SAFE SPAT REARING TRIALS – FINAL REPORT TO OYSTERS AUSTRALIA 17th JUNE 2014

Operational model

To prevent mortality of *Crassostrea gigas* due to OsHV-1 µVar in hatcheries it is recommended that incoming water for breeding oysters be treated. Eggs, larvae and spat should be in contact with and kept only in seawater that has been treated using either: i) aged and settled for at least 48 hours, or ii) filtered to 5 µm nominal pore size, usually effected via prefiltration (100 or 55 µm) to remove coarse particles.

Summary

Trials were undertaken with spat in upweller systems to determine a method to treat incoming seawater to prevent mortality due to POMS. The findings of the trials described here have extremely important practical implications for the farming of Pacific oysters. Firstly, it appears to be possible to treat water using simple physical methods such as aging water in a holding tank for 48 hours, or filtering water to 5 µm, and in this way render the water safe for rearing of spat. Secondly, the findings should be applicable to design of water intakes for hatcheries. Thirdly, it may be possible to devise shore-based or pontoon-based upweller systems to hold spat within an OsHV-1 infected estuary during the seasonal risk period for POMS. Fourthly, the water treatments described here should also be applicable for holding broodstock and other valuable resources because based on research from France, the spat used in these trials are believed to be the most susceptible stage of all stages, post settlement of larvae. The caveat is that artificial nutrition will be required for optimal growth of oysters kept in treated water. It is also very important to note that it was not possible with this trial design to confirm that infectious, virulent OsHV-1 had been eliminated from treated water, due to sample sizes, duration of trials and the nature of PCR data. The mortality-prevention effect observed with successful treatments might be a result of different nutrition or physiological state rather than exclusion of virus or prevention of transmission of infection. It is reasonable to assume that the severe mortality in these trials was due to the virus. However, PCR testing is ongoing of samples from treatments and times when mortality was minor or was not observed. These additional results are required before any comments about the effectiveness of treatments in eliminating OsHV-1 from water can be made. Furthermore, to confirm freedom of infection in spat raised in treated water would require a different trial design, and long term monitoring. The mechanism of action of aged water and filtration treatments was not studied. The reason for the prevention of mortality (POMS) in the 5 µm filtration and aged water treatments may be related to the elimination of OsHV-1 attached to particles that were removed from the water column by filtration or sedimentation. Funding from Oysters Australia/Seafood CRC complemented funding from FRDC to enable field work components of these trials. Further results will be reported to FRDC.

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Introduction

Disease associated with OsHV-1 occurred for the first time in Australia in November 2010 in Botany Bay and Port Jackson and by January 2013 had spread from an unknown source to the Hawkesbury River system (Jenkins *et al.*, 2013; Paul-Pont *et al.*, 2014). It caused cessation of the farming of Pacific oysters at these locations. The disease in Australia was named Pacific oyster mortality syndrome (POMS). A national survey conducted in 2011 suggested that OsHV-1 was absent from Pacific oysters in the waterways in which commercial Pacific oyster culture was practiced throughout Australia (Animal Health Australia, 2011). The national response to the threat of OsHV-1 includes two main research and development objectives: production of genetically resistant lines of triploid Pacific oysters using strategies including an infection challenge model, and developing understanding of the biological behavior of the virus and the epidemiology of the disease so that husbandry systems can be designed to farm Pacific oysters in the face of the disease. These strategies are complementary.

The production cycle of triploid Pacific oysters in Australia currently depends on hatchery production of spat, mainly in Tasmania. Spat are shipped for growout to farms located principally in Tasmania, New South Wales and South Australia. Experience in France and other European countries is that OsHV-1 affects mainly hatchery spat and juvenile oysters, with near total losses of affected batches being common. However, the observations in Australia and New Zealand are that oysters from spat through to adult stages are susceptible. For this reason research at the University of Sydney on ways to avoid losses has been directed at the entire production cycle. The results from trials conducted in FRDC projects 2011/053 and 2012/032 have already revealed that mortality in adult stock can be kept below 50% by placing stock in cultivation structures 300 mm above standard growing height (Paul-Pont *et al.*, 2013a). The trials described in this report are the first to examine strategies to enable survival of spat and juvenile oysters.

