure or capital to invest, the long line system on high poles being most adaptable. Step 7 requires further development of genetics. Step 8 is in use in the Hawkesbury River.

9. Active surveillance is required in regional oyster farming locations in Australia to both monitor the spread of OsHV-1, and to use as an early warning system for the oyster industry. There may be a substantial lag period between first signs of infection with OsHV-1 in a population and mass mortality, to enable an orderly emergency harvest and rational business decisions (for example whether or not to purchase spat). However, so far it has not been possible to devise a reliable active surveillance system. SAOGA has commissioned a study to do this.

Objective 4. To identify the natural reservoir(s) for the virus

Outbreaks of POMS have been explosive, with sudden appearance of the virus in high quantities among farmed oysters. Where sufficient samples were collected prior to outbreaks, such as in the Hawkesbury River prior to January 2013 and the longitudinal studies in the Georges River in 2011-2012 and 2012-2013, it became clear that trace quantities of OsHV-1 could be detected intermittently prior to an outbreak, often months before. However, there was no evidence of a progressive build up of virus in the farmed population before the outbreak. These observations strongly suggest the presence of an external, environmental source, with intermittent contamination of farmed stock at varying rates over time. The natural reservoir for OsHV-1 exists in the environment because there is seasonal recurrence in farmed oysters stocked into affected waters, and such oysters are free of OsHV-1 when stocked (they are OsHV-1 certified test negative hatchery spat from Tasmania). The environmental source could be local or distant. Given that OsHV-1 is unequivocally a molluscan virus, the possible environmental sources include:

i) local shellfish populations, with local spread to farmed oysters
ii) distant shellfish populations, with virus carried to the estuary by current and tide

In a prior project FRDC 2011/053 we sampled wild molluscs between December 2011 and February 2012 to determine potential reservoir hosts for POMS virus in the environment. Wild molluscs and also other organisms (algae and crustaceans) were sampled (n=30 per species per site) from opportunistically selected sites in Woolooware Bay, Quibray Bay and locations closer to oceanic influence in the Botany Bay/Georges River estuary (Table 22). Detection of OsHV-1 by PCR was performed on pools of 5-10 individuals. All pools tested in qPCR were negative for OsHV-1. These results indicate that the wild organisms were not infected with OsHV-1, or the level of infection was below the detection limit of the qPCR. Barnacles, which often occurred in large populations as overcatch on oyster trays containing OsHV-1 infected oysters from November 2011, were not contaminated/infected with OsHV-1. Sampling was mainly performed in January 2012, outside of the period of a mortality event, and in February 2012, during a mild mortality event at site B. As demonstrated by the mortality pattern of our experimental oysters, the distribution of disease was clustered and highly variable in time and space. Therefore, it is reasonable to assume that the time of collection, as well as the location, are important factors in the detection of OsHV-1 in the environment. We proposed that more intensive wild mollusc sampling at times closer to the mortality events may be required to identify potential reservoir hosts for POMS virus. Consequently, none of the species sampled were ruled out as potential reservoirs for POMS virus at that time (Whittington, et al., 2013).

Table 23. Wild molluscs and other wild species tested for OsHV-1 in FRDC project 2011/053. Extracted from Whittington, et al. (2013)

Date	Site	Common name	Phylum	Class	Family	Potential species	Number of pools/individuals tested
4/12/2011	A - trays	Barnacle	Arthropoda	Cirripedia	Balanidae	Various species	3 pools of 10 individuals
4/12/2011	B - trays	Barnacle	Arthropoda	Cirripedia	Balanidae	Various species	3 pools of 10 individuals
14/12/2011	B - trays	Barnacle	Arthropoda	Cirripedia	Balanidae	Various species	3 pools of 10 individuals
20/12/2011	B - trays	Barnacle	Arthropoda	Cirripedia	Balanidae	Various species	3 pools of 10 individuals
10/01/2012	4	Hairy mussel	Mollusca	Bivalvia	Mytilidae	*Trichomya hirsuta*	4 pools of 5 indviduals
10/01/2012	4	Hermit crab	Arthropoda	Decapod	Paguridae	Uncertain	5 indviduals
10/01/2012	4	Southern mudwhelk	Mollusca	Prosobranch	Batillaridae	*Batillaria australis*	20 individuals
10/01/2012	2	Sydney cockle	Mollusca	Bivalvia	Arcidae	*Anadara trapezia*	6 pools of 5 indviduals
10/01/2012	4	Sydney Rock Oyster	Mollusca	Bivalvia	Ostreidae	*Saccostrea glomerata*	6 pools of 5 indviduals
10/01/2012	6	Sydney Rock Oyster	Mollusca	Bivalvia	Ostreidae	*Saccostrea glomerata*	6 pools of 5 indviduals
10/01/2012	5	Sydney Rock Oyster	Mollusca	Bivalvia	Ostreidae	*Saccostrea glomerata*	6 pools of 5 indviduals
10/01/2012	1	Sydney Rock Oyster	Mollusca	Bivalvia	Ostreidae	*Saccostrea glomerata*	6 pools of 5 indviduals
10/01/2012	7	Sydney Rock Oyster	Mollusca	Bivalvia	Ostreidae	*Saccostrea glomerata*	6 pools of 5 indviduals
10/02/2012	B - trays	Barnacle	Arthropoda	Cirripedia	Balanidae	Various species	3 pools of 10 individuals
13/02/2012	8	Barnacle	Arthropoda	Cirripedia	Balanidae	Various species	3 pools of 10 individuals
13/02/2012	8	Blue periwinkle	Mollusca	Gastropoda	Littorinidae	*Austrolittorina unifasciata*	1 pool of 30 individuals
13/02/2012	5	Brown algae "Neptune's neckless"	Heterokontophyta	/	Hormosiraceae	*Hormosira banksii*	2 pools (~ 50g per pool)
13/02/2012	8	Eastern black crow	Mollusca	Gastropoda	Neritidae	*Nerita melanotragus*	5 pools of 5 individuals
13/02/2012	8	Ischnochitons	Mollusca	Polyplacophora	Ischnochitonidae	Uncertain	2 pools of 15 individuals
13/02/2012	8	Limpet	Mollusca	Gastropoda	Uncertain	Uncertain	3 pools of 10 individuals
13/02/2012	8	Nodular periwinkle	Mollusca	Gastropoda	Littorinidae	*Nodilittorina pyramidalis*	4 pools of 5 individuals
13/02/2012	4	Seaweed 1	Heterokontophyta	/	Sargassaceae	Uncertain	2 pools (~ 50g per pool)
13/02/2012	11	Sydney Rock Oyster	Mollusca	Bivalvia	Ostreidae	*Saccostrea glomerata*	6 pools of 5 indviduals
13/02/2012	10	Sydney Rock Oyster	Mollusca	Bivalvia	Ostreidae	*Saccostrea glomerata*	6 pools of 5 indviduals
13/02/2012	8	Tunicate	Chordata	Ascidiacea	Ascidiidae	Uncertain	2 pools of 5 individuals

Studies of environmental reservoirs of pathogens are inherently difficult, particularly in terms of ensuring that samples are representative. In this study a much more intensive sampling program was undertaken than in the previous project. Wild Pacific oysters and wild Sydney rock oysters from several locations contained detectable amounts of OsHV-1. Overall about 37% of the pooled samples of Pacific oysters were positive, compared to about 23% of pooled samples of Sydney rock oyster. About 14% of pooled or individual samples of other mollusc species (Sydney cockle, blue mussel, hairy mussel, Australian mud whelk, periwinkles), barnacles cnidarians, and other types of samples such as seaweed and sediments also contained detectable OsHV-1. However, viral loads in positive samples were very low. Consequently the significance of these findings for the epidemiology of OsHV-1 in farmed oysters is unknown. It is possible that the virus detected in these samples was merely filtered from water (in the case of bivalves) or ingested by or attached to the samples that were examined. It seems unlikely that the virus could actually infect such a wide range of species given the generally accepted high degree of host specificity displayed by herpesviruses. Furthermore it would not be possible to test the viability of the virus detected because of the lack of a cell culture system, and because of the very low viral load in the samples, transmission trials using a bioassay could not be undertaken. Further observational research is required to determine whether there are additional local or more distant environmental sources of OsHV-1.

