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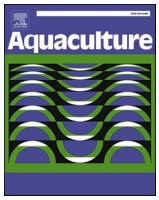

Investigations of the involvement of *Vibrio* species with *Ostreid herpesvirus-1* in mass mortality events in the Pacific oyster *Crassostrea gigas*

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ABSTRACT

Pacific oyster mortality syndrome (POMS) in Pacific oysters *Crassostrea gigas* is defined by mass mortality with *Ostreid herpesvirus I* (OsHV-1). In this study the association of *Vibrio* species with oysters before, during and after POMS events was investigated at population level to clarify their involvement with OsHV-1 in mass mortality events in the Georges and Hawkesbury River estuaries, Australia. In the POMS affected Georges River estuary, three patterns were observed concurrently at different sites: i) *Vibrio* counts and OsHV-1 DNA concentrations increased in adults and spat as mortalities started then decreased as mortalities stopped ii) bacterial counts increased in spat but not in adults associated with mortalities due to OsHV-1 iii) bacterial counts increased in the absence of mass mortality or high concentrations of OsHV-1 DNA. Although almost half of the 120 bacterial isolates were identified as an unknown *Vibrio* species belonging to the Splendidus clade with two predominant biotypes (A and H), there was no association between biotype and mortality. Concurrently in the Hawkesbury River estuary, neither mortality nor OsHV-1 were found, *Vibrio* counts fluctuated significantly and of 84 isolates identified, *V. alginolyticus, V. parahaemolyticus* and unidentified *Vibrio* sp. dominated; only five Splendidus clade isolates were identified. However, when POMS emerged in the Hawkesbury River estuary, the Splendidus-clade isolate became the dominant bacterial species. Mortality also occurred there without OsHV-1 and the dominant bacteria then were *V. alginolyticus* and *V. harveyi,* but the Splendidus-clade isolate was not detected. None of the bacterial species were specifically associated with POMS, although the Splendidus clade isolate increased in prevalence after POMS emerged in the Hawkesbury River estuary. *V. aestuarianus* was not detected in this study. While the findings suggest that bacterial proliferation follows infection with OsHV-1 in POMS, this was not a consistent feature. The number and diversity of bacteria within oyster tissues varied over time, between estuaries, between sites within an estuary and were associated with flooding, growing height, the age of oysters, mortality and OsHV-1.

1. Introduction

In terms of the global volume and value of production, the Pacific oyster *Crassostrea gigas* is an important aquaculture species. Summer mortality in this species has been reported for decades, but since 2008 has been associated with a particular variant of *Ostreid herpesvirus 1* (OsHV-1 µVar) in Europe (EFSA, 2010; Martenot et al., 2011; Martenot et al., 2013; Segarra et al., 2010). The disease outbreaks have been devastating in many areas due to near-total losses particularly of young oysters. Mass mortalities of farmed *C. gigas* associated with OsHV-1 began in 2010 in Australia and the disease was called Pacific Oyster Mortality Syndrome (POMS). The first estuary to be affected was the Georges River in New South Wales (Jenkins et al., 2013). Then in 2013, mass mortalities began in the Hawkesbury River estuary which is approximately 45 km distant from the Georges River (Paul-Pont et al., 2014) (Fig. 1). Multilocus sequence typing of the strains of OsHV-1 in

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Australia revealed that they are different from those in Europe (Trancart et al., 2022).

The epidemiology of POMS in both estuaries has been investigated, providing information about risk factors for the disease in Australia. Important findings were (i) age and size of oysters, growing height, water temperature and water treatments in hatcheries are important risk factors (ii) viral infection and disease expression are highly seasonal (iii) the outbreaks are point source epizootics with negligible horizontal transmission and iv) the strains of the virus are unique to each estuary (Hick et al., 2018; Paul-Pont et al., 2013a, 2013b; Paul-Pont et al., 2014; Trancart et al., 2022; Whittington et al., 2015a; Whittington et al., 2018; Whittington et al., 2015b; Whittington et al., 2019). Another important finding was that OsHV-1 does not act independently, as it was often detected in live oysters in both estuaries despite favourable environmental conditions for POMS (Paul-Pont et al., 2014; Whittington et al., 2019). This suggests a role for other factors in triggering the disease/mortality.

Bacteria have long been implicated in mortality in *C. gigas* (Lipp et al., 1976) with *Vibrio* sp. featuring with OsHV-1 (Keeling et al., 2014) or without this virus (De Decker and Saulnier, 2011; Garnier et al., 2007; Go et al., 2017; King et al., 2019; Yang et al., 2021; Zhang et al., 2023). Furthermore, many vibrio species have been confirmed to be pathogenic through experimental infection in challenge studies: *V. splendidus* (Lacoste et al., 2001; Le Roux et al., 2002; Pernet et al., 2012), *V. aestuarianus* (Garnier et al., 2008; Saulnier et al., 2009), *V. harveyi* (Saulnier et al., 2010), *V. natriegens* (Zhang et al., 2023) and *V. alginolyticus* (Yang et al., 2021; Zhang et al., 2023). Vibrios rapidly colonised oysters before OsHV-1 did so in an outbreak reported by Petton et al. (2015) but in contrast, de Lorgeril et al. (2018) demonstrated immunosuppression and subsequent bacteraemia in oysters infected with OsHV-1 µVar. Irrespective of the primary cause, Lasa et al. (2019) observed shifts in microbial community composition towards less diverse genera with *Vibrio* and *Arcobacter* species and OsHV-1 in oyster mortality outbreaks. These findings support a polymicrobial pathogenesis in POMS. In this study the association of vibrio species with oysters before, during and after POMS events was investigated at population level to clarify their involvement with OsHV-1 in mass mortality events.

2. Methods

2.1. Study design

A longitudinal investigation was conducted in two estuaries, the Georges River, which was affected by POMS from late 2010, and the Hawkesbury River which was used as a control site until January 2013 when it was first affected by POMS and then became a POMS study site (Fig. 1).

The population of interest was *C. gigas* on commercial oyster farms. Sampling of oysters in the Georges River was conducted during trials with experimental designs that have already been described in detail (Paul-Pont et al., 2013b; Whittington et al., 2015a). Briefly, *C. gigas* were stocked into three oyster leases (Fig. 1) in Woolooware Bay. At each site two growing heights and two age groups created four treatment groups: adult high and low; spat high and low. Oysters were placed in October 2011, before the seasonal onset of POMS, and were regularly sampled by the researchers at all three sites during the mortality event that occurred at two of the three sites in Nov-Dec 2011 (Table 1). Additional samples were collected from the third site in Feb-2012 when POMS occurred there.