The mode of spread of OsHV-1 over long geographic distances is unknown, although movements of infected spat from hatcheries are almost certainly responsible for some of the spread observed in Europe (EFSA, 2010). As it is unclear how OsHV-1 arrived in Australia, or how it spread to the Hawkesbury River system, hatcheries in OsHV-1 free zones must be considered to be at risk of infection. If outbreaks of POMs occur on hatcheries there will be severe impacts on spat production, and almost certainly there will be restrictions placed on sales of any successful batches into disease free growing areas. These two factors will have an immediate and devastating economic impact on production of triploid Pacific oysters throughout Australia.

If OsHV-1 spreads throughout Australia the trade barriers imposed on movement of stock between regions based on OsHV-1 status logically would be relaxed. Under those circumstances continued hatchery production would be desirable. However, no data exist on how this might be achieved in hatcheries, and an integrated disease control strategy involving both hatcheries and growers would be needed to continue farming. As no husbandry or genetic advances yet allow for reasonable survival of small spat (<3 month old) during the seasonal window of infection, the need for identification of safe rearing techniques that may enable hatcheries and farmers to hold spat in land-based facilities for prolonged periods becomes increasingly important.

It has been established through laboratory challenge experiments in Europe and Australia that OsHV-1 is shed from dying oysters into the surrounding water and that it can be transmitted in this way to healthy oysters (Schikorski *et al.*, 2011), or can be transmitted to healthy oysters by inoculation of the water in which they are kept. Logically some kind of water treatment method would be required to prevent infection of oysters. However, there are no published data on the viability of OsHV-1 in seawater or on means of disinfection. As part of the research trials conducted in Australia in 2011-2102 (FRDC project 2011/053), epidemiological observations were made of the patterns of mortality during POMS outbreaks. These observations led to an hypothesis that OsHV-1 was borne on particles and was not freely or uniformly distributed in water (Paul-Pont *et al.*, 2013b). This raises the possibility that

OsHV-1 could be removed from seawater using simple procedures such as filtration or sedimentation. Anecdotal reports exist from France that suggest spat raised in ponds in the system known as “pousse en claire” do not suffer from the disease but the mechanisms are unknown (Richez, 2012). Factors such as high water temperature, natural UV irradiation in shallow water and sedimentation of particles are possible explanations. Certainly in France OsHV-1 has been reported to be a problem only when water temperatures are in the range 16-24^{o}C, and in the ponds temperature can exceed 24^{o}C (Richez, 2012). A threshold temperature of 17^{o}C for onset of mortality due to OsHV-1 has been well established through observational research in France (Pernet, 2012).

Aim

The aim of this study was to obtain information about simple and practical methods to treat/disinfect water so as to develop a method fro hatcheries to safely produce and rear spat in infected estuaries during the risk period for disease expression, and for hatcheries or farmers to hold spat in land based facilities during the seasonal window of infection.

Materials and methods

Culture system
Land-based upweller tanks to hold/rear spat were designed and installed in a large enclosed shed adjacent to the Hawkesbury River at Mooney Mooney in March 2012. The entire system was dismantled and rebuilt in a different shed in September 2013 (Figure 1). For both systems, four main treatment tanks, each containing 4 upwellers (see below) were installed within a large fibreglass holding tank (depuration tank), which was connected to waste for discharge of all water from the upwellers to the river.

Time gaps between some trials and selective application of disinfection were included in the design to enable some conclusions to be made about physical and chemical inactivation of OsHV-1 in aquaculture contexts. In addition, as all of the treatment tanks were in close proximity to one another and it was impossible to control aerosols from the tanks, or movement of personnel and other equipment into and within the shed during the experiments, some observations about aerosol and cross-contamination were thought to be possible.

The upwellers were disinfected with Virkon ® (Antec International Limited) prior to Trials 2, 3 and 4, treatment tanks were rinsed out, mixing tanks were cleaned to remove sediments and the pipework was physically shocked to remove adherent debris. There was a three week gap between trials 3 and 4 during which time the treatment and mixing tanks were dry. There was an 11 day gap between trials 4 and 5 and tanks were cleaned prior to trial 5, but Virkon was not used prior to Trial 5. No disinfection or cleaning was undertaken prior to Trials 6 and 7.

