FINAL REPORT

aquatic animal health subprogram

2011/053 Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) – understanding biotic and abiotic environmental and husbandry effects to reduce economic losses

Richard Whittington, Ika Paul-Pont and Navneet Dhand

January 2013

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OBJECTIVES:

1. To correlate biotic and abiotic environmental factors with POMS occurrence in selected oyster populations

NON TECHNICAL SUMMARY

OUTCOMES ACHIEVED TO DATE

This project will assist in ensuring the sustainability and profitability of the aquaculture industry and the health of natural resources by providing new data on the epidemiology of Pacific oyster mortality syndrome (POMS). Practical management measures based on increasing growing height to reduce adult oyster losses due to POMS are possible but require confirmation through a second season of study because the summer of 2011-2012 was unusually mild and wet, and it is possible that POMS disease expression will be different in a typical hot dry summer. There is a broader responsibility towards the Australian community to ensure the sustainability of Australian aquatic natural resources. This was achieved through the promotion of information about oyster health in general and POMS in particular. This project assisted industry to strengthen biosecurity practices: there were proposals from specific sectors of the oyster industry for voluntary restrictions on oyster movements between estuaries, and objective laboratory testing of oysters for specific pathogens prior to movement to decrease the risk of disease spread. This will protect commercial aquaculture. The need for genetic selection to improve oyster health was addressed by flow of data from this project to the POMS genetic selection program, whereby optimal experimental design for field trials was confirmed to enable identification of a genetic component in resistance. Communication of the most recent findings of the project, namely confirmation that growing height could beneficially affect survival of adult oysters in the face of an outbreak, will be ongoing, including through a fully illustrated website that was established during the project and has proven to be very popular with industry (www.oysterhealthsydney.org).

There is a disturbing pattern of emerging diseases in commercial molluscs nationally that has required a succession of government/industry responses. Pacific oyster mortality syndrome (POMS), which appeared in NSW in 2010, is an internationally significant disease that has severely impacted Pacific oyster production in Europe and New Zealand. This project was based on the

premise that the oyster industry will need to learn to live with POMS by managing husbandry.

We investigated the epidemiology of POMS during its second summer in Australia to identify factors which may be exploited to reduce the impact of this viral infection. We describe the outbreak of POMS in Woolooware Bay near Sydney NSW, which started in November 2011 and in which virus associated mortalities were observed until late April 2012. The distribution of disease was non uniform, clustered, highly variable in time and space, and clearly dependent on the age of oysters and their growing height or position in the water column. Implementation of different farming practices, in this case modification of the growing height, could play a role in disease management and help reduce mortality of adult oysters during an OsHV-1 outbreak. The pattern of infection and disease was different on different leases suggesting that underlying environmental factors influence disease expression. Differences in mortality among sites, ages and growing heights were evaluated in relation to the intensity and prevalence of viral infection and the environmental data recorded during the outbreak. The epidemiological observations are of considerable importance and inform future strategies to control OsHV-1, including the methodologies to be applied in the genetic selection program.

C. gigas were stocked into 3 different oyster leases in Woolooware Bay and allocated into groups to study growing height and age. Two heights were used: a standard growing height, and one 300mm higher than this. Oysters comprised adults (12 month old; 67-93 mm length) and spat (2-3 month old; 21-38 mm) and were placed in plastic trays with lids. Other oysters were kept in floating baskets at each site. The sites were managed by oyster growers with the assistance of researchers and all sampling was conducted by the researchers. Oysters were placed during the late spring in October 2011, before POMS had recrudesced. The level of OsHV-1 virus, *Vibrio* sp., mortality rates and environmental parameters such as temperature and salinity were studied in detail before, during and after the outbreak.

Spat were highly susceptible to the virus and all those kept in trays died regardless of growing height. In contrast, the high growing height reduced the deaths of adult oysters by 40%. The pattern of results was consistent at all three sites. In a second smaller experiment, spat survived in floating baskets but not in trays.

Environmental factors may affect POMS disease events. We found a slight decrease in salinity and variable changes in water temperature just before three mortality events in Woolooware Bay in the Georges River. Importantly, salinity and temperature readings in the Hawkesbury River system were similar to those in the Georges River and therefore may be suitable for POMS to establish.

Overall the observations suggest that OsHV-1 is a necessary but insufficient factor in the mortality event or that OsHV-1 is a sufficient cause but with a very strong dose effect. The virus was detected in oyster tissues up to 2

months before mortalities commenced therefore other factors may be required. These factors could include environmental conditions/triggers or other as yet undiscovered pathogens. *Vibrio spp.* bacteria, which have been suggested to be involved with OsHV-1 virus in POMS disease in France, did not appear to be involved in this outbreak in Woolooware Bay because their intensity did not increase until after the POMS outbreak had started, the species of *Vibrio* present did not change over time and a similar number of *Vibrio* spp. bacteria were present in tissues of healthy oysters in the Hawkesbury River.

Importantly, the virus did not appear to be transmitted free in water. There was considerable epidemiological evidence that its distribution was clustered in Woolooware Bay, and that it behaved as if it was moving together with still to be defined planktonic particles. These important results have already been used by other researchers to confirm the optimal design of field experiments to study the resistance of different *C. gigas* family lines, to support a genetic selection program.

During the outbreak up to 92% of the oysters tested positive for OsHV-1 but afterwards the infection prevalence decreased over time suggesting that surviving adults can clear the virus. Some appeared to be resistant as they survived three separate mortality events during the summer 2011-2012. This suggests possible immunological mechanisms and underlying potential for genetic resistance to the infection.

It can be concluded from this study that husbandry factors may strongly influence the survival of adult *C. gigas* during an outbreak of POMS. It is possible that measures to reduce the level of exposure of oysters to OsHV-1 as distinct from preventing exposure may be sufficient to prevent mortalities.

Recommendations were made for further development. Further studies are indicated to confirm the effect of growing height on mortality rates, as the present research trial was conducted during an unusually wet and cool summer. If the same results are obtained when the trial is repeated in a more typical summer, oyster growers can confidently take steps to reduce the risk of losses of valuable adult oysters should POMS spread in Australia. Further studies are also required to investigate why some oysters appear to be resistant and how some clear the virus from their organs, to precisely identify the seasonal window of infection, to confirm the mode of transmission of the virus in the environment, to evaluate the risk of transmission with equipment and by handling oysters, to understand how environmental factors combine with the virus to cause mortality, whether a certain level of viral load in the environment is needed to initiate mortalities, and to identify potential wild mollusc hosts for the virus.

Note added in press: a new outbreak of POMS was detected in the Hawkesbury River on 21st January 2013

KEYWORDS: POMS, ostreid herpes virus, Pacific oyster, Crasostrea gigas, aquaculture, disease control

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BACKGROUND

POMS disease struck the Pacific oyster industry in Botany Bay at the end of 2010 and caused an immediate governmental response to prevent spread to unaffected areas of Australia. A national survey was conducted which confirmed that ostreid herpesvirus (OsHV-1), which is the cause of the disease, was not present outside Botany Bay and Sydney Harbour. The origin of the virus (endemic or exotic) and its mode of spread to or between these two sites is unknown. The reason for the immediate response to contain the disease was because of evidence from Europe and New Zealand that this virus had the potential to devastate Pacific oyster aquaculture.

Infectious diseases in molluscs can be due to a wide variety of pathogens including protozoan and metazoan parasites (genera *Perkinsus*, *Marteilia*, *Bonamia*, *Microcytos, Mytilicola*), bacteria (*Vibrio* spp., *Nocardia*) and viruses (*Iridoviridae*, *Papoviridae*, *Togaviridae*, *Reoaviridae*, *Birnaviridae*, *Picornaviridae* and *Herpesviridae*). Viruses and particularly herpes-like viruses are of particular concern due to their economical and ecological impact on cultured marine molluscs for the past 20 years (Renault, 2011). From 2008 massive mortalities of *C. gigas* were reported in several farming areas in France, United Kingdom, Jersey, Ireland, Spain, Netherlands and the United States. These outbreaks were attributed to complex interactions between oysters, pathogens (*Ostreid herpesvirus 1* or OsHV-1, and bacteria belonging to the genus Vibrio) and environmental parameters (Davison et al., 2005; Friedman C. S., 2005; Renault, 1994; Renault and Novoa, 2004; Schikorski et al., 2011b). Outbreaks occur in summer preferentially in sheltered habitats, rising to 80-100% especially in younger stages of oysters (larvae > spat > juveniles > adults). Existing data support the influence of seawater temperature on the disease onset.

Although the syndrome of summer mortality of juvenile Pacific oysters had been recognised for decades in the northern hemisphere, it was not until 2008 that epizootic mortality occurred in association with a variant of a common virus. The mutated virus was named OsHV-1 uvar.

FRDC convened a national workshop to discuss and document what was known about OsHV-1 uvar. The workshop was held in Cairns on 9-10 July 2011. The recommendations from the workshop included a key activity (recommendation 15) to support epidemiological analysis of the role of different purported risk factors in causing the disease, with the aim of identifying possible control mechanisms or predicting periods of high risk for disease outbreaks. This application was developed following the open round for pre-proposals and addressed this key research priority. There was an urgent need to obtain data during the approaching summer when the disease was likely to be most active. The application contained a number of integrated objectives, but because of the urgency one was promoted for immediate research: to correlate biotic and abiotic environmental factors with POMS occurrence in selected oyster populations. The remaining objectives were deferred to a later application (which was funded as project "2012/032 Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) – risk

mitigation, epidemiology and OsHV-1 biology″). FRDC advised that the scope for the new project “2011/053: Pacific oyster mortality syndrome (POMS) – understanding biotic and abiotic environmental and husbandry effects to reduce economic losses” should include a retrospective study component that uses previously collected environmental data from data loggers in areas where the disease outbreak has occurred with the view of identifying possible environmental factors that may lead to disease. Unfortunately the only outbreaks that had occurred were in Sydney harbour in wild oysters where there was no data logger, and in the Georges River at a time when the only data logger was off line or out of calibration. There were no prior outbreaks or sites that would enable use of retrospective data. For this reason a prospective study was required. The proposal was widely discussed, and letters of support were provided from industry.

Management of POMS is a high priority for *C. gigas* aquaculture sustainability and the main responses are restricted to active surveillance, rigorous biosecurity protocols and mollusc breeding programs targeting the production of OsHV-1 resistant diploid animals. Overall, the effects of aquaculture practices on mortality outbreaks in molluscs are still poorly understood even though some studies show that farming practices (density, depth, rearing structures) can play a role in disease management and mortality (Peeler et al., 2012; Pernet, 2012). Therefore an important objective of the present study was to assess the effect of husbandry practices on OsHV-1 associated mortality in *C. gigas* in Woolooware Bay during the summer 2011/2012 in the context of an epidemiological study.

NEED

There is a disturbing pattern of diseases in commercial molluscs nationally. They have all required a succession of government/industry responses, with no clear solutions: QX disease, Sydney rock oysters, NSW and QLD; Winter mortality Sydney rock oysters, NSW; Pacific oyster mortality syndrome, NSW; Abalone viral ganglioneuritis, VIC; Oyster oedema disease, pearl oysters, WA; Winter mortality, Sydney rock oyster, NSW. Economic impacts have been substantial or devastating. Wild fisheries and aquaculture have been impacted. In NSW, the primary impact of QX disease led to replacement of Sydney rock oysters by triploid Pacific oysters to reestablish the industry in some estuaries, but this is now threatened by POMS. In every case the new disease has spread. It has not been possible to devise an intervention strategy that would halt disease spread or ensure the recovery of the industry due to lack of available scientific knowledge.

Investigating the behaviour of POMS during its predicted recrudescence during its second summer in Australia provides an opportunity to identify factors which may be used to reduce the impact of the infection. This epidemiological project seeks to investigate the effect of host, environmental and husbandry factors on OsHV-1 prevalence and mortality rate in Pacific oysters during summer 2011-autumn 2012 with the objective of discovering aspects of epidemiology which can be manipulated by oyster growers. The project is based on the premise that the oyster industry will need to learn to live with POMS by managing husbandry and will need to continue farming despite the presence of POMS. The project fits within FRDC strategic R&D theme 1 - biosecurity and aquatic animal health, and Aquatic Animal Health Subprogram priority - Nature of disease and host-pathogen interaction - immunology of aquatic invertebrates.

OBJECTIVES

1. To correlate biotic and abiotic environmental factors with POMS occurrence in selected oyster populations

METHODS

The main objective in this project required the use of specific and diverse methodologies. To avoid confusion and because methodological development was required, the detailed methods are provided adjacent to appropriate sections of the results. However, a summary of the design and the methods are provided in this section of the report.

This project was designed to investigate POMS during Oct 2011-April 2012, a period during which the disease was predicted to appear. There were three studies within the project.

A. An historical review of POMS outbreak in 2010.
Information about the current/past husbandry conditions of each lease were recorded at the start of the study. This was done by the researchers during the initial visit to the lease and in subsequent discussions with oyster growers. A brief report was prepared and the growers were asked to check it for accuracy.

B. Intervention study - Georges River
Pacific oysters were stocked in 3 leases and allocated into treatment groups for two risk factors (growing rack height and age). Distinct sites were used to provide a contrast in background environment: temperature, salinity, water quality. The three main factors were analysed statistically. Differences in mortality observed between the three sites were expected to lead to hypotheses about environmental effects (such as temperature and salinity) which could be tested in laboratory-based trials in a subsequent project. Leases were purposively selected in the Georges River estuary, where the virus was already present, based on observed environmental differences between sites. As there was no commercial production of *C. gigas* at each location, infrastructure was installed to hold trays and baskets. The sites were managed by oyster growers with the assistance of researchers. All sampling was conducted by the researchers. Oysters were placed during the late spring in October 2011, before POMS had recrudesced in sentinel oysters already at the site. Oyster sampling was conducted at the time of placement, and then at appropriate intervals mindful of observed mortality rate. Viral load was measured using qPCR. *Vibrio* sp. and other pathogens were monitored by bacteriological culture.

Data loggers (temperature) were placed in representative experimental trays/baskets at each site (i.e. intertidal data collection). A salinity/temperature probe was placed sub-tidally at each site. At each sampling the researchers randomly picked oysters from a randomly selected segment of each experimental tray. Other mollusc/invertebrate species were collected opportunistically from leases and on trays to determine the load of OsHV-1 in the environment at the time the experimental oysters were at risk of exposure.

C. Observational study - Hawkesbury River
The potential spread of OsHV-1 from Sydney Harbour/Botany Bay to the nearest estuary where commercial Pacific oyster aquaculture exists was

studied through a longitudinal observational study of a spat cohort on 4 leases. Environmental data in the Hawkesbury river were contrasted with those from the Georges River to identify major variation that could explain disease absence, to use as a baseline for ongoing study, and to determine whether environmental conditions in this river might be conducive to disease expression if the virus was to spread there from the Georges River or Sydney Harbour. Oyster sampling was conducted fortnightly at each selected lease using systematic random sampling. A temperature logger was placed in representative trays/baskets and marked with a fluorescent tape. A sub-tidal temperature/salinity logger was placed at each site.

RESULTS

A. History of the first POMS outbreak in Botany Bay in 2010

POMS occurred for the first time in Australia in 2010 in commercial oyster leases in Woolooware Bay, which is part of the Georges River-Botany Bay estuary. Three commercial oyster growers were affected. OsHV-1 infection was detected by PCR tests conducted by NSW DPI, confirmed at AAHL and a response plan was implemented which involved quarantine and prevention of oyster movements from the Georges River-Botany Bay to contain the disease. Pacific oyster aquaculture became impractical and in 2011 and 2012 there was effectively no commercial Pacific oyster production at this location. The following details pertain to commercial Pacific oyster production in Botany Bay at the time of the first POMS outbreak.

Grower 1 obtained two large batches of commercial spat on 15th October (512,500 oysters) and 8th November 2010 (250,000 oysters). They were placed in a nursery and then put into leases in Woolooware Bay (at Site C, see below) on 16th November. By 24th November all these spat were dead. A third batch of 478,000 spat was obtained on 1st December and placed in Quibray Bay, which is also part of Botany Bay, and all were dead by 15th December.

A small number of adult oysters were then obtained and placed as sentinels in baskets at 4 sites on 7th January 2011 (74 – 100 per site). Within about 2 weeks the majority of these were dead at two of four different sites in Woolooware and Quibray Bays. This oyster placement was repeated on 22nd January and by 4th February the majority were dead at two of the four sites.

Grower 2 had 220,000 6 month old 70 mm Pacific oysters (co-cultivated with 30 mm Sydney rock oysters) in baskets on long lines opposite Site C (see below) in Woolooware Bay. Only 200 oysters survived a mortality episode in November 2010. A second crop of 600-800,000 spat were placed on trays opposite Site C in October 2010 and all died within 1 to 2 weeks. Prior to this a crop of 50,000 mid-to-late 2009 origin adult Pacific oysters which were stocked in floating baskets experienced 30% mortality in August 2010 and another 30% in September 2010, possibly associated with overstocking and rough weather as baskets were found on the sea floor. A further 10-20% died in October 2010. Overall 50% of this batch died, but the cause was not confirmed.

Grower 3 (not able to be contacted by the researchers; information provided by Grower 2) had small numbers of adult Pacific oysters in baskets on long lines near Site A (see below) during November 2010 and at least some of these were still alive a year later.

The observations made by oyster growers suggest that OsHV-1 infection caused POMS in Botany Bay over a period of weeks to months during summer 2010-2011. Multiple locations in Botany Bay were affected, and all ages of Pacific oysters appear to have been killed.

B. Intervention study - Georges River

Methods

Design. The experiment was set up in three different oyster leases located at least 1000m apart in Woolooware Bay (Figure 1). From October 2011 healthy oysters were sampled from Porto Bay in the Hawkesbury River and a sample tested negative for OsHV-1 by PCR (Martenot et al., 2010). From October 2011, oysters were transferred to Woolooware Bay and placed at sites A, B and C (Figure 1). Oysters comprised 4250 adults (12 month old; 67-93 mm length, Shellfish Culture SPL10GSD) and 4250 spat (2-3 month old; 21-38 mm, Shellfish culture SPL11D). These were divided randomly and used in two experiments conducted in parallel across the three oyster leases. The different systems used in experiment 1 and 2 are schematically presented in Figure 2. Experiment 1 started on the 20th of October 2011 whereas experiment 2 began on the 24th November 2011.

Figure 1. Experimental sites (Site A: 34.0118 S;151.1463 E / Site B: 34.0254 S;151.1400 E / Site C: 34.0329 S;151.1466 E) in Woolooware Bay (Botany Bay NSW Australia).

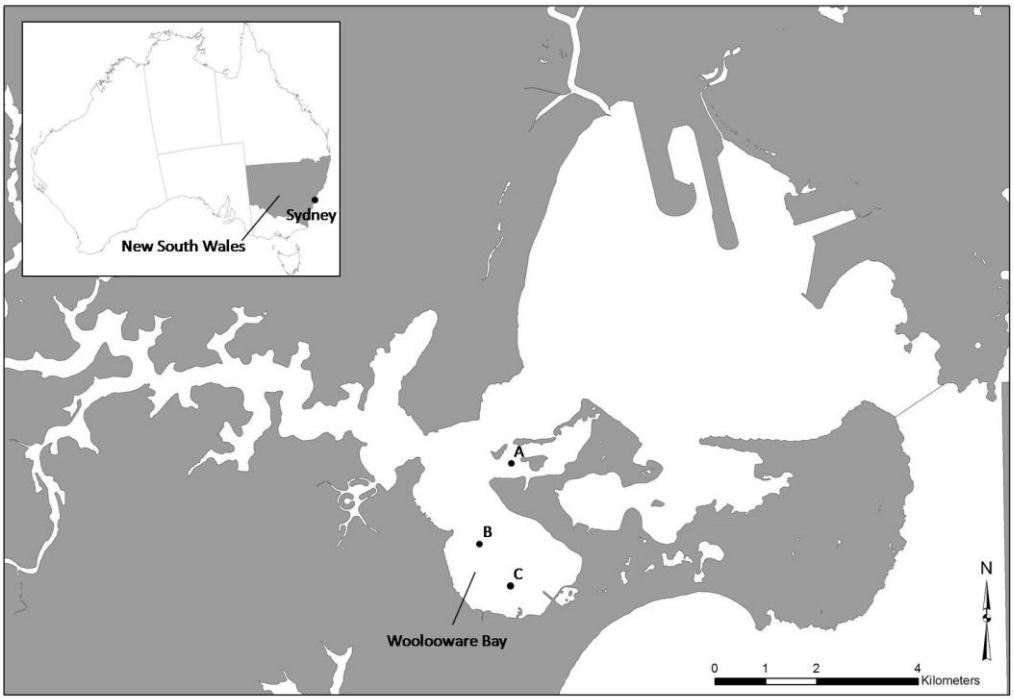
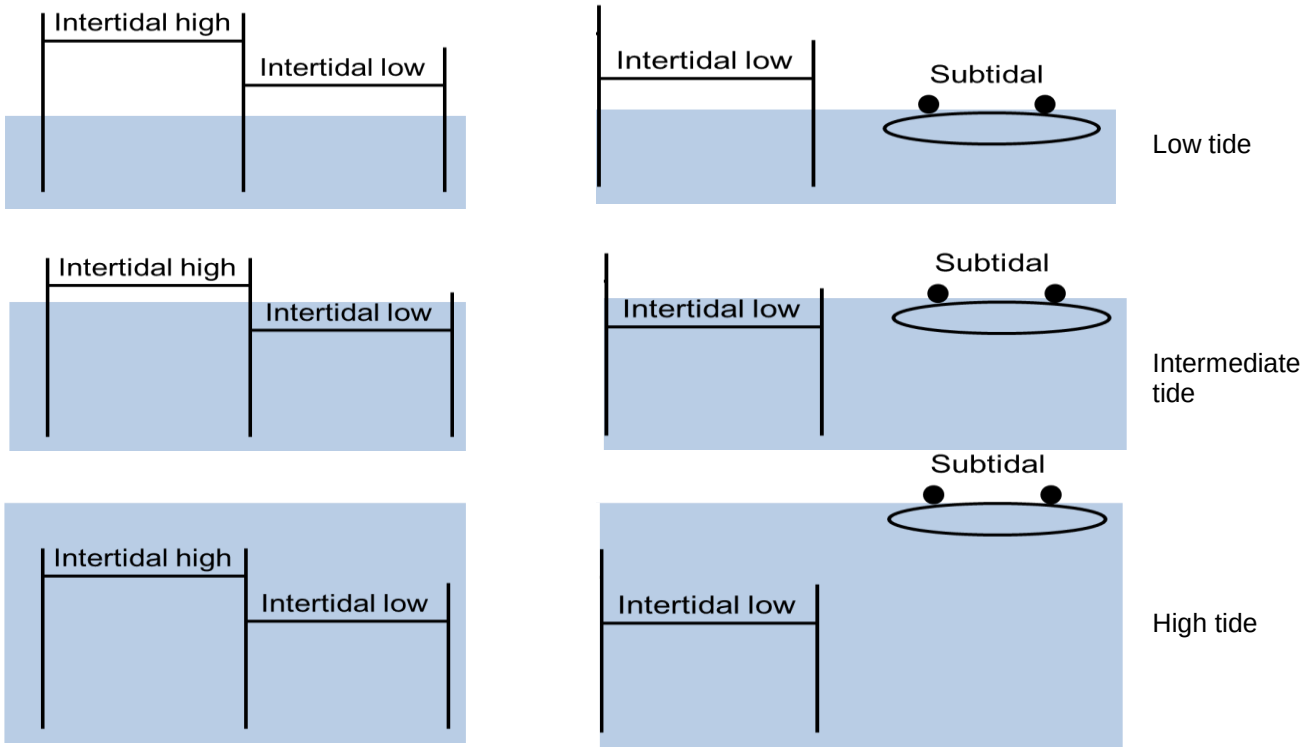


Figure 2. Schematic representation of the two experiments in different tide scenarios. Left panel, intertidal trays at high and low heights in Experiment 1; Right panel, intertidal trays at low height and subtidal floating baskets in Experiment 2.



Experiment 1 was designed to compare mortality rate as a function of the growing height, leading to different immersion times and air/UV/heat exposures. Adult oysters and spat were placed intertidally in plastic trays (2m x 1m) fixed on wooden racks at the standard height used by the local oyster farmers, and at a height 300 mm above standard rack height (Figure 2). The standard and higher heights were called “low” and “high”, respectively. This experimental design created 4 treatments per site: high adult, high spat, low adult and low spat. The initial stocking density was 320 oysters per tray, with two trays per treatment, so that there were 640 oysters per treatment per site.

Random sampling of 25 live oysters per treatment per site was performed at intervals for OsHV-1 µvar detection. In addition, dead oysters were systematically removed from trays and were not replaced. Therefore, the total number of oysters and the stocking density declined over time, and this was allowed for when calculating the cumulative mortality (see below). Random sampling for pathogen detection was stopped when the total number of oysters left in trays reached 10% of the initial population size; this was at different times for sites A, B and C.

The growth rate was assessed at intervals by measuring the shell length, width and depth (defined as the greatest anteroposterior, lateral and dorsoventral lengths, respectively) using a digital Vernier calliper (Kincrome) on a subsample of 10 individuals per treatment per site.

Experiment 2 was designed to compare the mortality rates among spat and adult oysters placed in two different cultivation systems: intertidal trays fixed on wooden racks at standard height (low) and subtidal floating baskets (Figure 2). These structures led to different immersion/emersion patterns and absolute position in the water column. The initial stocking density was lower in experiment 2 than in experiment 1 as the size of each floating basket did not allow more than 15 adults per basket. Consequently, 15 oysters were placed in each floating basket with two basket replicates per age class (spat/adult) per site (A, B and C). Additionally, 30 oysters of each age class (spat/adult) were placed in one intertidal tray at each site. Therefore, the total number of oysters at the beginning of the experiment on 24th November 2011 was 360. Dead oysters were systematically removed from trays and baskets. In contrast to experiment 1, dead batches of spat were replaced by later transfers of healthy individuals in order to determine the window of infection for OsHV-1: two additional batches of healthy spat (n=180 x 2, called “S2” and “S3”) were deployed in trays and baskets on the 20th December 2011 and 16th March 2012, respectively.

Mortality estimates. The mortality was assessed by manually counting dead and live oysters in each treatment. Additionally, in experiment 1 the number of dead/alive oysters was carefully recorded in each segment/square of each tray (8 segments/squares per tray) in order to assess the spatial distribution of mortality. In order to take into account the proportion of oysters sampled for pathogen detection (experiment 1) the cumulative mortality was expressed after correction for sampling according to the following equation:

Cumulative mortality $_t$

$$= \frac{(Observed\ mortality\ rate\ _t * Adjusted\ number\ of\ live\ oysters_{t-1}) + Cumulative\ \ mortality_{t-1}}{Initial\ population\ size\ _{t_0}}$$

Where:

$$Observed\ mortality\ rate_t = \frac{Number\ of\ dead\ oyster_t}{Actual\ population\ size_t}$$

and

$$Adjusted\ number\ of\ live\ oyster_{t-1}$$
$$= Adjusted\ number\ of\ live\ oyster_{t-2} - (Observed\ mortality\ rate_{t-1} * Adjusted\ number\ of\ live\ oyster_{t-2})$$

Where t is the observation time and t-1 and t-2 refer to the first and second previous observation times, respectively.

Detection of OsHV-1. Detection of OsHV-1 was performed on pools of 5 oysters, and 5 pools per treatment per site were analysed (corresponding to n=25 oysters). Infection prevalence was estimated from the proportion of positive pools (Cowling et al., 1999). Estimated prevalence is summarised in table 1 for each possible number of positive pools.

Table 1. Estimated prevalence for pooled samples (http://epitools.ausvet.com.au - method of Cowling et al (1999)).

Number of positive pools out of 5	Estimated prevalence	95% confidence limits
1	4.4%	0.1-22.3%
2	9.7%	1.0-31.2%
3	16.7%	3.1-44.5%
4	27.5%	6.5-65.3%
5	/	/

Additionally, a subsample of gills and mantle (20-50 mg) of each individual was kept separately in individual tubes at -80°C in order to assess the individual infection prevalence if required.

Tissue homogenisation. Oysters were thawed and gills and mantle were dissected from each oyster using separate instruments that were soaked in a disinfection solution (Virkon®), washed thoroughly and sterilised between uses. Tissue homogenates were prepared on pools at a dilution rate of one part tissue plus 4 parts sterile saline (1:4 W/V) using a stomacher. An aliquot of 500 µL of each homogenate was disrupted by bead-beating (Fastprep System, MP Biosciences, USA). Tissue homogenates were then clarified by centrifugation at 3000 × *g* for 10 min in a microcentrifuge. Clarified tissue homogenates were stored at −80°C until required.

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

Polymerase Chain Reaction (PCR). The detection of OsHV-1 DNA by PCR was adapted from a previously published PCR protocol (Martenot et al., 2010). Briefly, amplification was performed in 25 µl reactions containing 5 µl of template DNA, 1.25 µl of forward primer and reverse primer (18 µM), 1.25 µl of TaqMan probe (5 µM), 12.5 µl of 2X RT-PCR Buffer, 1 µl of RT-PCR Enzyme Mix (AgPath-ID™ One-Step RT-PCR - Applied Biosystems) and 2.75 µl of molecular grade water. Primer pairs and TaqMan probe sequences are listed in Table 2. The qPCR assay was performed using an Mx3000P Multiplex Quantitative PCR System (Stratagene) and run under the conditions described in Table 3. All reactions were run in duplicate and a positive and a no template control was included in each run.

Table 2. Nucleotide sequence of the primer pairs and the TaqMan probe used in the polymerase chain reaction (PCR) assay.

Primers	Sequence
OsHV1BF	5'- GTC GCA TCT TTG GAT TTA ACA A -3'
OsHV1B4	5'- ACT GGG ATC CGA CTG ACA AC-3'
Probe	
OsHV1probeB	5'- 6FAM-TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC-TAMRA -3'

Table 3. Temperature and cycle conditions for the polymerase chain reaction (PCR) assay.