Objective 5. To determine the stability of the virus in the environment

In the present study we determined that OsHV-1 remained stable and infective for less than 48 hours at 20^{o}C in seawater. OsHV-1 is a member of the order Herpesvirales (Davison, et al., 2009); it is a double stranded DNA virus with a lipid envelope (Hwang, et al., 2013; Renault, et al., 1994b). In general enveloped viruses are less resistant than non-enveloped viruses to damage due to environmental exposure (Quinn, Markey, 2001). The only prior data available on the stability of a pathogenic marine herpesvirus concerned abalone herpesvirus AbHV, which was stored in seawater under ideal conditions in a laboratory; it retained infectivity and virulence for abalone for only a few days at 15 °C (Corbeil, et al., 2012a). The large enveloped double stranded DNA virus white spot syndrome virus, a pathogen of shrimp, was infective for up to 12 days in seawater (Satheesh Kumar, et al., 2013). In freshwater, koi herpesvirus lost infectivity within approximately 3 days at temperatures above 15^{o}C (Shimizu,

et al., 2006). Studies of other aquatic pathogenic viruses such as viral hemorrhagic septicemia virus, suggest that factors such as temperature and salinity are likely to have substantial effects on viral survival and infectivity in seawater (Hawley, Garver, 2008). In the case of AbHV, survival was significantly less at 25^{o}C compared to 15^{o}C (Corbeil, et al., 2012a).

The stability of OsHV-1 in seawater has not been examined prior to this study, which involved storage of water without exposure to UV light, and used a much higher concentration of virus than is typically found in naturally contaminated seawater, even in a disease outbreak situation (Evans, et al., 2014). Therefore the stability of the virus is possibly less than that reported here. However, caution is required in the interpretation of these results because organic material is likely to affect persistence of infectivity, as indicated by the longer survival seen in tissues compared to artificial seawater in this study, and it is possible that organic or inorganic material present in natural seawater might provide a protective effect.

The tissue preparations used in this study were realistic with respect to the quantity of virus present, i.e. were consistent with a disease outbreak situation (Paul-Pont, et al., 2014). Dried and moist tissue from oysters acutely infected with OsHV-1 remained infectious for at least one week at 20^{0}C, and the end point for duration of viral survival was not determined in this study. Paul-Pont, et al. (2015) reported some stability data for the virus in homogenised and filter purified oyster tissue placed in the laboratory: infectivity persisted for at least 3 months at 4^{0}C but not at room temperature. In a more recent study (Martenot, et al., 2015), the stability of OsHV-1 in tissue homogenates at 15 and 25^{o}C was compared using an injection bioassay; OsHV-1 µVar remained infectious and induced mortality for up to 33 h at 25 °C and 54 h at 16 °C. Unfortunately these authors extrapolated their results to seawater, which was not valid.

The results of the laboratory trials were confirmed in the on-farm experiments in which spat were exposed to water that had been removed from the estuary and stored for 48 hours prior to use (so called “aged water”). The aged water treatment was different to other treatments in this series of experiments as it used water that was intermittently sourced from the river (duration about 3 hours every 2 days to fill the holding tank) while the other treatments were based on a continuous supply of water from the river. It is known that OsHV-1 is not uniformly distributed in river water, either spatially or temporally (Paul-Pont, et al., 2013b). It is possible that water obtained from the river at the times the pump was activated to fill the holding tanks may not actually have contained OsHV-1. For this reason the trial was repeated six times in order to provide many opportunities for the treatment to fail. In five of the six trials in which aged water was included as a treatment, the river control oysters died. However the river controls may not be a complete proxy for the water used to fill the holding tanks as the spatial and temporal clustering of OsHV-1 in the river could be at a very fine level (meters, hours). For example in the first trial the river controls died about a week after the upweller controls, suggesting exposure of the two treatments at different times, despite the close proximity of the submersible pump and the floating basket in the river. Nevertheless, these on-farm experiments, repeated multiple times, seem to confirm the results of the laboratory experiments.

Persistence of the virus on surfaces such as boots, clothing, boats and cultivation equipment could render them reservoirs of infectivity. This needs to be considered in addition to the common consideration of translocation of live oysters in the movement of OsHV-1. Therefore disinfection of OsHV-1 would be important to reduce the chances of spread of the virus on equipment and fomites. This was studied in the next objective.

Objective 6. To identify physical and chemical means for viral inactivation

OsHV-1 is a pathogen of great economic significance globally, but no data on its stability or disinfection are available. Studies to measure the inactivation of viruses are difficult and complex to conduct but the information they provide can be very important for disease control. Guidelines for viral inactivation studies have been published, for example Sattar, Springthorpe (2001) but in the absence of a cell culture system for OsHV-1, such studies are extremely challenging. Without cell culture it is seemingly impossible to undertake replicated trials to measure reduction kinetics through viral titration. However, a precedent for use of a bioassay to study one molluscan virus, abalone herpesvirus was recently published (Corbeil, et al., 2012a).

This is the first study to report data for physical and chemical inactivation of OsHV-1. Consequently, prior to this study generic disinfection measures inferred from studies of similar viruses have been recommended. However, inadequate disinfection is likely without due consideration of the matrix in which the virus is present. In the case of chlorine, a widely available and commonly used disinfectant, 50 ppm available chlorine for 15 min effectively disinfected OsHV-1 in seawater, but the addition of organic material (10% foetal bovine serum) inhibited disinfection at this dose. Disinfection with a dose of 200 ppm available chlorine is commonly quoted for inactivation of viral pathogens for example by the Centres for Disease Control (Rutala, Weber, 2008) (e.g. and doses as low as 1 ppm have been reported for koi herpesvirus (Kasai, et al., 2005; Yoshimizu, et al., 2005). However, chlorine doses in this range were not effective for AbHV (Corbeil, et al., 2012a). The New South Wales Government Aquatic Biosecurity (NSW DPI, 2014) recommend dipping potentially infected materials in a 1% available chlorine solution (10 000 ppm). Such conservatively high doses are required in the absence of data. Cleaning of surfaces to remove organic matter is also required to enable chlorine activity.

Several physical and chemical procedures were confirmed to be effective by this study: heating to 50^{o}C and exposure to a high dose of ultraviolet light; a quaternary ammonium compound (Virkon-S; Livingston); sodium hydroxide; iodine; and formalin. There were also some unsuccessful treatments. For example a surfactant disinfectant product inactivated AbHV (Corbeil, et al., 2012a), but an alkaline detergent (Pyroneg) did not inactivate OsHV-1.

The results of the on-farm experiments described above to study the stability of the virus also revealed that filtration of seawater to 5 µm was effective in preventing mortalities in spat held in land based upwellers. The mechanism may involve removal of OsHV-1 that is attached to particles.

Objective 7. To develop an infectivity model for POMS suitable for selection of resistant oysters and pathogenesis/environmental research

Note: Objective 7. Development of an infectivity model for POMS suitable for selection of resistant oysters was incorporated into a new FRDC project, 2012/052, *Development of a laboratory model for infectious challenge of Pacific Oysters (Crassostrea gigas) with ostreid herpesvirus type-1*. The final report for this project was completed in 2015. The remaining part of Objective 7, related to development of an infectivity model for POMS suitable for pathogenesis/environmental research, is documented in this final report.

This work was the first attempt to achieve a reproducible laboratory-based experimental infection model, or bioassay, using OsHV-1 µVar in Australia. It is the first report to confirm that OsHV-1 µVar remains viable in oyster tissues stored at -80°C, and such oysters are suitable as a source of virus for infection trials.

For all bioassay experiments, intramuscular (IM) injections of pure viral inoculums prepared from Pacific oysters naturally infected with OsHV-1 µVar led to >90% cumulative mortalities

associated with high viral loads in oysters. These results confirm active replication of the virus in experimentally infected animals as previously reported in studies using the French OsHV-1 µVar strain (Schikorski, et al., 2011a; Schikorski, et al., 2011b; Segarra, et al., 2014).

Even though intramuscular injection does not reflect a "natural" infection as it overcomes the natural barriers (mucus, epithelia), this method was preferred over a cohabitation assay for the initial research trials in order to obtain a standardized protocol, i.e. having all oysters infected at the same time with a similar dose of viral particles. The overall kinetics of mortality were similar among all individuals that received a fresh viral inoculum of similar concentration ($1.3 – 5.1 \times 10^6$ copies mL^{-1} with an onset of mortality at 48h pi and a cumulative mortality exceeding 90% after 7 days of exposure. This result is also in accordance with previous studies using the intramuscular injection method and conducted on 7-12 month old Pacific oysters (Schikorski, et al., 2011b; Segarra, et al., 2014). No mortality was observed at 24h pi, which might be due to the time needed for viral replication in oyster tissues leading to irreversible cell damage. Segarra, et al. (2014) detected OsHV-1 mRNAs in tissues as soon as 2, 4 and 18h post injection suggesting that the virus starts replicating early but the first mortalities were only observed at 42h pi.