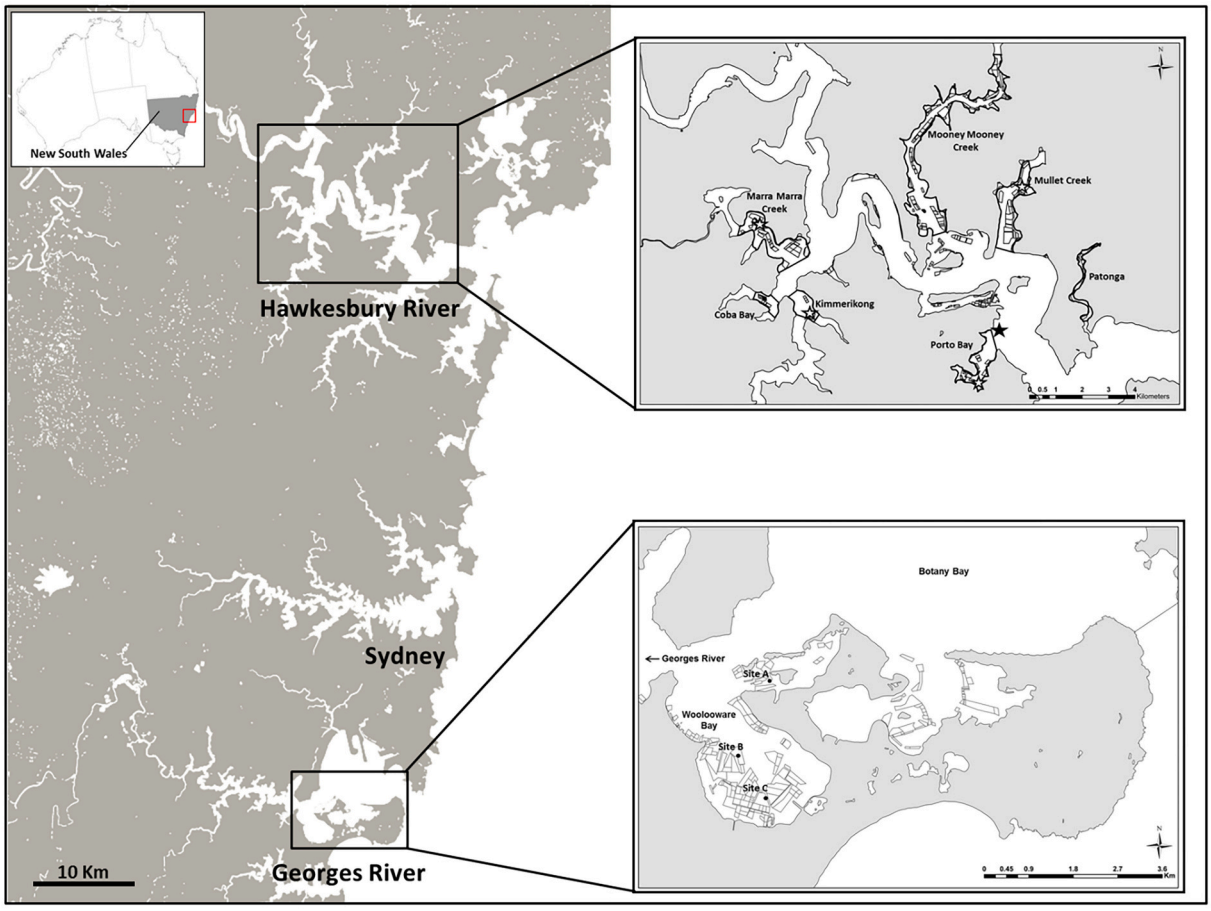

Fig. 1. Study sites in the Georges River and Hawkesbury River estuaries, New South Wales, Australia.

Table 1
Sampling dates, locations and analyses performed in this study. Sites of sample collection: A, Site A; B Site B; MC, Mullet Creek; PB, Porto Bay; CO, Coba Bay; MA, Marra Marra Creek.

Date collected	Sample reference	Oyster length mm[1]	Context	Sites with mortality observations and OsHV-1 qPCR	No. of pooled samples (no. oysters/pool)	Sites with total bacteria and vibrio counts	Sites with vibrio identification (no. cultures)	Splendidus clade biotype (no. isolates tested)	*Vibrio aestuarianus* PCR (no. tissue homogenates tested)
Georges River longitudinal study samples							$n = 120$	$n = 51$	$n = 144$
02–11-11	11/220	28–78[1]	POMS	A,B,C	20/site (5)	A,B,C	A(10)	7	–
16–11-11	11/234	31–75[1]	POMS	A,B,C	20/site (5)	A,B,C	A(12)	9	20
21–11-11	11/241	34–79[1]	POMS	A,B,C	20/site (5)	A,B,C	A(10)	6	20
24–11-11	11/245	30–78[1]	POMS	A,B,C	20/site (5)	A,B,C	A(10), B(9)	7	35
30–11-11	11/252	30–77[1]	POMS	A,B,C	20/site (5)	A,B,C	A(10), B(11)	6	30
05–12-11	11/254	nr[2]	POMS	A,B,C	20/site (5)	A,B,C	A(7)	4	5
14–12-11	11/259	35–76[1]	POMS	A,B,C	20/site (5)	A,B,C	A(10)	6	5
20–12-11	11/264	35–70[1]	POMS	A,B,C	20/site (5)	A,B,C	A(10)	4	5
10–02-12	12/025	nr	POMS	B	10 (5)	–	B(11)	1	14
13–02-12	12/027	nr	POMS	B	10 (5)	–	B(10)	1	10
Hawkesbury River longitudinal study samples							$n = 134$	$n = 28$	$n = 22$
07–12-11	11/255	26–37[2]	Control	MC,PB	10/site (6)	MC,PB	MC(10), PB(10)	MC(−), PB(5)	–
04–01-12	12/002	38–48[2]	Control	MC,PB	10/site (6)	MC,PB	–	–	–
18–01-12	12/008	nr	Control	MC,PB,CO,MA	5/site (6)	MC,PB,CO,MA	–	–	–
15–02-12	12/031	41–65[3]	Control	MC,PB,CO,MA	5/site (6)	MC,PB,CO,MA	MC(11), PB(10)	–	10
15–03-12	12/057	50–73[2]	Control	MC,PB,CO,MA	5/site (6)	MC,PB,CO,MA	MC(12), PB(10)	–	–
20–04-12	12/080	51–81[2]	Control	MC,PB,CO,MA	5/site (6)	MC,PB,CO,MA	MC(10), PB(11)	–	–
10–05-12	12/093	65–77[2]	Control	MC,PB,CO,MA	5/site (6)	MC,PB,CO,MA	–	–	–
05–06-12	12/103	nr	Control	MC,PB,CO,MA	5/site (6)	MC,PB,CO,MA	–	–	–
Hawkesbury River opportunistic samples									
05–11-13	13/197	41–67[4]	POMS[5]	CO	6 (5)	–	CO(24)	23	6
21–01-14	14/016	100–120[4]	Unexplained mortality	PB,CO	6 (1)	–	CO(3)	–	6
29–01-14	14/025	100–120[4]	Unexplained mortality	MA	2 (3)	–	MA(23)	–	–
Total							254	79	166

[1] Range, treatment group means; [2] not recorded [3] range, site means; [4] range, individual lengths. [5]Freshly dead when collected; whole oysters were homogenised.

