Future Oysters CRC-P: Advanced aquatic disease surveillance for known and undefined oyster diseases

Marty R Deveney & Kathryn H Wiltshire (Editors)

March 2020

FRDC Project No 2016-806

ISBN: 978-1-876007-24-9

Future Oysters CRC-P: Advanced aquatic disease surveillance for known and undefined oyster diseases. Project 2016-806 2020.

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Researcher Contact Details

Advanced aquatic disease surveillance for known and undefined oyster diseases

Name:	A/Prof Marty Deveney
Address:	SARDI Aquatic Sciences
	South Australian Research and Development Institute
	2 Hamra Avenue, West Beach, SA 5024
Phone:	08 8429 0742
Email:	marty.deveney@sa.gov.au

FRDC Contact Details

FRDC Contact Details

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

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

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Acknowledgments

This project was conducted as part of the Australian Governments Cooperative Research Centres Program. This Cooperative Research Centre Project (CRC-P 2016-553805; Future Oysters), was led by Australian Seafood Industry Pty Ltd in partnership with a number of Australian research organisations, including CSIRO, the Department of Primary Industries and Regions (South Australia), the South Australian Research and Development Institute (SARDI), the Department of Primary Industries, Parks, Water and Environment (Tasmania), the Fisheries Research and Development Corporation, Flinders University, Macquarie University, New South Wales Department of Primary Industries, Oysters Australia, Select Oyster Company, The Yield Technology Solutions, University of Adelaide, University of Newcastle, University of Sunshine Coast, University of Tasmania, and the University of Technology Sydney.

The work in Chapter 1 received additional support from SARDI Aquatic Sciences, CSIRO Australian Animal Health Laboratory and the Department of Primary Industries, Parks, Water and Environment. We are grateful to the South Australian Oyster Growers Association (SAOGA) for their cooperation and support. Dr Brian Jones, Dr Henry Lane and Ms Cara Brosnahan (Ministry for Primary Industries New Zealand Animal Health Laboratory, Wallaceville), Prof Sarah Culloty and Dr Sharon Lynch (University College Cork, Ireland), Dr Isabelle Arzul (IFREMER, France), Dr Peter Kirkland and Dr Cheryl Jenkins (Elizabeth Macarthur Agricultural Institute, Sydney, Australia) provided advice on tests for OsHV-1. Ms Mandee Theil and Mr Ian Moody processed samples and Dr Danièle Giblot-Ducray and staff at the SARDI Molecular Diagnostic Centre extracted the samples and tested them for Pacific Oyster DNA. Dr Sarah Catalano supported us at the molecular laboratory at SARDI Aquatic Sciences. Additional material was provided by Assoc Prof Christine Crawford and Dr Sarah Ugalde at the Institute for Antarctic and Marine Studies (IMAS) Tasmania.

For Chapter 2 we thank Graeme Knowles from the Animal Health Laboratory, Department of Primary Industries, Parks, Water and Environment, Tasmania and SARDI Aquatic Sciences for sourcing and providing oyster samples infected with OsHV-1 for research to be conducted on. We also thank Associate Professor Jill Carr from the College of Medicine and Public Health, Flinders University for providing *Dengue virus* and *Influenza A virus* samples to conduct initial experiments. This project was led by Professor Jim Mitchell from Flinders University. Laboratory research was undertaken by Dr James Paterson, Dr Lisa Dann, Dr Silvia Sapula, Ms Sarah Giles, Mr Connor McIvor and Mr Jack Blaikie.

For Chapter 3 we are grateful to the South Australian Oyster Growers Association (SAOGA) for their cooperation and support and to members who responded to the survey. Gary Zippel, Lester Marshall, Judd Evans and Simon Turner provided information and support, and reminded members about the project.