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

Figure 2. Physical design of the facility constructed to investigate treatment of water to inactivate OsHV-1. Treatments differed between experiments (see table 1 for details).

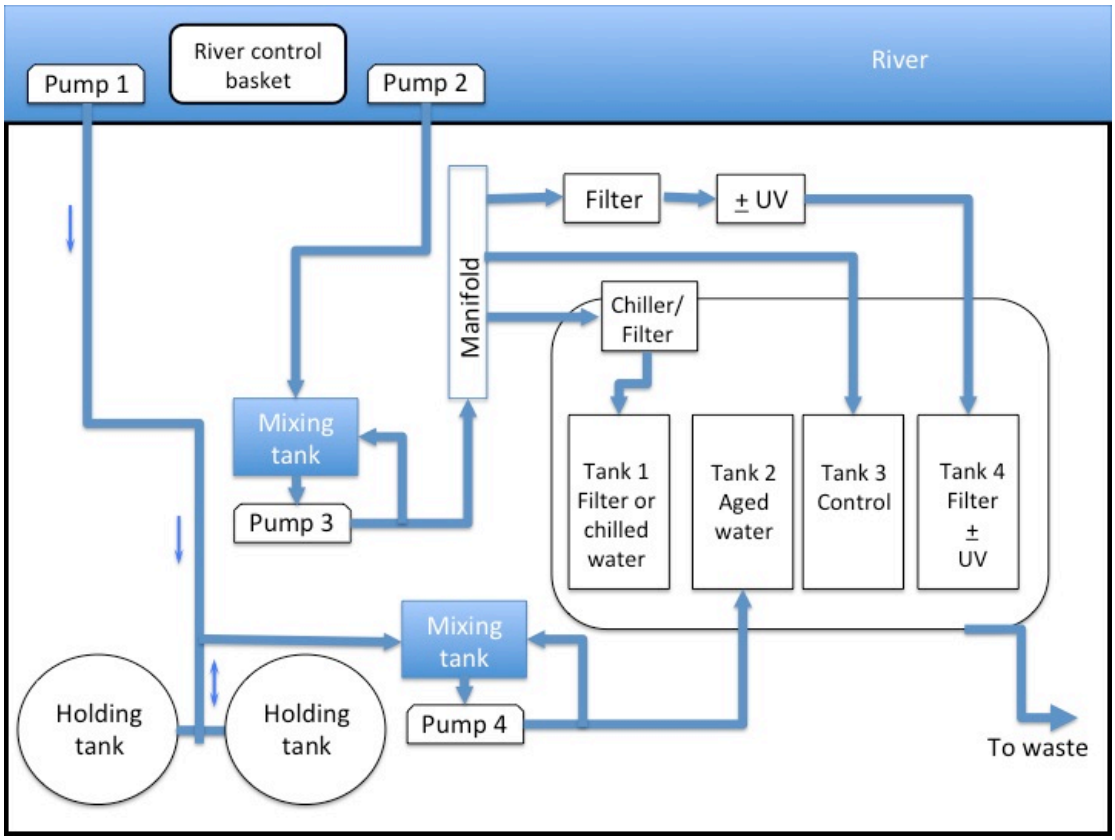


Figure 3. Examples of 5 µm pleated paper filter cartridges. Left, after washing; right, after 1 day in use.

Figure 4. Visual examination of spat from one of the treatments

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

Each mixing tank (118 x 91 x 43 cm, L x W x D, 460 L) had a separate pump to regulate flow to the upwellers (Figure 2). Flow was controlled by partial recirculation of water from the off-take back into the mixing tank, and a ball valve on the outlet to the treatment tank containing the upwellers. This prevented excess flow and pressure through filters.

Upwellers were small scale 140 x 100 mm commercial units with fine mesh screen. Four were included per treatment tank (81 x 60 x 35 cm, L x W x D, 170 L) (Figure 1). Typical flow rates used in all trials except Trial 4 were 0.5 to 1L per min per upweller. For upweller controls and filter treatments in Trial 4a, flow rates of 1 to 4 L per min per upweller were used, while in Trial 4b flow rates of 3 to 6 L per min per upweller were used.

Water flow rates in filtration treatments were nominal; they were adjusted after cleaning filters, but as the filters became obstructed the flow rate reduced. Flow rates were adjusted so as not to exceed the maximum rated flow for the filters of 38 L/min. Filters were either cleaned or replaced each day (Figure 3). Cleaning was achieved using water under moderate pressure from a domestic water supply.