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

Primers	direction	Gene target	Sequence	Reference
Ostreid herpesvirus				Martenot et al. (2010)
OsHV1BF	Forward	B region	GTC GCA TCT TTG GAT TTA ACA A	
OsHV1B4	Reverse		ACT GGG ATC CGA CTG ACA AC	
OsHV1probeB	TaqMan probe		TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC	
Vibrio aestuarianus				Saulnier et al. (2009)
DNAj aes F1	Forward	*dnaJ*	GTA TGA AAT TTT AAC TGA CCC ACA A	
DNAj aes R1	Reverse		CAA TTT CTT TCG AAC AAC CAC	
DNAj probe	TaqMan probe		TGG TAG CGC AGA CTT CGG CGA C	
Vibrio alginolyticus				Luo and Hu (2008)
AlgF1	Forward	*gyrB*	TCA GAG AAA GTT GAG CTA ACG ATT	
AlgR1	Reverse		CAT CGT CGC CTG AAG TCG CTG T	
Vibrio campbellii				Haldar et al. (2010)
Vca-hly5	Forward	*hly*	CTA TTG GTG GAA CGC AC	
Vca-hly3	Reverse		GTA TTC TGT CCA TAC AAA C	
Vibrio harveyi				Haldar et al. (2010)
Vh-hly1F	Forward	*hly*	GAG TTC GGT TTC TTT CAA G	
Vh-hly1R	Reverse		TGT AGT TTT TCG CTA ATT TC	
Vibrio parahaemolyticus				Haldar et al. (2010)
Vp-tlh1	Forward	*hly*	GAT TTG GCG AAC GAG AAC	
Vp-tlh2	Reverse		CGT CTC GAA CAA GGC G	
Vibrio splendidus		*splendilysine*		Prol et al. (2009)
VSF	Forward		CAC AGC GAT AAC CGC TAC AA	
VSR	Reverse		GTA CGT TGG CGA AGA CAT GA	

In the Hawkesbury River, longitudinal samples were collected from a spat cohort that was placed at downstream sites Mullet Creek and Porto Bay in 2011. As these oysters grew, some were moved upstream to Marra Marra and Kimmerikong (Fig. 1), then back to Porto Bay when flooding affected the upper river. Sampling was conducted fortnightly to monthly in 2011 and 2012 (Table 1). POMS occurred in Jan-2013 and has been described in detail (Paul-Pont et al., 2014). Other cohorts were opportunistically sampled due to mortality in upstream areas (Coba, Marra) in Nov-2013 and Jan-2014 (Table 1; Fig. 1).

2.2. Oyster collection and processing

Table 1 shows the dates of sampling of *C. gigas* in each estuary together with the types of analyses that were conducted. Systematic random sampling was performed except for opportunistic samples collected in 2013 and 2014. Dead and moribund oysters, if present, were removed prior to sampling live oysters, except where otherwise indicated (Table 1).

Oysters were processed on the day of collection or were placed at 4 °C overnight and processed the next day using an aseptic technique to minimise cross contamination of individual oysters. Whole oysters were processed in one instance in 2013 (Table 1). For each sampling time in 2011 and 2012, tissue homogenates were prepared from pools of five (Georges River) or six (Hawkesbury River) individual oysters. Twenty pools were cultured from oysters from each site each time in the Georges River (5 per age-height treatment) until the end of 2011, after which only Site B was sampled (5 pools per height-treatment, adult oysters only). Ten pools per site were cultured in the Hawkesbury River until 04-01-2012, then 5 pools per site until June 2012; in 2013 and 2014, pooled samples or individual oysters were cultured (Table 1). Tissue samples were homogenised in a stomacher (ratio tissue:sterile saline = 1:4). The stomaching bag (BagPage®, Interscience) had a 250 μm sieve and 5 mL of filtrate was collected for bacterial culture then stored at −80 °C until DNA extraction.

2.3. DNA extraction

Tissue homogenates (500 μL) were subjected to bead-beating (Fastprep System, MP Biosciences, USA) then centrifugation (3000 *g*,10 min). DNA was purified from 50 μL sub-samples with the MagMax-96 Viral Isolation Kit (Ambion, USA) and the MagMax Express-96 magnetic particle processor (Applied Biosystems, USA) according to the manufacturer's protocols.

2.4. Bacteriology

Standard methods for clinical microbiology were applied. A bacteriological loop was used to streak the clarified tissue homogenate (10 μL) onto 9 cm Petri dishes containing either marine salt agar with horse blood (MSA-B) (Buller, 2004) or thiosulfate-citrate-bile salts-sucrose medium (TCBS cholera medium, Difco); the plates were incubated aerobically (23 °C,24 h). Colonies on each plate were enumerated from digital images; bacterial counts were expressed as colony forming units per gram of tissue (CFU/g). Colony morphology was described using conventional terminology and representative colony types from each site/treatment/time were subcultured (only from TCBS) into nutrient broth containing 2% NaCl with incubation as described above; glycerol 15% *v*/v was added as a cryoprotectant and broths were placed at −80 °C for later identification.

2.5. Identification of bacterial cultures

Pure cultures of cryoprotected glycerol broths were subcultured on MSA-B plates and identified using conventional methods (biochemical tests, matrix-assisted laser desorption time of flight mass spectrometry) as described (Buller, 2014). PCR was used for confirmation of *V. aestuarianus*, *V. alginolyticus* (Luo and Hu, 2008), *V. campbellii*, *V. harveyi*, *V. parahaemolyticus* (Haldar et al., 2010) and *V. splendidus*

Table 3
Classification scheme for biotypes of the Splendidus clade isolate.

Biotype	TCBS	Sucrose	ONPG	Dnase
A	Y	pos	++	pos
B	Y	pos	+	pos
C	G	neg	+++	weak
D	G	neg	neg	pos
E	Y	pos	+	neg
F	Y	pos	neg	pos
G	Y	pos	neg	neg
H	Y	pos	+++	neg
I	G	neg	neg	neg

ONPG, o-nitrophenyl-β-D-galactopyranoside; Dnase, Dnase agar plate; G, green (sucrose-negative) colonies on TCBS; Y, yellow (sucrose-positive) colonies on TCBS.