Process	Temperature (°C)	Time	Cycles
Reverse transcription	50	20 mins	1
Hot start activation	95	10 mins	1
Denaturation	95	15 secs	40-45
Annealing and extension	60	45 secs	

Vibrio detection. An aliquot of 10 µL of clarified homogenate from each pool of homogenised oyster tissue was streaked onto marine salt agar with horse blood (MSA-B) (Buller, 2004) and thiosulfate citrate bile salt sucrose medium (TCBS cholera medium, TCBS) (Buller, 2004) in 10cm diameter Petri dishes using a standard microbiological loop. Cultures were incubated at 23°C for 24h, the plates were photographed and the number of colonies as well as their morphologies were carefully recorded. A subsample of 10 colonies per site, chosen to represent each colony type, was selected from the TCBS plates, individually placed in nutrient broth with 2% NaCl and incubated at 23°C overnight. The broth culture was diluted in glycerol to a final concentration of 15% v/v and stored at -80°C until required. A selection of 120 putative *Vibrio sp.* isolates was sent to the Animal Health Laboratory, Department of Agriculture and Food, Western Australia for identification. These bacterial samples were purified from oysters sampled before, during and after the outbreak at sites A and B and are listed in Appendix 4.

Wild molluscs and other potential reservoir hosts. Sampling of wild molluscs was undertaken to determine potential reservoir hosts for POMS virus in the environment at the time the experimental oysters were at risk of exposure. 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. Barnacles were collected from oyster trays at the experimental sites. The nature of the collected organisms, the date and sites of sampling are presented in Appendix 3. Detection of OsHV-1 by PCR was performed on pools of 5-10 individuals.

Environmental monitoring - temperature and salinity. Temperatures were recorded every 15 min using Thermocron® temperature loggers (Thermodata Pty Ltd) placed inside the trays at all sites (one temperature logger per treatment corresponding to two temperature logger replicates per height per site). Consequently, the temperature loggers were recording both air and

water temperature depending on the tidal emersion/immersion pattern. Temperature data were downloaded using the Thermodata 3.2.23 software (Thermodata Pty Ltd). Additionally, a subtidal HOBO U24-002 conductivity/salinity logger (OneTemp Pty Ltd - Onset®) was deployed at each site to monitor the water salinity (and temperature) every 15 min. This probe was placed at a deeper position in the water column and was constantly immersed. Salinity probes were systematically calibrated fortnightly using a portable conductivity meter (Waterproof CyberScan CON 400 – InstrumentChoiceTM) and data were downloaded using the HOBOware® Pro 3.2.1 software (Onset®).

Statistical analyses.

Mortality. Mortalities recorded for oysters over time were compared using survival analyses, taking synchronous interval censoring into account (Hosmer and Lemeshow, 1999; Radke, 2003). Analyses were conducted using a complementary log–log link function employing the SAS LOGISTIC procedure. Oysters that died or were sampled for other purposes were considered to be censored at the respective observation times. All oysters surviving at the end of the experiment were also censored. A binary outcome was created representing oyster status at each time point (alive or dead). The effect of age and height on survival was evaluated using univariable and multivariable models. Hazard ratios and their 95% confidence intervals were presented along with survival curves. Cumulative mortality was compared between age and height groups for each observation period and site (and for each rearing system for the second experiment) by using a Chi-square test or the Fisher's exact test as appropriate. These analyses were conducted using the SAS statistical program (release 9.2 © 2002–2008, SAS Institute Inc., Cary, NC, USA).

Spatial analysis. Spatial analysis was undertaken with commercial software (SatScanV9.1.1, Information Management Services Inc., Silver Spring, Maryland) (Kulldorff, 1997) using Cartesian coordinates for each of the 8 segments in each tray at each site, cases defined as the number of dead oysters, the population defined as the number of live plus dead oysters at each time point, a discrete Poisson scan statistic, and with the covariates of age and height. To confirm site effects, spatial analyses were conducted across all sites at selected time points: 16th November 2011, 21st November, 24th November 2011, 10th February 2012 and 13th February 2012. To look for within site effects, analyses were conducted for site C on 16th and 21st November, site A at 21st and 24th November, and site B at 10th and 13th February 2012. Contingency tables of the frequencies of dead and live oysters for each age-height group and replicate were prepared at selected time points: the 16th November 2011 for site C and the 21st and 24th November 2011 for site A. Chi-square and Fisher's exact tests (as appropriate) were conducted to compare proportional mortality between the left and right sides of trays and between the two replicates using SAS.

OsHV-1 infection determined by qPCR. Threshold cycle (Ct) values obtained from pooled samples were compared between age and height groups at each observed time for each site using general linear models. Ct values were log transformed to meet the assumptions of normality and homoscedasticity. Predicted means from the model were exponentiated for presentation.

Pool positive and negative status was compared between age and height groups using a logistic regression approach or Fisher's exact test, as appropriate. In addition to the pooled samples, a subsample of oysters of the high adult group collected over 6 observation times from Site A were individually analysed by real time PCR. Individual oyster samples of different age and height groups from Site B were also tested by real time PCR for only one time period (time = T3; 21st November 2012) to compare the effect of age and height on their infection status. Infection prevalence was compared among 6 observation times (Site A) and between age and height groups (Site B) using a similar logistic regression approach or Fisher's exact test.

Growth. Similar general linear model analyses were conducted for growth of oysters over time (length, width and depth). However, log transformation, and hence back transformation of the predicted means, was not required as the assumptions of normality and homoscedasticity were met.

Results

Experiment 1.

Three discrete mortality events were observed in Woolooware Bay: November 2011, February 2012 and April 2012. The presence of OsHV-1 was confirmed by qPCR.

The first mortalities were detected at site C on the 16th November (Figure 3) with mortalities of spat placed at high and low height, as well as, to a lesser extent, adult oysters placed at low height (mortality rate of 46%, 99% and 27%, respectively). By the 21st November, all spat were dead at site C regardless of the height while adult oysters started dying at high height (4%). Cumulative mortality in adult oysters placed at low and high heights reached 72% and 32% by the 30th November, respectively (Figure 3). After this date, no significant increase of mortality was observed for adult oysters until the end of the experiment.

A similar pattern was observed at site A with high mortalities of spat detected first on the 21st November leading to 100% mortality in this age class in 3 days, regardless of the height. Adult oysters placed at high height demonstrated a lower cumulative mortality than the ones placed at low height (final cumulative mortality of 50 and 72% in high and low adults, respectively) (Figure 3).

The mortality pattern displayed at site B was different from the other sites as no significant mortality was recorded until the 10th February 2012 (Figure 3).

In February, all spat exhibited a mortality rate of 100% regardless of the height while adult oysters demonstrated a maximum mortality of 35-36% with no difference between heights.

Mortality patterns are summarised in Table 4.

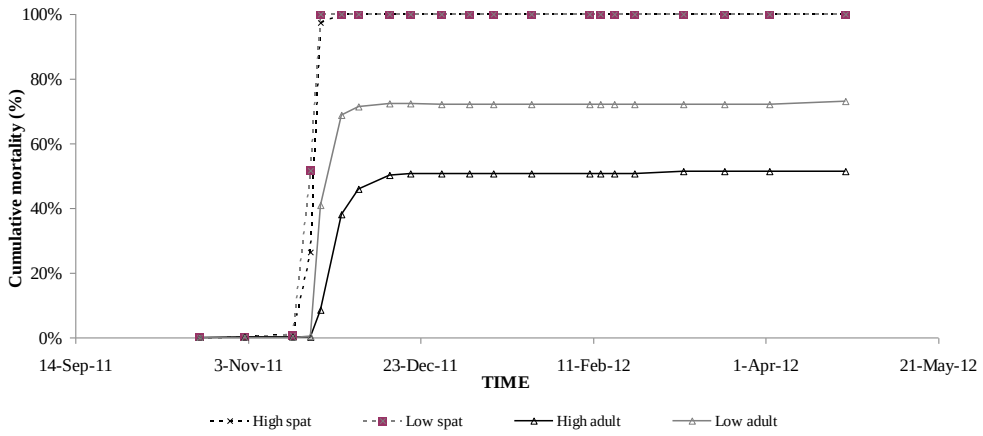
Table 4. Cumulative mortality (percentage) of oysters at sites A, B and C in Woolooware Bay in 2011-12 as a function of age and height of trays (Experiment 1). The initial stocking density was 640 individuals per treatment.

Rearing structure	Treatment	A			B			C		
		Dec 2011	Feb 2012	May 2012	Dec 2011	Feb 2012	May 2012	Dec 2011	Feb 2012	May 2012
Intertidal trays	High spat	100	100	100	3	100	100	100	100	100
	Low spat	100	100	100	1	100	100	100	100	100
	High adult	51	51	52	1	35	40	38	40	45
	Low adult	72	72	73	1	36	53	72	77	80

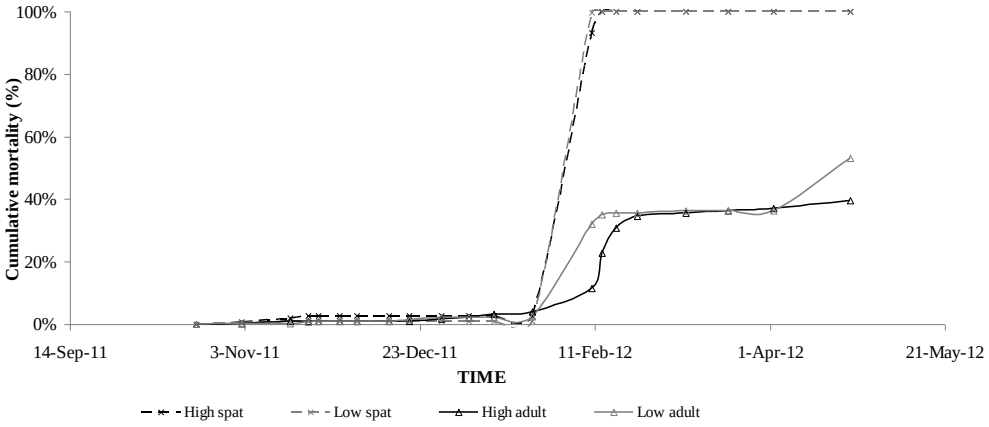
After the first mortality in November, there were no further substantial mortalities recorded in the adult population left alive at site A as shown by similar cumulative mortality in December, February and May ($P>0.05$) (Table 4). Site C exhibited a slight increase in mortality over time for high and low adults ($P=0.3$ and $P=0.003$, respectively).

Figure 3. Cumulative mortality over time for different age and height groups at sites A, B and C.

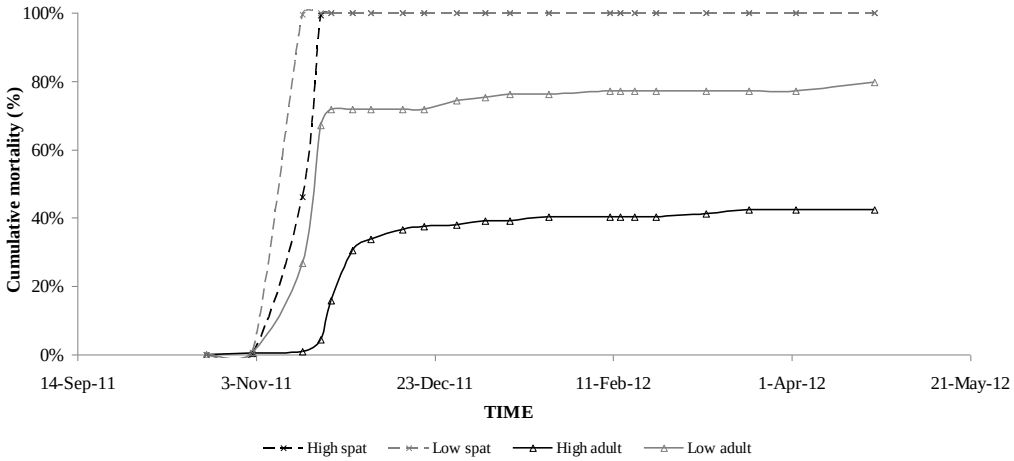
Site A



Site B



Site C



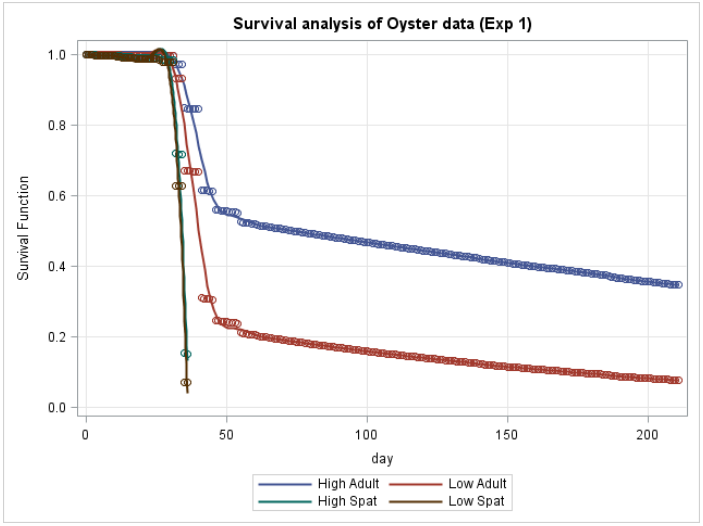
Results of survival analyses conducted to compare mortality between age and height groups are presented in Figure 4 and Table 5. At all sites, high spat had >10 times the hazard of death compared to high adults and low spat had 6-12 times the hazard compared to low adults. Low spat had 1.4-4 times the hazard of death compared to high spat and low adults had 1.5-5 times the hazard compared to high adults.

Table 5. Hazard ratios based on the survival analyses conducted to compare hazard of death between age and height groups in a field experiment conducted at Woolooware Bay, NSW, Australia, during the summer of 2011-2012.

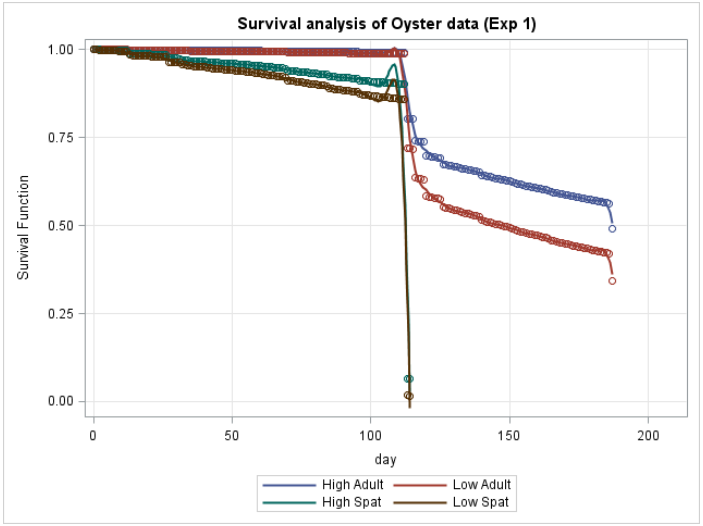
Group	Comparisons	Hazard ratio (95% CI) A	B	C
Age	High spat versus high adult	11.40	12.53	11.93
	Low spat versus low adult	6.66	12.32	9.15
Height				
	Low spat versus high spat	1.41	1.48	4.02
	Low adult versus high adult	2.41	1.5	5.24

Figure 4. Survival analysis for different age and height groups at sites A, B and C.

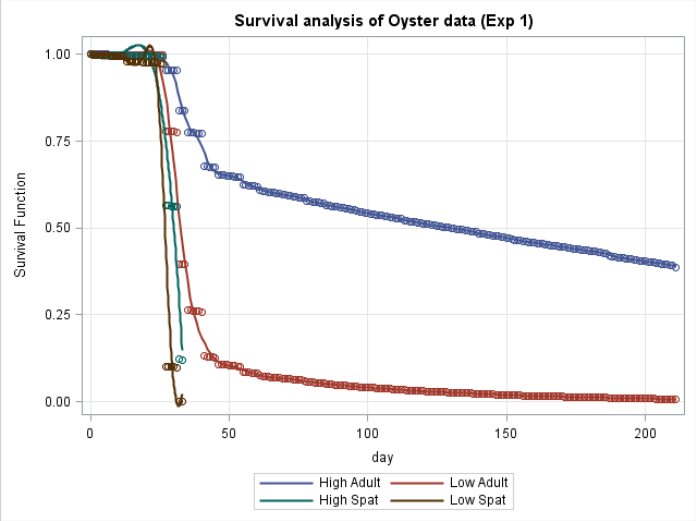
Site A



Site B

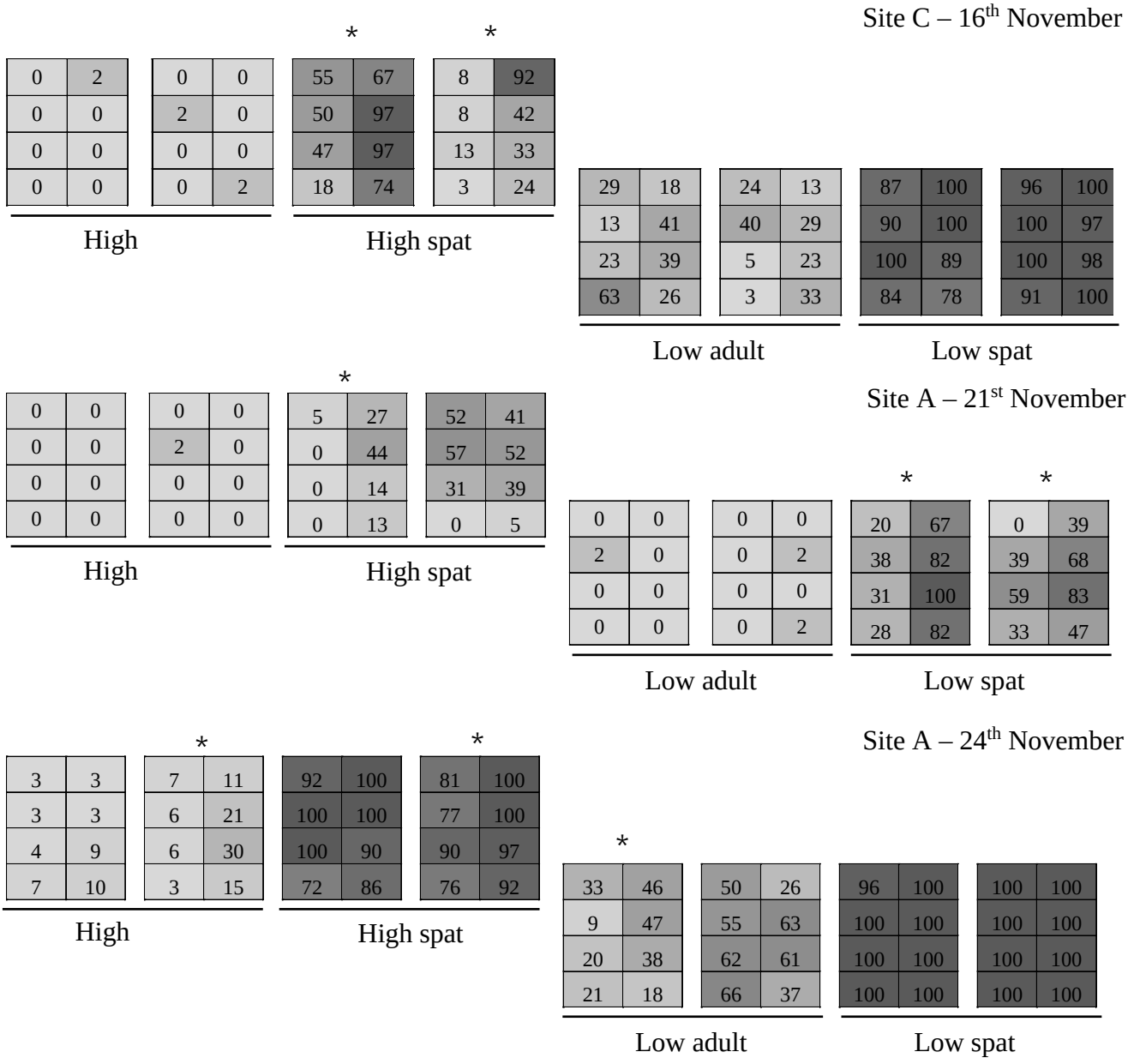


Site C



In retrospective spatial analysis across the three sites there was highly significant clustering of mortality at Site C on 16th November (P<0.001) which persisted until 21st November (P<0.001) by which time there was a secondary cluster at Site A (P<0.001). By 24th November 2011, mortality was clustered primarily at Site A (P<0.001) with a secondary cluster at Site C (P<0.001). Observed mortalities were about 2.3 to 3.5 times greater than expected within these clusters. On 10th and 13th February 2012 mortality was clustered at Site B (P<0.001) with observed mortality 2.1 to 4.8 times greater than expected. During November 2011, the mortality distribution within every segment (n=8 segments per tray) of each tray (n=8 trays per site) was closely observed at sites A and C. Spatial analysis revealed non random distribution of mortality with important clusters between and within trays (Figure 3). Within site C on 16th November the mortality was different between replicates and was clustered in parts of trays, with clusters identified among both spat and adults (P<0.001). Within Site A on 24th November mortality was clustered between and in parts of trays (P<0.001). Chi-square analyses revealed that mortalities were significantly different between left and right sides of trays at sites A and C (Figure 5).

Figure 5. Spatial distribution of mortality in trays at sites A and C for selected time points (experiment 1). Each number represents the observed mortality (%) in each segment of the tray.* indicates trays wherein the mortalities are significantly different between left and right hand sides.



OsHV-1 detection and infection prevalence

As sites A and C demonstrated high mortalities in November – December 2011 in all treatments, random sampling of oysters for pathogen detection stopped in late December for both sites. While a significant number of oysters remained at site B by the end of 2011, no sampling was performed during January, as no significant mortality was detected in January (Figure 3), and in order to maintain a sufficient number of oysters on site. When the second mortality event was detected at site B in February, random sampling of

oysters started again until the population size reached the limit of 10% of the initial number of oysters. Therefore, PCR data are available for all sites in November - December, and for site B only in February (Figure 6).

November - December 2011. Ct values (mean ± SD) and infection prevalence for pooled samples for different age and height groups at sites A, B and C are presented in Figure 6 and statistical analyses conducted to compare the mean Ct values are presented in Table 6. OsHV-1 was not detected in any of the pooled samples until the 16th November at site C and the 21st November at sites A and B. Overall, the range of Ct values for oysters from site B was higher than those measured at sites A and C, while the latter showed similar patterns (Table 6, Figure 6).

At sites A and C spat exhibited lower mean Ct values (i.e. higher viral load) than adults (Table 4). This was true for both high and low groups for Site A and C, but not for Site B, wherever sufficient samples were available to estimate the differences. Mean Ct values were not significantly different between low and high groups, except between high and low adults sampled on the 30th November at Site A, on the 16th and 30th November at site B and on the 24th November at site C (Figure 6, Table 4).

Mean Ct values were also compared over time in each age-height group. At Site A, compared to the mean Ct value at the start of the outbreak, mean Ct values at all successive observation times were significantly different for all groups where sufficient samples were available to make comparisons. At site B, successive mean Ct values (24th November, 30th November and 14th December) were significantly higher than those measured on the 21st November for the low adult group (Figure 6, Table 6). For the low spat group, all mean Ct values were lower than the last observation time in 2011 (28th December). At Site C, mean Ct value for the high adult group on the 24th November was significantly lower than those measured at the previous and later sampling points. For the low adult group, the mean Ct value measured on the 24th November was significantly higher than those measured on previously (16th and 21st November). No differences were observed over time for high and low spat.

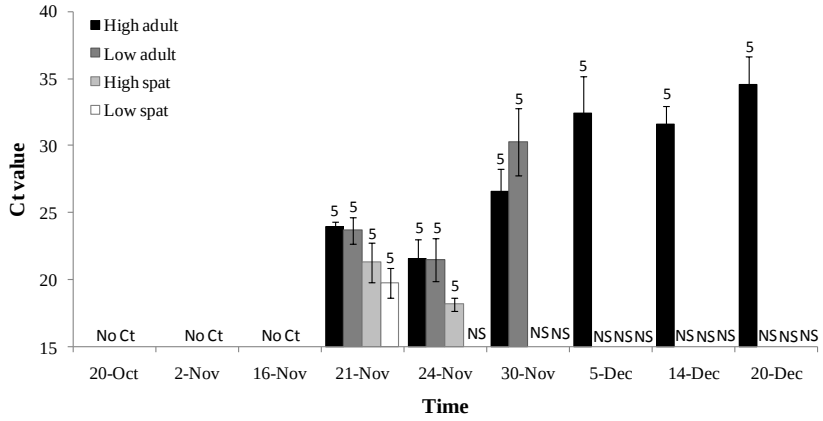
In terms of infection prevalence, logistic regression analyses to compare pool positivity could not be conducted for Site A and C, as almost all pools were positive for all age-height groups for all observation times (Figure 6). As it is not possible to estimate the infection prevalence when 5/5 pools are positive (Table 1), individual PCR were performed in addition to the pooled samples on a subsample of oysters of the high adult group collected over 6 observation times for Site A. The infection prevalence (i.e. the percentage of infected individuals per treatment) for high adults at site A is presented in Figure 6. The probability of an individual oyster being infected varied across time with the proportion of infected oysters increasing from 72% to 92% in 9 days and then decreasing to 60%, 48% and 56% by the 5th, 14th and 20th December, respectively (Figure 8). The infection prevalence on the 24th November (84%) was significantly higher than those measured on the 14th and 20th December

and the proportion infected on the 30th November (92%) was significantly higher than those recorded at a later time (Figure 8).

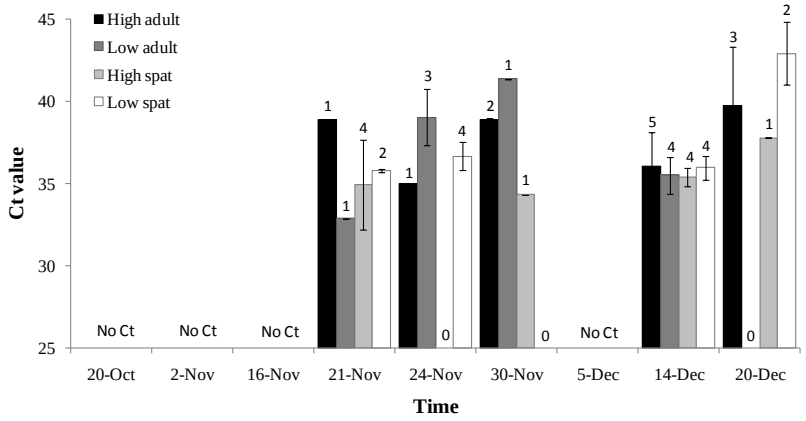
For Site B, the proportion of positive pools was significantly different over time for each age-height group (all P-values <0.05). However, proportions were not significantly different between age-height combinations for any observed time, except on the 24th November, when significantly higher proportions of pools were positive for low spat compared to high spat (P= 0.047) (Figure 7).

Figure 6. Ct values (mean ± SD) for pooled samples for different age and height groups at sites A, B and C in 2011. The number of positive pools per treatment is shown above each bar. The total number of pools per treatment was 5, except for two groups at site C annotated with *: "low spat" 21st November (n = 2 pools) and "low adult" 30th November (n=1 pool), where there were 2/2 and 1/1 positive pools, respectively. No Ct: no virus was detected by PCR. NS: no sampling, all oysters in the treatment were dead.

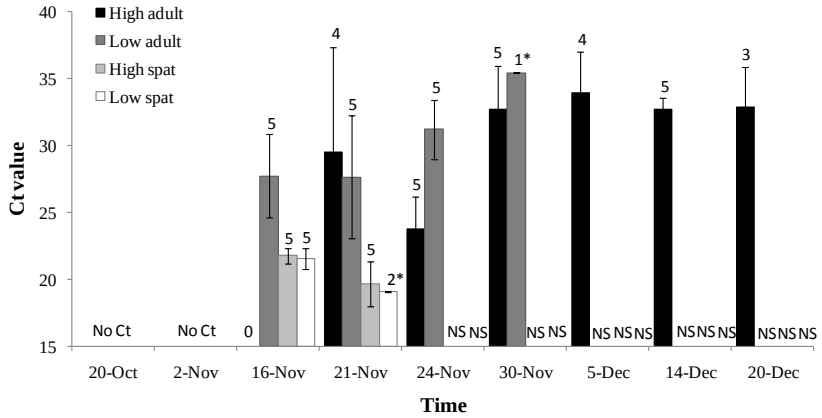
Site A



Site B



Site C



February 2012. Ct values (mean ± SD) and infection prevalence for pooled samples for different age and height groups at site B are presented Figure 7. On the 10th February, spat exhibited lower mean Ct values (i.e. high viral load) than adults. However, the mean Ct values were not significantly different between low and high adults and there were no differences over time.

Figure 7. Ct values (mean ± SD) for pooled samples for different age and height groups at site B in 2012. The number of positive pools per treatment is shown above each bar. The total number of pools per treatment was 5. No Ct: no virus was detected by PCR. NS: no sampling, all oysters in the treatment were dead.