Experimental treatments
Specific water treatments were tested. Treatments varied from trial to trial (Table 1).

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

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

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

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

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

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

Trial 4 was extended on day 15 (29 January 2014) when most of the spat in the upweller control, river control and 100/30 treatment had died (this phase was termed Trial 4a); after sample collection, 50 spat were removed from each upweller pot in the 55/5 um filter and aged water treatments, mixed together and placed in control upwellers (100 per upweller) to provide upweller controls for further monitoring. There were no river controls for this part of the experiment (this phase was termed Trial 4b).

Due to high mortality by day 23, the river control in Trial 6 was replenished with new spat on day 28.

The spat used for Trial 7 were received at the commencement of Trial 6 and were held in untreated river water in the mixing tank for the control and filtration upwellers, from 26th March, i.e. throughout Trial 6.

The factors monitored in the spat were survival, OsHV-1 infection and growth.

Duration of trials
In general the duration of each trial was determined by the time of onset of mortality in controls and the progressive depletion of oysters due to sampling.

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

Detection of OsHV-1
Oysters were dissected (if >6 mm length, see below), homogenised using mechanical disruption, DNA was extracted using a magnetic bead protocol and OsHV-1 DNA was detected using a quantitative real time polymerase chain reaction based on (Martenot *et al.*, 2010) as described previously (Paul-Pont *et al.*, 2013a). For Trial 1, ten pools of oysters were tested per treatment; each pool contained 300 mg of whole spat if they were < 6 mm in length, or tissue removed from 3 oysters if they were >6 mm long. For Trials 2 to 7, five pools of oysters were tested per treatment; each pool contained 300-500 mg of whole spat if they were <6 mm in length, or 500 mg of tissues removed from the shell if they were >6 mm long; the number of spat per pool ranged from 6 to 12.

Growth rate
The average shell length for each batch was assessed at the time of analysis of the oysters in the laboratory, n=10 pools (Trial 1) or 5 pools (Trials 2 to 7) per treatment per time. Due to the small size of the oysters, the pools of spat were placed on 2 mm square ruled graph paper, photographed and then individual spat were measured after enlarging the images.

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

Results

Treatments and trial dates
Seven experiments were conducted between April 2013 and May 2014 inclusive. The treatments examined in each trial are shown in Table 1. Each trial was conducted during the window of infection for disease caused by OsHV-1 in the Hawkesbury River (unpublished data). As the pattern of infection at any specific site in the river is unpredictable, trials were repeated throughout the second season (summer 2013-2014) to obtain repeated test outcomes for treatments that appeared to be beneficial. Treatments were dropped from the design if results indicated that they did not prevent mortality of spat. The controls in upwellers and the river were included in each trial except trial 4b where there were no river controls. Aged water was included as a treatment in 6 of 7 trials. UV irradiation treatment was included in Trials 1 and 2 only as it was shown not to be necessary in the second trial. The chiller treatment was used only once. The duration of each trial was determined by the appearance of mortality or by the reduction of the number of spat due to sampling.

Mortality patterns
In Trials 1, 2, 3 and 4 severe mortality occurred in both the river controls and the upweller controls. Although spat in the river control died in Trials 6 and 7, no mortality occurred in spat in control upwellers in trials in Trials 5, 6 and 7, therefore no conclusive data were obtained on the effectiveness of water treatments in preventing mortality of spat in trials 5, 6 and 7.

In trials 1, 2, 3 and 4 there were striking differences in mortality patterns of spat, and consistent results were obtained between trials (Table 1). There was a high mortality rate in controls; it developed suddenly and peaked within a few days. No mortalities were observed in spat that were kept in water that had been aged for 48 hours. No mortalities were observed in spat that were kept in water filtered to 5 µm, except in Trial 4b using high flow rates where 4.3% mortality was observed (see below). In the third trial spat kept in water filtered to 30 µm survived but in the fourth trial 30 µm filtration failed to prevent mortality. Filtration to 55 µm did not prevent mortality in the third trial. UV irradiation of water did not appear to be necessary to prevent mortality after filtration of water to 5 µm, based on a comparison of filtration treatments with and without UV irradiation in the second trial.