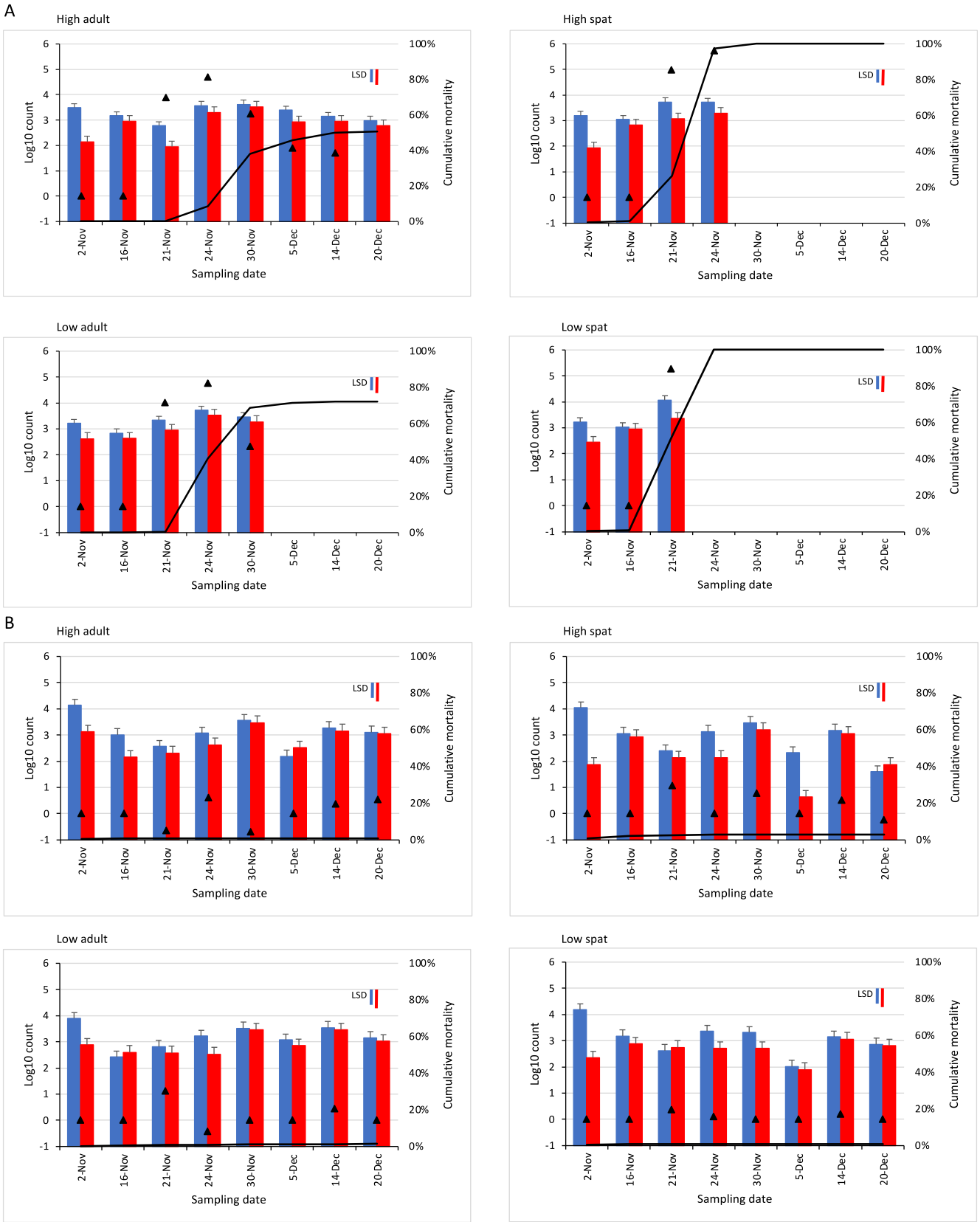

Fig. 2. Total bacteria, vibrio and OsHV-1 concentrations in oyster tissues and cumulative mortality over time in the Georges River estuary. Panel A, Site A; Panel B, Site B; Panel C, Site C. Blue bar, total bacteria; red bar vibrio; black triangle, OsHV-1; black line, mortality. Data are predicted means and standard errors of bacterial colony forming units per gram oyster tissue from REML models and mean OsHV-1 genome copies per mg oyster tissue. Mean total bacteria or vibrio counts differing over time by more than their respective least significant differences (LSD) are significantly different ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

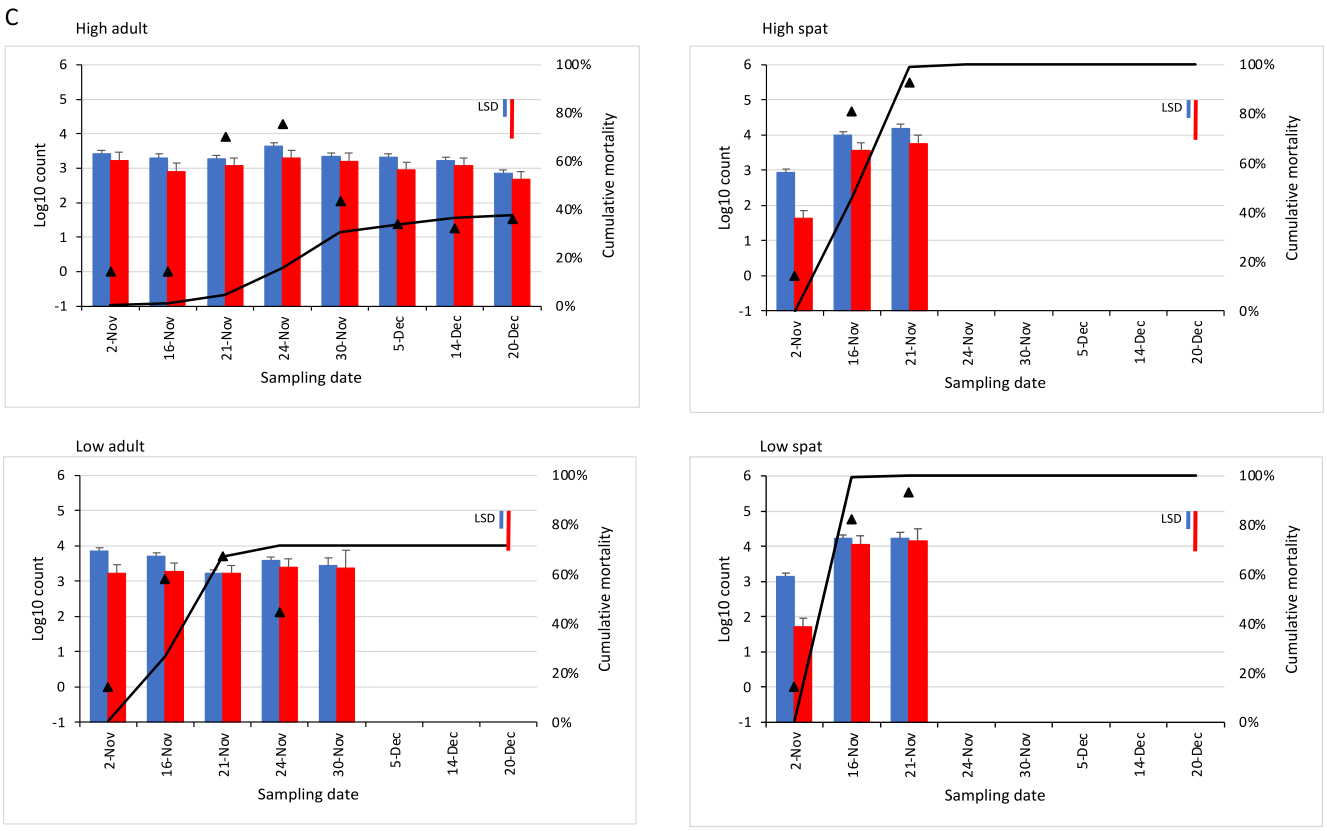

Fig. 2. (*continued*).

(Prol et al., 2009); primers are described in Table 2 and positive controls were DNA extracted from NCIMB 2236, ATCC 1507, ATCC 25920, ATCC 35084, NCTC 10884 and ATCC 33789, respectively. Isolates almost matching descriptions of named taxa were described as taxon-like. *Vibrio splendidus* is a complex of subtypes (Nasfi et al., 2015); isolates identified in this study as Splendidus clade were assigned a biotype number based on differences in biochemical reactions for sucrose, DNAse and ONPG (*o*-nitrophenyl-*β*-D-galactopyranoside) (Table 3).

2.6. *Vibrio aestuarianus* PCR

Strains of *V. aestuarianus* ssp. *aestuarianus* and *V. aestuarianus* ssp. *francensis* are pathogens of oysters (Coyle et al., 2023; Garnier et al., 2008; Mesnil et al., 2023) and the latter is slow growing compared to *V. aestuarianus* ssp. *aestuarianus* and does not grow on TCBS (Garnier et al., 2008). To overcome the possibility of non-detection by culture, the DNA extracts from oyster tissue homogenates were tested using a qPCR for *dnaJ* of *Vibrio aestuarianus* (Saulnier et al., 2009). Amplification was carried out on a Rotor-Gene Q 5 plex (Qiagen) real-time thermocycler with the Rotogene Multiplex Master Mix Kit using 5 μL of template DNA, the primers and probes in Table 2 at concentrations (0.5 and 0.2 μM, respectively) recommended in the kit handbook. An internal control utilising the 18S rRNA gene was included (0.3 μM primers, 0.2 μM probe). DNA from the Type strain NCIMB 2236 was used as a positive control. DNA extracted from a culture was diluted ten-fold with five dilution points tested. The cycling conditions were: 10 min at 95 °C (1 cycle), 15 s at 95 °C, 90 s at 60 °C (40 cycles). Amplification was achieved at 108 ng/μL through to 0.08 ng/μL but a limit of detection was not assessed. Results from the internal control indicated no PCR inhibition.