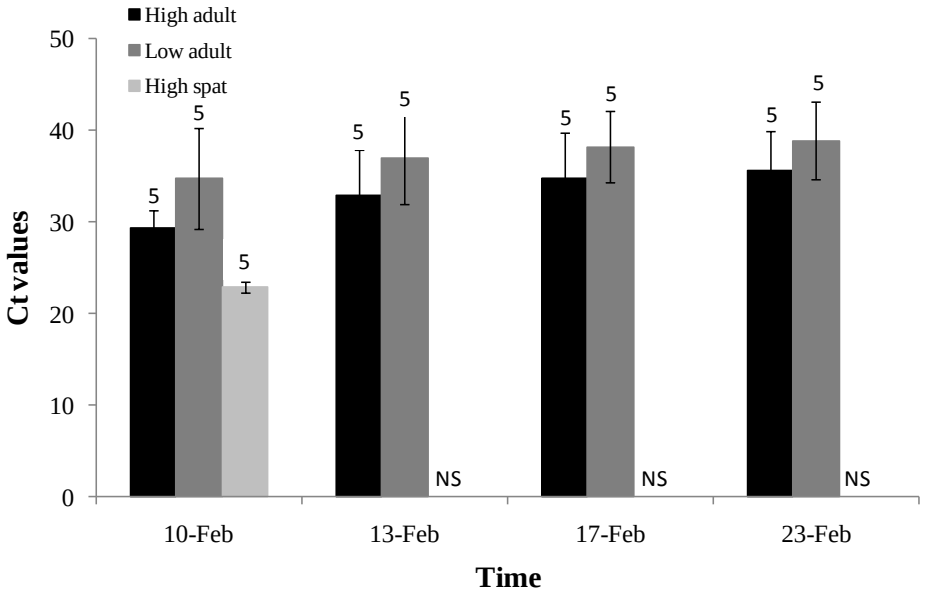


Figure 8. Infection prevalence (%) for high adults at site A before and during the mortality event in November-December. No Ct: no virus was detected by qPCR.

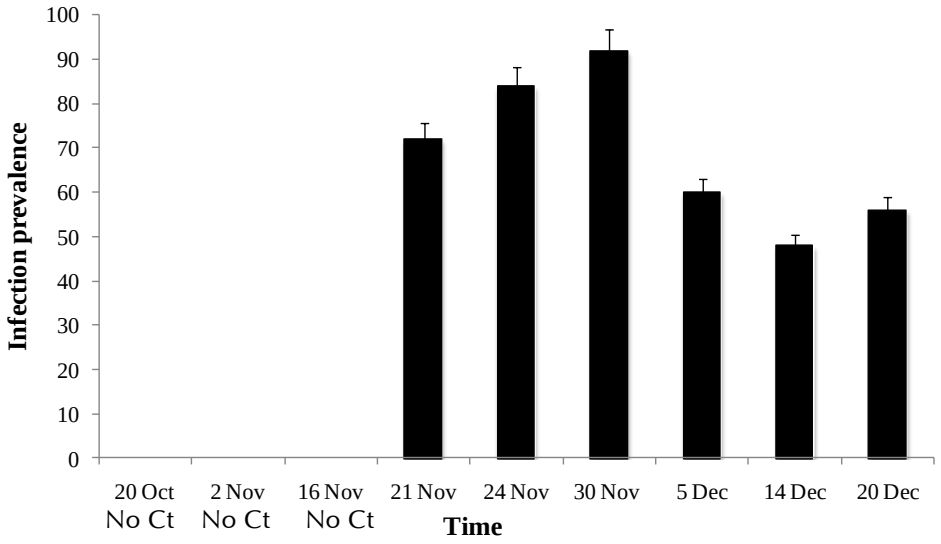


Table 6. Comparison of mean threshold cycle (Ct) values between age and height groups over time at three sites in a field experiment conducted at Woolooware Bay, NSW, Australia, during the summer of 2011-2012.

Site	Date	Mean predicted Ct values			
		High Adult	High Spat	Low Adult	Low Spat
Site A	16 Nov 11	No Ct	No Ct	No Ct	No Ct
	21 Nov 11	24.04[a]	21.21[b]	23.66[a]	19.77[b]
	24 Nov 11	21.64[a]	18.14[b]	21.49[a]	NS
	30 Nov 11	26.55[a]	NS	30.20[b]	NS
	5 Dec 11	32.29	NS	NS	NS
	14 Dec 11	31.58	NS	NS	NS
	20 Dec 11	34.48	NS	NS	NS
Site B	16 Nov 11	No Ct	No Ct	No Ct	No Ct
	21 Nov 11	38.86[b]	34.84[ab]	32.85[a]	35.77[ab]
	24 Nov 11	35.00[a]	No Ct	38.98[a]	36.64[a]
	30 Nov 11	38.84[ab]	34.31[a]	41.35[b]	No Ct
	5 Dec 11	No Ct	No Ct	No Ct	No Ct
	14 Dec 11	35.97[a]	35.39[a]	35.48[a]	35.93[a]
	20 Dec 11	39.41[a]	37.75[a]	No Ct	42.88[a]
Site C	16 Nov 11	No Ct	21.75[a]	27.59[b]	21.54[a]
	21 Nov 11	28.95[a]	19.55[b]	27.05[a]	19.00[b]
	24 Nov 11	23.63[a]	NS	31.13[b]	NS
	30 Nov 11	32.53[a]	NS	35.44[a]	NS
	5 Dec 11	33.84	NS	NS	NS
	14 Dec 11	32.69	NS	NS	NS
	20 Dec 11	32.83	NS	NS	NS

Mean Ct values sharing a common superscript across rows (i.e. for each observed time) are not significantly different. No Ct: Samples not positive; NS: No sampling done, all oysters dead.

Vibrio spp. bacteria in oyster tissues

The identity of putative *Vibrio* species was determined at the Animal Health Laboratories Perth. Of the 120 isolates submitted, the dominant organism (more than 50% of isolates) was *V. splendidus* or organisms most closely resembling this species (Table 7). Unidentifiable *Vibrio* sp. and *V. alginolyticus* were the next most commonly identified organisms. As sample selection was based on representative colonies, the frequency of occurrence of each species shown in Table 7 may not accurately represent that in the oyster population, although *V. splendidus* is most probably the most common organism present. Some of the species present in small numbers in Table 7 may have been the dominant organisms in particular individual oysters.

There did not appear to be a pattern of isolation of any of the species identified over time (Appendix 4). None of the dominant species identified were absent before mortality commenced and present after mortality commenced, suggesting lack of involvement of particular *Vibrio* species in the mortality event. *V. splendidus* was the dominant organism both before and after the mortality event.

Each of the species *V. splendidus, V. harveyi* and *V. alginolyticus* has been isolated from diseased molluscs. In order to determine whether these or other *Vibrio* sp. were associated with the outbreaks of mortality a temporal analysis of vibrio count and mortality rate was undertaken. The hypothesis was that counts of *Vibrio* sp. would increase prior to observed mortality, rather than after observed mortality if *Vibrio* sp. were involved in disease causation. The total bacterial load and total *Vibrio* spp. counts over time in relation to the patterns of mortality are presented for each treatment at each site in Figure 9. At site A the quantity of bacteria and *Vibrio* spp. increased markedly as the mortalities started (21st - 24th November) and for treatments where there were sufficient samples (high adults), a decrease in bacterial load was observed as the mortalities stopped. At site C no significant difference in *Vibrio* load was observed in adult oysters before, during or after the mortality event, in contrast to spat which exhibited higher numbers of bacteria in their tissues on the 16th and the 21st November when the cumulative mortalities were between 46% and 100%. At site B, a significant increase in the bacterial load was observed on the 30th November and the 14th December for all treatments but no mortality event was detected at this site. Overall, no increase in *Vibrio* load was observed prior to the mortality event, with the only exception being for high spat at site A which exhibited high *Vibrio* load on the 16th November, 5 days before significant mortalities started (Figure 9).

Table 7. Species identity and number of isolates of putative *Vibrio* species which were identified at the Animal Health Laboratories, Perth, in decreasing order of occurrence.

Species	No. of isolates
V. splendidus	51
Vibrio sp.	26
V. alginolyticus	18
V. harveyi	6
NT	5
? *V. splendidus*	4
V. parahaemolyticus	2
? *V. parahaemolyticus*	2
Photobacterium damselae	2
? *V. fischeri*	1
? *V. harveyi*	1
? *V. pelagius* II	1
? V. alginolyticus	1
Total	120

Figure 9. Pattern of mean bacterial loads (total bacteria and *Vibrio* spp.) and cumulative mortality over time for sites A, B and C. Mean bacterial counts sharing a common superscript (a,b,c) over time are not significantly different (total bacteria and *Vibrio* spp. data were analysed separately).

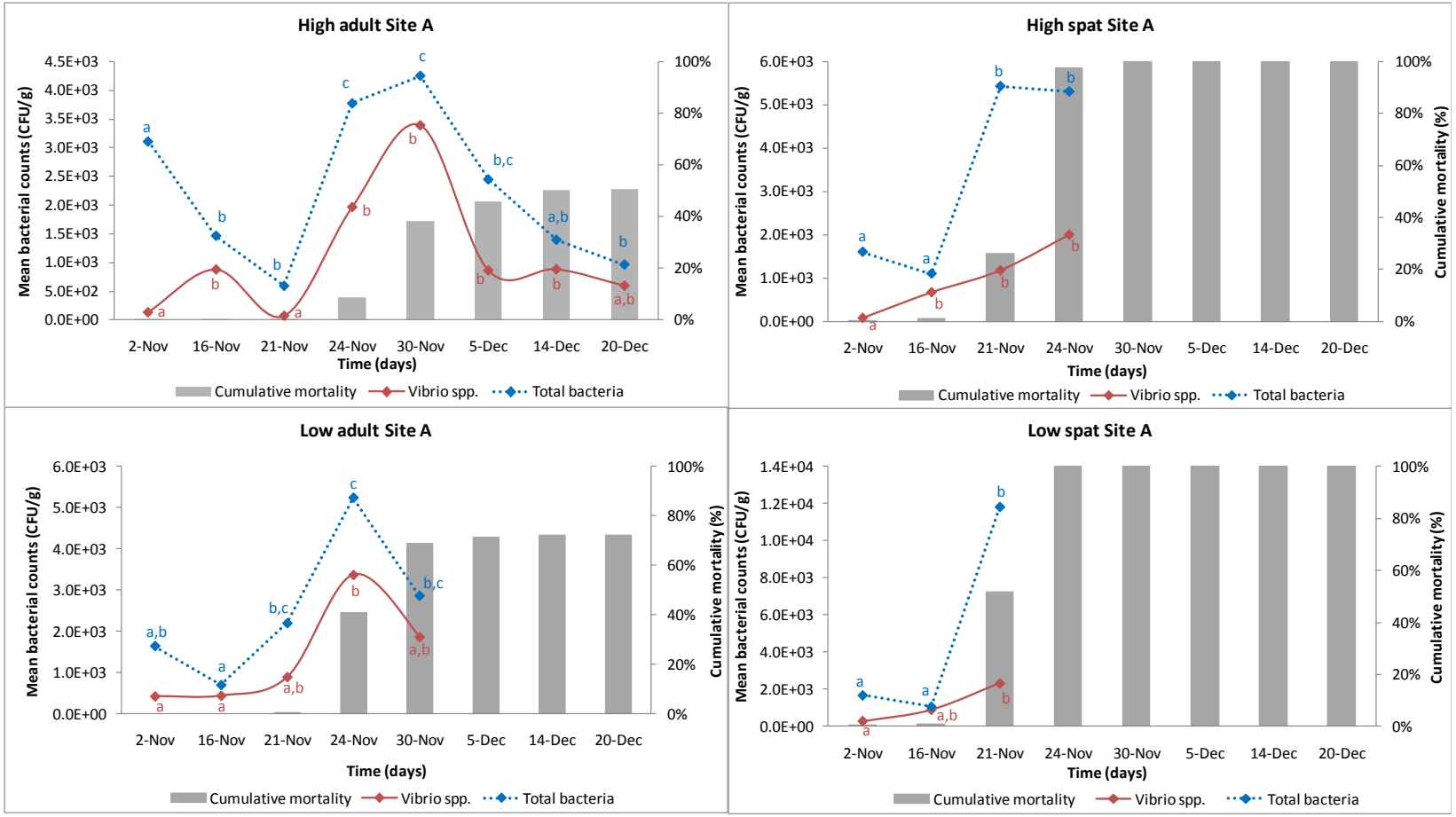


Figure 9 continued

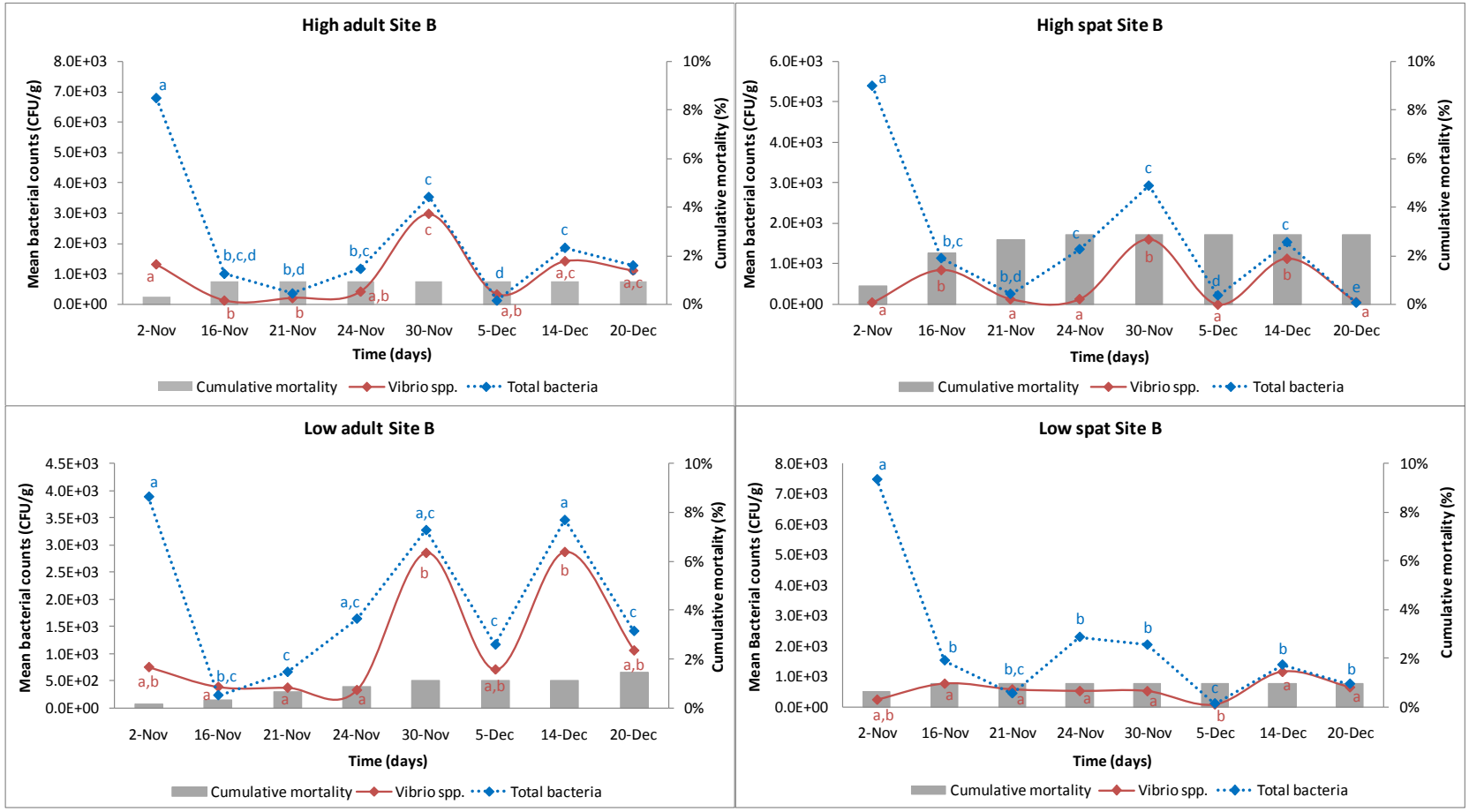
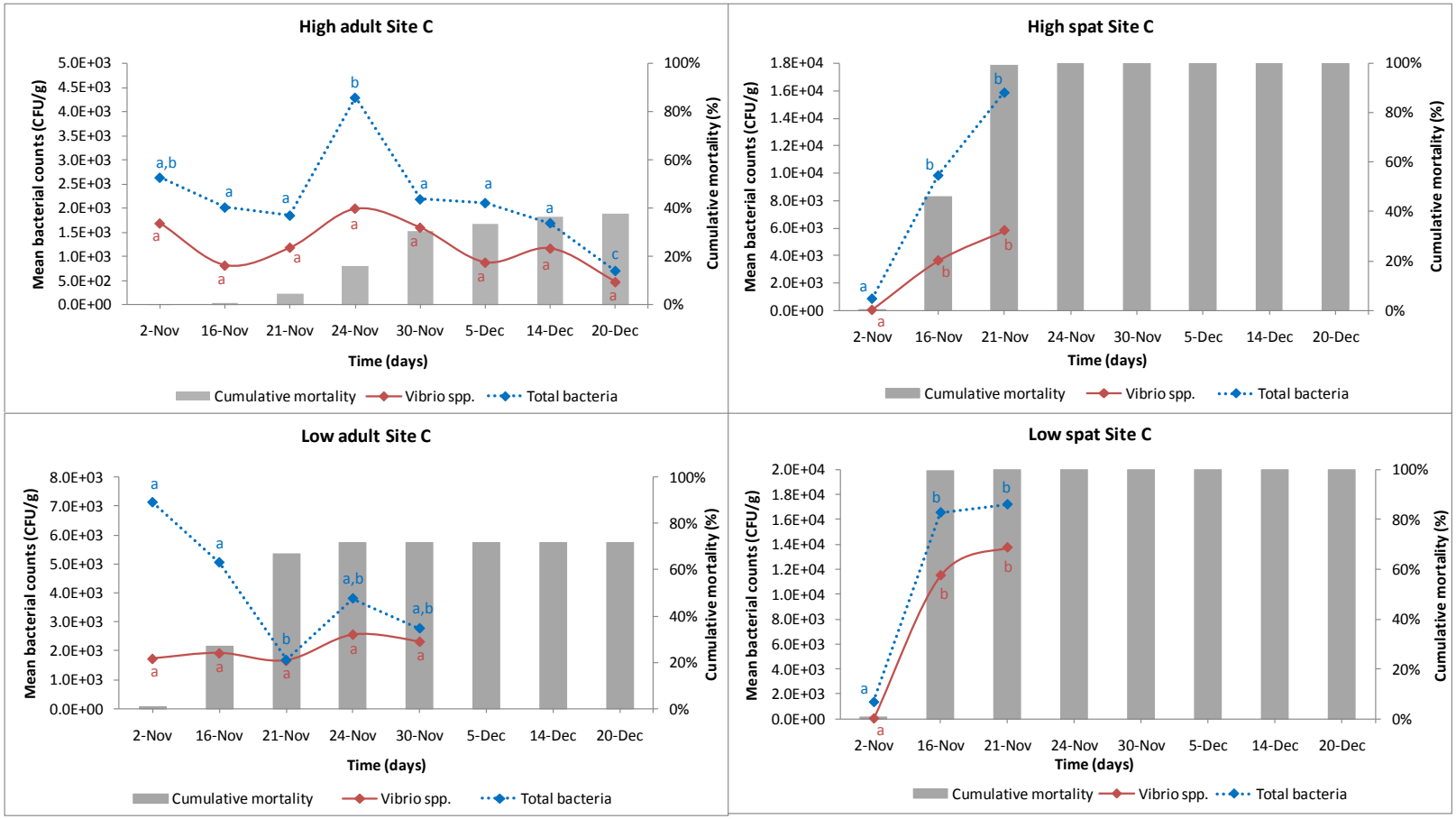


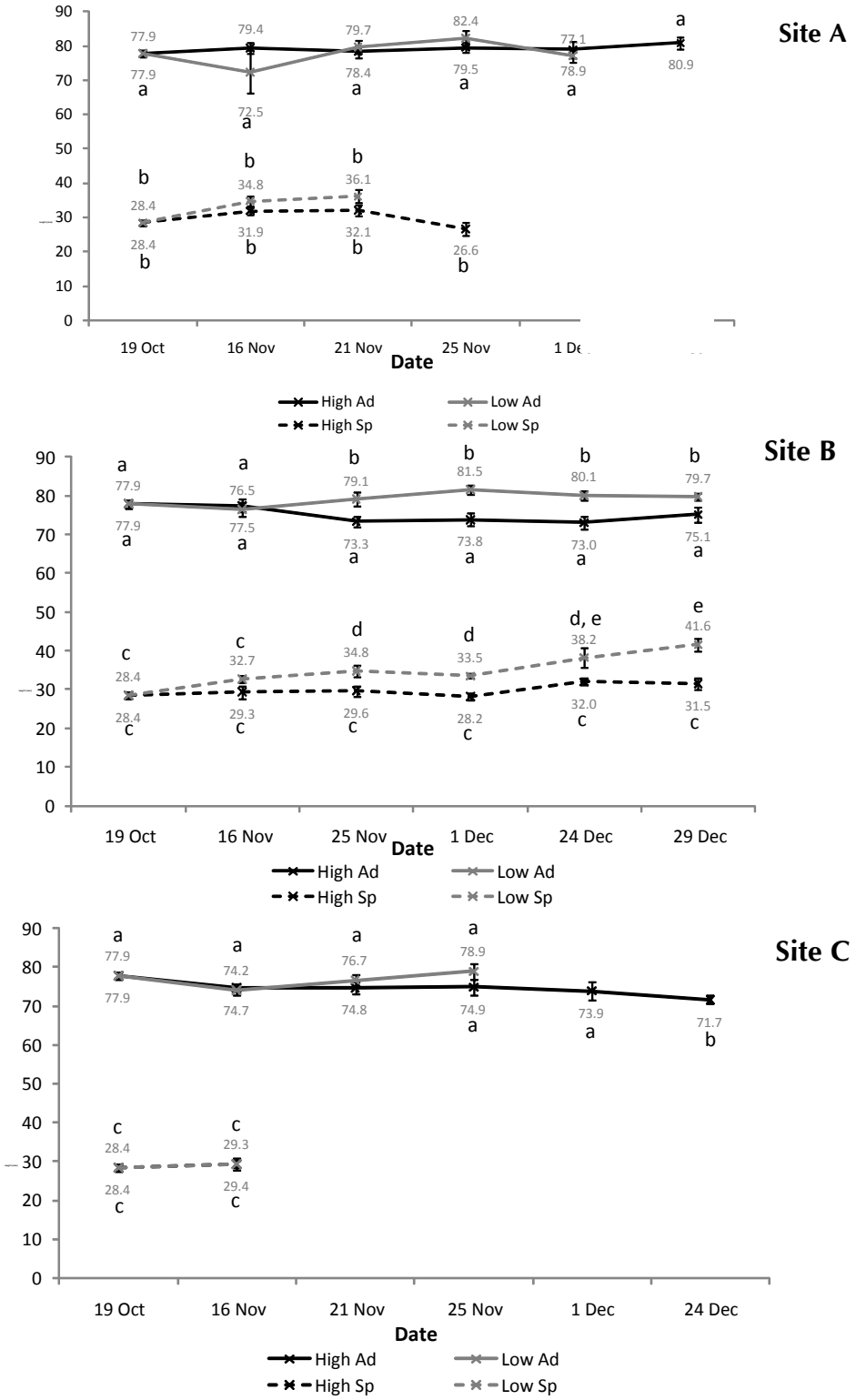
Figure 9 continued



Growth

The growth of oysters over time, measured by the increase of shell length, is shown in Figure 10. No significant difference in length was observed between low and high groups (for spat and adults) and over time at sites A and C. At site B, a significant difference in length was observed between high and low height (for spat and adults) from the 24th November, oysters placed at low height demonstrating a better growth than oysters placed at high height. Also, a significant increase in length over time was observed for oysters placed at site B at low height, from the 24th November for low adults and from the 24th December for low spat. No increase in length was observed over time for the spat and adult oysters placed at high height. No difference in growth in terms of shell width or depth was observed over time within each treatment or between the two heights at all three sites (data not shown).

Figure 10. Length (mean ± standard deviation) of oysters as a function of time at three sites A, B and C in Woolooware Bay. Mean values sharing the same letters are not significantly different (p>0.05). Each site is considered separately.



Wild molluscs and other potential reservoir hosts

All pools tested in qPCR were negative for OsHV-1. These results indicate that the wild organisms collected (described in Appendix 3) were not infected with OsHV-1, or the level of infection was below the detection limit at the time of collection. 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.

Environmental data

Salinity. Daily mean, minimum and maximum salinity values averaged for each week are presented for the three sites in Figure 11. Overall, the three sites demonstrated similar salinity patterns over time. Salinities recorded at site A had high variation, with extreme minimum daily values (13.6 ppt around the 2nd April) and higher maximum daily values than sites B and C. There was no significant difference between sites B and C in terms of mean daily salinities recorded over time. The higher range of variation recorded at site A is likely to be due to its topography (it is very shallow) and its location on the external and oceanic part of the bay, leading to more exchanges between the oceanic and freshwater inputs at each tide cycle. In contrast, sites B and C may be subjected to less fresh/seawater exchanges due to their location in the inner part of the bay. As the incubation period of OsHV-1 is between 3 to 10 days (Renault, 2011; Schikorski et al., 2011b), a focus for analysis was the salinity pattern at all three sites during the two weeks preceding each mortality event (highlighted in light pink in Figure 11) in order to determine (i) local environmental parameters, and (ii) whether a difference in one of these parameters could be associated with the pattern of mortality observed over 2011 and 2012 in Woolooware Bay.

Prior to the November mortality event, mean salinity values decreased slightly at all sites, from 35.9 – 36.0 ppt at the end of October to 34.0 - 34.7 ppt during the week of the 14th November 2011 when the first mortalities were detected. No significant differences were detected among the three sites during the two weeks prior to the mortality event, while significant spatial differences were observed at that time in terms of disease expression (site B was free of any sign of disease while sites A and C exhibited high mortalities). The only difference was detected in the week of the 21st November 2011 when salinity at site B appeared to be significantly lower than at sites A and C ($P < 0.001$). In the week of the 28th November, significantly higher salinity was recorded at site A in comparison with sites B and C.

Figure 11. Daily mean (A), minimum (B) and maximum (C) salinity values over time (weekly means) at sites A, B and C in Woolooware Bay. The periods when OsHV-1 associated mortality was observed are shaded.