A dose-response relationship between the concentration of viral particles in the inoculum and the mortality at 10d pi was seen. Cumulative mortality > 85% was observed in oysters injected with concentrated inoculums ($5.1 \times 10^4 – 5.1 \times 10^6$ copies mL^{-1}), while intermediate and insignificant (<10%) cumulative mortalities were recorded in oysters injected with less concentrated inoculum (5.1×10^3 and 5.1×10^2 copies mL^{-1}, respectively). This result allows the identification of an appropriate inoculum concentration ($1\text{-}5 \times 10^3$ copies mL^{-1}) leading to intermediate cumulative mortality, which will be necessary in order to conduct research on the pathophysiological pathways, environmental influences or to measure genetic effects.

There are a number of issues related to a standardized infection model: (i) variation in the host due to the utilization of different oyster batches for each experiment; (ii) experimental parameters such as water renewal, density of infected host and nutrition; and (iii) variation in the viral inoculum (strain, virulence). Natural variation in the resistance to pathogens is commonly observed in Pacific oyster populations and is related to age, physiology, individual genetic basis and life history (Degremont, et al., 2013; Dégremont, 2013; Normand, et al., 2014; Pernet, et al., 2012). The density of the population within a tank may also affect the kinetics of the disease. In the present study a density of 8-10 oysters for 25 L of seawater (without food supply) was used whereas others have used 40 -140 oysters in 20L -25L tanks (Normand, et al., 2014; Schikorski, et al., 2011a). The water renewal regime was also different among studies. Therefore precautions must be taken when comparing data from different studies.

The storage of the viral inoculum at +4°C for a period of 3 months allowed the viral particles to remain highly infectious. In contrast Corbeil, et al. (2012b) demonstrated a loss of infectivity over time when AbHV inoculum was held at +4°C. In addition, AbHV was stable in liquid nitrogen storage for at least 21 months. This is different from our results with OsHV-1. However, as no cryoprotectant was employed, this experiment should be repeated to test of a range of cryoprotectants and protocols for progressive reduction of temperature. This was undertaken and achieved in FRDC project, 2012/052, *Development of a laboratory model for infectious challenge of Pacific Oysters (Crassostrea gigas) with ostreid herpesvirus type-1*.

To our knowledge it is the first time that frozen tissues have been used as a source of infective viral particles for a repeatable infection model (Burge, Friedman, 2012; Green, et al., 2014; Normand, et al., 2014; Renault, et al., 2011; Schikorski, et al., 2011a; Schikorski, et al., 2011b). The use of archived frozen oysters as source of infective particles allows the conduct of experimental infections all year long even when the disease is not active in the field and fresh infected oysters are not available.

The concentration of OsHV-1 measured in the gills and mantle of dead oysters were high and ranged between 1.1 x 10^5 and 3.0 x 10^7 copies mg-1, regardless of the infection trial, the time of collection and the initial concentration of viral particles in the inoculum, with a few exceptions. Regardless of the time post infection and the quantity of viral particles injected into the animal, the quantity of viral products must reach a threshold in oyster tissues before inducing death. This is consistent with previous field and lab studies demonstrating that viral concentrations exceeding 10^6 - 10^7 copies mg-1 are systematically associated with moribund/dying/dead oysters (Oden, et al., 2011; Paul-Pont, et al., 2014; Pepin, et al., 2008). The low viral load found in the survivors is in accordance with Oden, et al. (2011) who defined a viral load threshold of 8.8 x 10^3 copies mg^{-1} below which the risk of mortality due to the disease was nil.

The concentration of viral DNA measured in water remained relatively stable over the first 120h pi and ranged between 2.2 x 10^3 and 1.8 x 10^6 copies mL-1, consistent with other studies (Schikorski, et al., 2011a). Viral loads in water in infection trials conducted in tanks are orders of magnitude higher than those observed in natural water bodies during outbreaks (Evans, et al., 2014).

The intramuscular injection bioassay methodology was able to be adapted to an immersion model, i.e. infection of oysters by bathing them in a solution of virus. This was required to evaluate the stability of the virus as well as to conduct the research on physical and chemical disinfection procedures that is described in this report. The relative sensitivity of the IM injection bioassay and the immersion bioassay were compared prior to the stability experiments. IM injection of juvenile oysters was the most sensitive method of detecting viability of OsHV-1; the limit of detection in the bioassay using immersion of spat was one to two 10-fold dilutions less sensitive. Both the IM injection and immersion approaches will be useful in the future, but for different purposes.

In conclusion, the present work demonstrated that infection trials can be successfully developed under local conditions. However precautions must be taken regarding (i) the source for the viral inoculum and its stability over time; (ii) the characteristics (age, physiology, genetics, life history) of the oysters used for the infection trials; and (iii) the experimental conditions (nutrition, infected host density, water renewal) in order to be able to claim a reproducible and transferable infection model. Conservation of the viral inoculum at +4°C provided the best outcome. A clear dose-response relationship for OsHV-1 was identified and further research is recommended to determine the most appropriate dose of OsHV-1 to use for the development of an infection model for different purposes. Both intramuscular injection of oysters and immersion of oysters in inoculum are valid approaches, but for different purposes. For the first time an infective inoculum was produced from frozen oysters enabling infection trials to be conducted at any time of year. However, we raise a question about the potential for persistence of live OsHV-1 in fresh-frozen commercial oysters, and whether this could be a mean of translocation of virus. Importing frozen oysters from infected areas/states/countries to non-infected locations is a possible means for spread of virus. Oysters may be diverted from the human food chain to be used as bait, or shells may be discarded in coastal waters from pleasure boats. Studies are required in Australia to assess the viability of OsHV-1 in imported fresh-frozen oysters.

The infectivity model developed in this study informed development of a model for the genetic improvement program in project FRDC 2012/012, as well as enabling direct research in the present project on OsHV-1 transmission mechanisms, the effect of water temperature on expression of POMS, the stability of the virus in seawater, and means for physical and chemical inactivation of OsHV-1. In turn, this study was informed by the development of an immersion model for challenge with OsHV-1 developed in project FRDC 2012/052 which was the first to use a standard, cryopreserved inoculum for challenge of oysters without the need for injection.

Objective 8. To address future shortages of technical expertise through the training and supervision of at least 1 PhD student

Ms Olivia Evans, first class honours graduate in Animal and Veterinary Biosciences and recipient of a competitive Australian Government Australian Post Graduate Award scholarship received a top-up scholarship and undertook her PhD candidature at the University of Sydney from August 2012 - August 2015 associated with the team of researchers in this project. Her project investigated aspects of OsHV-1 in seawater, particularly transmission factors. Two honours students, Mr Richard Looi (Bachelor of Veterinary Science degree) and Mr Max de Kantzow (Bachelor of Animal and Veterinary Biosciences degree) were trained in techniques of field and laboratory science during this project.

Conclusion

It is unknown how POMS arose in Australia but the strain of herpesvirus that caused the first outbreaks, in the Georges and Hawkesbury Rivers, is OsHV-1 µVar. It is very similar to the virus in New Zealand. Research in Europe, China and Japan has shown that there are many strains of OsHV-1 µVar. However, the limited sequence diversity among the samples from NSW suggests that the virus may have been introduced to Australia very recently. Sequencing of additional samples and additional parts of the genome is recommended to confirm this.

OsHV-1 µVar is necessary for initiation of POMS, but it is not sufficient on its own. Therefore study of other factors is necessary. OsHV-1 can be transmitted in water between infected and healthy oysters in the laboratory and in FRDC project 2011/053 we provided evidence that OsHV-1 may be transmitted with plankton. In the present study we determined that OsHV-1 remained stable and infective for less than 48 hours at 20^{o}C in seawater in the laboratory and this result was confirmed in on-farm experiments.

Analysis of field outbreaks revealed that passage of virus from one oyster to an adjacent oyster in a tray or basket was probably not important in the genesis of an outbreak. Instead the oysters were exposed to virus coming from a more distant source. This was an unexpected finding.