2.7. *OsHV-1 qPCR*

OsHV-1 DNA was detected and quantified by real-time qPCR using the protocol of Martenot et al. (2010) as previously described (Paul-Pont et al., 2013b).

2.8. *Identification of the Splendidus clade isolate and Vibrio splendidus ATCC 33789*

An investigation was conducted to determine the identification of the Splendidus clade isolate and *V. splendidus* ATCC 33789 that was used as a positive control for the biochemical and PCR methods. ATCC 33789 genome accession number NZ_AFWG00000000 was used to determine similarity to members of the Splendidus clade (detailed in Supplementary Table 1). Isolate AS-12-1149#1 was selected as a representative of the 120 isolates for Illumina sequencing at the Australian Genome Research Facility (AGRF), Melbourne, Australia according to their methods. Assembly was done by mapping to ATCC 33789 using Geneious Prime (Dotmatics, New Zealand). The contig of AS-12-1149#1 and ATCC 33789 accession number NZ_AFWG00000000 were analysed for average nucleotide identity (ANIb and ANIm) using JSpeciesWS (Richter et al., 2016). Then, AS-12-1149#1 and ATC 33789 were sequenced at DPIRD using Oxford Nanopore Technologies (ONT). Whole genome sequencing libraries were prepared with the ONT Ligation Sequencing Kit (SQK-LSK110). Sequencing was undertaken using ONT R9 flow cells (FLO-MIN106D) on the GridION instrument with onboard base calling and demultiplexing performed via the Guppy basecall server (v6.4.6). Adapter trimming was completed using Porechop_ABI v0.5.0 (Bonenfant et al., 2022) and quality filtering (min length 500; keep percent 90) undertaken with Filtlong v0.2.1 (https://github.com/rrwick/Filtlong). The Flye assembler v2.9.3 (Kolmogorov et al., 2019) was used for De Novo assembly with long read polishing in Medaka v1.11.3 (https: github.com/nanoporetech/medaka) to produce the final draft assembly.

OrthoANI (Yoon et al., 2017) was calculated with the online EZBioCloud tool (https://www.ezbiocloud.net/tools/orthoaniu). Isolates identified as *Vibrio splendidus* by PCR and biochemical testing are subsequently referred to as the Splendidus clade isolate following the results of genome sequencing.

2.9. Data analysis

The involvement of *Vibrio* sp. in the mortalities was explored by assessing bacterial counts and identities before and during outbreaks in the Georges River estuary in 2011–2012 and comparing these with the control site in the Hawkesbury River before OsHV-1 was first detected there. Results were then compared with subsequent POMS and other mortality events in the Hawkesbury River in 2013–2014.

The bacterial count data were log transformed for statistical analyses. For the 2011 Georges River data, separate general linear models (GLM) were built for each site because one site was not affected by POMS; total bacterial and vibrio counts were separate outcomes while treatment group (age and height), date and their interactions were explanatory terms. For the Hawkesbury River data, GLM were fitted on site, date and their interaction. Residual plots were assessed for normality and equal variances. Treatment group predicted means between times were compared using REML least significant differences. Effects were tabulated and their upper and lower 95% confidence limits calculated using average standard errors. The reported *p*-values are two sided and model outputs are provided in Supplementary Tables 2 and 3. The association between the number of Splendidus clade isolates in samples from oysters with and without mortality/detection of OsHV-1 was assessed using Fisher's exact test (Prism, Graphpad Software Inc). The association of Splendidus clade biotype A with mortality was assessed using a two-sample binomial test of proportions (Genstat, VSN International). Significance was assessed at $p < 0.05$.

3. Results

3.1. Georges River – Longitudinal study - POMS outbreak 2011–2012

High mortality (between 35% and 100%) occurred at two of the three sites (A and C) during the longitudinal study in 2011. At site A, total bacterial and *Vibrio* sp. counts fluctuated over time but both increased significantly and exceeded 10^3 CFU/g when there were POMS mortalities in both age groups at both heights (21st - 24th November) (Fig. 2, panel A and Supplementary Table 2). Mean OsHV-1 loads increased and were $> 10^4$ genome copies/mg during the week prior to the onset of mortalities in adults and $> 10^5$ genome copies/mg coincident with the mortalities in spat. All the pooled samples ($n = 5$ oysters per pool) contained detectable OsHV-1 DNA. The proportion of oysters with OsHV-1 at this site was reported previously from tests on both pooled and individual oysters and was estimated to be 56–80% in the high adult group (Paul-Pont et al., 2013b). After the mortality event, lower bacterial counts, OsHV-1 load and OsHV-1 prevalence were observed in treatments in which there were still sufficient oysters available for sampling (high adults). At site C, there was a different pattern because the counts of vibrios did not change significantly throughout the period in the adults despite there being very high cumulative mortalities especially in the low adult group (>70%) (Fig. 2, panel C and Supplementary Table 2). In contrast, in the young oysters, there were greater total bacteria and vibrio counts between 16-Nov and 21-Nov along with mortality of 46–100%. OsHV-1 DNA concentrations and prevalence were similar to Site A.

A third pattern occurred at Site B, where mortality remained very low (<4%), viral loads were $<10^1$ genome copies/mg (below the limit of quantification) (Fig. 2, panel B and Supplementary Table 2) and a maximum of 56% of oysters had detectable OsHV-1 (on 14th December) (Paul-Pont et al., 2013b). Despite this, total bacterial and vibrio loads fluctuated over time and significant increases were observed in both age groups and at both growing heights between 30-Nov to 14-Dec.

The total bacterial and vibrio counts at each site were significantly associated with the age of oysters, the time of sample collection, and at Site C also with the growing height, and there were interactions between these factors at each site because of time differences in peak counts in the different treatments (Supplementary Table 2).

Of the 120 bacterial isolates identified by phenotypic and genotypic tests, Splendidus clade isolates were dominant (55 of 120 isolates, 46%) (Table 4). There were unidentified *Vibrio* sp. and *V. alginolyticus* bacteria among the most frequently identified bacteria. *V. aestuarianus* bacteria were not detected by PCR from any of the oyster tissue homogenates.

There did not appear to be an association between any particular *Vibrio* species and mortality (Table 4). Splendidus clade isolates and *V. alginolyticus* were the dominant organisms regardless of mortality. As demonstrated in Table 4, the Splendidus clade isolate was identified in 19/40 (47.5%) of the pooled samples from live oysters collected outside of a mortality event compared to 32/80 (40%) of the pooled samples of live oysters collected from a mortality event, and 33/89 (37.1%) of

Table 4
Species and number of isolates of *Vibrio* species identified in pooled samples of *C. gigas* collected from the Georges River in 2011–2012, in decreasing order of occurrence. Refer to Table 1 for the sites and the dates of sampling. Splendidus clade isolate biotypes are shown in Table 5.