Figure 11.A Mean salinity

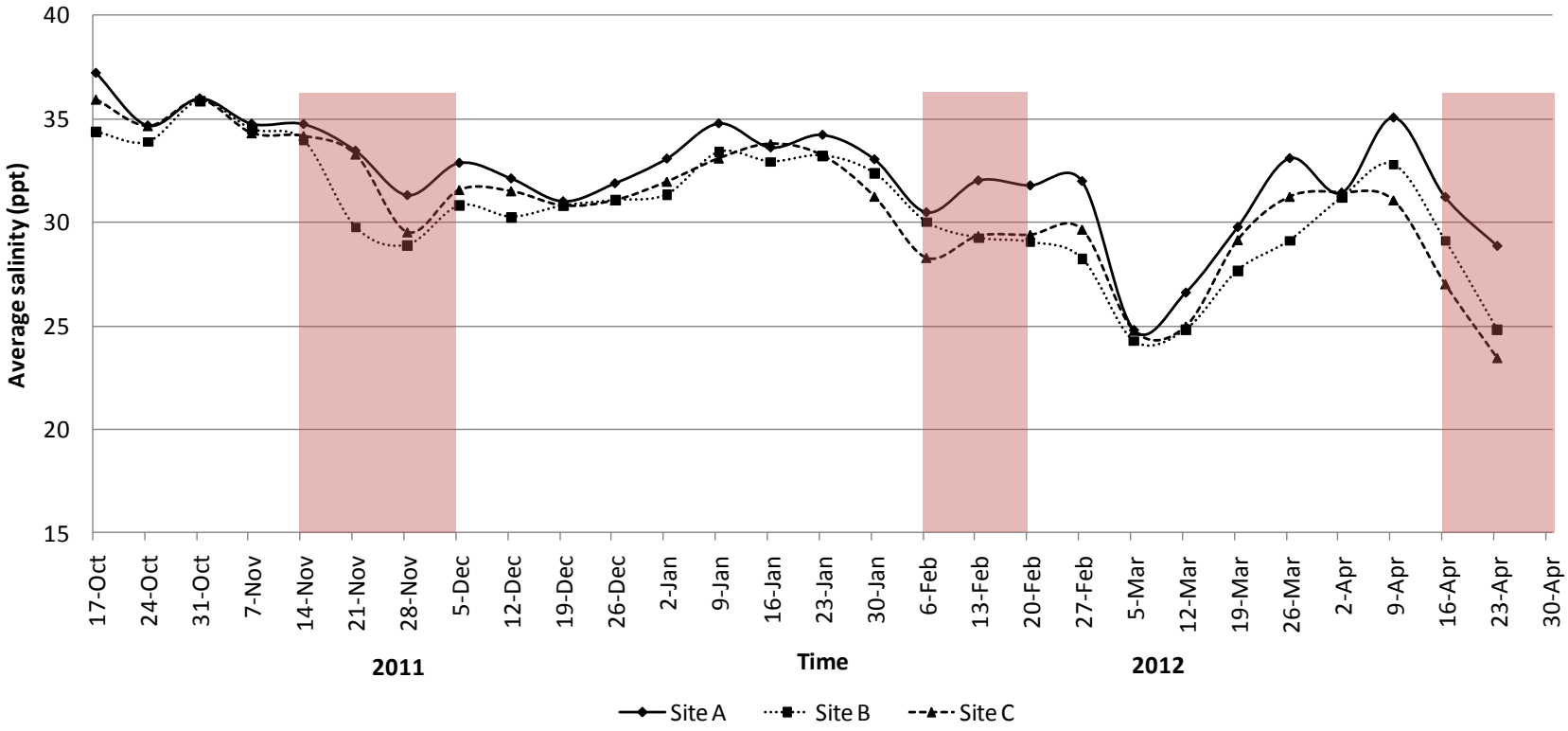


Figure 11.B Minimum salinity

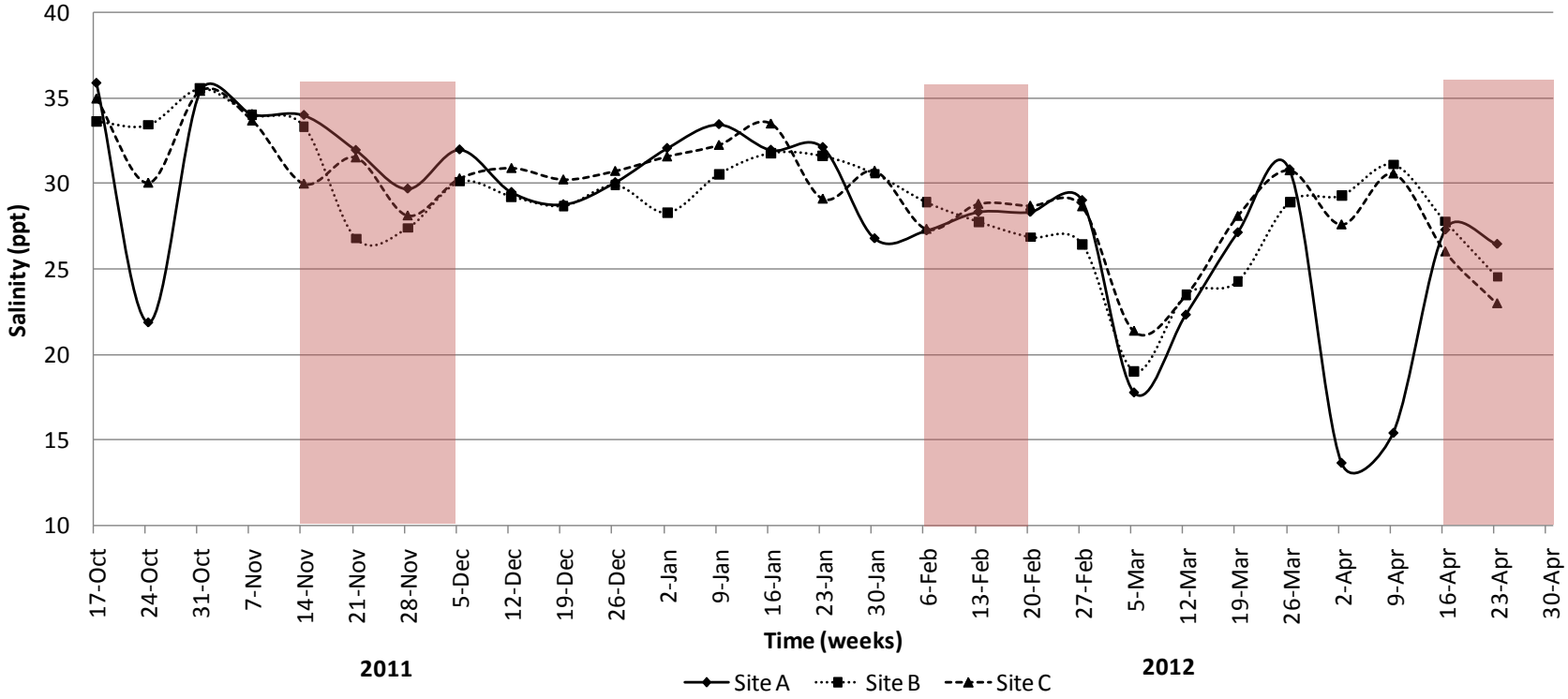
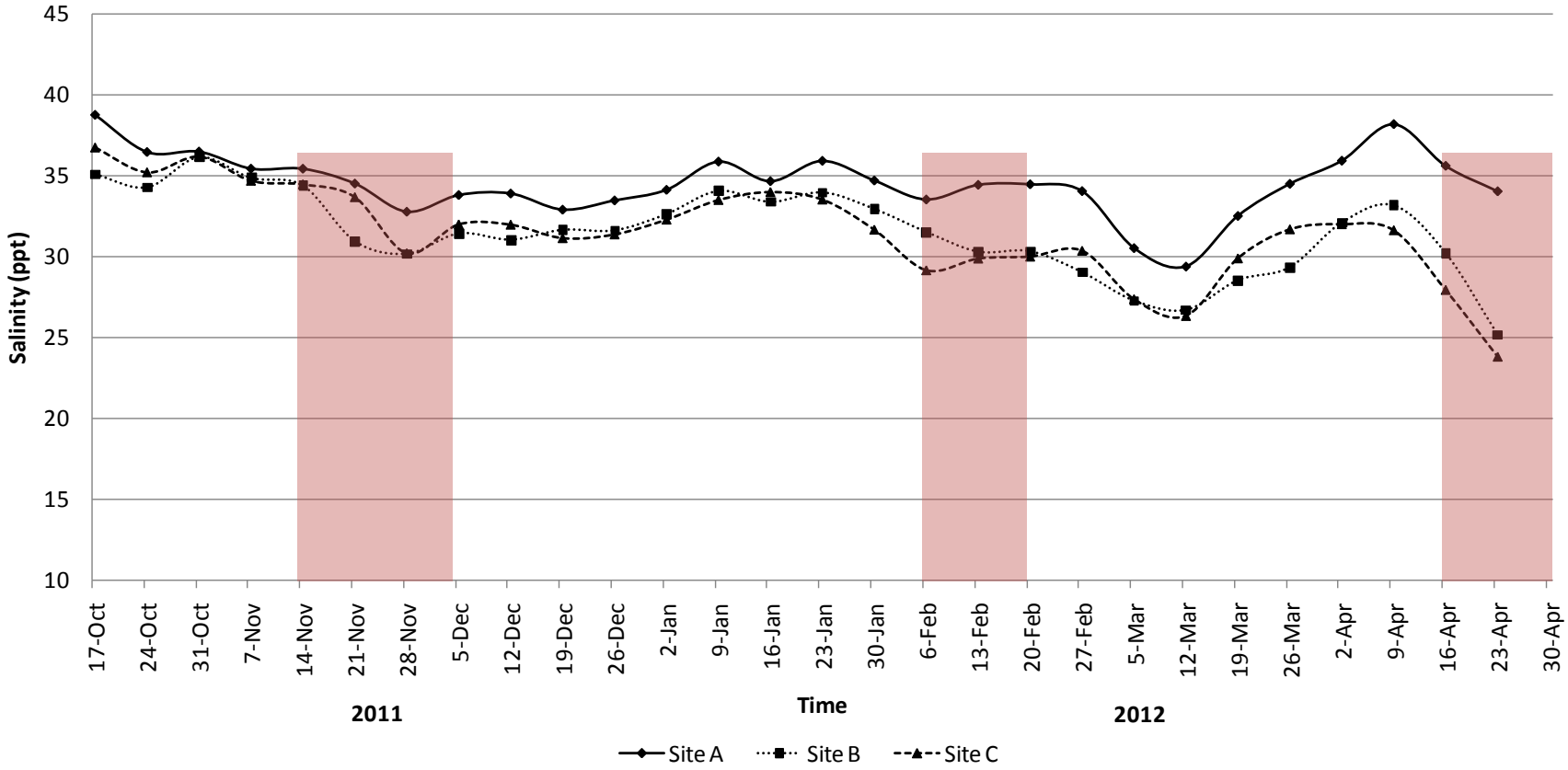


Figure 11.C Maximum salinity



During the two weeks prior to the February 2012 mortality event which affected sites B and C only, mean salinity values decreased by 3.1, 2.9 and 5.5 ppt at sites A, B and C, respectively. From the beginning of February 2012 site A exhibited significantly higher weekly mean salinity values than sites B and C and the difference remained significant until the beginning of March. No significant difference was observed between sites B and C over this period of time.

The last mortality event was detected in April 2012 (19th April) and affected all sites. There was a decrease of mean daily salinity values at all sites; the difference in weekly mean salinity values ranged from 6.2 to 8 ppt between the weeks of the 9th April and the 23rd April (Figure 11). Site A exhibited higher mean daily salinity values than sites B and C during the week of the 9th April. No difference between sites B and C was demonstrated until the 23rd April.

Overall, the difference in weekly salinity means among the three sites cannot explain the different patterns of mortality observed sequentially at site A, B and C. However, it is interesting to note a decrease in salinity at all sites prior to each mortality event. The amplitude of the drop in salinity differed from time to time (November *vs.* February *vs.* April) and could be associated with an increase in rainfall leading to a diminution of the salinity by direct and indirect effects (direct diminution of the salinity on site, increase in the runoff from the catchment area through the Georges River and local creeks). There is no prior evidence to suggest that salinity plays any important role in OSHV-1 expression (Anon, 2011). However, it is well known that variation of salinity and extreme values can significantly impair the immune systems of oysters and other bivalve molluscs, making them more susceptible to infection (Gagnaire et al., 2006; Li et al., 2009). During major rain events, inflows to an estuary can contain a wide variety of organic and metallic pollutants which significantly affect bivalve immunity even at very low concentrations (Canesi et al., 2003; Canesi et al., 2012; Galloway and Depledge, 2001; Wootton et al., 2003). These factors were not measured in the present study.

Water temperature (subtidal probes). The mean daily water temperature recorded by the subtidal salinity probe and averaged for each week is presented in Figure 12. Overall, site A exhibited a slightly lower mean temperature than sites B and C (0.5°C difference on average) and this difference was significant at all times (all P-values < 0.0001) while no significant difference was observed between temperatures recorded at sites B and C. The pattern of temperature at all sites increased and decreased over time leading to significant differences observed between all time points within each site.

From the 31st October 2011 to the 7th November 2011 the water temperature increased rapidly from 20.3 - 20.8°C to 22.9 - 24.1°C (difference of 2.7, 3.3 and 3.4°C at sites A, B and C, respectively). After this rise, the water temperature progressively decreased to 19.8 – 20.0°C by the 5th December. Over a four week period (5th Dec – 2nd Jan) the mean water temperature increased slowly to 24.0 - 24.9°C at all sites and stabilised until late February 2012, showing only slight weekly variations. From the end of February 2012 until the beginning of April 2012, the pattern of water temperature showed significant undulations between 22 and 24°C on average. A sudden decrease in water temperature by 3.6-4.0°C (depending on

the site) was observed between the weeks of the 2nd and 9th April 2012, before a slight increase of 1.2-1.4°C by the week of the 16th April.

In Europe OsHV-1 associated mortalities occur when the water temperature is between 16 and 24°C and often happen after a sudden rise in water temperature (Garcia et al., 2011; Le Deuff et al., 1996). Therefore, the sudden increase in temperature observed in Woolooware Bay in early November 2011 may have triggered the onset of the disease. As significant mortalities were detected in February and April without any major increase in water temperature, it is reasonable to assume that the link between water temperature and disease onset is indirect and might involve other environmental factors. It is also interesting to note that the disease started at the same time of year in November 2010 and 2011, within a day. It is currently not known whether the patterns of water temperature were identical among these years and whether indirect biological processes linked to the temperature pattern at the beginning of the Australian summer are involved in the onset of OsHV-1 disease.

Water temperature (temperature probes in trays). The mean temperatures (averaged for each week) recorded by the probes which were placed in high and low trays are presented in Figure 13. At each site, the temperature patterns over time recorded in trays were almost identical to the ones recorded by the salinity probes (Figure 12). The major difference concerns the absolute values (mean, max and min) which were consistently higher in the data set recorded by the salinity probes. This difference might be due to the location of the salinity and tray temperature probes, *i.e.* constantly immersed in water *vs.* intermittently exposed to air, respectively, as the summer was particularly cool and rainy. This difference could also be due to inherent variability of the two sets of probes, characterized by different accuracy and calibration methods.

No significant difference was observed in mean temperature values between probes placed at the same height within each site (low spat tray *vs.* low adult tray; high spat tray *vs.* high adult tray). Temperature probes placed at low height exhibited consistently higher mean temperature records than the probes placed at high height within each site (Figure 13). However the differences of mean temperatures between low and high probes were small and ranged from a minimum of 0.1 to a maximum of 1.4°C. Given the relatively low internal accuracy of the temperature probes (±1°C), these differences, while interesting, cannot be considered to be significant.

The temperature probes which were placed in trays enabled the difference in immersion time between low and high trays to be determined. On average high trays were immersed for ~2 hours less per tide cycle (~4 hours per day) than high trays. This is illustrated in Figure 14.

Maximum temperatures recorded were higher for high trays than low trays during certain conditions, specifically when low tide occurred during hot weather with high incident solar radiation. This is illustrated for one two week period in Figure 15; temperature differences of more than 10°C were seen between low and high trays, and temperatures over 35°C were recorded in the high trays.

Figure 12. Mean water temperature recorded over time by the subtidal salinity probes at sites A, B and C. The periods when OsHV-1 associated mortality was observed are shaded.

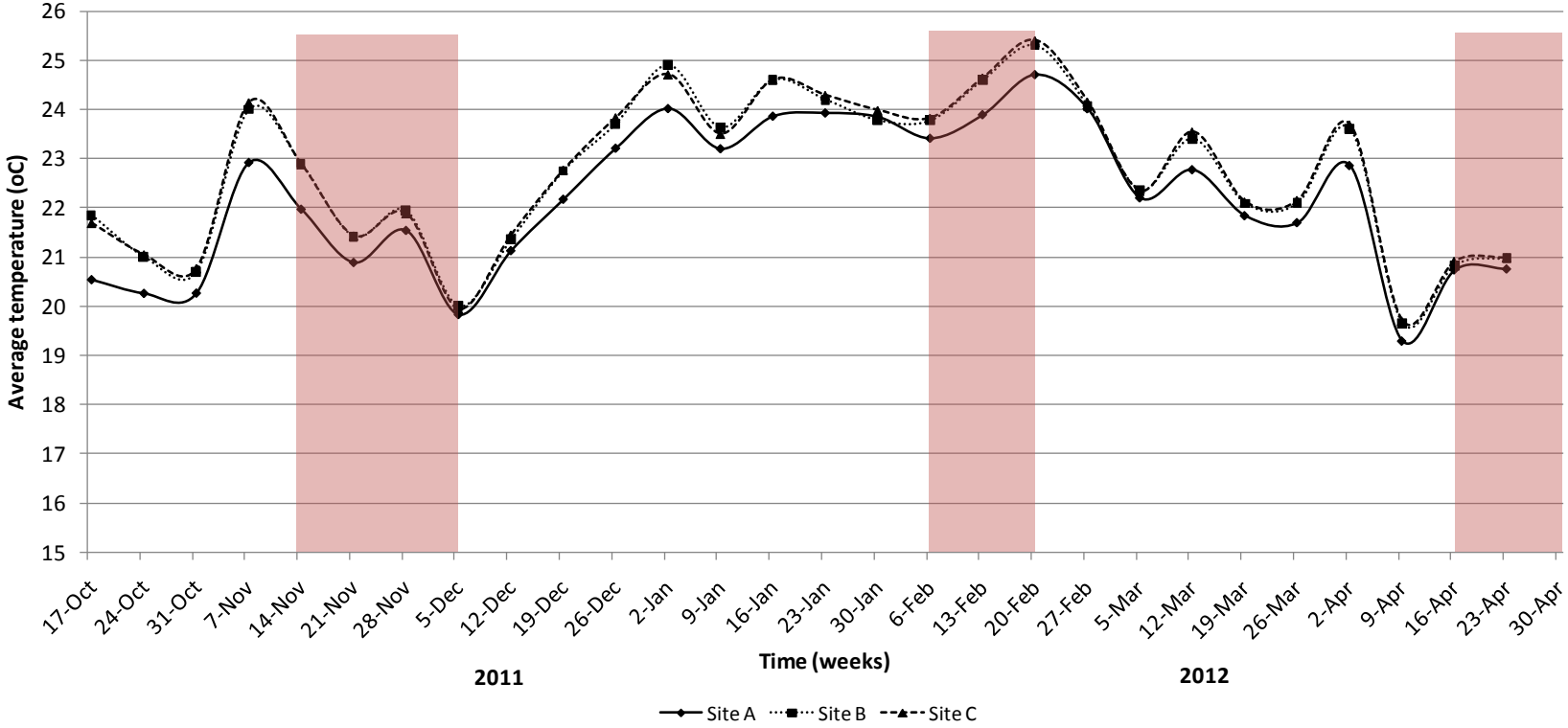
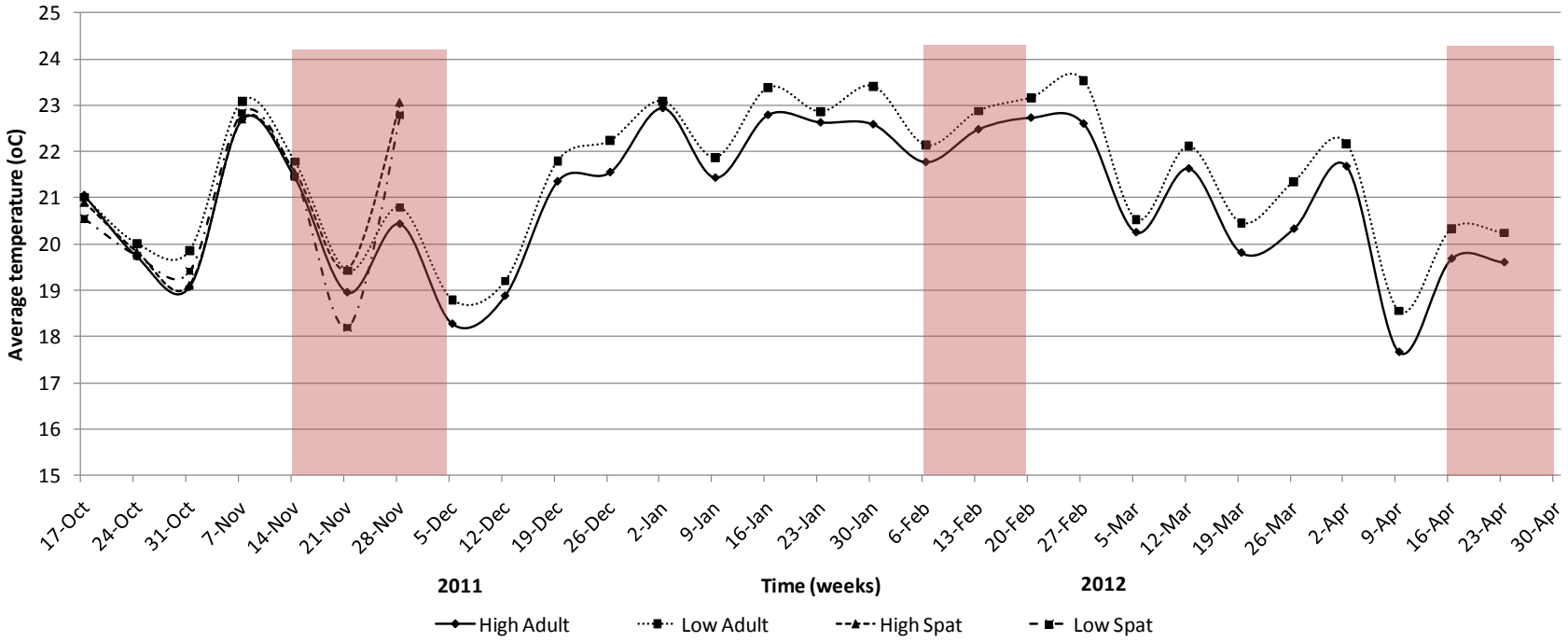
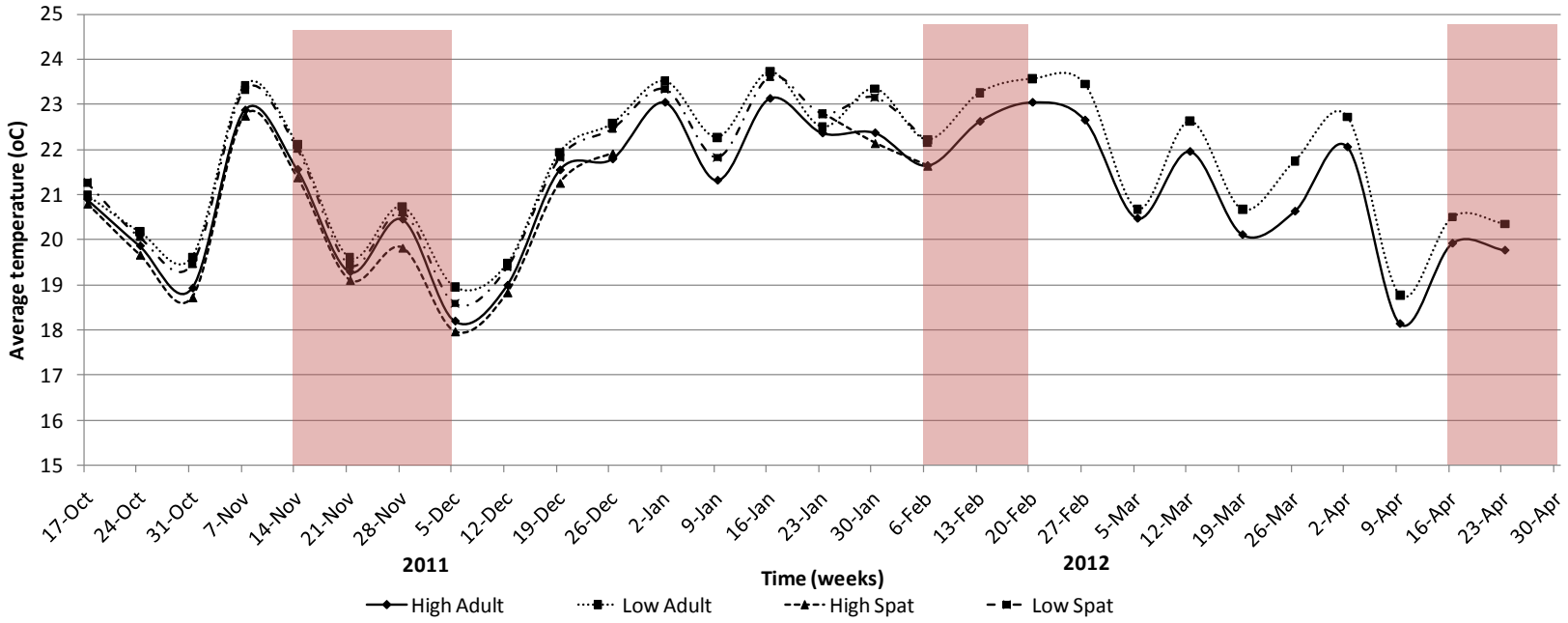


Figure 13. Mean temperature recorded by the temperature probes placed in trays at the three sites (A , B and C). The periods when OsHV-1 associated mortality was observed are shaded.

Site A



Site B


25
24
23
22
21
20
19
18
17
16
15
Average temperature (oC)
17-Oct
24-Oct
31-Oct
7-Nov
14-Nov
21-Nov
28-Nov
5-Dec
12-Dec
19-Dec
26-Dec
2-Jan
9-Jan
16-Jan
23-Jan
30-Jan
6-Feb
13-Feb
20-Feb
27-Feb
5-Mar
12-Mar
19-Mar
26-Mar
2-Apr
9-Apr
16-Apr
23-Apr
30-Apr
2011
2012
Time (weeks)
High Adult
Low Adult
High Spat
Low Spat

Site C

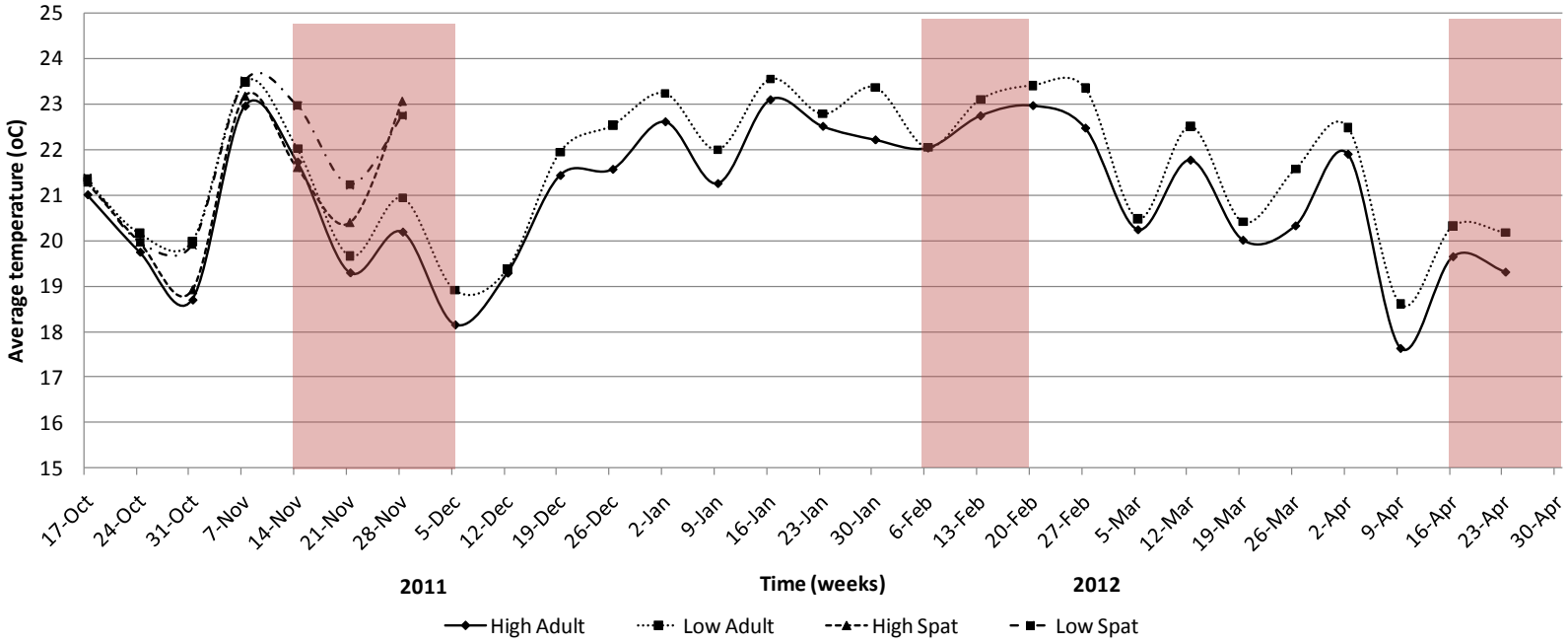

Average temperature (oC)
25
24
23
22
21
20
19
18
17
16
15
17-Oct
24-Oct
31-Oct
7-Nov
14-Nov
21-Nov
28-Nov
5-Dec
12-Dec
19-Dec
26-Dec
2-Jan
9-Jan
16-Jan
23-Jan
30-Jan
6-Feb
13-Feb
20-Feb
27-Feb
5-Mar
12-Mar
19-Mar
26-Mar
2-Apr
9-Apr
16-Apr
23-Apr
30-Apr
2011
Time (weeks)
2012
High Adult
Low Adult
High Spat
Low Spat

Figure 14. Variation of temperature between low and high trays over 48h (31/10/11 - 2/11/11) at site C. Each vertical gridline represents an hour. Red arrows represent the immersion time for high trays. Purple arrows represent the immersion time for the low trays. The difference in immersion time between low and high trays was 2 hours on average.