OsHV-1 was present for months prior to the massive outbreak in the Hawkesbury River, without leading to significant mortalities. There was no evidence that the infection built up in the population over time before triggering the outbreak. Therefore the most likely explanation was synchronous exposure of most oysters at the same time to a high infectious dose from an external source. The incubation period for mass mortality was less than 4 days. Similar to observations in the Georges River, oyster to oyster transmission in trays and between leases in the same bay was inefficient and did not lead to progression of mass mortality. Subclinical infection was common and did not lead to mortality, confirming that OsHV-1 may persist in sub-clinically infected oysters. However, prevalence and viral loads decreased over time, confirming that surviving oysters can eliminate viral DNA from their tissues. Further research 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, and whether persistent infection rather than repeated infection is the reason for positive tests for OsHV-1 in oyster populations over periods of time.

Collectively these results suggest that OsHV-1 is transmitted to susceptible oysters via water, and that a factor other than the close presence of dead and dying oysters is necessary to initiate a field outbreak. The geographically and temporally clustered nature of disease occurrence is strongly suggestive of an environmental source (reservoir host in the environment rather than an on-farm source) of OsHV-1, and a transmission mechanism involving particulates in the water column.

A survey was conducted of potential reservoir hosts. OsHV-1 was detected in wild Pacific oysters (37%), Sydney rock oysters (23%) and several other mollusc species (14%), as well as some barnacles cnidarians, and other types of samples such as seaweed and sediments, but levels of virus were low. It is unclear whether there is any link between wild reservoirs of OsHV-1 and disease outbreaks on farms.

Other environmental risk factors for POMS were identified. These included the site/location. Exposure of oysters to OsHV-1 at any given site or time was highly variable. This has major implications for surveillance and for prediction of risk. A higher growing height was beneficial for survival of adult oysters. Overall the final cumulative mortality over the summer risk period was reduced by about 50% when adult oysters were raised by 300 mm in the intertidal zone. However the final mortality figures for spat remained extremely high and raising the growing height cannot be used for increasing spat survival. Growing height in the intertidal zone is a proxy for time spent in the water exposed to the risk of contact with the virus and for the time spent feeding.

Cultivation structure did not affect the mortalities. In both the Georges and Hawkesbury River outbreaks and field trials, oysters in floating baskets, hanging baskets and trays all succumbed to POMS.

In comparison with French farming sites, the density of cultured Pacific oysters is much lower in NSW coastal areas and especially in Woolooware Bay where there has been no commercial *C. gigas* farming activity since 2010. This could explain why outbreaks in France are continuous in summer, while they are intermittent in NSW.

In contrast to France where the disease is active throughout the summer, the pattern of mortality in Woolooware Bay and the Hawkesbury River was discontinuous. Based on data from field experiments pooled across years and rivers, the window of infection for OsHV-1 began in October and ended in May each year. Susceptible oysters placed in these estuaries in these months would be at risk of POMS, but discrete rather than continuous mortality events occur in this window.

Water temperature may be an important risk factor. In Australia POMS occurred in the warmer months from November onwards when water temperatures were >20^{o}C, which is about 4^{o}C warmer than the threshold in France.

Host risk factors were also important determinants of mortality. Age was unequivocally important. Spat had two to four times the chance of death compared to adults. Further controlled experiments are required to determine whether practical applications exist for exploiting age/size based resistance. It has been suggested that food quality/quantity, which influences host energy reserves, may have major implications for OsHV-1 epizootics. This was not investigated directly in the present study. However, the increased susceptibility of spat compared to adults may relate to the energetic status of young oysters. There is evidence that host resistance to the effects of OsHV-1 µVar infection is heritable, and this will form the basis of a breeding program in Australia.

This is the first study to report data for physical and chemical inactivation of OsHV-1. Several physical and chemical procedures were confirmed to be effective: heating to 50^{o}C and exposure to a high dose of ultraviolet light; a quaternary ammonium compound (Virkon-S; Livingston); sodium hydroxide; iodine; and formalin. There were also some unsuccessful treatments. For example a surfactant disinfectant product inactivated AbHV (Corbeil, et al., 2012a), but an alkaline detergent (Pyroneg) did not inactivate OsHV-1. Even high concentrations of chlorine may be ineffective if organic matter is present.

A reproducible laboratory-based experimental infection model, or bioassay, was developed using OsHV-1 µVar purified from frozen oysters. A dose-response relationship was seen, which will allow for the identification of an appropriate inoculum concentration to conduct research on pathophysiological pathways, environmental influences or to measure genetic effects. The use of archived frozen oysters as source of infective particles allows the conduct of experimental infections even when fresh infected oysters are not available. The intramuscular injection bioassay method was adapted to cohabitation and immersion models. These were used in other parts of the project to study the effects of feeding, and to study viral stability in seawater and disinfection.

Finally, this project enabled training of one PhD student and two honours students in relevant research methods in field and laboratory science.

Implications

It is premature to assess the impact of this research even though findings have been adopted.

Recommendations

Further development

Farming *C. gigas* in the face of POMS requires improvements in both husbandry and genetics. Genetically resistant stock will not be available commercially until 2018, with partial resistance (Appendix 2). We identified that POMS was not continuously present, was transmitted associated with particles, could be removed from seawater by simple treatments and that reduced immersion time of adult oysters benefited survival; however, solutions for culture of juvenile oysters which remain at risk and fully susceptible must still be developed by improving underlying knowledge of disease seasonality periodicity, risk and resistance mechanisms/tolerance. If successful this will enable completion of commercial production cycles in POMS endemic areas. Improved husbandry is needed at all stages of the production cycle. Mortality can be reduced by 50% in adult stock by raising the growing height. However, many growers do not have infrastructure for this.

A strategy is required to enable growers in POMS affected estuaries to manage oysters that must be held through the summer risk period. While large/adult oysters will benefit from placement at a high growing height, this does not benefit young/small oysters. Age was unequivocally important but the age/size at which the benefits of high growing height manifest is not yet clear. Controlled experiments are required to determine whether practical applications exist for exploiting age/size based resistance, in particular to disentangle the correlations between age and size as determinants of natural resistance.

OsHV-1 can be present in wild molluscs in an estuary where POMS occurs. However, it remains unclear whether there is any link between these potential wild reservoirs of OsHV-1 and disease outbreaks on farms. Further observational research is required to determine the environmental sources of OsHV-1 that are suspected to exist to explain mass mortalities. The identification of the main environmental reservoir for OsHV-1 as well as the existence of a potential planktonic vector remains of high priority in order to improve understanding of the ecology of the disease and enable mitigation strategies to be improved.

Further research 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, and whether persistent infection is always cleared or whether it leads to amplification and initiation of outbreaks.

Exposure of oysters to OsHV-1 at any given place or time is highly variable. This has major implications for surveillance and for prediction of risk. Active surveillance is required in regional oyster farming locations in Australia to use as an early warning system. There may be a substantial lag period after first infection in a population prior to the onset of mass mortality. The lag would enable an orderly emergency harvest and rational business decisions (for example whether or not to purchase spat). A reliable active surveillance system needs to be devised based on understanding of the spatial dynamics of OsHV-1 discovered in this project.

Temperature patterns in POMS in Australia differ from those in Europe; the onset of the disease is considerably delayed in NSW relative to the start of the spring-summer in France, where a seasonal threshold water temperature of 16 C has been well documented. In addition a remarkable periodicity of POMS mortality was observed at Mooney Mooney in this project. While temperature may not be the trigger for POMS, it may be a proxy for some other factor that acts as a trigger. All outbreaks of POMS in Australia have occurred in relatively warm water. Further study of the consistency of seasonal patterns of POMS, the periodicity of infection within season, inter-estuary temperature variation, and integration of this information to predict POMS behaviour is warranted. Temperature data are required from estuaries in NSW, Tasmania and South Australia to enable prediction of risk periods in places where POMS has not yet occurred.