Species	No mortality		Mortality		Total
	OsHV-1 not detected	OsHV-1 detected	OsHV-1 not detected	OsHV-1 detected	
Splendidus clade isolate	14	7	6	28	55
V. sp	6	3		18	27
V. alginolyticus	2	5		17	24
V. harveyi	1			5	6
V. parahaemolyticus				2	2
Photobacterium damselae subsp. *damselae*		1		1	2
V. fischeri-like			1		1
V. harveyi-like				1	1
V. parahaemolyticus-like				1	1
V. pelagius II-like	1				1
Total	24	16	7	73	120

Table 5
Splendidus clade isolate biotypes in tissue homogenates ($n = 79$) of *C. gigas* collected from the Georges River in 2011–2012 and the Hawkesbury River in 2011–2014 based on their association with mortality and OsHV-1 detection. Refer to Table 1 for sites and the dates of sampling.

Biotype	No mortality		Mortality		Overall
	OsHV-1 not detected	OsHV-1 detected	OsHV-1 not detected	OsHV-1 detected	
Georges River					
A	3		2	9	14
H	4	2		4	10
E			1	3	4
I	1			3	4
L	2	1		1	4
C	1	1		1	3
D		1		2	3
G		1		1	2
K				1	1
Not typed	2		2	2	6
Total	13	6	5	27	51
Hawkesbury River					
H	3			12	15
C	2			3	5
D				3	3
F				2	2
I				2	2
E				1	1
Total	5			23	28

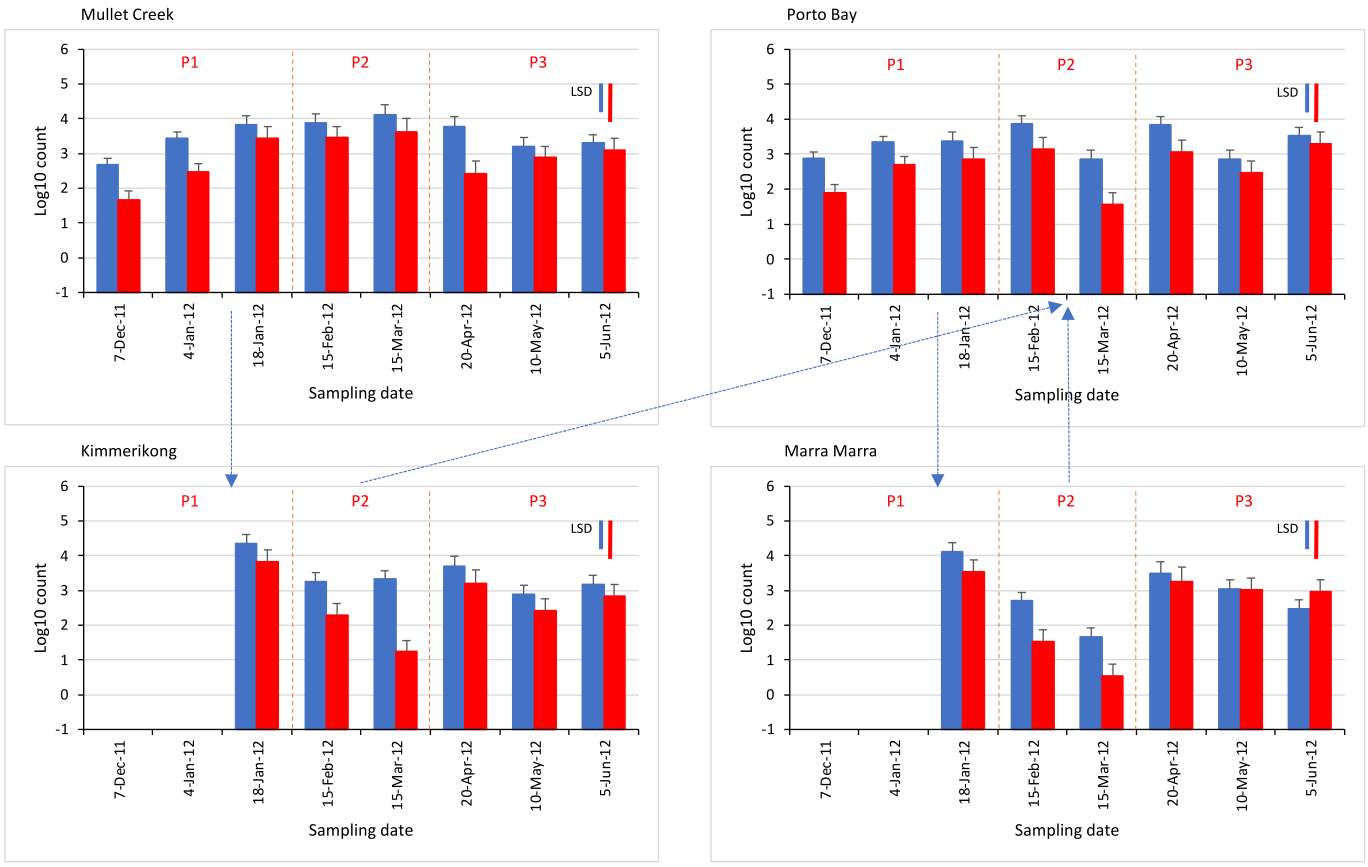

Fig. 3. Total bacteria and vibrio concentrations in oyster tissues at four sites in the Hawkesbury River estuary. Some oysters were transferred from Mullet Creek and Porto Bay to Marra Marra Creek and Kimerikong Bay, and then back to Porto Bay (arrows with dashed lines). The red dotted lines define periods P1, P2 and P3 (see text). Blue bar, total bacteria; red bar vibrio. Data are predicted means and standard error of bacterial colony forming units per gram oyster tissue from the REML model. Mean total bacteria or vibrio counts differing over time by more than their respective least significant differences (LSD) are significantly different ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
Species identity and number of isolates of *Vibrio* species identified in pooled samples of *C. gigas* collected from the Hawkesbury River between 2011 and 2014, in decreasing order of occurrence. Refer to Table 1 for the sites and the dates of sampling. Splendidus clade isolate biotypes are shown in Table 5.

Species	No mortality	Mortality		Total
	OsHV-1 not detected	OsHV-1 not detected	OsHV-1 detected	
V. alginolyticus	19	11		30
Splendidus clade isolate	5		23	28
V. sp	18	2		20
V. parahaemolyticus	17			17
V. harveyi	1	9		10
V. fortis-like	4			4
Photobacterium damselae subsp. *damselae*	4			4
Aeromonas hydrophila	3			3
V. fluvialis	3			3
V. mediterranei		3		3
V. fluvialis-like	2			2
V. pacinii-like	2			2
V. (*Listonella*) *anguillarum*	2			2
Aeromonas molluscorum	1			1
Aeromonas sp.	1			1
V. toranzoniae		1		1
Shewanella or *Arthrobacter* sp.	1			1
Unidentified			1	1
No growth	1			1
Total	84	26	24	134

pooled samples of live oysters with OsHV-1 compared to 18/31 (58.1%) in which it was not detected; these differences were not significant (Fisher's exact test, $P > 0.05$). Similarly, there was no significant difference in the proportion of pooled oyster samples with *V. alginolyticus* before (7/40, 17.5%) or during mortality (17/80, 21.3%). However, the proportion of pooled oyster samples with *V. alginolyticus* was significantly greater when OsHV-1 was present (22/89, 24.7%) compared to absent (2/31, 6.5%) (Fishers's exact test, $p = 0.036$) (Table 4).