For Chapter 4 we acknowledge the assistance of Dr Paul Worden who undertook microbiome analyses and performed the bioinformatics components of this study. Thanks to Dr Zoe Spiers for conducting the histopathological examination of the samples. We also thank Melinda Niedermayer and Emily Onizawa who assisted in oyster processing, sample collection and molecular testing. We also appreciate the assistance of Mike Dove and Jeffrey Go in helping to coordinate the sample submissions and for useful discussions. Finally, many thanks to the oyster growers who participated by submitting samples for this study.

Executive Summary

This project improved understanding of methods for surveillance for several diseases of farmed oysters. Surveillance is a critical component of biosecurity and aquatic animal health activities. Surveillance is used to assess health status of animal populations, and provides evidence to support claims of freedom from disease or to determine disease prevalence. Effective surveillance also increases the likelihood that a new or emergent disease can be detected early and controlled.

Mollusc diseases are less well understood than terrestrial animal and and many finfish diseases and this project sought to develop understanding of three oyster diseases of substantial economic impact in the Australian edible oyster aquaculture industries: Pacific Oyster mortality syndrome (POMS), South Australian Mortality Syndrome (SAMS), and Winter Mortality (WM).

POMS is caused by the virus OsHV-1 microvariant, and has emerged worldwide since 2008. Information on virus transmission was however lacking, and diagnostic sensitivity and specificity of tests used for OsHV-1 detection were undefined. Improvements in time-to-diagnosis were also required to support management and business continuity when responding to mortalities in OsHV-1 free areas. Development of the *National Biosecurity Guidelines for Australian Oyster Hatcheries* required rapid diagnostic capacity to provide real-time understanding of risk and surveillance for hatcheries producing OsHV-1 free stock in disease-affected zones. Mortalities of Pacific Oysters in POMS-free areas in South Australia (SA), termed SAMS, have been described since the 1980s but remain poorly understood, with no causative agent or major risk factors identified, despite development of a case definition and investigation of mortality events. WM is a disease of Sydney rock oysters, but despite disease characteristics being described nearly 100 years ago, had no case definition or identified causative agent. The lack of information about SAMS and WM created confusion about what management actions could decrease losses and disruption to business activities, ultimately limiting capacity to act positively to manage these syndromes.

The project included activities with four main aims:

- Improving understanding of tests for OsHV-1 and investigation of using these tests for area surveillance
- Development of a low-cost, rapid test for OsHV-1
- Refinement of the case definition and investigation of the cause of SAMS in Pacific Oysters
- Development of a case definition and improving understanding of the cause of Winter Mortality in Sydney Rock Oysters.

The project provided diagnostic sensitivity (DSe) and specificity (DSp) data for two qPCR tests for OsHV-1 when testing oyster tissue and showed that these tests could also be applied to environmental samples, providing a non-destructive approach for OsHV-1 area surveillance. This information enables improved design and interpretation of surveys for OsHV-1, including improved sampling and testing criteria for hatchery and zone or compartment certification, understanding disease status in OsHV-1 affected areas and monitoring transmission during outbreaks.

A flow cytometry test to detect and quantify OsHV-1 was developed and optimised. The test is particularly applicable to testing seawater. Analysis of infected oysters using this test showed that OsHV-1 concentrations in infected oysters from the Port River are up to $3x10^7$ mL^{-1}, indicating that the viral load released from infected areas would be at least in the 10^{10}-10^{15} viruses per day range. The critical applications of this test are to assess water in hatcheries after intake treatment and to test outflow water prior to discharge. This testing can contribute to making self-declarations of freedom and protect surrounding environments from outbreaks where a hatchery might be the first site of infection and therefore the primary source of virus in a previously free area.

One SAMS event occurred during the study period, hampering investigations. The project identified reference ranges for biochemical values in Pacific Oysters, however, which provide a baseline for understanding ‘normal’ Pacific Oyster health. These biochemical values also provide information for cases in which mortality or disease is observed but where conventional tools cannot identify a causative

agent. SAMS and other mortalities were characterised by few consistent pathological signs, but changes in bacterial communities in oysters were detected during mortalities. SAMS is likely to have multifactorial causes, but the outcomes of this project contribute to refining knowledge about the factors that contribute to SAMS.