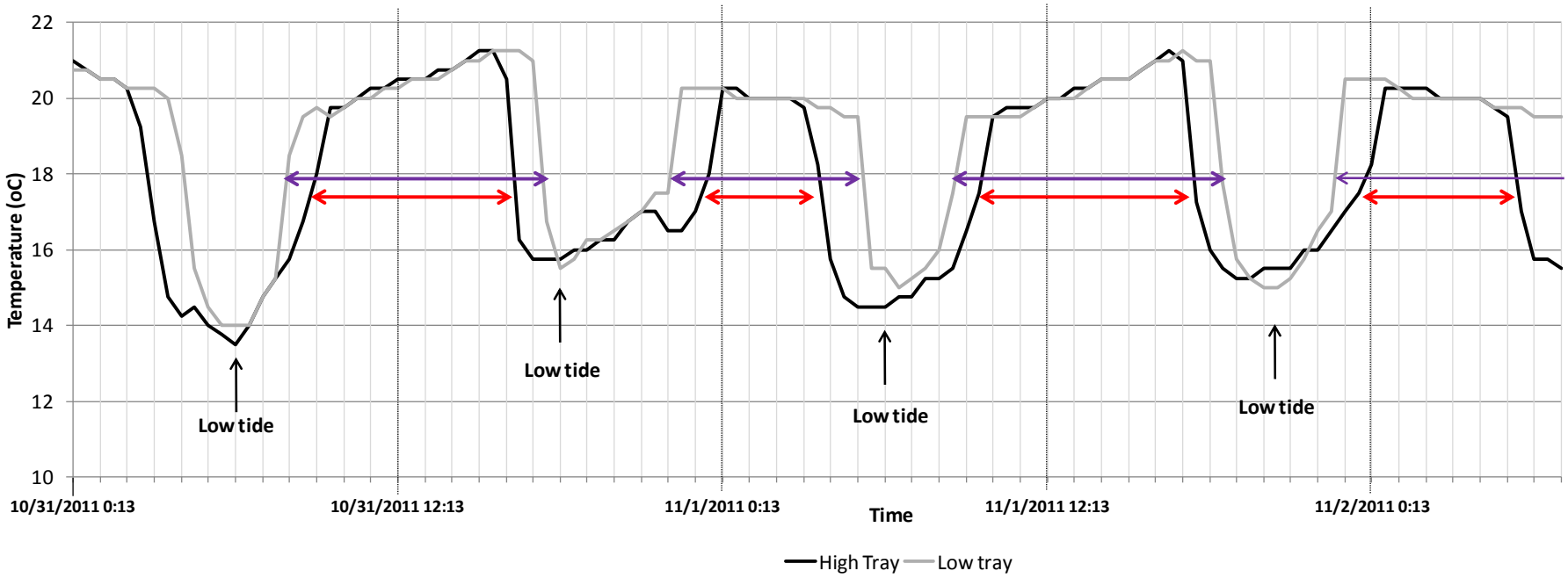
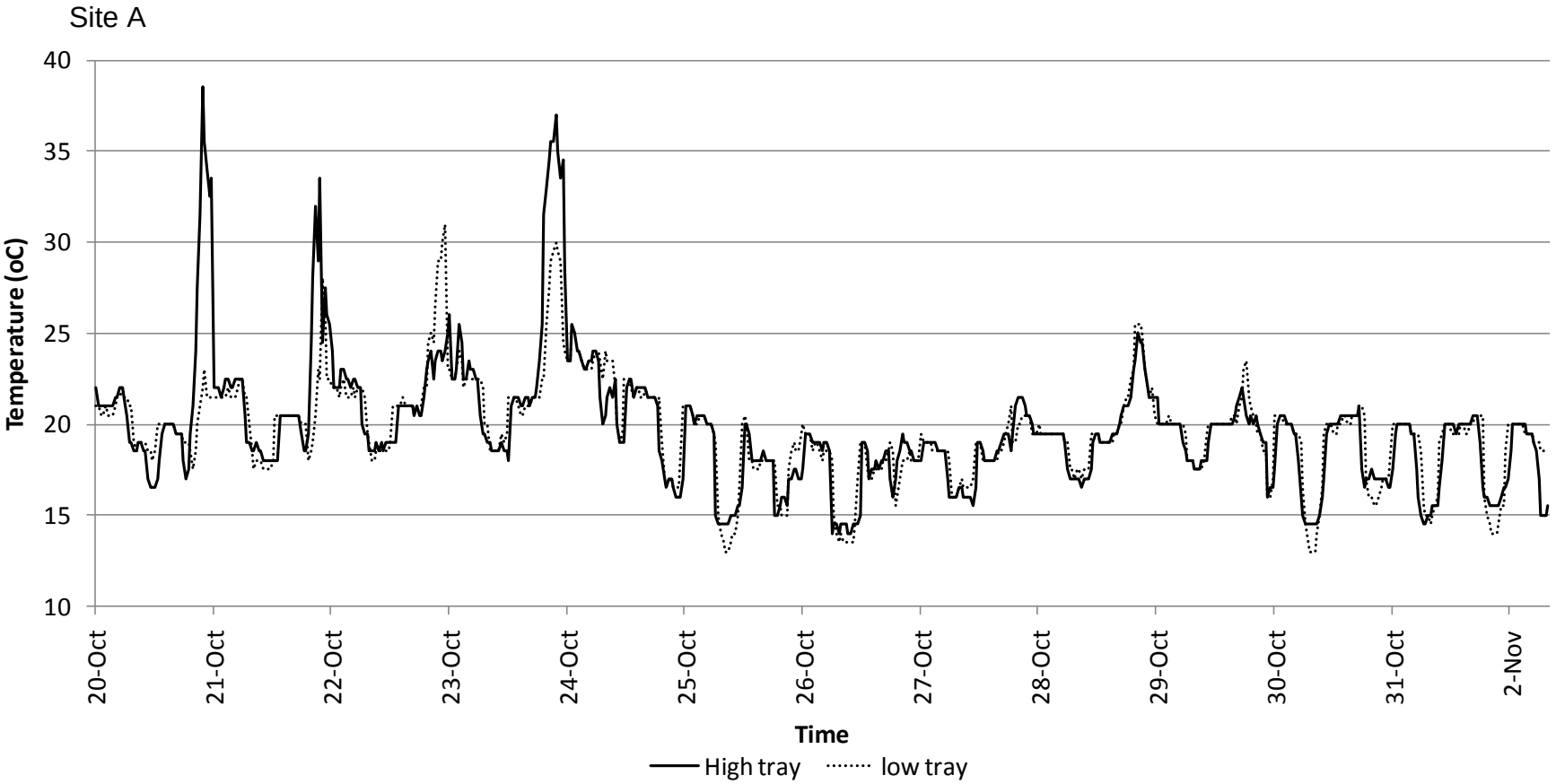
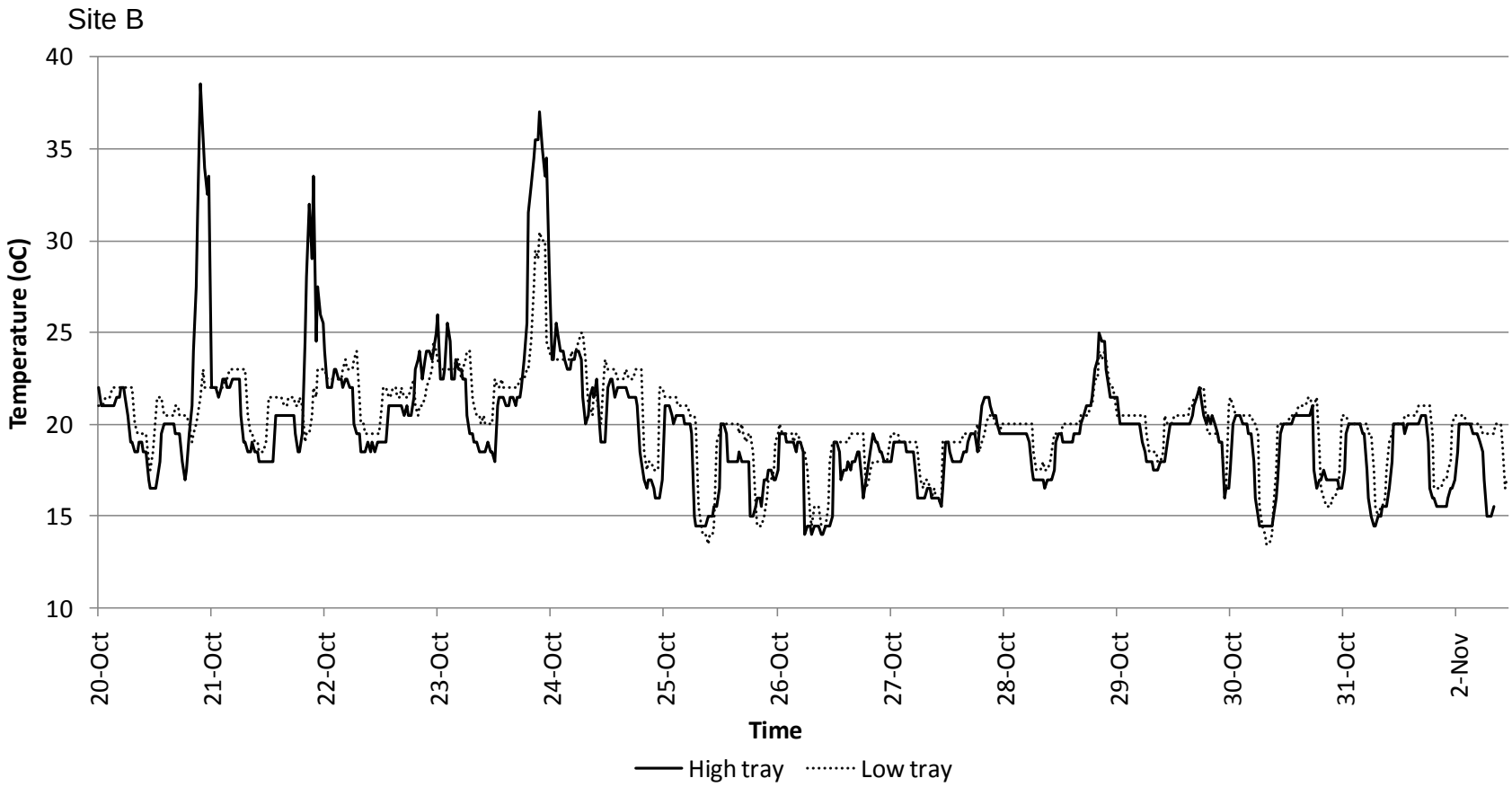
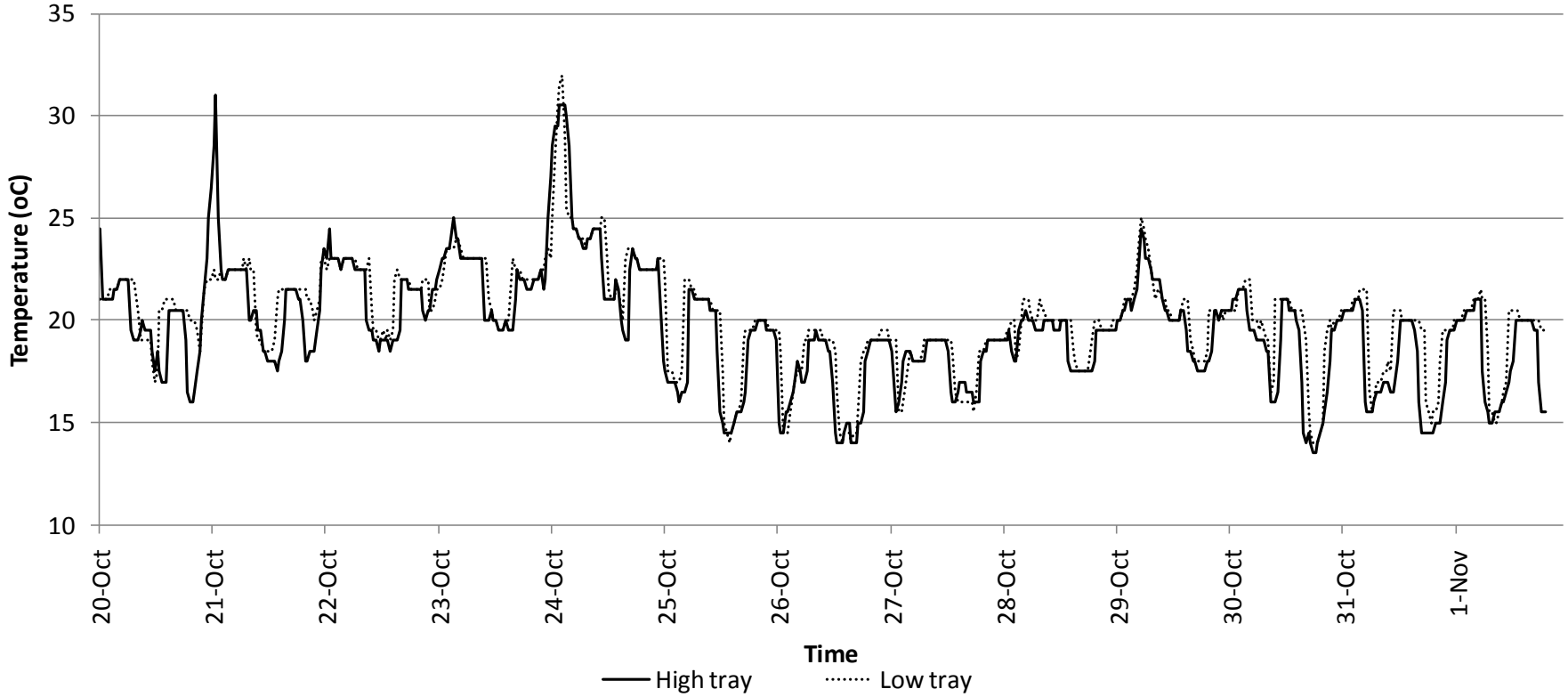


Figure 15. Temperatures recorded at 15 minute intervals by probes placed beneath oyster shells in high and low trays at Sites A, B and C during October and November 2011. Trays were covered by lids, so that there was partial shade over the oysters.




Site B
40
35
30
25
20
15
10
Temperature (oC)
20-Oct
21-Oct
22-Oct
23-Oct
24-Oct
25-Oct
26-Oct
27-Oct
28-Oct
29-Oct
30-Oct
31-Oct
2-Nov
Time
High tray
Low tray

Site C


35
30
25
20
15
10
Temperature (oC)
20-Oct
21-Oct
22-Oct
23-Oct
24-Oct
25-Oct
26-Oct
27-Oct
28-Oct
29-Oct
30-Oct
31-Oct
1-Nov
Time
High tray
Low tray

Experiment 2

In the second experiment oysters were deployed in both intertidal trays and floating baskets on the 24th November 2012. Spat experienced a significantly higher level of mortality (48%) than adults (26%) (P<0.001) (Table 8). Overall, the mortality levels were different at the three sites (p=0.03). At the end of December only site A exhibited significant mortalities while oysters deployed at sites B and C remained free of any sign of disease until February 2012 (Table 8). All spat from the initial batch (S1) died in trays at all sites while the mortality rate remained relatively low in floating baskets (17-47% depending on the site) (Table 8). As the mortality rate for intertidal spat at site A reached 100% in December, a new batch (S2) was introduced at the end of the month at this site only (Table 8). While no mortality was observed in this second batch at site A in February, the first batch deployed at sites B and C then demonstrated mortality rates ranging from 3 to 100% (Table 8). Consequently, a new batch of spat (S3) was deployed at sites A, B and C in March. In May 2012, there was mortality of oysters at all sites (Table 8).

Table 8. Cumulative mortality of oysters at sites A, B and C in Woolooware Bay according to the age and cultivation systems (Experiment 2). The initial stocking density was 30 individuals per treatment and per batch. Adult 1 and spat 1 were placed on sites on the 24/11/11 whereas additional spat called “additional batch 1” and “additional batch 2” were placed on the 20/12/11 and 16/03/12, respectively. The symbol “/” means that no oyster was deployed at that time.

Rearing structure	Oyster batch	A			B			C		
		Dec 2011	Feb 2012	May 2012	Dec 2011	Feb 2012	May 2012	Dec 2011	Feb 2012	May 2012
Intertidal trays	Spat S1	100	100	100	0	100	100	0	100	100
	Spat S2	/	0	77	/	/	/	/	/	/
	Spat S3	/	/	53	/	/	94	/	/	100
	Adult A1	17	17	43	0	50	63	0	43	43
Floating baskets	Spat S1	47	47	47	0	0	17	0	3	33
	Spat S2	/	0	20	/	/	/	/	/	/
	Spat S3	/	/	5	/	/	3	/	/	0
	Adult A1	3	17	63	0	20	30	0	27	30

Synthesis of results between experiments 1 and 2: variation of mortality among oysters exposed to different strata of the water column. The position of trays and floating baskets relative to strata of the water column at three tide levels is illustrated in Figure 2. Adult oysters kept at lower height had a significantly higher mortality (74.3%) than those kept at higher levels (64.2%) at Site A and C at all three time points (all P<0.001) (Table 8). As the outbreak started later at site B a significant difference in mortality between low and high trays was observed only in May 2012 (P<0.001). In the second experiment, there was a significantly lower mortality in oysters kept in baskets (18%) than those kept in trays (60.9%) (P<0.05) (Table 8). This observation was consistent for all additional batches of spat (S2 and S3) at all sites. As the absolute mortality rate was lower for adult oysters (A1) than for spat (S1, S2, S3) the difference between subtidal and intertidal systems for this age class was less outstanding and only significant at site B in February and May 2012 (P<0.05) (Table 8).

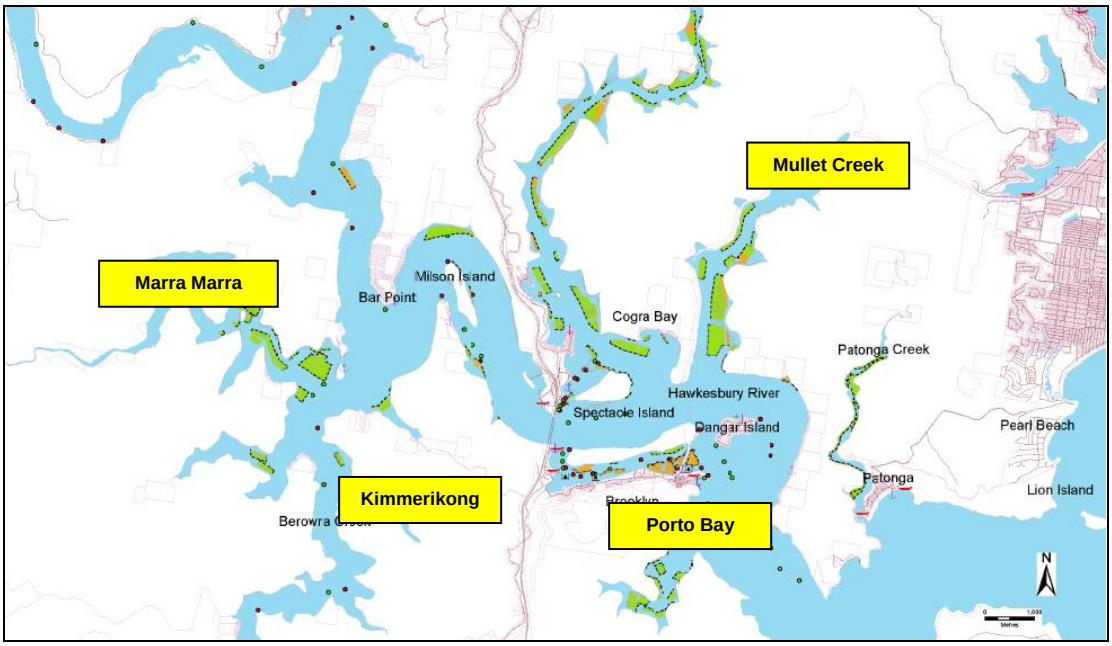
C. Observational study - Hawkesbury River

The Hawkesbury River, where POMS has not been detected constitutes the control site. A batch of triploid spat was closely monitored for mortality, presence of OsHV-1 and bacterial counts from the time of their arrival on the river when they were stocked in floating baskets. Water temperature and salinity were also recorded.

Methods

Study sites and oyster management. The baskets were placed at two different leases for the first 4 months (Mullet Creek and Porto Bay, both nursery areas) before being graded in January 2012 and placed into four sites (Mullet Creek and Porto Bay, and 2 growing areas upstream – Marra Marra and Kimmerikong) (Figure 16). However, due to the floods in February 2012 and very low salinity in the Hawkesbury system, all the mature oysters which were placed upstream were moved down into Porto Bay between the 5th and the 8th March 2012.

Figure 16. Experimental sites in the Hawkesbury River: Mullet Creek and Porto Bay (nursery areas downstream), Marra Marra and Kimmerikong (growing areas – upstream). Base map courtesy of NSW Department of Primary Industries.



Sampling. Every 2 weeks (28th September - 15th January) and then once a month (15th January – 5th June) a random sample was collected from a series of randomly selected baskets which were part of normal production runs. The mortality was recorded at each sampling point and a random sample of 30 oysters per site was collected for OsHV-1 testing by PCR to confirm the absence of the virus in farmed *C. gigas* in the Hawkesbury River. An additional 10 oysters were collected and archived in 10% formalin seawater.

Microbiology. Bacterial cultures were performed to quantify the bacterial load (total bacteria and *Vibrio* species) among the different sites. The methods used were described above. For logistic reasons, the analysis of the bacterial loads could not be performed for all time points in 2011 and data were available for the following dates: 7th December 2011, 4th January 2012, 18th January, 15th February, 15th March, 20th April, 10th May and 5th June.

Environmental monitoring - temperature and salinity. Temperatures were recorded every 15 min using Thermocron® temperature loggers (Thermodata Pty Ltd) placed inside the baskets/trays at all sites (five temperature loggers per site). Temperature data were downloaded using the Thermodata 3.2.23 software (Thermodata Pty Ltd). Additionally, a subtidal HOBO U24-002 conductivity/salinity logger (OneTemp Pty Ltd - Onset®) was deployed at each site to monitor salinity (and temperature) every 15 min. This probe was placed at a deeper position in the water column and was constantly immersed. Salinity readings were systematically calibrated every 2 weeks using a portable conductivity meter (Waterproof CyberScan CON 400 – InstrumentChoiceTM) and data were downloaded using the HOBOware® Pro 3.2.1 software (Onset®).

Statistical methods. Summary statistics were calculated and box-and-whisker plots created for growth data in order to make preliminary comparisons and to check for data entry errors. General linear models were then built to compare average length, width and depth over time at four sites. Predicted means were calculated and plotted for presentation. Survival analyses were conducted to compare mortality across the four sites, taking synchronous interval censoring into account (Hosmer and Lemeshow, 1999; Radke, 2003). Analyses were conducted using a complementary log–log link function employing the SAS LOGISTIC procedure. Oysters that were sampled for other purposes (40 at each site at each time) were considered to be censored at the respective observation times. Similarly, all oysters surviving at the end of the experiment (10 May 2012) were also censored. A binary outcome was created representing oyster status at each time point (alive or dead). Hazard ratios and their 95% confidence intervals were presented along with survival curves.

Results

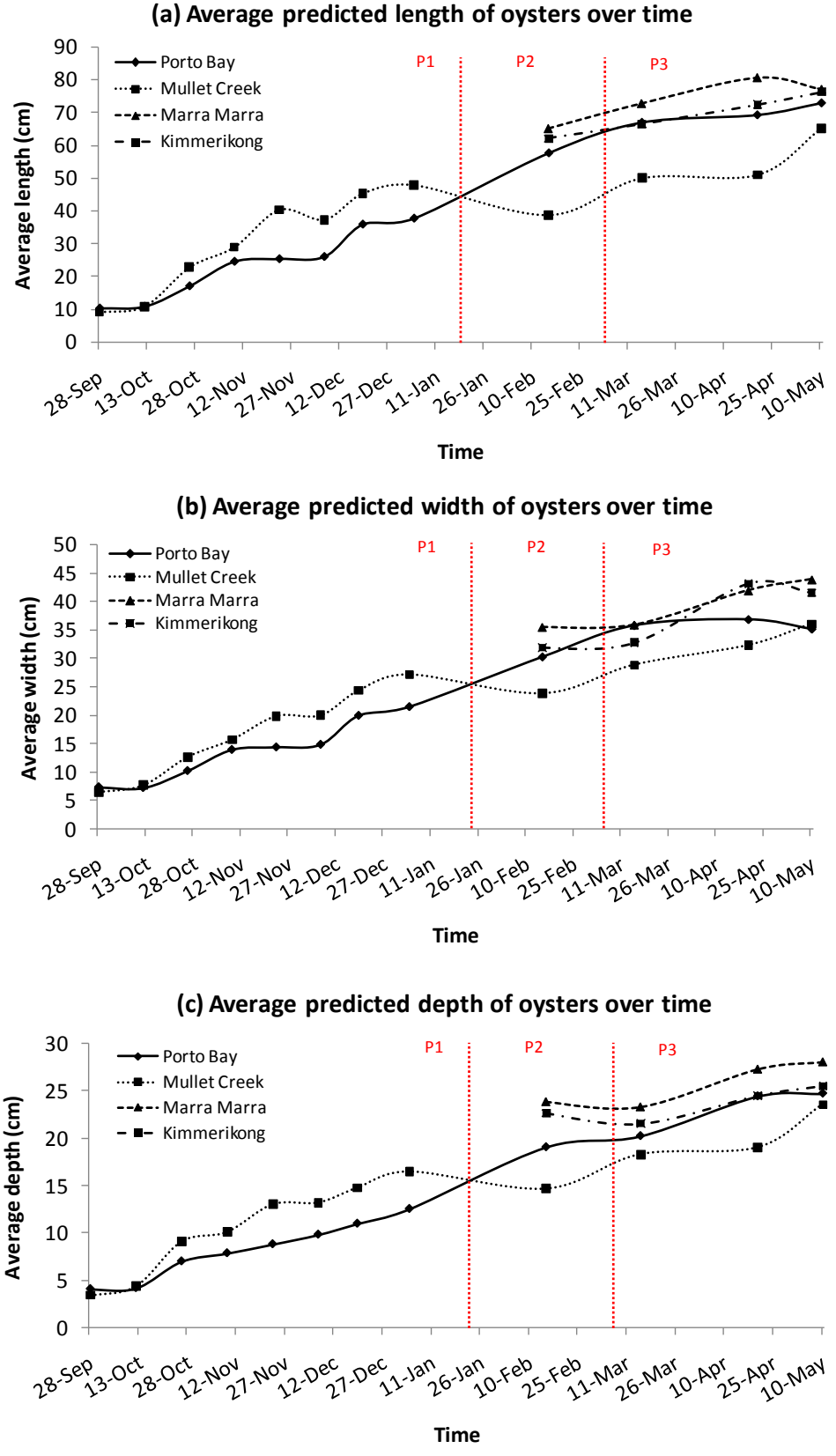
OsHV-1 detection. OsHV-1 was not detected at any sites or time as listed in Table 9. The monitoring of OsHV-1 was performed downstream near the oceanic part of Broken Bay, which is subject to frequent pleasure boating activities/transfers. Monitoring was also conducted upstream as the mechanism of transmission of OsHV-1 from an estuary to an upstream area is unknown. The status of farmed *C. gigas* in the Hawkesbury River being apparently free of OsHV-1 infection was confirmed from October 2011 to June 2012.

Table 9. Sampling sites and times for OsHV-1 detection in the Hawkesbury River from October 2011 to June 2012. Sample size per site and time was 30; oysters were tested in pools of 6.

Date	Sites Mullet creek (1), Porto Bay (2), Marra Marra (3), Kimmerikong (4)	OsHV-1 status
26 Oct 2011	1,2	Negative
7 Dec 2011	1,2	Negative
19 Dec 2011	1,2	Negative
4 Jan 2012	1,2	Negative
18 Jan 2012	1,2,3,4	Negative
15 Feb 2012	1,2,3,4	Negative
15 Mar 2012	1,2	Negative
20 Apr 2012	1,2	Negative
10 May 2012	1,2	Negative
5 Jun 2012	1,2,3,4	Negative

Growth. Due to the oyster movements required by management or flood events, the growth rate must be analysed over three distinct periods P1, P2 and P3 (Figure 17). P1 corresponds to the initial phase when spat were grown in the two nursery areas Mullet Creek and Porto Bay (28th September 2011 – 4th January 2012). During this first period, oysters grew at a steady rate with a better growth rate observed at Mullet creek (all P-values <0.01) (Figure 2). On the 13th January 2012 (start second period, P2) the oysters were graded and the different sizes were split into the four sites: the fast growing oysters (front runners) were transferred to Marra Marra and Kimmerikong, the slow growing individuals (runts) were placed in Mullet creek while the intermediate sizes were transferred to Porto Bay. As a consequence of the grading event a sudden change of shell length, width and depth was observed at the four sites (Figure 17). The third period P3 corresponds to the flood period, from the 5th - 8th March 2012 when all mature oysters from the upstream sites were moved downstream to Porto Bay where the growth rate was very low due to the poor environmental conditions. There was a large influx of fresh water leading to particularly low salinity values and modification of the food quantity and quality. Although average lengths, widths and depths of oysters generally appeared to be higher at Marra Marra compared to Kimmerikong, they were not significantly different at any observation time (all P-values >0.05).

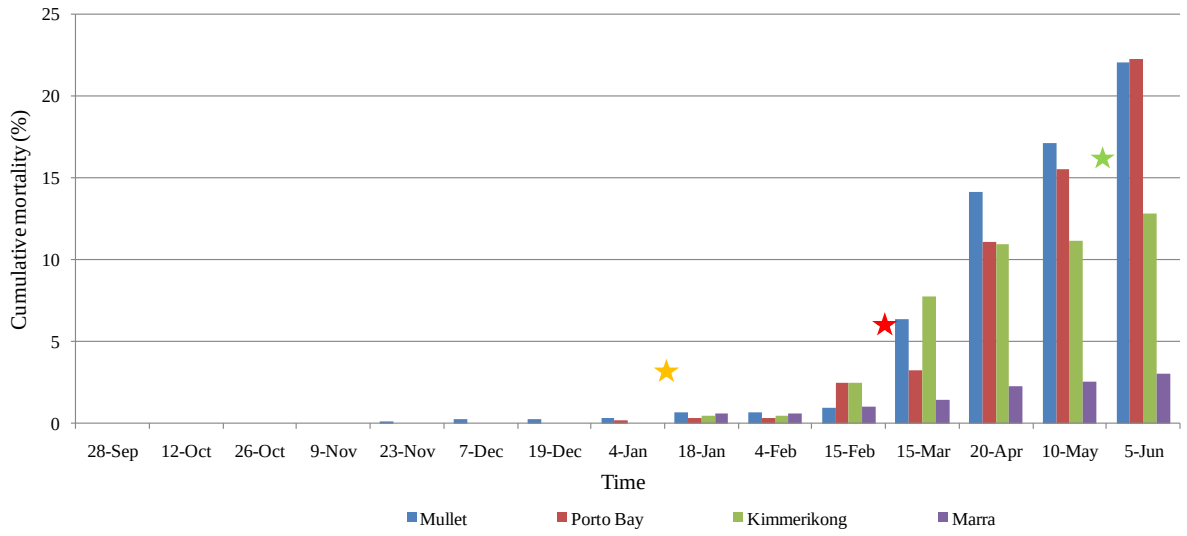
Figure 17. Growth of oysters over time in the Hawkesbury River. There were initially two sites (from 28 September 2011 to 18 January 2012) but after grading on 18 January 2012 four sites were used. (a) Predicted mean length; (b) Predicted mean width; (c) Predicted mean depth.



Mortality. Overall the cumulative mortality of the oyster cohort monitored over an 11 month period in the Hawkesbury River ranged from 3 to 22.3% depending on their growth performances (front runners, runts, intermediate sizes) and their locations in the river. From September 2011 to January 2012 mortality of spat at Mullet Creek and Porto Bay was negligible, confirming the favourable status of the Hawkesbury River in terms of environmental conditions, water quality, food supply and absence of POMS disease, as well as good management strategies. From January 2012 to March 2012 the mortality was also negligible, with the highest cumulative mortality values of 6.5 and 7.8% being recorded in Mullet Creek and Kimmerikong, respectively (Figure 18). Major flood events occurred in late-February/March leading to a forced movement of mature oysters from upstream to downstream (red star) to prevent consequent mortality events due to low salinity (salinity was almost nil upstream – see salinity data below). Oysters from Kimmerikong and Marra Marra were moved down to Porto Bay during the first week of March 2012. We kept the 3 different batches separated and identified in Porto Bay from March 2012. Following the flood events a progressive increase in oyster mortality was observed in the slow growing oysters and intermediate sizes which had been left in Mullet Creek and Porto Bay (final cumulative mortality values of 22 - 22.3% on the 5th June 2012) (Figure 18).