Currently there is no strategy available to protect juvenile oysters from POMS. However, field observations from both the Hawkesbury and Georges Rivers are that: i) some oysters become infected with the virus well before an outbreak of POMS, and they appear to be healthy ii) some oysters survive an outbreak of POMS, often with a persistent infection iii) as the proportions seem to be similar, oysters that survive an outbreak may be the same ones that had non-lethal infection prior to the outbreak, and iv) longitudinal field studies have identified cohorts of oysters that survive a POMS outbreak that have subsequently had very low mortality during subsequent outbreaks, suggesting long term immunity. Laboratory and field experiments are recommended to study the conditioning of oysters to withstand a field challenge with OsHV-1. More understanding of exposure, pathogenesis, immunity, tolerance or latency is required. Methods of conditioning could include exposure of spat to the virus, killed virus and/or fractionated viral components, restricting growth rate and using oysters of particular age and size to avoid mortality during juvenile growout. Detailed controlled experiments using the infectivity model and exploring combinations of conditioning method/dose and time are required prior to field validation. Tolerance if confirmed/induced would be aimed to benefit juveniles as well as adult stages in a range of cultivation systems including standard non-elevated rack and rail or floating systems. The overall approach would be consistent with approaches used in other animals to set up tolerance or immunity to specific diseases at population level.

It is important to determine whether water treatments prevent OsHV-1 infection of spat or merely prevent mortality, and whether they can be applied for biosecurity of hatchery effluent. In order to produce OsHV-1–free spat in an endemic region, and have batches certified for movement to disease free regions, greater confidence than that provided in the current project is needed to assess whether the virus can be excluded from hatcheries. Long term trials are required in which spat are exposed to infected estuarine water before (controls) and after water treatment (48 hour aging or 5 µm filtration). Protected oysters would be tested by qPCR to provide 95% confidence of detecting infection at 2% prevalence. To test effluent biosecurity, sentinel oysters would be held in treated water (filtered, aged 48 hours) downstream from control oysters in which POMS develops and their infection and survival would be monitored.

There is a question about the potential for persistence of infective OsHV-1 in fresh-frozen commercial oysters, and whether this could be a means of translocation of virus. In this study we found that the virus survives for years in oysters frozen at -80 °C, but for less than 3 months in oyster homogenates at -20 °C. Depending on the temperatures used by commercial seafood processors, moving frozen oysters from infected areas/states/countries to non-infected locations could be a possible means for spread of virus. Oysters may be diverted from the human food chain to be used as bait, or shells may be discarded in coastal waters from pleasure boats. Studies are required in Australia to assess the viability of OsHV-1 in imported fresh-frozen oysters, including those from New Zealand.

Sequencing of additional samples of OsHV-1 and additional parts of the genome of samples from Australia is recommended to further understand infection and disease spread patterns.

Extension and Adoption

Extension

This project was linked to a national emergency response for POMS, there were many stakeholders and therefore the extension required was complex and multidirectional.

The researchers were in regular contact with local oyster growers in the Hawkesbury, Georges and Shoalhaven Rivers as well as with the entire industry through Oysters Australia, Oysters Tasmania, South Australian Oyster Growers Association and other oyster associations. Select Oyster Company provided information about this research project in its newsletter, as well as links to media as did NSW DPI through the Aquaculture Newsletter. The project team was also in regular contact with growers in New Zealand, Aquaculture New Zealand and institutional stakeholders in both Australia and New Zealand. Richard Whittington was in regular contact with researchers at NSW DPI EMAI, Port Stephens and regulatory officers in NSW DPI Biosecurity and Aquaculture sections, as well as officers in the Department of Agriculture Canberra.

International engagement was also important to bring Australian findings into context with international work, and to look for useful outcomes in the international arena that could be adopted in Australia. Richard Whittington, Paul Hick and Ika Paul-Pont were members of the five person Australian delegation that attended the KBBE Workshop on Disease Mitigation and Prevention in Mollusc Aquaculture in Nantes France from the 11th to 15th of June 2012. Richard Whittington and Paul Hick attended the KBBE workshop on Mollusc Disease Diagnosis in Geelong 21-24 October 2013. In August 2012 Richard Whittington and Ika Paul-Pont visited a number of French oyster research laboratories and field stations to ascertain current research direction and results, funded by the University of Sydney International Program Development Fund. Ika Paul-Pont presented a paper on POMS at the 13th International Conference on Veterinary Epidemiology and Economics, Maastricht Netherlands in August 2012. At this conference Richard Whittington and Dr Paul-Pont had detailed discussions with researchers and consultants from Australia, France and UK on POMS. Richard Whittington and Ika Paul-Pont visited Cawthron Institute New Zealand in April 2013. Richard Whittington visited the Cawthron Institute again in March 2015. Ika Paul-Pont presented papers on POMS research at the Disease and the Environment session at the National Shellfisheries Association meeting in Jacksonville, Florida 29th March – 2nd April 2014, and discussed the issues with US researchers.

The project team was active in presenting findings and research plans directly to Australian industry. Richard Whittington addressed the South Australian Oyster Growers Association meeting on SAMS in April 2013 regarding the POMS outbreak in the Hawkesbury River. Ika Paul-Pont presented a paper on the research findings to the NSW Oyster Conference at Port Stephens 31 July -1 August 2013. Richard Whittington, Paul Hick and Olivia Evans presented papers on POMS research at the World Aquaculture Society Conference Adelaide 7th to 11th June 2014. A feature of the program was a special session for oyster farmers at which research highlights and applications were presented. Richard Whittington presented a research update at the South Australian Oyster Growers Association annual meeting at Streaky Bay on 7th August 2014. Richard Whittington presented a research update at the Oysters Tasmania annual conference at Smithton on 18th October 2014.

The project team actively promoted the need for national coordination of research on POMS. Outcomes from technical visits and research findings from this project were presented to the POMS Research and Development Coordination Group meetings which were convened at EMAI on 10th December 2012, Port Stephens on 2nd August 2013 and Sydney Airport on 10th April 2014. This was an ad hoc committee managed by the Principal Investigators of the three

extant POMS research programs. There was a rotating chairperson. Through this group Richard Whittington led the development of a consensus statement for industry on research outcomes and expected timelines. This is provided as Appendix 2. It was intended that it would be updated periodically.

Richard Whittington was a member of the Pacific Oyster Health Management Working Group, under the auspices of the Sub-Committee for Aquatic Animal Health (SCAAH), Department of Agriculture Canberra. Research updates were provided to this forum, which had industry members.

Extension of findings from this project will continue for the foreseeable future through the website www.oysterhealthsydney.org.

Adoption

There has been immediate adoption of the findings related to safe rearing of spat in hatcheries in both Australia and New Zealand. The researchers have had direct feedback from industry in Tasmania where one leading hatchery has strategic development plans for implementation to protect the water supply from a threat of POMS and another has already implemented a protected water supply. In New Zealand, the leading hatchery has modified treatment of incoming water to prevent a threat from POMS – this is estimated to have resulted in 25% increased availability of safe water with commensurate reduction in costs.

Responses to POMS including diversification of the species under culture have been recommended through this project as part of the national discussion about sustainability of oyster farming and appear to be under adoption nationally.

Project coverage

As POMS was a major emergency issue this project was reported from time to time in newspaper and radio media, sometimes by news aggregation services, and it was not possible for the researchers to track this. Examples of media reports include:

FRDC Fish June 2013. POMS strikes another Australian Estuary

ABC Rural Country Hour. 9 June 2014 interview with Richard Whittington

ABC Landline 15 June 2014. Lengthy interview with Richard Whittington

ABC 7pm TV News 27 July 2014. Hawkesbury River oyster farmers team up with researchers for survival

Bloomberg 14 Dec 2014. Oyster herpes' killer virus threatens $4 billion industry. http://www.bloomberg.com/news/2014-12-14/oyster-herpes-killer-virus-threatens-4-billion-industry.html

Project materials developed

Peer reviewed scientific papers in international journals

Summaries and links to obtain the full text articles are provided on the website below

Evans, O., Paul-Pont, I., Hick, P., Whittington, R.J., 2014. A simple centrifugation method for improving the detection of Ostreid herpesvirus-1 (OsHV-1) in natural seawater samples with an assessment of the potential for particulate attachment. Journal of Virological Methods 210, 59-66.

Paul-Pont, I., Dhand, N.K., Whittington, R.J., 2013. Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. Aquaculture 412, 202-214.

Paul-Pont, I., Evans, O., Dhand, N., Whittington, R., 2015. Experimental infections of Pacific oyster *Crassostrea gigas* using the Australian OsHV-1 µVar strain. Diseases of Aquatic Organisms 113, 137-147.

Paul-Pont, I., Evans, O., Dhand, N.K., Rubio, A., Coad, P., Whittington, R.J., 2014. 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. Aquaculture 422–423, 146-159.