Examinations of WM in Sydney Rock Oysters identified a case definition based on historical information and cases investigated during this project:

> *Mortalities in 18+ month old* Saccostrea glomerata *between July and November, from Port Stephens to the southerly reaches of the range of* S. glomerata*, in water with higher than average salinity. Affected cohorts display gross lesions consisting of brown or yellow pustules on the external surfaces of the gonad, mantle, palps or gills or internally in the adductor muscle or gut epithelium. The lesions are focal and consist of accumulations of haemocytes.*

Investigations of these events indicated that microcells such as *Bonamia* spp. and *Mikrocytos* spp. that have been proposed as causative agents were not consistently associated with mortalities, and *Perkinsus* spp. were not detected in any oysters. Prevalent *Vibrio splendidus* isolates from WM cases, however, contain mobile genetic elements that are linked to capacity to cause disease, and the environmental conditions in which WM occurs induces physiological changes in these bacteria. Changes in bacterial communities, however, are inconsistent and may be symptomatic rather than causative. WM is complex and is probably multifactorial, but the case definition and pathology identified in this project facilitate better surveillance and disease investigations, including refining understanding of the contributing factors.

Keywords

Pacific Oyster, *Crassostrea gigas*, POMS, Ostreid herpesvirus, OsHV-1, eDNA, flow cytometry, mortality, Sydney Rock Oyster, *Saccostrea glomerata*; winter mortality; *Mikrocytos*, *Bonamia*; microbiome *Vibrio*; *Vibrio splendidus* diagnostic performance, aquatic disease surveillance, aquaculture.

Chapter 1 Advanced surveillance for OsHV-1 microvariant

Marty R Deveney, Kathryn H Wiltshire, Y Nhu Lieu, Jessica J Buss, Nicholas JG Moody, Peter G Mohr, Kevin A Ellard

Summary

Infection with ostreid herpesvirus type 1 microvariant (OsHV-1 microvariant), a specific genotypic group of ostreid herpesvirus type 1 (OsHV-1), causes Pacific Oyster mortality syndrome (POMS) and substantial acute mortality in juvenile and adult Pacific Oysters (*Crassostrea gigas*). OsHV-1 microvariant was first detected in Australia in 2010. By June 2014, it occurred in Australia in the Georges River–Botany Bay, Port Jackson–Sydney Harbour and Hawkesbury River–Brisbane Water estuaries in New South Wales (NSW). The disease was detected in Tasmania (TAS) in 2016 following investigation of mortality in farmed Pacific Oysters (Ugalde *et al.* 2018) and in Port Adelaide, South Australia (SA) where substantial mortality was observed in wild Pacific Oysters in February 2018 during surveillance that formed part this project. POMS has a substantial impact on the viability of businesses and regional productivity where it occurs.

Surveillance is critical for maintaining evidence of freedom and for managing and containing outbreaks of disease where they occur. OsHV-1 is primarily diagnosed using quantitative polymerase chain reaction (qPCR) assays applied to oyster tissue. These assays are sensitive and facilitate quantification of viral copies in target tissue, but such testing is destructive, and data were difficult to interpret in some cases because the diagnostic sensitivity (DSe) and specificity (DSp) of the tests were not established. Measures of DSe and DSp are required to design surveys with known confidence of detection, to estimate true disease prevalence, and to calculate predictive values and likelihood ratios which describe the robustness of the test for detection or providing evidence to support claims of freedom from disease. In some cases sampling oysters or deploying sentinels is difficult or impossible for safety or security reasons or because increasing the biomass of Pacific Oysters at a site is not permitted or not socially acceptable. Disease surveillance using eDNA has several advantages over surveillance based on testing oysters: samples can be collected cheaply and easily, the method is non-destructive, and provides information on pathogen reservoirs and transmission pathways.