As *Vibrio splendidus*, previously associated with epidemics in various aquatic animals, is a complex of subtypes (Nasfi et al., 2015), there is a possibility that the *V. splendidus* population structure may change either in response to mortality or even as an antecedent to mortality if there is a causative role. Therefore, biotypes of the Splendidus clade strains were identified (Table 5). Biotypes A (14 isolates) and H (10 isolates) were the most commonly identified biotypes. Although 34% (11/32) of the Splendidus clade isolates from pooled oyster samples in a mortality event were biotype A compared to 16% (3/19) from oysters not involved in a mortality event (Table 5), this difference was not significant ($p = 0.15$). Nevertheless, the majority of biotype A isolates were obtained from oysters in a mortality event where OsHV-1 was detected (9/14 isolates) (Table 5).

3.2. Hawkesbury River – Longitudinal study - control oysters 2011–2012

Samples were collected over time from oysters at two downstream sites, Mullet Creek, Porto Bay Initially there were similar total bacterial and vibrio counts in oysters at both site (P1, Fig. 3). These batches were divided on 13-Jan and some oysters were moved to upstream sites, Kimmerikong and Marra Marra. On 18-Jan total bacterial and vibrio counts were similar at all four sites, however over the following month they fell significantly at the two upstream sites ($p < 0.05$) (P2, Fig. 3, Supplementary Table 3). On 3-Mar some oysters from Kimmerikong and Marra Marra were moved back to Porto Bay due to heavy rainfall and low salinity levels in the upstream river. Interestingly, on the 15-Mar, the total bacterial and vibrio loads in all batches then kept at Porto Bay were similar, while the batch at Mullet Creek had significantly higher total bacterial and vibrio counts. From 20-Apr there were similar counts in oysters at all sites (P3, Fig. 3 and Supplementary Table 3).

Of the 84 bacterial isolates submitted for identification in 2011–2012, i.e. before any signs of mortality in the oysters being monitored in the Hawkesbury River, the dominant organisms representing around 64% of the isolates were *V. alginolyticus* (19 isolates), unidentifiable *Vibrio* sp. (18 isolates) and *V. parahaemolyticus* (17 isolates) (Table 6). Only five Splendidus clade isolates isolates were identified then, all from pooled oyster samples collected in Porto on the 7-Dec 2011 and the biotypes were C (2/5) and H (3/5) (Table 6). None of the oyster tissue homogenates contained detectable *V. aestuarianus* DNA.

3.3. Hawkesbury River – Opportunistic study - POMS outbreak 2013–2014

In November 2013 there was 90% mortality in juvenile *C. gigas* at Coba, and OsHV-1 viral loads exceeded 10^5 per mg tissue. The Splendidus clade isolate was the dominant bacterial species (23/24) and the biotypes were H (12/23), D (3/23), C (3/23), F(2/23), I (2/23) and E (1/23) (Table 6). Only one other species was found, an unidentified organism (Table 6). None of the oyster tissue homogenates contained detectable *V. aestuarianus* DNA.

3.4. Hawkesbury River – opportunistic study - unexplained mortality 2014

In January 2014 mortality occurred in adult *C. gigas* at Coba and Marra (Fig. 1). Approximately 90% of the oysters died but OsHV-1 was not detected. *V. alginolyticus* (11/26 isolates) and *V. harveyi* (9/26 isolates) were the dominant species cultured from samples collected during the mortality event. *V. splendidus* was not isolated (Table 6) and none of the oyster tissue homogenates contained detectable *V. aestuarianus* DNA.

3.5. Presumptive identification of Vibrio species belonging to the Splendidus clade and investigation of identify of ATCC 33789

Isolate AS-12-1149#1 was not identified as *V. splendidus* according to the ANIb value (94.21% to *V. celticus*) while the ANIm value for *V. celticus* was just greater than the 95% cut off at 95.13%. Results for other known members of the Splendidus clade and a selected number of other phenotypically similar *Vibrio* species were all less than the 95% cut off for species delineation being <90.14% (ANIb) and < 90.99% (ANIm). These results were supported by the findings of the in-house ONT sequencing which produced a draft genome of AS-12-1149#1 consisting of 8 contigs.

V. splendidus ATCC 33789 accession number NZ_AFWG00000000 was not identified as *V. splendidus* or *V. tasmaniensis* as the ANIb and ANIm were less than the required 95%. The highest score was to *V. chagasii* (ANIb 94.12%, ANIm 94.63%). The scores to *V. tasmaniensis* LMG 20012 T were 84.08% and 86.70%, respectively, and to *V. splendidus* LMG 19031 T 84.07% and 86.65%, respectively.

OrthoANIu results from ONT sequencing (99.95%) indicated that the isolate of ATCC 33789 in the DPIRD culture collection is the same as accession number NZ_AFWG00000000.

4. Discussion

The objective of this study was to evaluate *Vibrio* species in oysters before the onset of mortality initiated by OsHV-1, during the mortality event and afterwards, to better understand the role of bacteria in POMS. Overall, three distinct patterns in the *Vibrio* fraction in oyster tissues were observed during POMS in the Georges River:

i) cultivable bacterial counts and OsHV-1 DNA concentrations increased in both spat and adults as mortalities began but decreased as mortalities stopped
ii) bacterial counts increased in spat but not in adults associated with mortalities due to OsHV-1
iii) bacterial counts increased in both spat and adults without mortality or high concentrations of OsHV-1. This pattern was also observed in healthy control oysters in the Hawkesbury River, where OsHV-1 was not present and where counts were strongly influenced by site.

While increased bacterial populations dominated by vibrios tended to accompany OsHV-1 induced mortality, this was not a requirement for such mortality. In fact the number and diversity of cultivable bacteria and vibrios within oyster tissues varied over time associated with the geographic location (estuary, site within estuary), age of oysters (adult, spat), growing height (high, low), presence or absence of mortality and occurrence of OsHV-1. Furthermore, none of the bacterial species identified were consistently present during POMS although the Splendidus clade isolate appeared along with OsHV-1 and may have become more common in oysters after the emergence of POMS in the Hawkesbury River estuary.

In both estuaries, the absence of OsHV-1 explained the absence of mortality at particular sites and times, despite elevations of vibrio counts. As there was generally no increase in vibrio load before the onset of mortality (the exception being young oysters at high height at one site in the Georges River), opportunistic bacterial colonisation of moribund oysters seems more likely than bacterial initiation of this disease syndrome. Other risk factors including water temperature and the oysters' age and size are required to precipitate the primary viral infection and any secondary bacterial infections (de Kantzow et al., 2016; Hick et al.,

2018; Pathirana et al., 2022; Whittington et al., 2019). The strain of OsHV-1 may also be important, some being less pathogenic than others (Cain et al., 2021). Those present in Australia are not OsHV-1 µVar or a microvariant related to this strain (Trancart et al., 2022). Despite the inconsistent bacterial proliferation in POMS observed in the present study, a plausible mechanism for bacterial involvement in OsHV-1 mortalities has been demonstrated through experimental infection: viral replication occurred in haemocytes which were destroyed, leading to immunosuppression and dissemination of opportunistic bacteria within haemolymph (de Lorgeril et al., 2018; Petton et al., 2021), a feature first described by Friedman et al. (2005).

Both OsHV-1 (Whittington et al., 2021) and vibrios (Le Roux et al., 2016) can be ingested with particles from the environment during filter feeding, a process that may facilitate a co-infection process, and the presence or absence of relevant particles may explain some of the variation in disease occurrence. While oysters are the natural host for a wide range of vibrios (Le Roux et al., 2016), during disease events these can be replaced by pathogenic varieties (Lemire et al., 2015). For example, vibrios in the Splendidus clade were present in healthy oysters at low water temperatures (<16 °C), but were replaced by pathogenic *V. crassotreae* at higher water temperatures when mortalities occurred (Bruto et al., 2017). The appearance of the Splendidus clade isolate with OsHV-1 in this study may be another example.