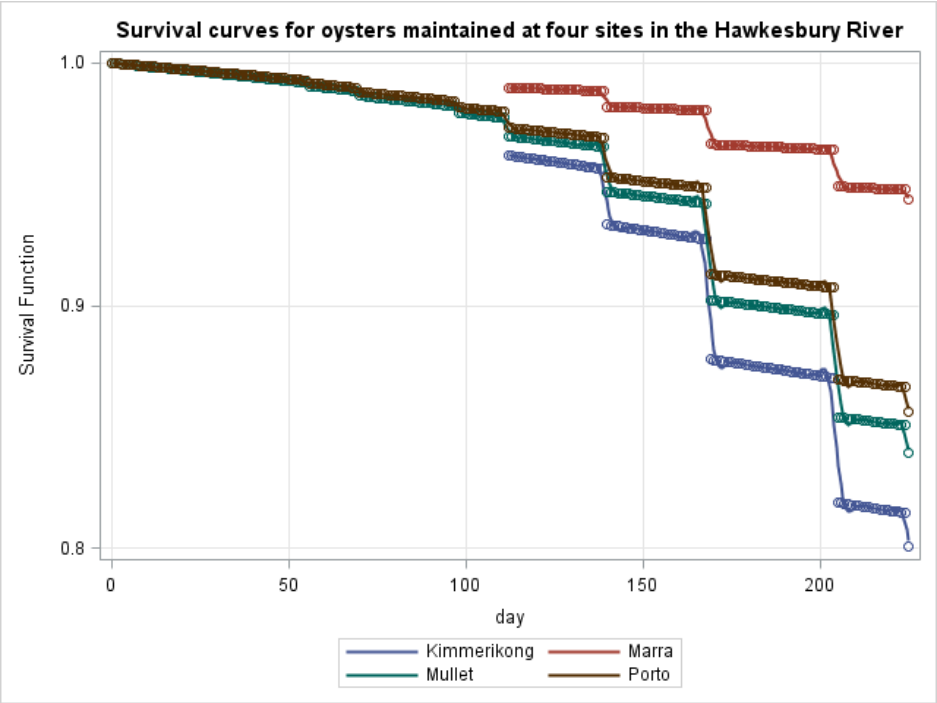
This increase in mortality can be related to the poor environmental conditions with particularly low salinity values recorded at all sites (see salinity data below), modification of the food quantity and quality, silting of the trays/baskets promoting mud worm proliferation and heavy fresh mussel overcatch. Also, due to the duration of the floods and the urgency of this situation in terms of management for the Hawkesbury River oyster farmers, the oysters were not able to be cleaned or graded as often as they should have been, which might also have led to an increase in the mortality especially in the context of sub-optimal environmental conditions. From March to June 2012, the oysters from Kimmerikong demonstrated a higher mortality rate than the oysters from Marra Marra even though both groups had apparently identical history (same initial batch, same growth rate, kept at the same site - Porto Bay). This result demonstrates that mortality of oysters is not only related to their immediate environment and their genetic performance (front runners *vs.* runts) but also to their life history. Environmental stressors can impair a wide range of physiological processes (immune system, filtration activity, nutrition) which weaken the oysters to such a point that the population is not able to recover or cope with any other stressors.

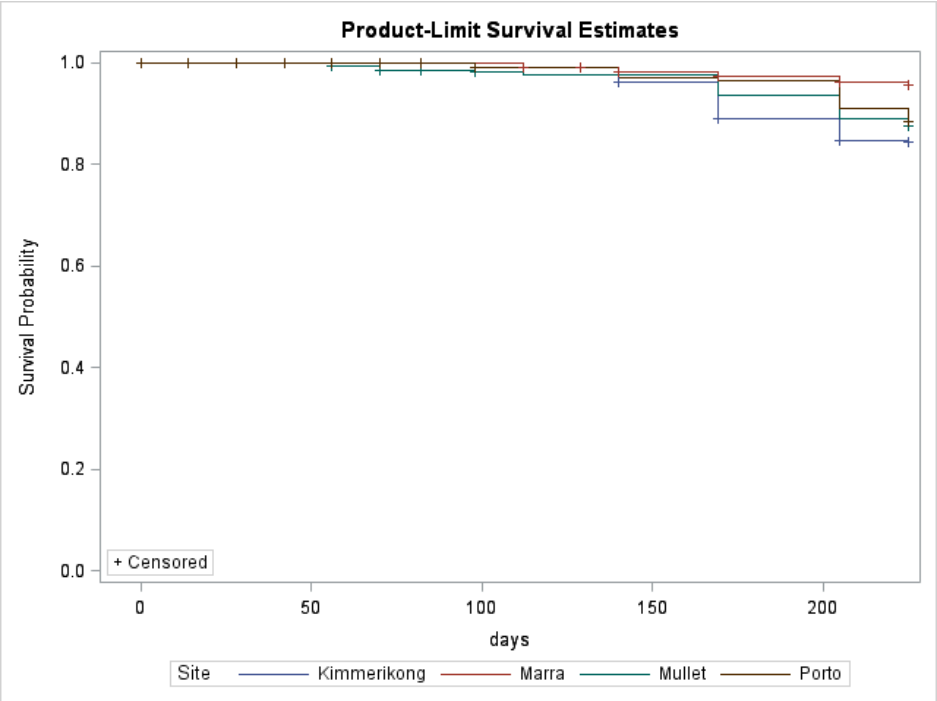
Figure 18. Cumulative mortality in oysters placed at four different sites in the Hawkesbury River. Yellow star: oysters movements on the 13th January 2012: the largest oysters (front runners) were transferred upstream to Marra Marra and Kimmerikong, intermediate sizes were transferred to Porto Bay, while runts were trasnferred to Mullet Creek. Red star: oyster movements on the 5th March due to flood events: front runners from upstream were transferred to Porto Bay on the 5th March 2012. Green star: oyster movements on the 1st June 2012: front runners were transferred back to Marra Marra and Kimmerikong.



Survival curves for four sites are shown in Figure 19. There was no significant difference in survival between Mullet Creek and Porto Bay, but compared to Porto Bay, the hazard of death was significantly higher at Kimmerkong (hazard ratio = 1.43; P= 0.003) and significantly lower at Marra Marra (hazard ratio (0.37; P <0.001).

Figure 19. Survival curves for oysters maintained at four sites in the Hawkesbury River, from 28 September 2011 to 10 May 2012. Oysters were initially reared at two sites (Porto Bay and Mullet Creek) but some were transferred to Marra Marra and Kimmerikong on 18 January 2012 after grading.





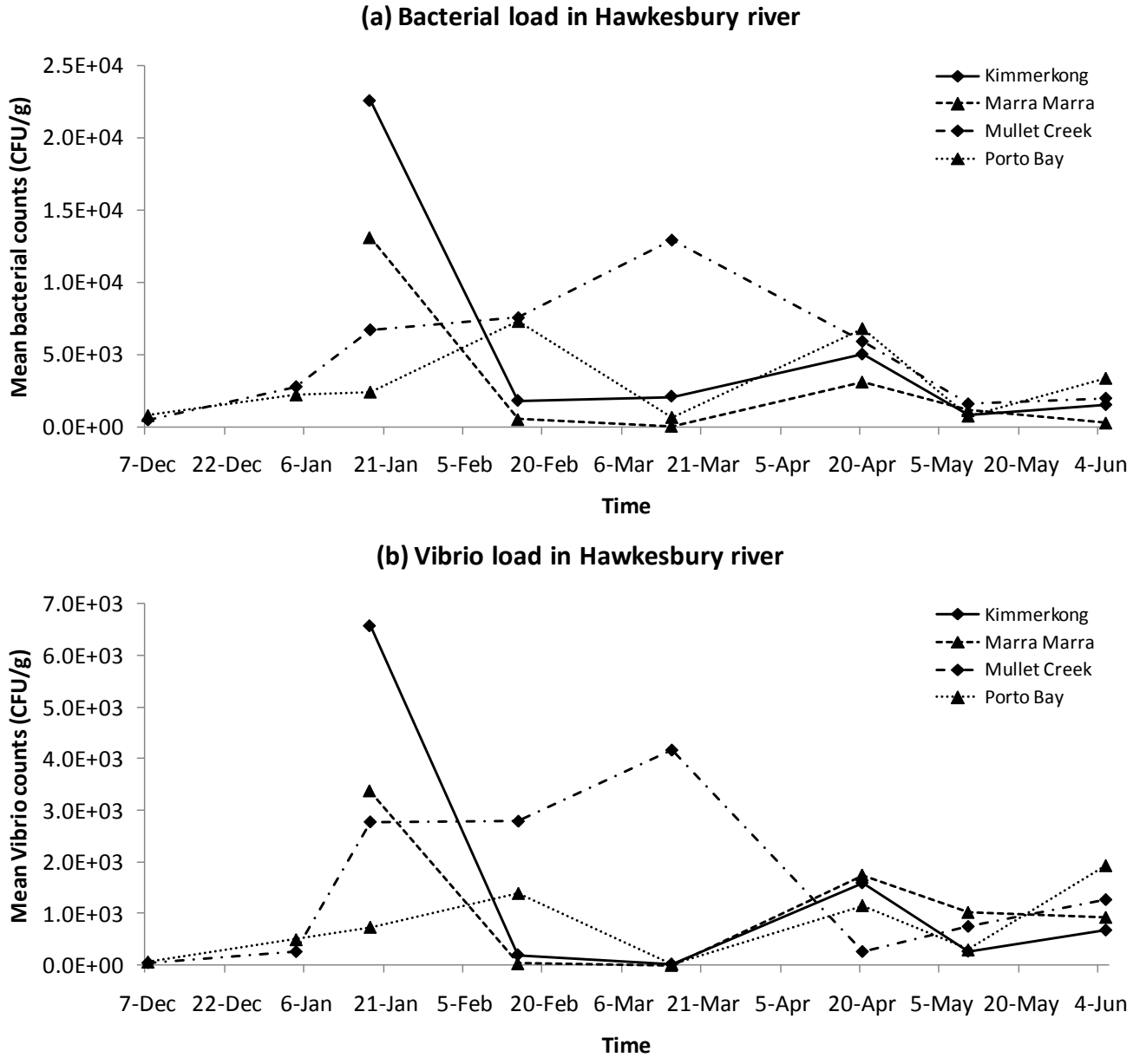
Bacterial load. The bacterial load (total bacteria and *Vibrio* spp.) over time for the four sites is presented in Figure 20. Overall, the patterns of total bacterial and *Vibrio* spp. loads were very similar over time. From December 2011 to January 2012 (period P1), oysters from Mullet Creek and Porto Bay demonstrated similar quantities of bacteria (total bacteria and *Vibrio* spp.) in their tissues. Five days after the oysters were split into four sites (18th January), no significant difference was demonstrated among all batches in terms of total bacterial and *Vibrio* spp. loads, however a month or so after (15th February) the oysters sampled from the two downstream nursery areas had significantly higher bacterial loads (total bacteria and *Vibrio* spp.) than the ones collected in the two growing sites located upstream (all P-values <0.01). Around the 3rd – 5th March 2012, the mature oysters from Marra Marra and Kimmerikong were transferred to Porto Bay due to the flood events. Interestingly, the total bacterial and *Vibrio* loads detected in the three batches kept at Porto Bay were similar on the 15th March, while the batch of oysters kept at Mullet Creek had significantly higher total bacterial and *Vibrio* loads. Finally, from April to June 2012 all the oyster batches kept downstream (in Mullet Creek and Porto Bay, period P3) demonstrated similar total bacterial and *Vibrio* loads.

In comparison to the Georges River, overall total bacterial counts in *C. gigas* in the Hawkesbury River were not significantly different (P=0.83) (Table 10). However, *Vibrio* spp. counts in the Georges River were significantly higher than those in the Hawkesbury River (P=0.003) due to the high counts observed in the Georges River during the POMS outbreak. Note that these comparisons were based on pooled data and ignored the substantial changes that were observed over time, for example increases in counts during the outbreak of POMS in the Georges River, and reductions of counts observed in the Hawkesbury River associated with the flood event. In a simple comparison, examination of Figures 9 and 20 confirms that total bacterial and vibrio loads in *C. gigas* were within similar ranges in both rivers.

Table 10. Total bacterial and *Vibrio* spp. counts (cfu/g) in *C. gigas* in the Hawkesbury and Georges Rivers. Estimates are based on data pooled from all sites, treatments and sample collection times within each location.

Location	Total bacteria		*Vibrio* spp.	
	Mean	SE	Mean	SE
Georges River	1791.3	154.1	654.6	72.8
Hawkesbury River	1853.4	238.6	362.9	60.4

Figure 20. Mean bacterial loads (total bacteria and *Vibrio* spp.) over time at the four experimental sites in the Hawkesbury River.



Environmental data

Salinity. Daily mean, minimum and maximum salinity values averaged for each week are presented for the three sites in Figure 21. At the beginning of the experiment (late September until mid October 2011), the mean salinity values recorded at Porto Bay were significantly higher than the ones recorded at Mullet Creek (difference between 2.6 and 4.9 ppt). Both sites stabilised around 29-31ppt from the 17th October until 21st November 2011 before a sudden decrease to 23-24 ppt for both sites on the week of the 28th November 2011. From that time until the beginning of 2012 the salinity progressively increased at both sites, with significantly higher salinity values recorded at Porto Bay in comparison with

Mullet Creek. Due to probe failures no data were available for Porto Bay between the 23rd January 2012 and the 19th March 2012, and between the 16th April 2012 and the 21st May 2012 for Mullet Creek. Wherever sufficient data enabled comparison between the two sites, all differences were significant (all P-values < 0.001). Mean salinities recorded at Marra Marra and Kimmerikong were similar over time and consistently lower than the mean salinity values measured downstream at Mullet Creek and Porto Bay. The differences in salinity between sites were driven by their proximity to the open ocean and the amount of water exchanges between freshwater and seawater inputs. In terms of temporal patterns, all sites demonstrated wide variation, especially during the flood events (February – March 2012) when a massive drop of salinity was observed at all sites (minimum value of 1.8 and 3.8 ppt recorded in the week of the 5th March at Marra Marra and Kimmerikong, respectively). Indeed, Warragamba dam overflowed on 2nd March 2012 reducing the salinities at the Hornsby Shire Council (HSC) Gunyah probe (at the entrance to Porto Bay) to less than 10 ppt, a value so low not having been seen during the last 15 years of monthly sampling by HSC (http://mhl.nsw.gov.au/projects/berowra/HistoricalData). Due to these extremely low salinities, mature oysters were transferred from Marra Marra and Kimmerikong to Porto Bay and were kept there until the end of the experiment. Consequently, after the date of transfer (3rd - 5th March 2012) the environmental parameters of interest were at Mullet Creek and Porto Bay only. From mid March 2012 to the beginning of April 2012 the salinity at Mullet Creek and Porto Bay slowly reverted to a normal range of 25-35 ppt.

Mortalities in the experimental oysters increased significantly from March 2012 onwards and this was likely to be due to the direct and indirect effects of the floods (extremely low salinity values, modification of food quantity and quality, silting of trays/baskets promoting mud worm proliferation and heavy fresh mussel overcatch) as previously documented in other estuaries (Matthews and Constable, 2004).

Figure 21. Daily mean (A), minimum (B) and maximum (C) salinity values over time (weekly means) in the Hawkesbury River

Figure 21.A Mean salinity

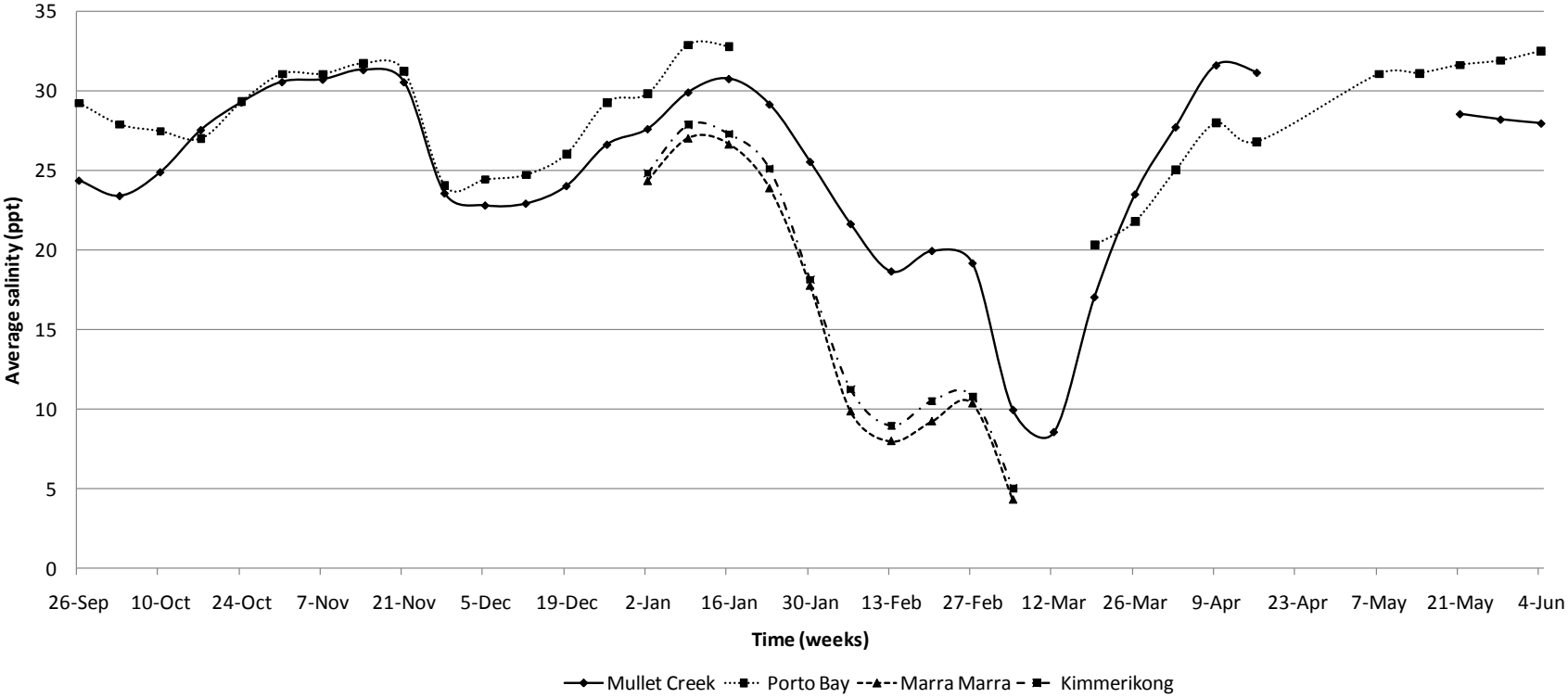


Figure 21.B. Minimum salinity

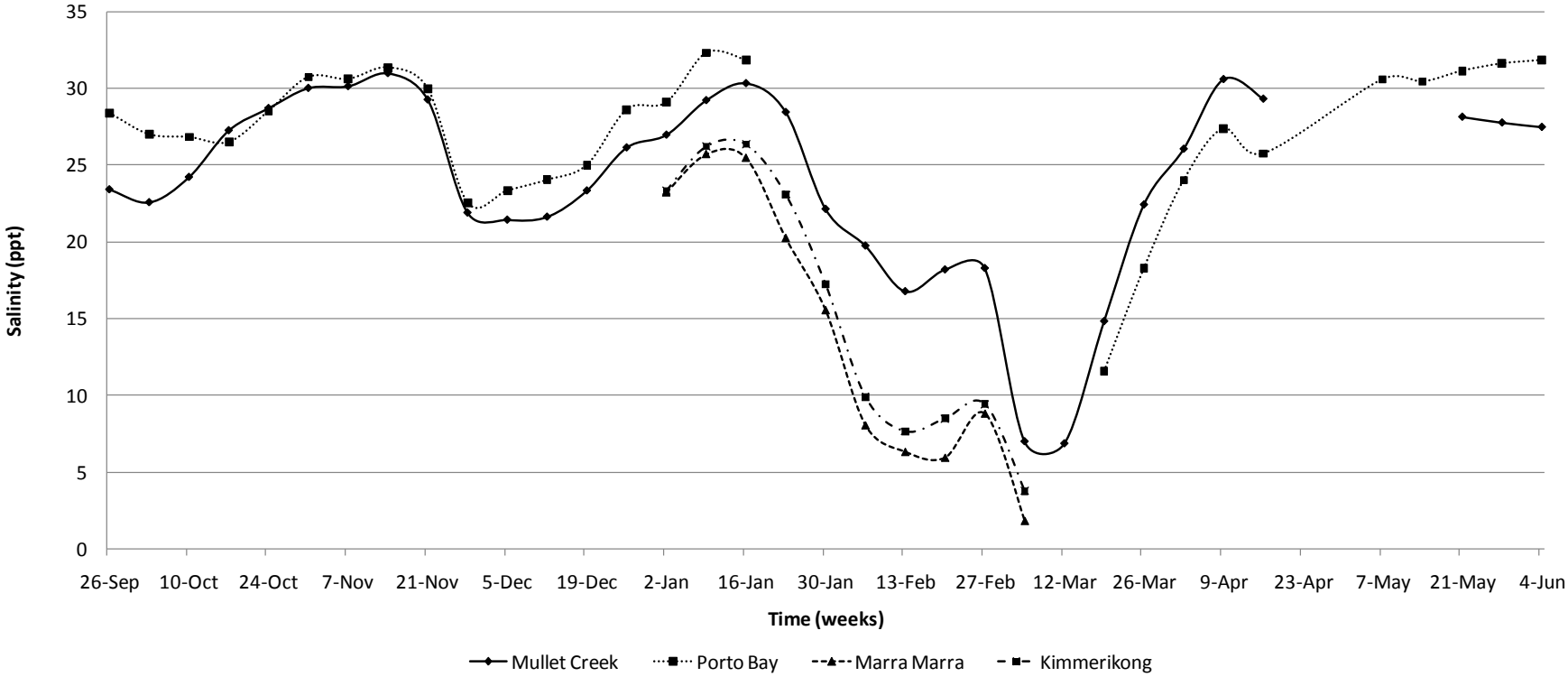
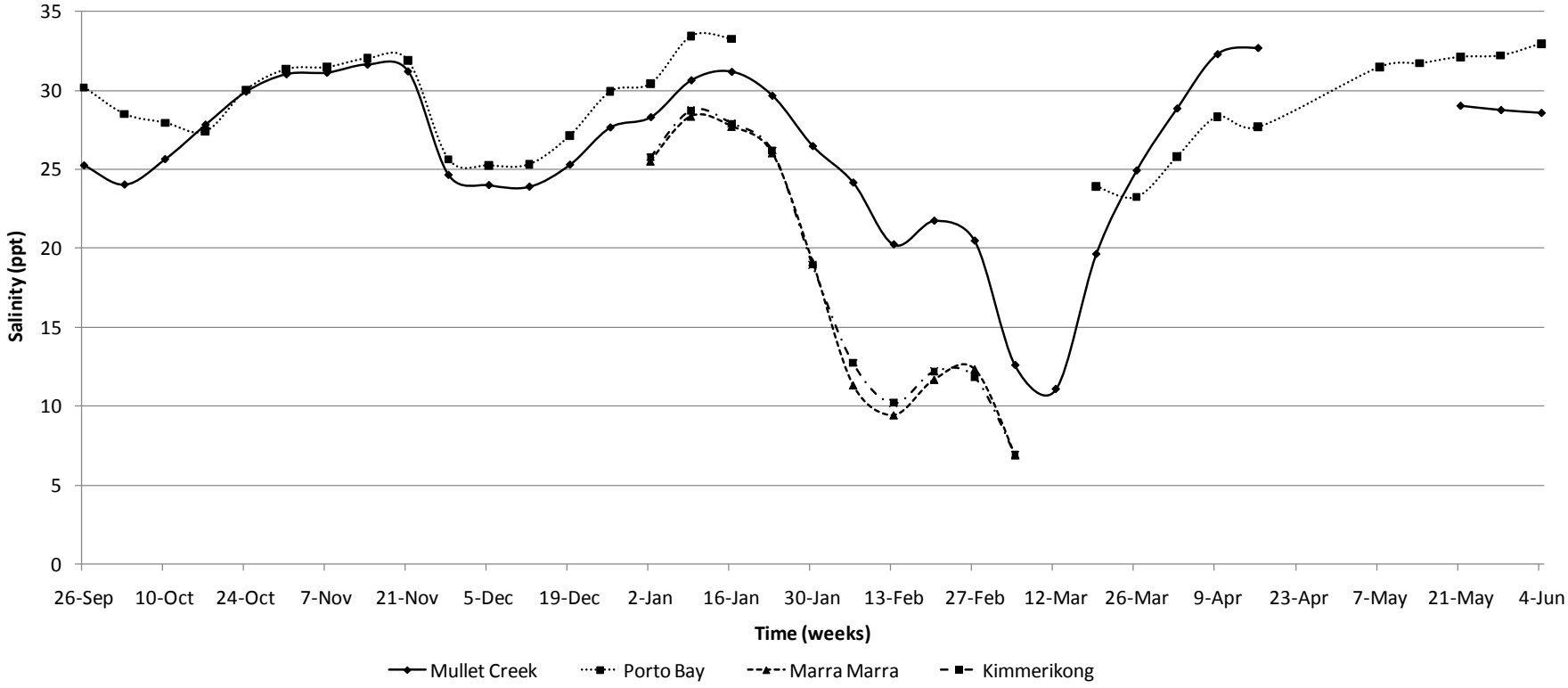


Figure 21.C. Maximum salinity



Temperature (measured using subtidal salinity probes). The mean daily water temperature recorded by the subtidal salinity probes averaged for each week is presented in Figure 22. Despite their relatively distant locations upstream and downstream the pattern of temperature among the four sites monitored in the Hawkesbury River appeared to be very similar over time (Figure 22). The water temperature progressively increased from around 17.0-17.5°C on the 26th September to 24.0-24.5°C around the 7th November 2011. After a slight decrease to 21°C by the beginning of December the water temperature increased again to above 25°C and remained relatively stable between 24-26°C over January and February 2012. From the end of February the water temperature started decreasing and progressively dropped to 17°C by the beginning of June 2012. This pattern of temperature over a year is quite usual in the Hawkesbury River and is consistent with previous annual observations recorded by Hornsby Shire Council.

Temperature (using temperature probes in trays). The mean temperatures (averaged for each week) recorded by the probes directly placed in baskets and trays are presented in Figure 23. Overall, the ranges of temperature as well as their pattern over time correspond to those recorded by the salinity probes, for each site. However, more variations/undulations were observed in these data than in salinity probe records of temperature due to their locations in subsurface (baskets) or intertidal (trays) presenting higher temperature variations (air/water, day/night, rain events) than the salinity probes which were subtidal.