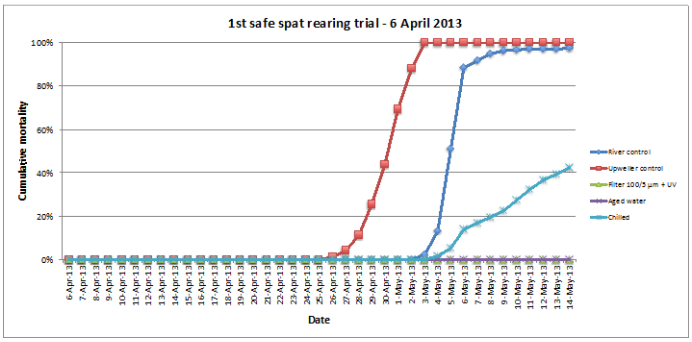
Table 1. Treatments included in each of 7 experiments conducted between April 2013 and May 2014. Red shading, high mortality; pink shading, moderate mortality, grey shading, low mortality, green shading, no mortality. The day when mortality was first observed is shown.

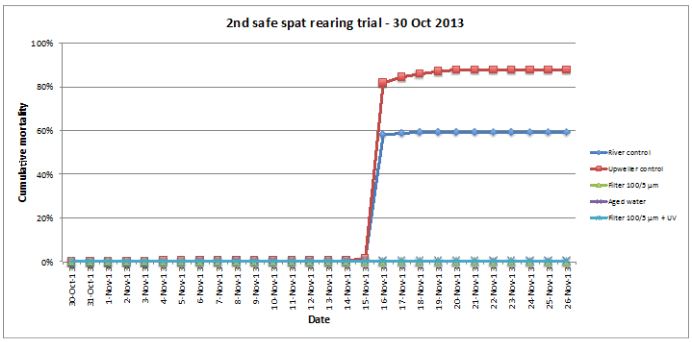
Trial No.	Start date	Duration (days)	River control	Upweller control	Aged water	Filtration					Chilled water
						100/5 µm + UV	100/5 µm	55/5 µm	100/30 µm	55 µm	
1	1 Apr 13	39	d27[1] 97.2%	d20 100%	0	0					d27 42.3%
2	30 Oct 14	28	d15 59.3%	d15 87.8%	0	0	0				
3	27 Nov 14	28	d21 99.9%	d20 99.8%	0				0	d25 60.2%	
4a	15 Jan 14	15	d14 100%	d14 100%	0			0	d14 90.0%		
4b	29 Jan 14	15	0	d8 73.0%	0		d8 4.3%				
5	26 Feb 14	28	0	0	0		0	0			
6	26 Mar 14	28+21[2]	d23[2] 95.0%	0	0		0	0			
7	14 May 14	15	d6 1.4%	0				0			

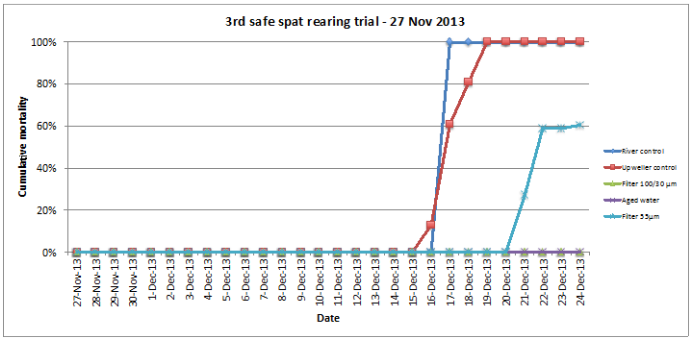
[1] this notation indicates that mortality commenced on day 27
[2] river control spat were replenished on day 28 and there was 0.1% cumulative mortality in these by day 49

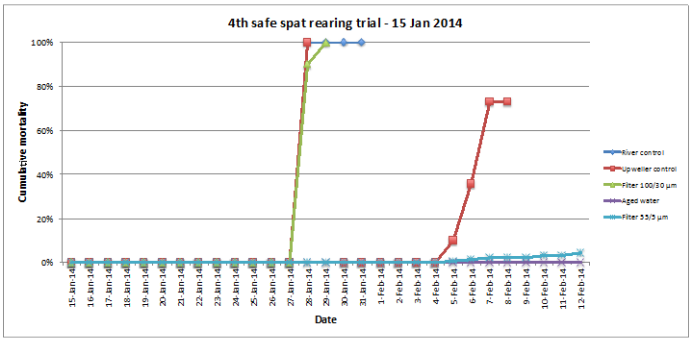
The patterns of cumulative mortality in each trial are shown in Figure 5. In general these conform to the patterns expected in OsHV-1 infection in spat, that is, a very rapid increase in mortality over a few days.