Snieszko (1974) noted the important role of both environmental conditions and infectious agents in the genesis of diseases in aquatic animals. Consistent with this multifactorial process, summer mortalities of *C. gigas* commonly reported in Europe typically involve both a seasonal increase in water temperature and pathogens including OsHV-1 and/or *Vibrio* sp. (Garnier et al., 2007; Saulnier et al., 2009). However, various studies highlight viral, bacterial or both groups of pathogens as being most important. Furthermore, the species of *Vibrio* reported to be associated with mortality varies from study to study, with or without OsHV-1 involvement. For example, *V. aestuarianus* has been associated with disease events in *C. gigas* in Europe (Garnier et al., 2008), including in oysters with OsHV-1 (Burioli et al., 2018; Lasa et al., 2019). However, it was not found at all in the present study, ruling out its involvement in the Georges and Hawkesbury Rivers in both POMS and oyster mortalities of unknown cause. Schikorski et al. (2011) demonstrated that OsHV-1 alone caused mortality, without *V. aestuarianus* or changes in the population of *V. splendidus* however, *V. splendidus* alone can be pathogenic if given at high enough doses (Gay et al., 2004). *V. alginolyticus* was pathogenic in *C. gigas* without OsHV-1 (Yang et al., 2021; Zhang et al., 2023), while *V. harveyi* had a combined virulence role with OsHV-1 (Oyanedel et al., 2023). The relationships between disease and populations of *V. splendidus* or *V. aestuarianus* were less certain in other studies (Dégremont, 2011; Pernet et al., 2012) or the role of *V. aestuarianus* in mortalities was conditional, being restricted mainly to adult oysters at certain locations with particular habitats (Fleury et al., 2020) or in conjunction with OsHV-1 detection (Lasa et al., 2019). Mass mortalities in 2013–2014 in *C. gigas* in Port Stephens, Australia were attributed to high water temperature and low salinity accompanied by increased tissue loads of *V. harveyi* and another vibrio, OsHV-1 being absent (Go et al., 2017; King et al., 2019). That the administration of antibiotics to oysters prevented the replication of *V. harveyi* and suppressed mortality provided experimental evidence for the role of these bacteria in the Port Stephens mortality syndrome (Green et al., 2019). While *V. harveyi* had a role in pathogenesis of mortality with OsHV-1 in Europe (Oyanedel et al., 2023), this was not the case in Australia.

Beyond the roles proposed for these cultivable bacteria, other *Vibrio* sp. and *Arcobacter* sp. have been revealed in diseased oysters using molecular tools (Lasa et al., 2019; Pathirana et al., 2022). Another molecular study revealed most pathogenic vibrios from recurrent oyster mortality episodes in Europe were from the Splendidus super-clade (*V. splendidus*, *V. tasmaniensis*, *V. cyclitrophicus*, *V. lentus* and *V. pomeroyi*) (Lasa et al., 2019). In a separate study of POMS in France, members of the Splendidus clade, followed by *V. harveyi* and *V. aestuarianus* were involved (Petton et al., 2021). Interestingly, the Splendidus clade isolate was uncommon in the Hawkesbury River estuary until it was affected by OsHV-1 and was not detected during mortalities unrelated to OsHV-1. Instead, the bacterial populations in these samples were dominated by *V. alginolyticus* and *V. harveyi*. Overall, the findings suggest that the Splendidus clade isolate may increase in prevalence after oysters in an estuary become infected with OsHV-1. Studies of other sites before and after emergence of OsHV-1 will be necessary to confirm this.

The above observations suggest two models of *Vibrio* pathogenesis in seasonal mortalities of *C. gigas:*

i) The first requires immune suppression due to OsHV-1 or a poor environment to permit commensal or opportunist environmental vibrios to overgrow and cause mortality
ii) In the second model, particular *Vibrio* species may be primary pathogens, but they are defined by virulence factors rather than species designation, and they can initiate disease without OsHV-1. However, an increase in bacterial load per se is insufficient, as was apparent in 2011 at site B in Georges River estuary. Apart from putative virulence factors, the pathogenicity of *Vibrio* populations also needs to be supported by environmental and host factors.

In the discussion above it is evident that there is wide disagreement between different oyster mortality studies in attributing a causal role to particular *Vibrio* species. This may merely be due the different environments in which these studies have been conducted. The contrasting *Vibrio* species composition in oysters sampled at approximately the same time from the Georges River and Hawkesbury River estuaries in 2011–2012 is an example of the effect of the immediate estuarine environment. In oyster mortality events in Australia that did not involve OsHV-1, Worden et al. (2022) found inconsistencies between the bacterial taxa identified in separate geographical locations. In oyster transfer experiments, there were strong effects of local environment on diversity and abundance of bacteria in haemolymph (Wendling et al., 2014) and the microbiome in oysters sampled from different Australian estuaries was different, even though all oysters originated from one hatchery (Pathirana et al., 2019). The data presented here on changing *Vibrio* counts with time and location in the Hawkesbury River shows the importance of these factors as drivers of *Vibrio* populations in the absence of OsHV-1.

There are several limitations in this study. There are non-cultivable members of the oyster microbiome such as *Arcobacter* that have been detected in abundance in the haemolymph of moribund oysters (Lasa et al., 2019; Lokmer and Wegner, 2015). In experimental infections, this organism increased in abundance in OsHV-1 infected oysters but not in control oysters (Pathirana, 2020). *Arcobacter* would have gone undetected with the conventional bacteriological methods and the targeted PCR assays employed in the current study. Furthermore, as traditional bacteriological methods were used, the frequencies of the species identified may not be representative. This is because colonies were selected based on their appearance and species and colony morphologies are not mutually exclusive. Furthermore, infrequent members of the population may have been missed. As this study involved the culture of samples pooled from different oysters, it may not accurately represent pathogenesis as may be revealed through the study of individual oysters. However, pools of 10 oysters were used to demonstrate immune suppression caused by OsHV-1 and the role of bacteria in the mortality of *C. gigas* during POMS (de Lorgeril et al., 2018), results which have not been challenged.

A report by Machado et al. (2017) stated that *Vibrio splendidus* ATCC 33789 was more closely related to *V. tasmaniensis* based on sequence similarity with the ferric-uptake regulator gene, *fur*. Further sequencing is required to resolve the genome and determine formal identification for both ATCC 33789 and AS-12-1149#1.

5. Conclusion

While the findings generally support the view that bacterial proliferation follows infection with OsHV-1 in the mass mortality syndrome known as POMS, this was not a consistent feature. None of the bacteria identified in this study were specifically associated with POMS. However, the Splendidus clade isolate may have increased in prevalence after POMS occurred for the first time in the Hawkesbury River estuary. During this observational study the number and diversity of bacteria within oyster tissues varied over time, between estuaries, between sites within an estuary and were associated with factors including flooding, the age of oysters, mortality and OsHV-1.

CRediT authorship contribution statement

Richard J. Whittington: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nicky Buller:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Erandi Pathirana:** Investigation, Writing – review & editing. **Navneet K. Dhand:** Formal analysis, Writing – review & editing. **Sam Hair:** Investigation, Methodology, Writing – review & editing. **Paul M. Hick:** Investigation, Writing – review & editing. **Ika Paul-Pont:** Conceptualization, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741090.

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