Figure 22. Mean water temperature recorded over time by the subtidal salinity probes deployed in the Hawkesbury River

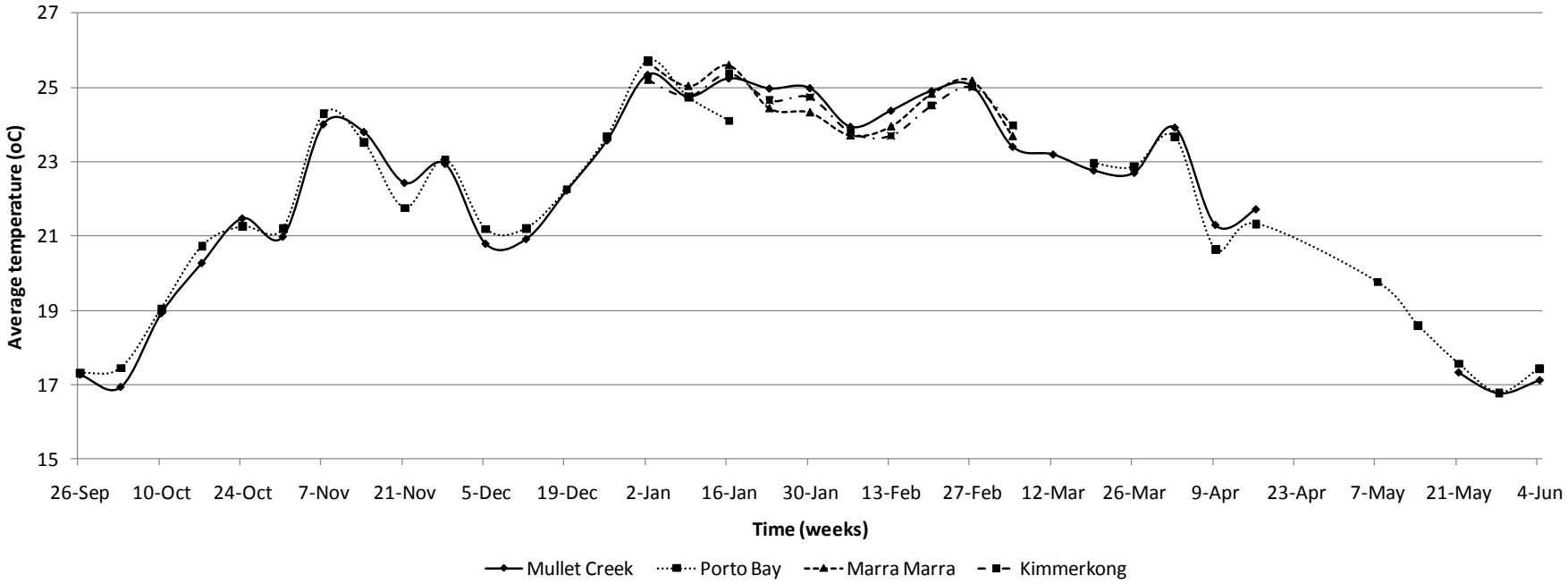
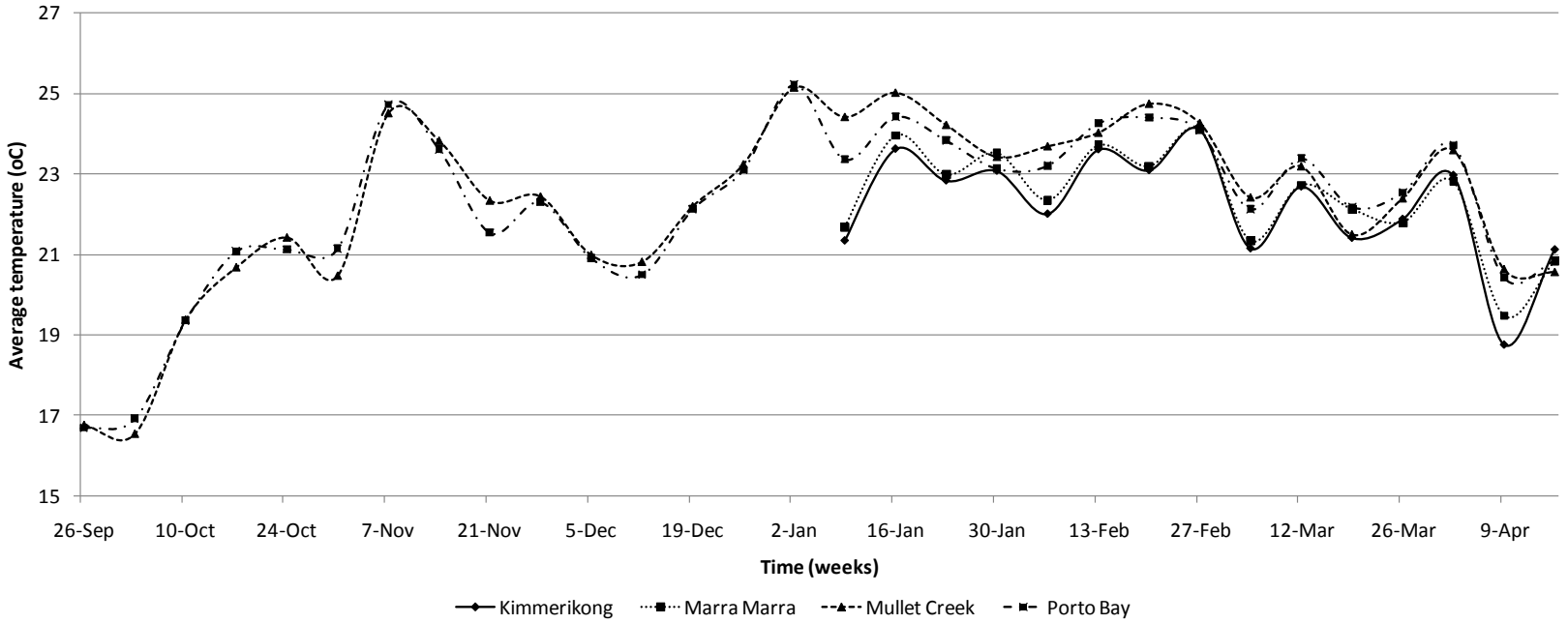


Figure 23. Mean temperature recorded by the temperature probes placed in baskets/trays at all sites - Hawkesbury River



Comparison of environmental data from the Georges and Hawkesbury Rivers. The mean daily temperatures recorded by the salinity probes were averaged for each week in the Hawkesbury and the Georges Rivers (all sites taken together within each River) and are presented in Figure 24. Although some significant differences can be observed in terms of mean temperatures between the two Rivers, similar temporal patterns as well as ranges of variations were observed in both Rivers (Figure 24). Consequently, the temperature pattern in the Hawkesbury River, being within the same range as the temperature pattern measured in Woolooware Bay, cannot explain the absence of OsHV-1 expression in the Hawkesbury River. Additionally, it is important to note that the mean water temperatures measured in the Hawkesbury River are within the optimum temperatures for OsHV-1 activity (16 -24°C) (Anon, 2011).

The mean daily salinities recorded by the salinity probes were averaged for each week in the Hawkesbury and the Georges Rivers (all sites taken together within each River) and are presented in Figure 25. The Hawkesbury River exhibited significantly lower salinities than the Georges River, as well as a wider range of variation over 2011 and 2012. The differences in weekly means between rivers ranged from 0.2 to 18.3 ppt with an average value of 8.1 ppt (Figure 25). However, it is important to note that this period was unusual in the Hawkesbury River in terms of flood events and extremely low salinity values upstream as well as downstream. To our knowledge there are no published data available on the viability of OsHV-1 at low or variable salinity. Therefore, there is no evidence suggesting that the salinity pattern over the Australian summer in the Hawkesbury River might have played a role in the absence of OsHV-1 in the system.

Figure 24. Mean water temperature recorded over time by the salinity probes in the Hawkesbury and Georges Rivers. The periods when OsHV-1 associated mortality was observed in the Georges River are shaded.

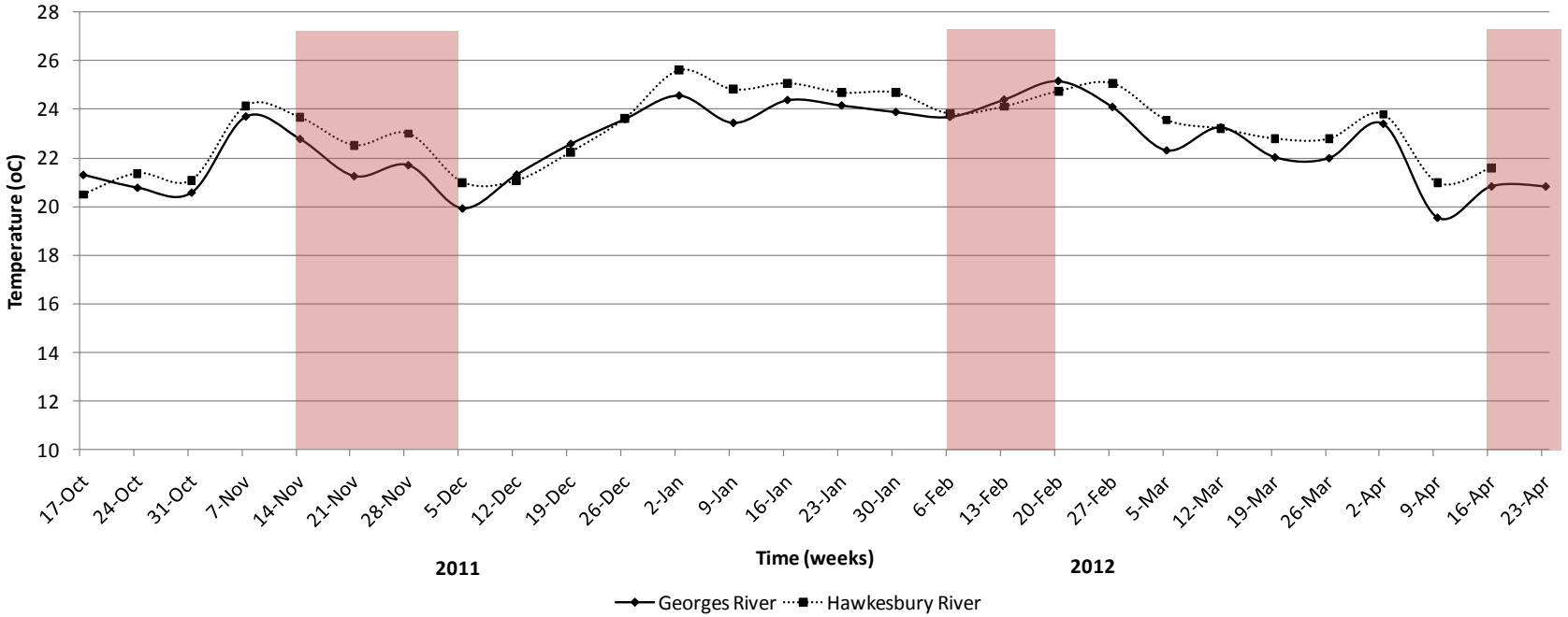
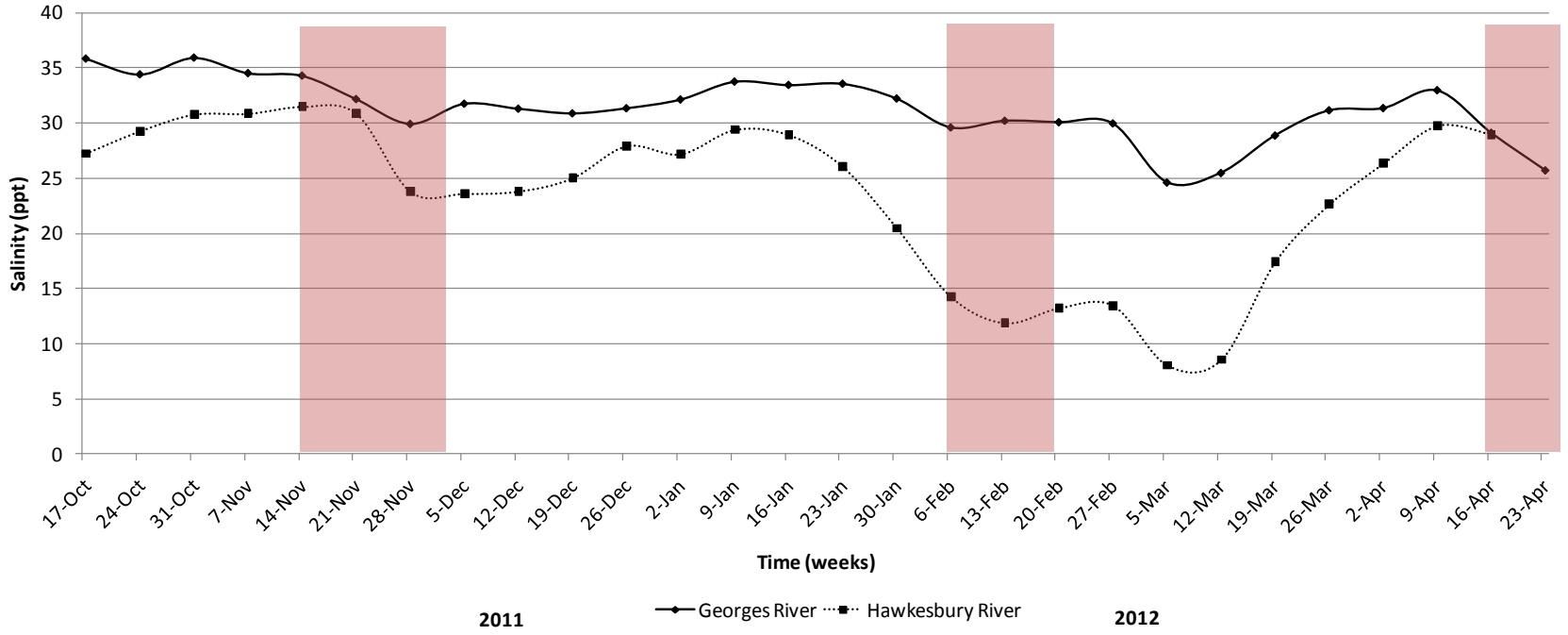


Figure 25. Mean salinity recorded over time by the salinity probes in the Hawkesbury and the Georges Rivers. The periods when OsHV-1 associated mortality was observed in the Georges River are shaded.



DISCUSSION

In this report we describe an outbreak of POMS in Woolooware Bay near Sydney NSW, which started in November 2011 and in which OsHV-1 associated mortalities were observed until late April 2012. The present work demonstrated that the distribution of OsHV-1 associated mortalities was non uniform, clustered, highly variable in time and space, and clearly dependent on the age of oysters and their growing height or position in the water column. It demonstrated that implementation of different farming practices, in this case modification of the growing height, could play a role in disease management and help reduce mortality of adult oysters during an OsHV-1 outbreak. The pattern of infection and disease was different on different leases suggesting that underlying environmental factors influence OsHV-1 disease expression. Differences in mortality among sites, ages and growing heights were evaluated in relation to the intensity and prevalence of OsHV-1 infection and the environmental data recorded during the outbreak. The epidemiological observations are of considerable importance and inform future strategies to manage OsHV-1, including the methodologies to be applied in the genetic selection program.

The effect of age

In this study juvenile oysters were consistently more susceptible to OsHV-1 disease than were adults. This has been demonstrated previously in Europe (Dégremont, 2011; Miossec, 2009; Renault, 1994; Schikorski et al., 2011b).

The effect of growing height

Although no difference was observed for spat, a significant decrease in mortality rate was demonstrated for adults placed at high height in comparison with low height. This observation was consistent among sites A and C with the reduction in mortality approaching 40% when oysters were grown at a higher height (Figure 3). A similar observation was recorded at site B later in April 2012 but to a lesser extent as the overall mortality rate was lower; the difference in mortality between the two treatments was 13% (Figures 3, 4).

The effect of height could be explained by a lower immersion time leading to lower exposure to viral particles in the water. Similar assumptions were made in France and Ireland after observation of reduced mortality when oysters were placed higher on the shore (Peeler et al., 2012). However, the present data do not support the hypothesis of a lower viral exposure as the mean Ct values (representative of the quantity of viral particles in tissues) were not significantly different between low and high groups, with a few exceptions. However, as two sites (A and C) suffered from rapid and severe mortality and the third (B) did not demonstrate massive mortality during November and December, the number of samples available to estimate the differences between the two heights might not be sufficient (n=3 and 6 observations for spat and adult oysters, respectively). As the PCR does not discriminate dead or damaged virus *vs.* infective particles, the analysis of Ct values cannot inform this hypothesis and further studies involving viral RNA activity analysis or experimental infection trials are required.

The effects of temperature and sunlight (UV) being stronger on trays placed at high height could also explain the difference of mortality between the two heights. Indeed, UV and high temperature can significantly damage OsHV-1 virus by destroying its lipid-containing envelope (Renault, 2011). No measure of UV radiation was performed during this study, however the temperatures recorded by the probes placed in trays clearly demonstrated (i) a longer exposure to the ambient air with a ~2 hour immersion time difference per tide cycle on average between low and high heights, and (ii) higher temperatures sometimes recorded in high trays, especially when low tide occurred between 12pm - 4pm.

The effect of the growth rate of oysters

A rapid growth rate may constitute a risk factor for oyster susceptibility to OsHV-1 as differences in mortality between oysters presenting contrasting growth rates (spat *vs.* adult; diploid *vs.* triploid; hatchery *vs.* wild; subtidal *vs.* intertidal) have been reported (Anon, 2011). However, the exact role of growth rate remains unclear as it is almost always confounded with other factors (genetics, age, location). Intuitively, oysters spending a longer time out of the water would stay closed for longer periods, feed less and grow more slowly. In our study, no difference in growth rate was observed at sites A and C between high and low groups of oysters, but this could be due to insufficient observation time, especially for the spat. In contrast, a significant difference in growth rate was observed for spat and adult oysters at site B, with a lower growth rate for the oysters kept at high height (Figure 10). However, this observation could not be associated with any increase in survival as all the spat died in February, regardless of the height. Overall, the present data do not shed light on the potential role of growth rate on OsHV-1 disease susceptibility.

The effects of temperature and salinity

There are observations from Europe to suggest that POMS disease expression is linked to water temperature increases as summer approaches (Anon, 2011), but salinity does not appear to have been linked to this disease. In this study water temperature changes before each mortality event were variable, comprising an increase before the first event and decreases before two later events. We also observed decreases in salinity just before each of three mortality events in Woolooware Bay in summer 2011-2012, but the magnitude of the reduction each time were quite small. Furthermore, disease expression at Site B was very different from that at Sites A and C, despite not having a different salinity or water temperature profile from the other sites. These observations suggest that salinity and water temperature may not be major factors in POMS disease expression in Woolooware Bay. Further study will be required to confirm this. Observations have already been reported in regions in Europe demonstrating presence of OsHV-1 and favourable environmental conditions but no sign of mortality (Dundon et al., 2011; Pernet, 2012), similar to site B in the first period of this study.

Survivors of an outbreak and resistance

Oysters that survived the first mortality event in experiment 1 (November 2011 for site A and C; February 2012 for site B; Table 1) had only a slight increase in mortality in subsequent OsHV-1 events. A similar pattern was observed for surviving oysters in experiment 2. This observation was consistent among sites and heights (Table 2). The prevalence of OsHV-1 infection measured by PCR declined over time indicating that oysters which survived were able to progressively clear the virus. Observations during summer mortalities of *C. gigas* in France suggested that oysters that survived a mass mortality event were then resistant to OsHV-1 (Dégremont, 2011; Dégremont et al., 2010; Pernet, 2012). The mechanism for this immunity is unknown. A key question is whether the apparent resistance of these oysters to OsHV-1 is genetically determined. It is possible that there is a strong dose effect with OsHV-1. Oysters that survived may have received a lower dose of virus by chance initially, and then progressively cleared the infection.

The type of disease outbreak

Did the outbreak of mortality due to OsHV-1 in Woolooware Bay in 2011-2012 arise from a common point source (*i.e.* infection derived from a source which is common to all individuals, eg the environment) or was it propagating (*i.e.* the first cases in oysters are the source of infection for subsequent cases in oysters) (Thrusfield, 2007)? Note that an environmental reservoir could initiate the latter type of outbreak. When this study was conducted there was no commercial Pacific oyster production in Woolooware Bay, and the experimental oysters used in the study were free of OsHV-1. This suggests the existence of an environmental reservoir. Two lines of evidence help explain the type of disease outbreak: changes in prevalence of infection over time, and patterns of infection among sites.

There was a sudden and dramatic increase of OsHV-1 infection prevalence in November 2011 (e.g. from 0% to 72% prevalence within 5 days in high adults at site A). Mortality and OsHV-1 infection was detected first at Site C, and within 5 days at Site A. Site B, lying between Sites C and A, became infected at the same time as Site A, but there was no mortality. Infection persisted at site B, but prevalence remained relatively low (0 to 17% on most occasions) throughout 6 sampling periods between 21st November and 20th December. For these reasons it appeared that the virus did not propagate from oyster to oyster and it is more likely that OsHV-1 came from a common environmental point source which affected all individuals at the same time (Thrusfield, 2007). However, the degree of exposure from the environment appeared to be less at Site B than at Sites A and C.

To examine this more closely, the nearly concomitant events at sites A and C in experiment 1 suggested either a simultaneous point source for the virus or very rapid dispersion of infective viral particles between both sites, but as there was no sign of mortality and a lower prevalence of OsHV-1 infection at site B the latter is unlikely. The situation of site B, located in the middle of Woolooware Bay between sites A and C, as well as the hydrodynamics of the bay where tidal flows from sites A and C must pass through site B, make propagation highly unlikely to explain the spatial distribution of mortalities

among sites in November 2011. As there was no progressive wave of infection or mortality from site A to B to C or from site C to B to A during three OsHV-1 events in the summer of 2011/2012, a simple spread of the disease from lease to lease due to tidal flow in and out of the bay is insufficient to explain the distribution of mortality.

Based on these observations it is reasonable to conclude that oyster to oyster transmission within an oyster lease was not important in disease transmission, and neither was direct spread of the virus due to simple water currents/tidal flows carrying infective material from infected oysters on one lease to naive oysters on another lease. This raises a very significant question about the mode of transmission.

The mode of transmission

Planktonic transmission hypothesis. We propose that OsHV-1 virus could be transmitted through planktonic organisms acting as a vector to explain the patchy distribution of mortality at large scale. Planktonic organisms are not distributed uniformly throughout the water but have a clustered (patchy) distribution in both time (day/night, summer/winter) and space (horizontal and vertical) across a wide range of spatial scales (from < 10 cm to > 1km) (Malone and McQueen, 1983; Suthers, 2009).

Data on the existence of a potential vector for OsHV-1 transmission in natural environments are lacking as is direct evidence to show whether OsHV-1 transmission mainly involves waterborne free virus or virus attached to or engulfed by particles. Sauvage et al. (Sauvage et al., 2009) analysed seawater samples from an artificial pond during a mortality outbreak and were able to detect a significant quantity of viral particles (10^3 viral DNA copies L^{-1}). It has already been demonstrated in experimental studies in aquaria that OsHV-1 can be a waterborne infection transmitted between infected and healthy individuals through cohabitation (Schikorski et al., 2011a). Herpes-like particles have been detected previously in unicellular organisms (thraustochytrid-like organism, *Schizochytrium* sp.) from the York River estuary (Virginia, USA) (Kazama and Schornstein, 1972, 1973). More recently, enveloped OsHV-1-like virions were detected in a marine fungoid protist which was present in experimental rearing tanks containing Pacific oyster larvae (Renault, 2003). These findings suggest that marine unicellular organisms may play a role as vectors for transmission of OsHV-1 in natural environments (Renault, 2011). However, the argument should not be restricted to unicellular life forms, as inanimate suspended particles and metazoans in the plankton may also be vectors.

Viruses, like other microbes, can attach to particles in the environment through complex reversible and irreversible interactions, and so their fate and transport is associated with that of the particles (Dhand et al., 2009; Tufenkji, 2007). French oyster farmers from Marennes-Oleron reported a significant decrease in mortality of spat kept in their inland ponds (called “Claires”) during the period of infection when the seawater was aged for 48h in an empty pond

before being used with their oysters, in comparison with freshly pumped seawater (Pers. Comm.). This observation could reflect a sedimentation process in the aged water that would support the particulate vector hypothesis. In the present study, the non uniform, non random and highly clustered distribution of mortalities over a range of spatial scales suggests the potential involvement of plankton particles in the dispersion and the transmission of OsHV-1 in the water. Note that in this report the term “plankton” is used in its broad sense, i.e. ranging from minute bacteria to microscopically visible phytoplankton, larger zooplankton and small invertebrate larvae.

The patterns of mortality within oyster trays. Substantial spatial differences were also observed within the cultivation systems at each site, specifically, between two tray replicates and among square divisions within a single tray (Figure 3). Therefore OsHV-1 is not uniformly distributed in the seawater even at a local scale, leading to different exposures of individuals which lie quite close together. The micro-hydrodynamics occurring around those rearing structures might have played a role. The physical dimensions of oyster trays and the walls between segments of trays would impede water flow, which may lead to sedimentation of suspended particles in proximity to oysters. The number, density and design of trays, their orientation in relation to currents and water flows may have influenced the circulation of water and therefore the accessibility to suspended particles in the water. Therefore the clusters of mortality observed at micro scale could also reflect the aggregation/patchiness behaviour of most marine pelagic organisms. Non random distribution of plankton organisms associated with aggregation even within a very small scale (<10cm) is a well recognised phenomenon (Benoit-Bird and McManus, 2012; Boltovskoy, 1988; Cassie, 1959).

The position in the water column. The vertical distribution of mortality in this study also showed an interesting feature. A significant decrease in mortality rate was demonstrated for adults placed in intertidal trays at high height in comparison with low height. High height is associated with lower immersion time. However, the low mortality rate of spat placed in floating baskets cannot be explained by a lower immersion time as these oysters were constantly immersed. Given the fact that 100% of spat in adjacent intertidal systems died regardless of the height, two main hypotheses are considered to explain the high survival in baskets. Firstly, oysters in subtidal floating baskets might have been exposed to a reasonably high quantity of viral particles but the virus may not have been infective. The same UV/heat deleterious impact mentioned above may also have happened in the first centimetres of the water column which has higher exposure to sunlight and higher temperature than the subjacent water layers. The second hypothesis is related to the possibility that oysters present in the superficial layer of water might not have been exposed to a quantity of viral particles sufficient to induce disease, which could be due to the vertical clustering behaviour of plankton which might carry OsHV-1. Malone & McQueen (Malone and McQueen, 1983) investigated zooplankton communities in lakes and demonstrated that they were all patchy in terms of both vertical and horizontal distributions, and the patches tended to be comprised of unique groups of species. If OsHV-1 was carried by plankton

cells that maintain a deep position in the water column, oysters remaining near the surface would then be less exposed than oysters placed at a fixed intertidal position. This is illustrated in Figure 2.

The prevalence and intensity of infection in relation to mortality

In November 2011, the infection prevalence in pools at the two affected sites (A and C) was high during but also after the mortality event (mortalities stopped around 30th November) as demonstrated by the number of pools remaining positive (5/5 in most cases). The high infection prevalence in pools was confirmed by PCR analysis of individual oysters (high adults at site A). During the outbreak up to 92% of these individuals tested positive for OsHV-1 (Figure 6) but this rate decreased over time suggesting that surviving oysters may be able to clear the virus (Figure 6). At the pool level, mean Ct values tended to increase over time as the mortalities stopped, consistent with a decrease of viral load in oyster tissues. A different pattern of infection was observed at site B where the virus was detected in oysters 2 months before mortalities commenced. There was no sign of disease at site B during the first mortality event in Woolooware Bay in November 2011. At that time the mean Ct values were relatively high (min-max: 32.9-42.9; mean ± SD: 37.1 ± 2.7) suggesting that OsHV-1 virus was present in oysters in relatively low quantities. The infection prevalence was also lower at site B at that time and ranged between 0 and 22.3% in 70% of the analysed pools (Figure 6).

In this study the highest viral loads in oyster tissues were seen only during the period of mortality, and the virus was clearly associated with the disease. But overall the observations suggest that OsHV-1 is a necessary but insufficient factor in the mortality event or that OsHV-1 is a sufficient cause but that there is a very strong dose effect. The presence of the virus in oyster tissue did not lead to mortality and other factors may be required. These factors could include environmental conditions/triggers or other as yet undiscovered pathogens.

It is possible that a threshold dose of OsHV-1 is required to cause mortality, and below this threshold infection merely persists or is cleared from the tissues. This requires confirmation through experimental infection trials. If this was found to be the case, no other factors may be needed to cause mortality.

Regardless of the reason, it is possible that measures to reduce the level of exposure of oysters to OsHV-1 as distinct from preventing exposure, and measures to alter the environment, may be sufficient to reduce mortalities.

Importantly, both the prevalence of infection in the population of surviving oysters and the intensity of infection in individual oysters declined over time, suggesting that some oysters clear the infection. This may be a form of resistance and may be determined genetically. Further studies are indicated to determine the pathophysiological and immunological mechanisms leading to clearance of virus from infected oysters.

The role of *Vibrio* spp.

It is generally accepted that many aquatic diseases result from a combination of stressors and infectious agents (Snieszko, 1974) and it is still not clear whether *Vibrio* spp. are primary pathogens, predisposing factors or secondary pathogens in the context of POMS. Summer mortalities of *C. gigas* in Europe have coincided with elevated detection frequency and quantity of OsHV-1 DNA and to a lesser extent some *Vibrio* spp. (*V. splendidus* and *V. aesturianus*) in oyster tissues, suggesting the involvement of these factors in triggering mass mortality. Schikorski et al. (Schikorski et al., 2011a) demonstrated that OsHV-1 alone and *Vibrio splendidus* alone can cause death in oysters under experimental conditions. Others demonstrated the pathogenicity to oysters of strains of the *V. splendidus* group isolated during mortality events (Gay et al., 2004). However, infection with these bacteria has not been unequivocally demonstrated to cause oyster mortality since experimental inocula were at artificially high doses (~10^7 CFU ml^{-1}). Several studies *in situ* have shown no or weak correlations between mortality and prevalence of *V. splendidus* and *V. aesturianus* (Dégremont, 2011; Pernet, 2012).

In this study, an increase in bacterial load was observed in association with the mortalities for treatments at sites A and C (except for high and low adult oysters at site C). However, no significant increase of *Vibrio* load was observed prior to mortalities (except for high spat at site A) suggesting an opportunistic bacterial colonisation while the oysters were moribund or dying rather than a causative involvement in the initiation of the disease. Interestingly, a significant increase in bacterial load (total bacteria and *Vibrio* spp.) was observed at site B but no mortality event occurred at this site in November/December 2011, suggesting the influences of other environmental parameters on the vibrio communities and their interactions with oysters. As well as lack of increase in vibrio numbers prior to the onset of mortality, we did not observe a change in the species assemblage of *Vibrio* spp., and we did not isolate *V. aesturianus.* Overall, in this field study we did not demonstrate any clear evidence for the role of *Vibrio* spp. in the initiation of the disease.

V. splendidus was commonly isolated in this study. This species is in fact a complex of subtypes, and it was beyond the scope of this study to undertake sub-typing. It is conceivable that the assemblage of *V. splendidus* subtypes changed prior to the mortality event in favour of more pathogenic varieties.

In comparison with the control site (Hawkesbury River), total bacterial loads in *C. gigas* in the George's River were no higher, and vibrio loads were also within similar ranges in both rivers.

Transmissibility of OsHV-1 and biosecurity precautions

Throughout this study the researchers observed a strict biosecurity protocol which included physical disinfection of all equipment in disinfectant (Virkon ®), wearing dedicated personal protective clothing and boots which were specifically laundered and disinfected upon leaving the oyster farm, commercial washing of vehicles after each field trip and analytical work on oyster samples in a PC2 laboratory. However, during field work at the infected

site, oyster trays from each site were collected and placed in one boat and were examined in the same location. For logistical reasons it was not possible to segregate materials from each site and so trays from one site were often stacked on top of trays from another site during transport or for many hours in the shed. During the outbreak this meant that it was possible for potentially infective material to be transferred between sites by the activities of oyster farmers or researchers. Despite the potential exposure in this manner of oysters from site B to infective materials from sites A and C at least weekly during November 2011, this was not sufficient to initiate a clinical outbreak at site B. Note that oysters at site B were infected with OsHV-1 from November. These observations suggest that it might not be possible to infect oysters with sufficient doses of OsHV-1 to cause death unless they are in water at the time of exposure. However, it might be possible to induce infection and a carrier state by exposure out of water. This is an issue that could be tested experimentally.