Whittington, R., Dhand, N., Evans, O., Paul-Pont, I., 2015a. Further observations on the influence of husbandry practices on OsHV-1 µVar mortality in Pacific oysters *Crassostrea gigas*: age, cultivation structures and growing height. Aquaculture 438, 82-97.

Whittington, R., Hick, P., Evans, O., Rubio, A., Alford, B., Dhand, N., Paul-Pont, I., 2015b. Protection of Pacific oyster (*Crassostrea gigas*) spat from mortality due to Ostreid herpesvirus-1 OsHV-1 µVar) using simple treatments of incoming seawater in land-based upwellers. Aquaculture 437, 10-20.

Website

www.oysterhealthsydney.org

Appendices

Appendix 1 - List of researchers and project staff

This project was led by Professor Richard Whittington (project leader) and other members of staff of the University of Sydney including Dr Ika Paul-Pont (Research Fellow, until August 2013), Dr Navneet Dhand (Senior Lecturer), Dr Paul Hick (Senior Lecturer) and Dr Ana Rubio (Research Fellow) as well as Ms Alison Tweedie (Research Assistant) and Ms Olivia Evans (Laboratory Assistant) who provided leadership in the laboratory throughout the project. Mr Bruce Alford and Mr Len Drake, oyster farmers in the Hawkesbury and Georges Rivers, respectively, were employed to ensure the successful conduct of the field-based experiments that were critical components of this research program.

Appendix 2 - POMS R&D Consensus Statement

Position statement to industry on POMS research - August 2013

This information was prepared by the POMS R&D Coordination Committee

Summary

Australian research is making rapid progress, has provided validated experimental data to complement information from overseas, and is in partnership with the New Zealand Pacific oyster industry. Use of genetically resistant Pacific oysters and husbandry practices that minimise mortalities throughout the production cycle will be required in order to farm Pacific oysters in the face of POMS.

Work on a laboratory infection model to support genetic selection is progressing well and will be trialled in early 2014. Commercially available diploid stocks of the best families (60 to 70% survival of juveniles and adults) are expected to be commercially available in mid to late 2018. As spat are more susceptible it will be critical to limit their exposure to high risk conditions. Water treatments to enable safe rearing of spat have been demonstrated while POMS induced mortality in adult oysters can be limited to 50% by growing at a high height. Proof of concept of an integrated production system is still required and is recommended as a new topic of research.

These projections assume funding will be available for selective breeding and, importantly, for operational trials to evaluate the combined effects of resistant stock and changed husbandry prior to commercial release.

Theme 1. Genetic selection for POMS resistance

Seafood CRC Project 2012/760 This project aims to understand patterns of genetic inheritance of POMS resistance and to begin the process of selecting for disease resistance. A series of trials (6 to date) have been done using the ASI Pacific oyster selective breeding population. Key findings so far are:

- The ASI selective breeding population has potentially useful levels of genetic variation for POMS. Resistance to POMS is heritable and a proportion of juveniles survive infection. Selective breeding for resistance is therefore possible.
- However, measuring resistance for an applied breeding program will need to be done using a laboratory challenge due to the vagaries and logistical difficulties of natural exposure in the field.
- The best families available now will not provide sufficient resistance to allow economic production of Pacific oysters in a POMS-affected region. Current data suggest that useful levels of resistance in juveniles and adults can be attained after a further three years of selection and testing, i.e. in mid 2016. The current population, which is unselected for POMS resistance, has a juvenile survival of about 10% (this appears to be comparable to other countries). Survival of the best families is expected to be 60 to 70% after a further 3 years of selection. Commercial diploid stocks from these families are expected to be available from hatcheries in mid to late 2018.
- Spat are known to be far more susceptible than juveniles or adults, and timelines to provide useful levels of resistance in spat are uncertain but are expected to be significantly longer. Timelines for resistant triploid stock will also be longer and the exact timing is dependent on the decisions of commercial hatcheries.

Selective breeding on its own will not provide a solution to POMS in the short or medium term. However, a workable medium term solution is expected through the use of stock with a moderate level of resistance and changed husbandry practices to limit disease severity. A critical aspect of husbandry in the medium term will be to limit the exposure of spat to high disease risk conditions.

Further information: Dr Peter Kube; Peter.Kube@csiro.au

Theme 2. Development of a laboratory infection model for OsHV-1

FRDC project 2012/052

The objective of this project is to develop a procedure to consistently and reliably infect oysters with OsHV-1, the virus that causes POMS. Applications include screening Pacific oysters of different genotypes for evidence of a genetic basis for resistance to disease. Good progress has been made in the first 6 months of this project including:

- Isolation of a strain of OsHV-1 from oyster tissues from the first POMS outbreak in the Georges River, its propagation in disease free oysters, and long-term storage of the virus in the laboratory.
- A method of preserving virus in an ultra-low temperature freezer so that a single virus preparation can be used at the same dose in different trials over a long time frame.
- Successful infection of spat by immersion in virus. This has not been achieved previously and will be an invaluable method as it more closely resembles natural infection than does injection of virus into oysters and it is the only practical way to do the large scale screening (thousands of animals each year) required by the selective breeding program.

The repeatability of this procedure will now be evaluated and then it will be tested in parallel with natural exposure of oysters in the field. It is expected that these trials will commence early in 2014 by testing families produced by the CRC genetics project. The experimental infection procedure will also be supplied to collaborators in New Zealand.

Further information: Dr Peter Kirkland; peter.kirkland@dpi.nsw.gov.au

Theme 3. Epidemiology and husbandry practices to reduce economic losses

FRDC project 2012/032

This project addresses the identity of the virus, risk factors for disease outbreaks, mechanisms of transmission, identification of environmental reservoirs for the virus, means of inactivating the virus and the effect of environment and husbandry practices on mortality. The objective is to discover new ways to grow oysters so as to minimise losses. Important findings so far include:

- Mortality of adult oysters can be reduced by 25-50% by raising growing height 300 mm (triploid; age class 11-12 month), confirmed in controlled experiments over 2 years. The maximum cumulative mortality observed at this height was 50%. Oysters must be placed at high growing height before exposure to the virus. There is no benefit of high growing height for spat.
- Spat have been protected from POMS in an infected estuary by using land-based tanks or ponds with simple water treatments (filtration with UV, or 48h sedimentation). This is crucial information for hatcheries threatened by the likely spread of POMS. Further research to confirm these findings is being undertaken in 2013-2014, with results expected by mid 2014.
- The danger period for POMS in NSW waters appears to be November to May inclusive, but this is the subject of further research in 2013-2014.

- An integrated production system involving safe rearing of spat, avoidance of the danger period, promotion of rapid growth in winter, and protection of adults at high height has been proposed but requires field validation.
- Mechanisms of transmission, virus distribution and incubation period were identified through intensive monitoring in Woolooware Bay and the Hawkesbury River (NSW). With appropriate surveillance for OsHV-1, rational business decisions can be made regarding purchase of spat, emergency harvest and stock management to mitigate the financial impact of POMS on oyster farming businesses.

Information on natural reservoirs for OsHV-1, mechanisms of infection, the window of infection, safe spat rearing and environmental factors associated with outbreaks will become available during 2014.

Further information: Professor Richard Whittington; www.oysterhealthsydney.org; richard.whittington@sydney.edu.au

Appendix 3 - References

References

Agreste, 2005. Agreste Cahiers conchyliculture n°1 – Recensement de la conchyliculture 2001. Février 2005 (http://agreste.agriculture.gouv.fr/publications/dossiers/article/recensement-conchyliculture-2001-1243).

Agreste, 2014. Agreste Cahiers conchyliculture n°316 – Recensement de la conchyliculture 2012. Juillet 2014 (http://www.agreste.agriculture.gouv.fr/IMG/pdf/primeur316.pdf).

Animal Health Australia, 2011. Animal Health in Australia 2011. Animal Health Australia, Canberra, 209 pp.

Arzul, I., Renault, T., Thébault, A., Gérard, A., 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic Crassostrea gigas adults. Virus Research 84, 151-160.

Belin, C., Claisse, D., Daniel, A., Fleury, E., Miossec, L., Ropert, M., 2015. Qualité du Milieu 517 Marin Littoral Synthèse Nationale de la Surveillance 2013. Ifremer Report.