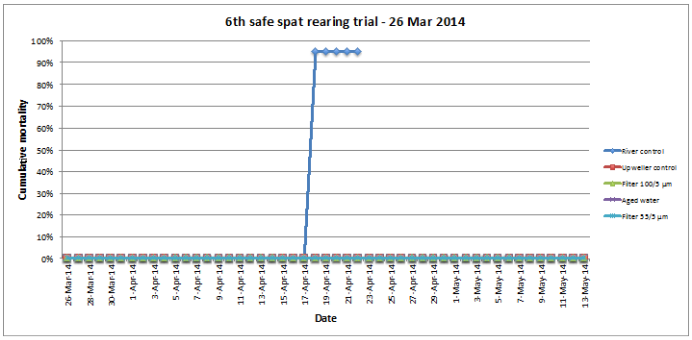
Figure 5. Mortality patterns of spat in each treatment in Trials 1, 2, 3, 4 and 6. Trial 7 not shown as final mortality was 1.4% in river control only.











Trial 1. Mortality started in the control tank (no water treatment) on day 28 (26th April) and reached 100% on day 28. On the same day first mortalities were observed in river control spat and the cumulative mortality increased rapidly to 88.3% (day 31) then 97.2% by the end of the experiment on day 39. In the chiller treatment there was progressive mortality from day 29 until the end of the experiment (final cumulative mortality 42.3%). No mortality was observed in the aged water or 100/5 µm filter + UV treatments.

Trial 2. Mortality commenced on day 15 in both river controls and upweller controls, reached 60% and over 80% within one day and then progressed very gradually.

Trial 3. Mortality commenced on day 20 in the upweller controls and day 21 in the river controls, and most spat were dead within 3 days. Onset of mortality was delayed by 4 days in the 55 µm filter upwellers and developed more gradually.

Trial 4. Mortality began on day 14 and reached 100% in both the river controls and the upweller controls within 1 day and 90% in the 100/30 µm upwellers within 2 days. In the second part of the experiment, upweller controls started dying on day 8 and within 3 days 73% had died; due to the number sampled each day it was not possible to continue observing that treatment group. A very low grade mortality occurred in the 55/5 µm treatment, commencing on day 8 and reaching 4.3% by day 15 when the trial ended.

Trial 5. No mortalities were observed in spat in any of the treatments during the 28 day trial.

Trial 6. Mortality began on day 23 in the river controls, with 95% dead within 1 day. The floating basket in the river was replenished with new spat on day 28. Thirteen dead oysters were observed in the river control between days 47-49, with the final cumulative mortality being 0.1% at the end of the trial. No mortality was observed in any other treatment.

Trial 7. The final mortality was 1.4% in the river control but no mortalities were observed in spat in any of the other treatments during the15 day trial.

Detection of OsHV-1 DNA by qPCR

Trial 1. PCR results confirmed that OsHV-1 was the cause of mortality in river control, upweller control and chiller treatments with viral loads up to 5.4 $x10^5$, 1.5 x 10^6 and 4.6 x 10^5 copies/mg, respectively, in spat in these treatments associated with the mortality.

Trial 2. PCR results confirmed that OsHV-1 was the cause of mortality in upweller controls and river controls; viral loads of up to 2 x 10^6 copies/mg were detected.

Trial 4. PCR results for samples collected on 29 Jan 2014 confirmed that OsHV-1 was present in spat in the upweller control and 100/30 µm filter treatments. Viral loads were relatively low (~10^1 to 10^3 copies/mg) as only empty shells were tested.

Determination and analysis of PCR results from all trials is ongoing.