Reservoir hosts for POMS

Studies of environmental reservoirs of pathogens are inherently difficult. 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. 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 can be identified or ruled out as potential reservoirs for POMS virus at this time.

Genetic studies for resistance to POMS

In Australia, POMS occurs only in the Botany Bay/Georges River and Port Jackson areas of Sydney. An Australian program to develop genetic resistance to POMS was requested by industry and has commenced within the Seafood CRC in response to the threat posed by POMS. To obtain data for this program in 2011 it was necessary to perform experimental challenge of family lines by natural exposure in the Georges River estuary. However, the results of this FRDC project which demonstrated strong clustering of OsHV-1 infection in the estuary would lessen the value of many study designs on genetic resistance by natural exposure. For this reason the results of this FRDC project were communicated in real time to the Seafood CRC research team. As a result of the preliminary assessments made for the 2009 year class (Kube et al., unpublished data) an experiment was designed and conducted under the auspices of the Seafood CRC with the aim of measuring the survival of ASI 2011 year class families in a field challenge. Data presented from this FRDC project to a closed meeting of the ASI Technical Committee on 10th February 2012 reinforced the critical need to assess the spatial variation in survival via a well planned field design to allow comparisons of genetic differences in the 2011 year class. The experiment was successful in demonstrating highly significant genetic variation and heritability ($h^2 = 0.38$), which suggested that there would be a strong

response to selection for resistance to POMS (Kube et al., 2012, unpublished data). Spatial effects were also confirmed, similar to those reported here. This study benefited from the knowledge and interpretation of the results of this FRDC project. It paves the way for future research trials and encourages the development of an experimental laboratory infection model for OsHV-1.

Risk of spread of POMS in Australia

Data from this project confirm that environmental conditions (water temperature and salinity) suitable for POMS disease expression are not unique to Woolooware Bay, Georges River. For example, the environment of the Hawkesbury River, our control site, appears suitable for the disease to establish.

The mode of spread of POMS over long distances is unknown, and even short term transmission mechanisms are unclear. Movement of infected oysters from one place to another would appear to be a major risk factor for viral spread. Current biosecurity regulations in New South Wales should prevent this. Spontaneous appearance of the disease (for example from an unknown environmental source) or its introduction from overseas (eg ballast water, imported shellfish) or through recreational boating are also possibilities. These risk factors require further consideration.

BENEFITS AND ADOPTION

The benefits and beneficiaries of this research project align with those identified in the project application, which was based on national consultation coordinated by FRDC. Commercial Pacific oyster growers in the states and hatcheries (principally in Tasmania) were kept informed of results throughout the project. Pragmatic adoption actions have been taken in two areas. Firstly, oyster growers in NSW have commenced planning possible infrastructure modifications to survive an outbreak of POMS, formed groups to monitor oyster movements between estuaries and mandated pre-movement testing for OsHV-1. Secondly, researchers in the Seafood CRC research project on genetic selection for POMS resistance adopted an experimental design appropriate to deal with non-uniform exposure in a field challenge of family lines leading to excellent outcomes and demonstration of genetic components in resistance to POMS (Kube et al 2012, unpublished). The national aquatic animal health laboratory network has benefited by provision of common control materials and standardisation of diagnostic test approaches for OsHV-1 at two laboratories (AAHL and USyd).

FURTHER DEVELOPMENT

At the completion of this project there remained a number of key knowledge gaps, that if closed, would provide greater confidence in recommendations to control POMS infection in oysters. Recommendations for further development include:

1. Further studies are indicated to confirm the results of this experiment, which was conducted during an unusually wet and cool summer. If the same beneficial effects of high growing height are obtained when the trial is repeated, oyster growers can confidently take steps to reduce the risk of losses of valuable adult oysters should POMS spread further in Australia.
2. Survivors from the mortality events in the 2011-2012 outbreak of OsHV-1 infection in Woolooware Bay seem to be resistant to POMS, having survived at least three challenges that killed other individuals in the cohort. It is unclear whether they possess innate resistance, whether they avoided exposure to the virus by some mechanism (including chance) or possibly, whether they developed adaptive immunity. Note that adaptive immunity is not a recognised phenomenon in molluscs. Detailed investigation of this population of survivors is warranted. Options include experimental challenge in a laboratory to determine whether they are resistant compared to oysters which have not been exposed to OsHV-1; sampling to determine whether individuals have been exposed or are carrying OsHV-1; immunological analyses; and, genetic analyses.
3. During the outbreak, up to 92% of the individuals tested positive for OsHV-1, but prevalence decreased over time suggesting that surviving oysters may be able to clear the virus. This should be confirmed experimentally. Detailed studies are indicated to determine the pathophysiological and immunological mechanisms leading to clearance of virus from infected oysters.
4. In view of the observations or resistance in survivors of an outbreak, it is essential to regularly place naive oyster in the environment of an oyster lease if an objective is to ensure effective monitoring of OsHV-1 outbreaks, including the number of events and also the seasonal window of infection. Window of infection studies are required to identify periods when it may be safe to place spat in a potentially infected estuary.
5. Regarding the mode of transmission of OsHV-1, more investigations are required to clarify the roles of both micro-hydrodynamics and plankton distribution on the strong clustering pattern of OsHV-1 associated mortalities at micro level. As no plankton sampling was performed in the present study to validate or invalidate the plankton hypothesis, it is evident that further work is required to clarify the possible involvement of a vector for OsHV-1 transmission. High frequency water samples using appropriate plankton nets and water samplers before, during and after an outbreak at different tide cycles and time (day/night) will be required to investigate this question. PCR and *in situ* hybridization will constitute relevant tools to detect and observe OsHV-1 particles in the water samples and, if found, its potential carrier. The importance of such a study is that it may be possible to ameliorate the impact of OsHV-1 by

disturbing the spatial distribution of a vector around an oyster farm, or by placing oysters in a husbandry system designed to avoid the vector.

6. We obtained evidence that it is difficult to initiate an outbreak of mortality due to OsHV-1 by manual handling/cross contamination of apparently healthy and affected oysters in a boat or shed. These observations suggest that it might not be possible to infect oysters with a lethal dose of OsHV-1 unless they are in water, but do not rule out the possibility that they can become infected. This should be tested experimentally. Other risk factors for introduction and spread also require investigation: examples include imported seafood and ballast water.
7. In this study the highest viral loads in oyster tissues were seen only during the period of mortality, and the virus was clearly associated with the disease. Overall the observations suggest that OsHV-1 is a necessary but insufficient factor in the mortality event or that OsHV-1 is a sufficient cause but that there is a very strong dose effect. The presence of the virus in oyster tissue did not lead to mortality and other factors may be required. These factors could include environmental conditions/triggers which can be tested through experimental infection trials, or other as yet undiscovered pathogens.
8. It is possible that a threshold dose of OsHV-1 is required to cause mortality, and below this threshold infection merely persists or is cleared from the tissues. This requires confirmation through experimental infection trials.
9. This study had limited power to detect OsHV-1 infection in wild molluscs and other potential reservoir hosts because the time of sample collection did not align with outbreaks when the virus was most likely to be present in the environment, low sample sizes and spatial influences in the distribution of the virus in the environment. More intensive studies are required.

Some of the factors mentioned above will be addressed in a new FRDC project "2012/032 Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) – risk mitigation, epidemiology and OsHV-1 biology".

The raw data from this project are stored at the Faculty of Veterinary Science University of Sydney in electronic form and may be accessed by contacting the authors.

Note added in press: subsequent to completing this research an outbreak of POMS commenced in the Hawkesbury River, on 21st January 2013. The outputs of this project are being used to address the emergency response in the Hawkesbury River, including recommendations for management to reduce the rate of spread, methodology for a survey of all leases for OsHV-1, interpretation of laboratory test results, and husbandry responses to reduce losses.

PLANNED OUTCOMES

The planned outcomes of this project were to include better understanding of why molluscs succumb to POMS, potentially leading to new management strategies. The important scientific observation that growing height and cultivation system can significantly affect survival rates for adult oysters when exposed to OsHV-1 provides some hope that oyster grower investments made in nurturing oysters from spat through to commercial size can be salvaged in the face of an outbreak, enabling some economic return. Longer term strategies to farm *C. gigas* in the presence of OsHV-1 are possible. The Pacific oyster aquaculture sector across Australia will benefit directly from this project. Indirect benefits and research methods/findings may flow to abalone, pearl and flat oysters where studies of this kind are uncommon. It is premature to claim potential public benefits such as better understanding of global impacts such as climate change and catchment-specific impacts on disease occurrence in estuaries. However, communication activities during this project, specifically discussion of POMS and the research program at conferences and industry meetings have led to greater awareness of biosecurity in disease prevention. This may inform development of land use and quarantine policies. The methods developed for this study are suitable for study of important diseases including QX and winter mortality.

A planned outcome was that practical management measures to reduce disease losses may be identified for adoption by oyster growers through this research. The results to date suggest that this might be possible, but the major findings of this project require confirmation through a second season of study. The reason for this is that the summer of 2011-2012 was unusually mild and wet, and it is possible that POMS disease expression will be different in a typical hot dry summer.

The planned outcomes of this project were in addition a broader responsibility towards the Australian community to ensure the sustainability of Australian aquatic natural resources. This overall outcome was achieved through the promotion of information about oyster health in general and POMS. This has led to proposals from specific sectors of the oyster industry, for example Broken Bay Oysters, for voluntary restrictions on oyster movements between estuaries, and objective laboratory testing of oysters for specific pathogens prior to movement, to reduce the chance of disease spread. This will protect commercial aquaculture in these areas.

Research on genetic selection for POMS resistance was initiated in Australia in 2011 because of the threat this disease poses to industry. The program commenced with natural challenge of family lines of *C. gigas* in the Georges River estuary. A meeting of the CRC research team with this project enabled the design of the CRC challenge trial to account for strong clustering of exposure/mortality in trays of oysters. This led to statistically significant outcomes and demonstration of a genetic component in POMS resistance among family lines in a trial which was completed in 2012.

Communication of R&D results to industry was achieved through presentations to the Australasian Aquaculture Conference 2011, and through industry meetings that were convened by state jurisdictions and local associations. Communication of the most recent findings of the project, namely confirmation that growing height can beneficially affect survival of adult oysters in the face of an outbreak, will be ongoing. A website to promote the research approach and main outcomes was established during the project and has proven to be very popular with industry (www.oysterhealthsydney.org).

CONCLUSION

POMS has severely impacted Pacific oyster production in Europe and New Zealand, where oyster growers who suffered severe looses have tended to cease production. This project was based on the premise that the oyster industry will need to learn to live with POMS by managing husbandry. The epidemiological observations are of considerable importance and inform future strategies to control OsHV-1, including the methodologies to be applied in the genetic selection program. By stocking *C. gigas* into 3 different oyster leases in Woolooware Bay and allocating them into groups to study growing height and age, we demonstrated that a growing height 300mm higher than standard could reduce mortality rates by 40% in adult oysters. Unfortunately spat were highly susceptible to the virus and all those kept in trays died regardless of growing height. These results were consistent at all three leases. Is a second smaller experiment, spat survived in floating baskets but not in trays.

Our observations suggest that OsHV-1 is a necessary but insufficient factor in the mortality event. An alternative is that OsHV-1 is a sufficient cause but that a threshold dose must be reached to initiate mortalities. The presence of the virus in oyster tissue did not lead to mortality and other factors may be required. These factors could include environmental conditions/triggers or other as yet undiscovered pathogens. *Vibrio sp.* bacteria, which have been suggested to be involved with OsHV-1 virus in POMS disease in France, may not be involved because their intensity increased only after the POMS outbreak had started, species assemblage did not appear to change and similar populations of bacteria were observed in healthy *C. gigas* in the Hawkesbury River. Water temperature and salinity effects require further investigation, but there was a small reduction in salinity before each mortality event in the Georges River.

Importantly the virus did not appear to be transmitted free in water but rather it behaved as if it was travelling with still to be defined planktonic particles, in clusters in the water column. This led to uneven exposure of oysters, even those within the same trays. These important results have already been used by CRC researchers to confirm the optimal design of field experiments to study the resistance of different *C. gigas* family lines, to support a genetic selection program.

OsHV-1 was present in oysters at low levels up to 2 months before mortality was observed. Up to 92% of the oysters tested positive for OsHV-1 during mortality events but the infection prevalence decreased over time suggesting that surviving adults can clear the virus. Some oysters survived three separate mortality events during the summer 2011-2012. These observations point to underlying immunological mechanisms and suggest possible genetic resistance.

It can be concluded from this study that husbandry factors may strongly influence the survival of adult *C. gigas* during an outbreak of POMS and it is

possible that measures to reduce the level of exposure of oysters to OsHV-1 as distinct from preventing exposure may be sufficient to reduce mortalities.

Recommendations were made for further development. Further studies are indicated to confirm the effect of growing height on mortality rates, as the present research trial was conducted during an unusually wet and cool summer. If the same results are obtained when the trial is repeated in a more typical summer, oyster growers can confidently take steps to reduce the risk of losses of valuable adult oysters should POMS spread further in Australia. Further studies are also required to investigate why some oysters appear to be resistant and how some clear the virus from their organs, to precisely identify the seasonal window of infection, to confirm the mode of transmission of the virus in the environment, to evaluate the risk of transmission with equipment and by handling oysters, to understand how environmental factors combine with the virus to cause mortality, and whether a certain level of viral load in the environment is needed to initiate mortalities. Some of these factors will be addressed in a new FRDC project “2012/032 Aquatic Animal Health Subprogram: Pacific oyster mortality syndrome (POMS) – risk mitigation, epidemiology and OsHV-1 biology”.

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APPENDIX 1: INTELLECTUAL PROPERTY

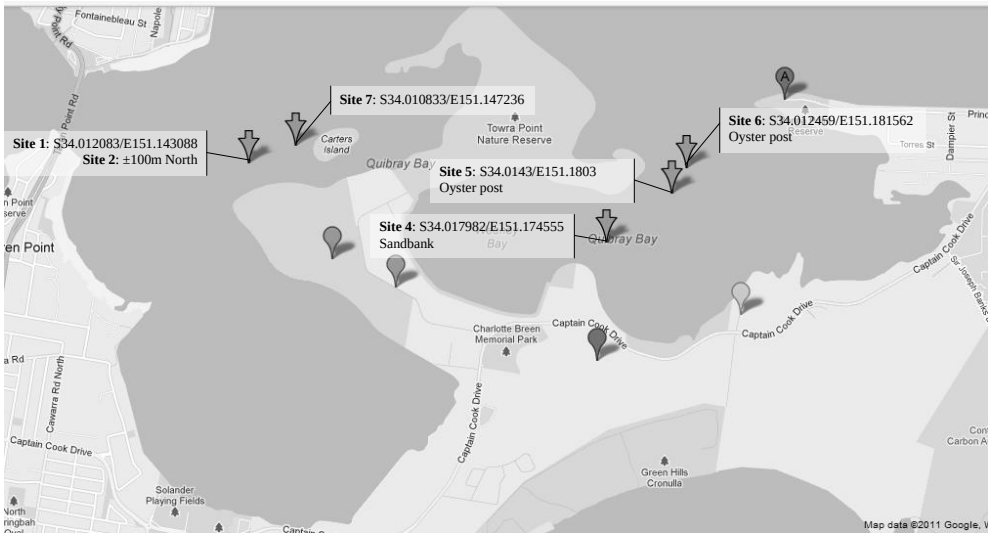
This project has not developed any intellectual property that requires legal protection.

APPENDIX 2: STAFF

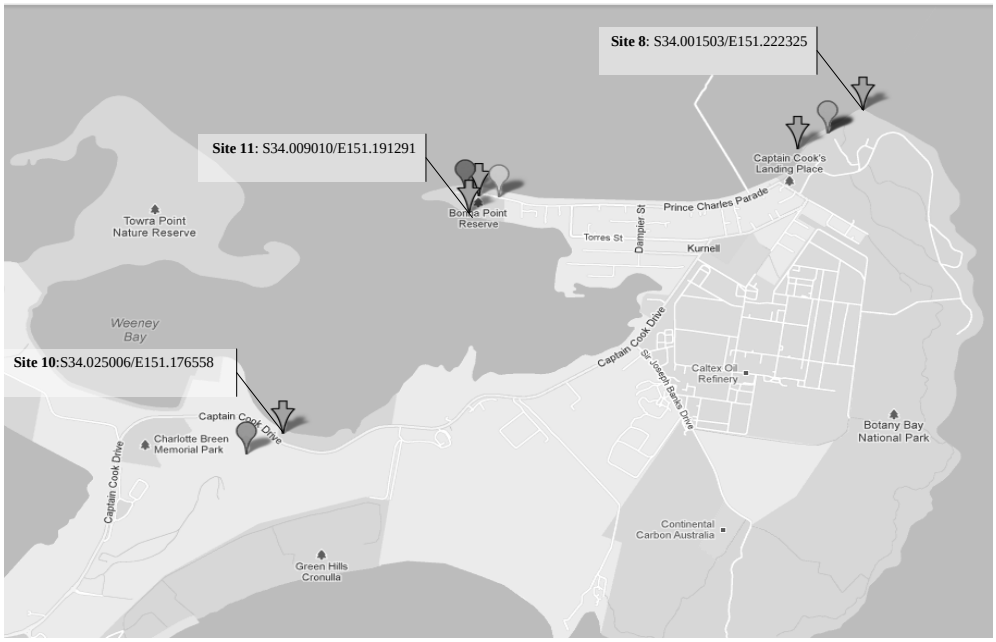
Many people contributed to the research described in this report. The core project team included Richard Whittington, Ika Paul Pont and Navneet Dhand (University of Sydney). Laboratory assistance was provided by Alison Tweedie, Anna Waldron, Vicki Patton and Ann-Michele Whittington.

APPENDIX 3: WILD MOLLUSCS AND OTHER POTENTIAL RESERVOIR HOSTS FOR POMS

WILD MOLLUSC COLLECTION 10/01/12



WILD MOLLUSC COLLECTION 13/02/2012



Base maps courtesy of Google

Table A3.1. Wild molluscs and other wild species tested for OsHV-1. Site is illustrated on maps on page 92.

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

APPENDIX 4: RESULTS OF BACTERIOLOGY

Table A4.1. Details of samples of putative *Vibrio* species which were sent to the Animal Health Laboratories, Perth for identification

Date	Isolate	Site	Treatment	Lab code		Identity
2-Nov-11	220-1	A	High Ad	AS-12-1509	#1	*? V. Pelagius II*
2-Nov-11	220-10	A	Low Sp	AS-12-1509	#10	*V. splendidus*
2-Nov-11	220-2	A	High Ad	AS-12-1509	#2	*V. splendidus*
2-Nov-11	220-3	A	High Ad	AS-12-1509	#3	*V. splendidus*
2-Nov-11	220-4	A	High Ad	AS-12-1509	#4	*V. splendidus*
2-Nov-11	220-5	A	Low Ad	AS-12-1509	#5	*? V. splendidus*
2-Nov-11	220-6	A	High Sp	AS-12-1509	#6	*V. splendidus*
2-Nov-11	220-7	A	High Sp	AS-12-1509	#7	*V. splendidus*
2-Nov-11	220-8	A	High Sp	AS-12-1509	#8	*Vibrio* species
2-Nov-11	220-9	A	High Sp	AS-12-1509	#9	*V. splendidus*
16-Nov-11	234-1	A	High Ad	AS-12-1149	#1	*V. splendidus*
16-Nov-11	234-10	A	Low Sp	AS-12-1149	#11	*? V. splendidus*
16-Nov-11	234-2	A	High Ad	AS-12-1149	#2	*V. splendidus*
16-Nov-11	234-3	A	High Ad	AS-12-1149	#3	*V. splendidus*
16-Nov-11	234-4	A	Low Ad	AS-12-1149	#4	*V. splendidus*
16-Nov-11	234-5	A	Low Ad	AS-12-1149	#5	*V. harveyi*
16-Nov-11	234-6	A	High Sp	AS-12-1149	#6	*V. splendidus*
16-Nov-11	234-7	A	High Sp	AS-12-1149	#7	*? V. fischeri*
16-Nov-11	234-8	A	Low Sp	AS-12-1149	#8a	*V. splendidus*
16-Nov-11	234-9	A	Low Sp	AS-12-1149	#9	*V. splendidus*
21-Nov-11	241-1	A	High Ad	AS-12-1149	#12	*? V. splendidus*
21-Nov-11	241-10	A	Low Sp	AS-12-1149	#21	*V. splendidus*
21-Nov-11	241-2	A	High Ad	AS-12-1149	#13	*V. splendidus*
21-Nov-11	241-3	A	Low Ad	AS-12-1149	#14	*V. alginolyticus*
21-Nov-11	241-4	A	Low Ad	AS-12-1149	#15	*Vibrio* species
21-Nov-11	241-5	A	Low Ad	AS-12-1149	#16	*V. splendidus*
21-Nov-11	241-6	A	High Sp	AS-12-1149	#17	*V. splendidus*
21-Nov-11	241-7	A	High Sp	AS-12-1149	#18	*V. splendidus*
21-Nov-11	241-8	A	High Sp	AS-12-1149	#19	*? V. splendidus*
21-Nov-11	241-9	A	Low Sp	AS-12-1149	#20	*V. splendidus*
24-Nov-11	245-1	A	High Ad	AS-12-1509	#11	*V. alginolyticus?*
24-Nov-11	245-10	A	High Sp	AS-12-1509	#21	*Vibrio* species
24-Nov-11	245-11	B	High Ad	AS-12-1509	#64	*V. alginolyticus*
24-Nov-11	245-12	B	High Ad	AS-12-1509	#65	*V. splendidus*
24-Nov-11	245-13	B	Low Ad	AS-12-1509	#66	*Photobacterium damselae*
24-Nov-11	245-14	B	Low Ad	AS-12-1509	#67	*V. splendidus*
24-Nov-11	245-15	B	Low Ad			
24-Nov-11	245-16	B	High Sp	AS-12-1509	#68	*Vibrio* species
24-Nov-11	245-17	B	High Sp	AS-12-1509	#102	*Vibrio* species
24-Nov-11	245-18	B	High Sp	AS-12-1509	#103	*V. splendidus*
24-Nov-11	245-19	B	Low Sp	AS-12-1509	#014	*V. alginolyticus*
24-Nov-11	245-2	A	High Ad	AS-12-1509	#12 &13	*Photobacterium damselae*
24-Nov-11	245-20	B	Low Sp	AS-12-1509	#105	*V. alginolyticus*
24-Nov-11	245-3	A	High Ad	AS-12-1509	#14	*V. harveyi*
24-Nov-11	245-4	A	Low Ad	AS-12-1509	#15	*V. splendidus*
24-Nov-11	245-5	A	Low Ad	AS-12-1509	#16	*Vibrio* species
24-Nov-11	245-6	A	Low Ad	AS-12-1509	#17	*V. splendidus*
24-Nov-11	245-7	A	Low Ad	AS-12-1509	#18	*V. alginolyticus*
24-Nov-11	245-8	A	High Sp	AS-12-1509	#19	*V. splendidus*
24-Nov-11	245-9	A	High Sp	AS-12-1509	#20	*V. splendidus*
30-Nov-11	252-1	A	High Ad	AS-12-1509	#22	*V. splendidus*
30-Nov-11	252-10	A	Low Ad	AS-12-1509	#31	*V. harveyi*
30-Nov-11	252-11	B	High Ad	AS-12-1509	#81	*Vibrio* species

Date	Isolate	Site	Treatment	Lab code		Identity
30-Nov-11	252-12	B	High Ad	AS-12-1509	#82	*V. splendidus*
30-Nov-11	252-13	B	Low Ad	AS-12-1509	#84	*V. splendidus*
30-Nov-11	252-14	B	Low Ad	AS-12-1509	#85	*V. alginolyticus*
30-Nov-11	252-15	B	High Sp	AS-12-1509	#86	*Vibrio* species
30-Nov-11	252-16	B	High Sp	AS-12-1509	#87	*V. alginolyticus*
30-Nov-11	252-17	B	Low Sp	AS-12-1509	#88	*Vibrio* species
30-Nov-11	252-18	B	Low Sp	AS-12-1509	#89	*V. alginolyticus*
30-Nov-11	252-19	B	Low Sp	AS-12-1509	#90	*Vibrio* species
30-Nov-11	252-2	A	High Ad	AS-12-1509	#23	*? V. harveyi*
30-Nov-11	252-20	B	Low Sp	AS-12-1509	#91	*Vibrio* species
30-Nov-11	252-3	A	High Ad	AS-12-1509	#24	*Vibrio* species
30-Nov-11	252-4	A	High Ad	AS-12-1509	#25	*Vibrio* species
30-Nov-11	252-5	A	High Ad	AS-12-1509	#26	*V. splendidus*
30-Nov-11	252-6	A	Low Ad	AS-12-1509	#27	*V. alginolyticus*
30-Nov-11	252-7	A	Low Ad	AS-12-1509	#28	*V. splendidus*
30-Nov-11	252-8	A	Low Ad	AS-12-1509	#29	*Vibrio* species
30-Nov-11	252-9	A	Low Ad	AS-12-1509	#30	*V. alginolyticus*
5-Dec-11	254-1	A	High Ad	AS-12-1509	#32	*V. alginolyticus*
5-Dec-11	254-10	A	wild site C	AS-12-1509	#41	*V. splendidus*
5-Dec-11	254-2	A	High Ad	AS-12-1509	#33	*V. splendidus*
5-Dec-11	254-3	A	High Ad	AS-12-1509	#34	*V. splendidus*
5-Dec-11	254-4	A	High Ad	AS-12-1509	#35	*V. splendidus*
5-Dec-11	254-5	A	High Ad	AS-12-1509	#36	*Vibrio* species
5-Dec-11	254-6	A	High Ad	AS-12-1509	#37	*V. alginolyticus*
5-Dec-11	254-7	A	High Ad	AS-12-1509	#38	*V. splendidus*
5-Dec-11	254-8	A	wild site C	AS-12-1509	#39	*V. splendidus*
5-Dec-11	254-9	A	wild site C	AS-12-1509	#40	*V. splendidus*
14-Dec-11	259-1	A	High Ad	AS-12-1509	#42	*V. splendidus*
14-Dec-11	259-10	A	High Ad	AS-12-1509	#52	*V. splendidus*
14-Dec-11	259-2	A	High Ad	AS-12-1509	#43	*? V. parahaemolyticus*
14-Dec-11	259-3	A	High Ad	AS-12-1509	#44	*V. splendidus*
14-Dec-11	259-4	A	High Ad	AS-12-1509	#45	*? V. parahaemolyticus*
14-Dec-11	259-5	A	High Ad	AS-12-1509	#47	*V. splendidus*
14-Dec-11	259-6	A	High Ad	AS-12-1509	#48	*Vibrio* species
14-Dec-11	259-7	A	High Ad	AS-12-1509	#49	*V. splendidus*
14-Dec-11	259-8	A	High Ad	AS-12-1509	#50	*V parahaemolyticus*
14-Dec-11	259-9	A	High Ad	AS-12-1509	#51	*V. splendidus*
20-Dec-11	264-1	A	High Ad	AS-12-1509	#53	*Vibrio* species
20-Dec-11	264-10	A	High Ad	AS-12-1509	#63	*V. splendidus*
20-Dec-11	264-2	A	High Ad	AS-12-1509	#54	*Vibrio* species
20-Dec-11	264-3	A	High Ad	AS-12-1509	#55	*V. splendidus*
20-Dec-11	264-4	A	High Ad	AS-12-1509	#56	*V alginolyticus*
20-Dec-11	264-5	A	High Ad	AS-12-1509	#58	*V. splendidus*
20-Dec-11	264-6	A	High Ad	AS-12-1509	#59	No DNA
20-Dec-11	264-7	A	High Ad	AS-12-1509	#60	*V. splendidus*
20-Dec-11	264-8	A	High Ad	AS-12-1509	#61	*V alginolyticus*
20-Dec-11	264-9	A	High Ad	AS-12-1509	#62	*Vibrio* species
10-Feb-12	025-1	B	High Ad	AS-12-1509	#69	*V. splendidus*
10-Feb-12	025-10	B	High Sp	AS-12-1509	#79	*V. parahaemolyticus*
10-Feb-12	025-2	B	High Ad	AS-12-1509	#70	*V. harveyi*
10-Feb-12	025-3	B	High Ad	AS-12-1509	#71	*Vibrio* species
10-Feb-12	025-4	B	High Ad	AS-12-1509	#72	*Vibrio* species
10-Feb-12	025-5	B	Low Ad	AS-12-1509	#73	*V. harveyi*
10-Feb-12	025-6	B	Low Ad	AS-12-1509	#75	*V. alginolyticus*
10-Feb-12	025-7	B	Low Ad	AS-12-1509	#76	*V. alginolyticus*
10-Feb-12	025-8	B	Low Ad	AS-12-1509	#77	*Vibrio* species
10-Feb-12	025-9	B	High Sp	AS-12-1509	#78	*Vibrio* species
13-Feb-12	027-1	B	High Ad			
13-Feb-12	027-2	B	High Ad	AS-12-1509	#93	*V. alginolyticus*

Date	Isolate	Site	Treatment	Lab code		Identity
13-Feb-12	027-3	B	High Ad	AS-12-1509	#94	*V. alginolyticus*
13-Feb-12	027-4	B	High Ad	AS-12-1509	#95	*Vibrio* species
13-Feb-12	027-5	B	High Ad	AS-12-1509	#96	*V. splendidus*
13-Feb-12	027-6	B	Low Ad	AS-12-1509		
13-Feb-12	027-7	B	Low Ad	AS-12-1509	#97	*V. harveyi*
13-Feb-12	027-8	B	Low Ad	AS-12-1509	#98	*Vibrio* species
13-Feb-12	027-9	B	Low Ad	AS-12-1509	#99	*Vibrio* species
13-Feb-12	027-10	B	Low Ad			No DNA