Bingham, P., Brangenberg, N., Williams, R., van Andel, M., 2013. Investigation into the first diagnosis of ostreid herpesvirus type 1 in Pacific oysters. Ministry for Primary Industries New Zealand, Surveillance 40, 20-24.

Burge, C.A., Friedman, C.S., 2012. Quantifying Ostreid herpesvirus (OsHV-1) genome copies and expression during transmission. Microb Ecol 63, 596-604.

Burge, C.A., Griffin, F.J., Friedman, C.S., 2006. Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. Diseases of Aquatic Organisms 72, 31-43.

Burge, C.A., Judah, L.R., Conquest, L.L., Griffin, F.J., Cheney, D.P., Suhrbier, A., Vadopalas, B., Olin, P.G., Renault, T., Friedman, C.S., 2007. Summer seed mortality of the Pacific oyster, *Crassostrea gigas* Thunberg grown in Tomales Bay, California, USA: The influence of oyster stock, planting time, pathogens, and environmental stressors. Journal of Shellfish Research 26, 163-172.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55, 611-622.

Corbeil, S., Williams, L.M., Bergfeld, J., Crane, M.S.J., 2012a. Abalone herpes virus stability in sea water and susceptibility to chemical disinfectants. Aquaculture 326–329, 20-26.

Corbeil, S., McColl, K.A., Williams, L.M., Mohammad, I., Hyatt, A.D., Crameri, S.G., Fegan, M., Crane, M.S.J., 2012b. Abalone viral ganglioneuritis: Establishment and use of an experimental immersion challenge system for the study of abalone herpes virus infections in Australian abalone. Virus Research 165, 207-213.

Corporeau, C., Tamayo, D., Pernet, F., Quéré, C., Madec, S., 2014. Proteomic signatures of the oyster metabolic response to herpesvirus OsHV-1 µVar infection. Journal of Proteomics 109, 176-187.

Cowling, D.W., Gardner, I.A., Johnson, W.O., 1999. Comparisons of methods for estimation of individual level prevalence based on pooled samples. Preventive Veterinary Medicine 39, 211-225.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E., 2009. The order Herpesvirales. Archives of Virology 154, 171-177.

Degremont, L., Guyader, T., Tourbiez, D., Pepin, J.-F., 2013. Is horizontal transmission of the Ostreid herpesvirus OsHV-1 in Crassostrea gigas affected by unselected or selected survival status in adults to juveniles? Aquaculture 408, 51-57.

Dégremont, L., 2011. Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. Aquaculture 317, 94-98.

Dégremont, L., 2013. Size and genotype affect resistance to mortality caused by OsHV-1 in *Crassostrea gigas*. Aquaculture 416–417, 129-134.
Dégremont, L., Boudry, P., Ropert, M., Samain, J.F., Bédier, E., Soletchnik, P., 2010. Effects of age and environment on survival of summer mortality by two selected groups of the Pacific oyster *Crassostrea gigas*. Aquaculture 299, 44-50.
Dundon, W.G., Arzul, I., Omnes, E., Robert, M., Magnabosco, C., Zambon, M., Gennari, L., Toffan, A., Terregino, C., Capua, I., Arcangeli, G., 2011. Detection of Type 1 Ostreid Herpes variant (OsHV-1 μvar) with no associated mortality in French-origin Pacific cupped oyster *Crassostrea gigas* farmed in Italy. Aquaculture 314, 49-52.
EFSA, 2010. Scientific opinion on the increased mortality events in Pacific oyster *Crassotrea gigas*. European Food Safety Authority Journal 8(11):1894, 1-59.
Evans, O., Paul-Pont, I., Hick, P., Whittington, R.J., 2014. A simple centrifugation method for improving the detection of *Ostreid herpesvirus-1* (*OsHV-1*) in natural seawater samples with an assessment of the potential for particulate attachment. Journal of Virological Methods 210, 59-66.
Farrell, H., Brett, S., Ajani, P., Murray, S., 2013. Distribution of the genus *Alexandrium* (Halim) and paralytic shellfish toxins along the coastline of New South Wales, Australia. Marine Pollution Bulletin 72, 133-145.
Fleury, É., 2014. RESCO - Réseau d'Observations Conchylicoles: Campagne 2013. Ifremer report.
Flye-Sainte-Marie, J., Pouvreau, S., Paillard, C., Jean, F., 2007. Impact of Brown Ring Disease on the energy budget of the Manila clam *Ruditapes philippinarum*. Journal of Experimental Marine Biology and Ecology 349, 378-389.
Gagnaire, B., Frouin, H., Moreau, K., Thomas, G., Renault, T., 2006. Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish Shellfish Immunol 20, 536-547.
Garcia, C., Thébault, A., Dégremont, L., Arzul, I., Miossec, L., Robert, M., Chollet, B., François, C., Joly, J.P., Ferrand, S., Kerdudou, N., Renault, T., 2011. Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. Veterinary Research 42.
Green, T.J., Montagnani, C., Benkendorff, K., Robinson, N., Speck, P., 2014. Ontogeny and water temperature influences the antiviral response of the Pacific oyster, Crassostrea gigas. Fish & Shellfish Immunology 36, 151-157.
Gustafson, L.L., Ellis, S.K., Beattie, M.J., Chang, B.D., Dickey, D.A., Robinson, T.L., Marenghi, F.P., Moffett, P.J., Page, F.H., 2007. Hydrographics and the timing of infectious salmon anemia outbreaks among Atlantic salmon (*Salmo salar* L.) farms in the Quoddy region of Maine, USA and New Brunswick, Canada. Preventive Veterinary Medicine 78, 35-56.
Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P., 2010. Effects of Alexandrium minutum exposure on nutrition-related processes and reproductive output in oysters Crassostrea gigas. Harmful Algae 9, 427-439.
Hawley, L.M., Garver, K.A., 2008. Stability of viral hemorrhagic septicemia virus (VHSV) in freshwater and seawater at various temperatures. Diseases of Aquatic Organisms 82, 171-178.
Honkoop, P.J.C., Bayne, B.L., 2002. Stocking density and growth of the Pacific oyster (*Crassostrea gigas*) and the Sydney rock oyster (*Saccostrea glomerata*) in Port Stephens, Australia. Aquaculture 213, 171-186.
Hosmer, D.W., Lemeshow, S., 1999. Applied Survival Analysis: Regression Modelling of Time to Event Data. John Wiley and Sons, New York.
Hwang, J.Y., Park, J.J., Yu, H.J., Hur, Y.B., Arzul, I., Couraleau, Y., Park, M.A., 2013. Ostreid herpesvirus 1 infection in farmed Pacific oyster larvae *Crassostrea gigas* (Thunberg) in Korea. Journal of Fish Diseases 36, 969-972.
Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S.A., Gu, X., Read, A., Go, J., Dove, M., O'Connor, W., Kirkland, P.D., Frances, J., 2013. Identification and characterisation of