Growth of oysters

In the first trial there was growth retardation of spat in the aged water and filtered water treatments relative to the river controls and upweller controls, and those in chilled water. The size differential is illustrated in Figure 6. In subsequent experiments similar effects were observed although some growth was observed in all treatments. Analysis of growth data for all experiments is ongoing.

Figure 6. Size comparison of spat in control and other treatments prior to the onset of mortality in Trial 1. Left panel, control upweller; middle panel, aged water; right panel filtration with UV.

Environmental data
Analysis of environmental records from the Hawkesbury river and temperature probe data from the treatment tanks revealed water temperatures were above 16 ^{o}C throughout the period of the trials. For each trial, the temperature profiles of treatments with and without mortality were quite similar. In general the average daily water temperature was between 20 and 25 ^{o}C. Maxima approaching 30 ^{o}C were recorded in water holding tanks, and between 30 and 35 ^{o}C in the river controls. In trial 2 there were no data able to be collected from 30/10/13 to 5/11/13 due to computer faults. Further analysis of water temperature data in relation to the onset of mortality will be undertaken.

Discussion

The findings of the trials described here have extremely important practical implications for the farming of Pacific oysters. Firstly, it appears to be possible to treat water using simple physical methods such as aging water in a holding tank for 48 hours, or filtering water to 5 µm, and in this way render the water safe for rearing of spat. Based on research from France, the spat used in these trials are believed to be the most susceptible stage of all stages, post settlement of larvae. Therefore the findings should be relevant to older and larger classes of oysters. Secondly, the findings should be applicable to design of water intakes for hatcheries. Thirdly, it may be possible to devise shore-based or pontoon-based upweller systems to hold spat within an OsHV-1 infected estuary during the seasonal risk period for POMS. Fourthly, the water treatments described here should also be applicable for holding broodstock and other valuable resources. The caveat is that artificial nutrition will be required for optimal growth of oysters kept in treated water. It is also very important to note that it was not possible with this trial design to confirm that infectious, virulent OsHV-1 had been eliminated from treated water, due to sample sizes, duration of trials and the nature of PCR data. The mortality-prevention effect observed with successful treatments might be a result of different nutrition or physiological state rather than exclusion of virus or prevention of transmission of infection.

PCR data confirmed the presence of high levels of OsHV-1 within the tissues of spat during periods of high mortality. The viral loads at these times in these treatment groups were above the threshold of 1 x 10^{4} copies/mg that is accepted to be associated with mortality in France and Australia (Oden *et al.*, 2011; Paul-Pont *et al.*, 2013a; Paul-Pont *et al.*, 2014). Therefore it is reasonable to assume that the severe mortality observed in some treatments and trials was due to the virus. However, PCR testing is ongoing of samples from treatments and times when mortality was minor or was not observed. These additional results are required before any comments about the effectiveness of treatments in eliminating OsHV-1 from water can be made. Even when these additional PCR results are available there will be uncertainty about

the interpretation of both positive and negative results because i) sample sizes of 40 spat per treatment per day were not sufficient to prove beyond reasonable doubt that virus was excluded; the daily confidence would be 95% that prevalence of infection was less than about 10% (Cannon and Roe, 1982), although with repeated daily testing the confidence would be much higher than this, and ii) the PCR provides no indication about viability of virus. Thus it is possible that DNA from inactivated virus would be detected. To confirm freedom of infection in spat raised in treated water would require a different trial design, and long term monitoring. This will be proposed to FRDC in a separate project.

The mechanism of action of aged water and filtration treatments was not studied. The reason for the prevention of mortality (POMS) in the 5 µm filtration and aged water treatments may be related to the elimination of OsHV-1 attached to particles that were removed from the water column by filtration or sedimentation. This is consistent with the hypothesis about particulate attachment of OsHV-1 (Paul-Pont et al 2013a). In the case of the aged water treatment, the protective effect might also be related to inactivation of OsHV-1 over 48 hours. OsHV-1 is an enveloped virus, and in general it is thought that viral envelopes are required for infection and are sensitive to damage due to environmental exposure. However, there are no published data on environmental persistence of pathogenic marine herpesviruses.