an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Diseases of Aquatic Organisms 105, 109-126.
Kasai, H., Muto, Y., Yoshimizu, M., 2005. Virucidal effects of ultraviolet, heat treatment and disinfectants against koi herpesvirus (KHV). Fish Pathol 40, 137-138.
Kristoffersen, A.B., Viljugrein, H., Kongtorp, R.T., Brun, E., Jansen, P.A., 2009. Risk factors for pancreas disease (PD) outbreaks in farmed Atlantic salmon and rainbow trout in Norway during 2003-2007. Preventive Veterinary Medicine 90, 127-136.
Lassudrie, M., Soudant, P., Nicolas, J.-L., Fabioux, C., Lambert, C., Miner, P., Le Grand, J., Petton, B., Hegaret, H., 2015. Interaction between toxic dinoflagellate *Alexandrium catenella* exposure and disease associated with herpesvirus OsHV-1µVar in Pacific oyster spat *Crassostrea gigas*. Harmful Algae 45, 53-61.
Le Deuff, R.M., Renault, T., Gérard, A., 1996. Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster Crassostrea gigas. Diseases of Aquatic Organisms 24, 149-157.
Martenot, C., Oden, E., Travaillé, E., Malas, J.P., Houssin, M., 2010. Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crassostrea gigas*. Journal of Virological Methods 170, 86-89.
Martenot, C., Travaille, E., Lethuillier, O., Lelong, C., Houssin, M., 2013. Genome exploration of six variants of the Ostreid Herpesvirus 1 and characterization of large deletion in OsHV-1mµVar specimens. Virus Res 178, 462-470.
Martenot, C., Denechere, L., Hubert, P., Metayer, L., Oden, E., Trancart, S., Travaille, E., Houssin, M., 2015. Virulence of Ostreid herpesvirus 1 mu Var in sea water at 16 degrees C and 25 degrees C. Aquaculture 439, 1-6.
Miossec, L., Le Deuff, R.-M., Goulletquer, P., 2009. Alien species alert: *Crassostrea gigas* (Pacific oyster), ICES Cooperative Research Report, pp. 42.
Normand, J., Li, R., Quillien, V., Nicolas, J.-L., Boudry, P., Pernet, F., Huvet, A., 2014. Contrasted survival under field or controlled conditions displays associations between mRNA levels of candidate genes and response to OsHV-1 infection in the Pacific oyster Crassostrea gigas. Marine Genomics 15, 95-102.
NSW DPI, 2014. POMS Oyster Equipment Movement Protocol, New South Wales Department of Primary Industry Prime Fact 1287, Second edition, http://www.dpi.nsw.gov.au/factsheets accessed 12-6-2015.
Oden, E., Martenot, C., Berthaux, M., Travaille, E., Malas, J.P., Houssin, M., 2011. Quantification of ostreid herpesvirus 1 (OsHV-1) in *Crassostrea gigas* by real-time PCR: Determination of a viral load threshold to prevent summer mortalities. Aquaculture 317, 27-31.
OIE, 2013. Infection with Ostreid Herpesvirus 1 microvariant. OIE, World Organisation for Aniaml Health, Paris, 14 pp.
Paul-Pont, I., Dhand, N.K., Whittington, R.J., 2013a. Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas*. Aquaculture 412, 202-214.
Paul-Pont, I., Dhand, N.K., Whittington, R.J., 2013b. Spatial distribution of mortality in Pacific oysters *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission. Diseases of Aquatic Organisms 105, 127-138.
Paul-Pont, I., Evans, O., Dhand, N., Whittington, R., 2015. Experimental infections of Pacific oyster *Crassostrea gigas* using the Australian OsHV-1 µVar strain. Diseases of Aquatic Organisms 113, 137-147.
Paul-Pont, I., Evans, O., Dhand, N.K., Rubio, A., Coad, P., Whittington, R.J., 2014. 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. Aquaculture 422–423, 146-159.
Peeler, E.J., Allan Reese, R., Cheslett, D.L., Geoghegan, F., Power, A., Thrush, M.A., 2012. Investigation of mortality in Pacific oysters associated with *Ostreid herpesvirus*-1 μVar in the Republic of Ireland in 2009. Preventive Veterinary Medicine 105, 136-143.

Pepin, J.F., Riou, A., Renault, T., 2008. Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. Journal of Virological Methods 149, 269-276.
Pernet, F., Barret, J., Marty, C., Moal, J., Le Gall, P., Boudry, P., 2010. Environmental anomalies, energetic reserves and fatty acid modifications in oysters coincide with an exceptional mortality event. Marine Ecology Progress Series 401, 129-146.
Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., Pépin, J.-F., Keck, N., 2012. Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquaculture Environment Interactions 2, 215-237.
Pernet, F., Lagarde, F., Jeannee, N., Daigle, G., Barret, J., Le Gall, P., Quere, C., D'orbcastel, E.R., 2014. Spatial and temporal dynamics of mass mortalities in oysters is influenced by energetic reserves and food quality. Plos One 9 (2), e88469. doi:88410.81371/journal.pone.0088469.
Petton, B., Pernet, F., Robert, R., Boudry, P., 2013. Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. Aquaculture Environment Interactions 3, 257-273.
Quinn, P., Markey, B., 2001. Disinfection and disease prevention in veterinary medicine. In: Block, S. (Ed.), Disinfection, Sterilization and Preservation. Lippincott Williams & Wilkins, Philadelphia, pp. 1069-1103.
Radke, B.R., 2003. A demonstration of interval-censored survival analysis. Preventive Veterinary Medicine 59, 241-256.
Renault, T., Cochennec, R., Le Deuff, M., Chollet, B., 1994a. Herpes-like virus infecting Japanese oyster (*Crassostrea gigas)* spat. Bulletin of the European Association of Fish Pathologists 14, 64-67.
Renault, T., Le Deuff, R.M., Cochennec, N., Maffart, P., 1994b. Herpesviruses associated with mortalities among Pacific oyster, *Crassostrea gigas*, in France - comparative study. Revue de Medecine Veterinaire 145, 735-742.
Renault, T., Faury, N., Barbosa-Solomieu, V., Moreau, K., 2011. Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, Crassostrea gigas, challenged with Ostreid herpesvirus 1. Developmental and Comparative Immunology 35, 725-735.
Renault, T., Moreau, P., Faury, N., Pepin, J.-F., Segarra, A., Webb, S., 2012. Analysis of Clinical Ostreid Herpesvirus 1 (Malacoherpesviridae) Specimens by Sequencing Amplified Fragments from Three Virus Genome Areas. Journal of Virology 86, 5942-5947.
Rico-Villa, B., Bernard, I., Robert, R., Pouvreau, S., 2010. A Dynamic Energy Budget (DEB) growth model for Pacific oyster larvae, *Crassostrea gigas*. Aquaculture 305, 84-94.
Rutala, W.A., Weber, D.J., 2008. Guideline for Disinfection and Sterilization in Healthcare Facilities, Centers for Disease Control and Prevention, Atlanta, GA 30333.
Salama, N.K.G., Murray, A.G., 2013. A comparison of modelling approaches to assess the transmission of pathogens between Scottish fish farms: The role of hydrodynamics and site biomass. Preventive Veterinary Medicine 108, 285-293.
Satheesh Kumar, S., Ananda Bharathi, R., Rajan, J.J.S., Alavandi, S.V., Poornima, M., Balasubramanian, C.P., Ponniah, A.G., 2013. Viability of white spot syndrome virus (WSSV) in sediment during sun-drying (drainable pond) and under non-drainable pond conditions indicated by infectivity to shrimp. Aquaculture 402–403, 119-126.
Sattar, S., Springthorpe, V., 2001. Methods of testing the virucidal activity of chemicals. In: Block, S. (Ed.), Disinfection, Sterilization and Preservation. Lippincott Williams and Wilkins, Philadelphia, pp. 1391-1412.
Sauvage, C., Pépin, J.F., Lapègue, S., Boudry, P., Renault, T., 2009. Ostreid herpes virus 1 infection in families of the Pacific oyster, Crassostrea gigas, during a summer mortality outbreak: Differences in viral DNA detection and quantification using real-time PCR. Virus Research 142, 181-187.

Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P., Pepin, J.F., 2011a. Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Veterinary Research 42.

Schikorski, D., Faury, N., Pepin, J.F., Saulnier, D., Tourbiez, D., Renault, T., 2011b. Experimental ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: Kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. Virus Research 155, 28-34.

Segarra, A., Faury, N., Pepin, J.F., Renault, T., 2014. Transcriptomic study of 39 ostreid herpesvirus 1 genes during an experimental infection. J Invertebr Pathol 119, 5-11.

Shimahara, Y., Kurita, J., Kiryu, I., Nishioka, T., Yuasa, K., Kawana, M., Kamaishi, T., Oseko, N., 2012. Surveillance of Type 1 Ostreid Herpesvirus (OsHV-1) Variants in Japan. Fish Pathol 47, 129-136.

Shimizu, T., Yoshida, N., Kasai, H., Yoshimizu, M., 2006. Survival of koi herpesvirus (KHV) in environmental water. Fish Pathol 41, 153-157.

Tamayo, D., Corporeau, C., Petton, B., Quere, C., Pernet, F., 2014. Physiological changes in Pacific oyster *Crassostrea gigas* exposed to the herpesvirus OsHV-1 µVar. Aquaculture 432, 304-310.

Thrusfield, M., 2007. Veterinary epidemiology. Blackwell Publishing Ltd, Oxford.

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

Whittington, R., Paul-Pont, I., Dhand, N., 2013. 2011/053 Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) – understanding biotic and abiotic environmental and husbandry effects to reduce economic losses. FRDC and University of Sydney, Sydney, 96 pp.

Yoshimizu, M., Shimizu, T., Kasai, H., 2005. Survivability of koi herpesvirus (KHV) in environmental water as well as physical and chemical inactivation. . Fish Pathol 40, 203.