The physical design of the experiment involved water distributed from a common manifold prior to filtration or chiller treatment. Therefore the exposures to OsHV-1 for upweller controls and filter/filter+UV/chiller treatments are directly comparable. There was delayed onset of mortality in spat in the chilled water treatment in Trial 1 which may relate to physiological influences on the pathogenesis of the disease. It is possible that a small reduction in water temperature (in this experiment it was about 3 degrees) may favour the oyster and not viral replication. This will require confirmation in an experimental infection model under controlled environmental conditions. In Trial 3, the delayed onset of mortality compared to controls in spat placed in 55 µm filtered water may relate to a reduction in exposure dose, infection from a different source (eg virus released from dying oysters in the river control) or attachment to a larger particle taken up less efficiently by oysters.

The aged water treatment was quite different to the others as it used water that was intermittently sourced from the river (duration about 3 hours every 2 days to fill the holding tank) while the other treatments were based on a continuous supply of water from the river. It is known that OsHV-1 is not uniformly distributed in river water, either spatially or temporally (Paul-Pont *et al.*, 2013b). For this reason the aged water treatment is not directly comparable to the other treatments. It is possible that water obtained from the river at the times the pump was activated to fill the holding tanks may not actually have contained OsHV-1. For this reason the trial was repeated in order to provide many opportunities for the treatment to fail. In five of the 6 trials in which aged water was included as a treatment, the river control oysters died. However the river controls may not be a complete proxy for the water used to fill the holding tanks as the spatial and temporal clustering of OsHV-1 in the river could be at a very fine level (meters, hours). For example in the first trial the river controls died about a week after the upweller controls, suggesting exposure of the two treatments at different times, despite the close proximity of the submersible pump and the floating basket in the river. For this reason it is recommended that the aged water treatment be examined in a laboratory infection model challenge experiment under controlled conditions. This will be proposed to FRDC in a separate project.

Water temperatures recorded throughout the trials exceeded the threshold for onset of mortality associated with OsHV-1 of 16 ^{o}C that has been observed in France (Pernet, 2012). Further analysis of records from these trials as well from probes in the main channel of the river will be undertaken in order to determine whether there are subtle temperature triggers for onset of mortality.

Although the experiments were of relatively short duration, growth rates were retarded in the aged water and filtration treatment groups compared to the controls. Consideration of spat nutrition will be required for long term husbandry if rearing spat in treated water is attempted commercially. An evaluation of compensatory growth of spat held in filtered water will be proposed in a separate FRDC project.

Some observations relevant to the stability of the virus are possible. During these trials there was considerable opportunity for cross contamination of treatments. The treatment tanks were in close proximity, there would have been aerosols from the tanks and pumps, there was movement of personnel and other equipment into and within the shed. Based on these considerations there was lack of cross contamination from controls sufficient to cause mortality across treatments.

The upwellers were disinfected with Virkon ® (Antec International Limited) prior to Trials 2, 3 and 4, treatment tanks were rinsed out, mixing tanks were cleaned to remove sediments and the pipework was physically shocked to remove adherent debris. Tanks were cleaned prior to trial 5 but Virkon was not used. There was an 11 day gap between trials 4 and 5. No disinfection or cleaning was undertaken prior to Trials 6 and 7.

Based on the interval of 11 days between trial 4 where POMS occurred in controls, and Trial 5 where there was no mortality in any treatment, it is reasonable to conclude that the persistence of infectivity (sufficient to induce mortality) in culture systems is less than 11 days.

There was no gap between the end of Trial 2 and the start of Trial 3. Virkon was used to disinfect equipment at the end of Trial 2. Based on the absence of mortality in control upwellers in Trial 3 within a recognised incubation period for OsHV-1 mortality (<10 days), it is reasonable to conclude that Virkon is an effective disinfectant for OsHV-1 when applied to aquaculture infrastructure.

A further consideration is that hatcheries at risk of OsHV-1 infection (for example due to importation of broodstock from other locations) may be required to implement effluent controls to prevent infection of local wild stocks of Pacific oysters. Effective methods to minimise the risk of OsHV-1 spread from hatcheries through effluent control are currently unknown and at present would have to be based on general principles of virology rather than actual data. The design of the experiments described in this report do not allow conclusion about effluent treatment, because particles to which OsHV-1 may attach may not be present in effluent water and therefore OsHV-1 may not be able to be removed by filtration or sedimentation. However, the findings suggest a useful experimental design for conduct research on effluent controls for hatcheries and this will be proposed to FRDC in a separate project.

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