SARDI has developed eDNA approaches for marine pest surveillance and this project facilitated initial testing of these methods for detection of an important marine pathogen. Plankton and oyster samples collected between January 2017 and February 2019 from areas with wild oyster populations in both South Australia (SA) and Tasmania (TAS), and from oyster farming areas in TAS where POMS has occurred were tested for OsHV-1 by two quantitative polymerase chain reaction (Jenkins and Martenot qPCRs) assays and plankton was tested by qPCR for Pacific Oyster DNA. Bayesian latent class models were used to provide estimates of DSe and DSp in both environmental samples and oyster tissue, and disease prevalence for affected areas or oyster populations. Both assays had high specificity in both oyster tissue and plankton, with no evidence of false positive detections. Sensitivity of both assays applied to oyster tissue was high (Jenkins: 0.72, Martenot: 0.85), but the Martenot assay (0.82) had greater sensitivity when applied to plankton than the Jenkins assay (0.22). eDNA methods can be used for OsHV-1 surveillance. The work in the Port River, detected OsHV-1 for the first time in SA in November 2017 from plankton eDNA. A POMS outbreak occurred in February 2018 and an emergency response was initiated, led by Biosecurity SA with assistance from PIRSA Fisheries and Aquaculture, Fisheries Operation and SARDI Aquatic Sciences. The surveillance provided by this project and the response and subsequent containment activities have maintained OsHV-1 freedom for the SA Pacific Oyster industry although the feral Pacific Oyster population in the Port River remains infected.

This project produced information that forms the basis for best practice surveillance for OsHV-1 using oyster tissue or plankton and surveillance activities can now be planned with high epidemiological certainty. In particular, the DSe and DSp data support proof-of-freedom programs, translocation health

assessment and analysis of surveillance data. These data should be used to inform surveillance for OsHV-1 and in particular they should form the basis for translocation surveillance, and management of free areas in Tasmania and in the South Australian Pacific Oyster growing areas.

1.1 Introduction

Infection with ostreid herpesvirus type 1 microvariant (OsHV-1 microvariant), a specific genotypic group of ostreid herpesvirus type 1 (OsHV-1), causes Pacific Oyster mortality syndrome (POMS), characterised by substantial acute mortality in Pacific Oysters (*Crassostrea gigas*). Pacific Oysters (and the Portuguese cupped oyster, *C. angulata*) are the only species that develop clinical disease when infected with OsHV-1 microvariant. In Australia, OsHV-1 microvariant was first detected in New South Wales (NSW) in 2010 (Jenkins *et al.* 2013; NSW DPI 2016). By June 2014, it was detected in three NSW estuaries: the Georges River–Botany Bay, Port Jackson–Sydney Harbour and Hawkesbury River–Brisbane Water estuaries. The disease was detected in Tasmania (TAS) in 2016 following investigation of mortality in farmed Pacific Oysters (Ugalde *et al.* 2018) and in the Port River, Port Adelaide, South Australia (SA) where substantial mortality was observed in wild Pacific Oysters in February 2018 during surveillance that formed part of this project.

POMS has a serious impact on the viability of businesses and regional productivity where it occurs due to high mortality, and due to control measures such as cessation of stock movements that are required to prevent the disease from spreading. The $35M+ SA Pacific Oyster industry developed based on translocation of juvenile oysters (spat) from TAS to SA. When OsHV-1 was detected in TAS in 2016 it caused substantial business losses there, with further severe impacts caused by closure of the trade in spat between TAS and SA. Surveillance for diseases such as POMS is important to provide information on disease occurrence, including distribution of endemic diseases and occurrence of notifiable outbreaks, and data for risk assessment and disease management. Surveillance is also required to provide evidence to support claims about the absence of disease. Improving the accuracy and cost-effectiveness of disease surveillance offers substantial benefits for governments, industry and stakeholders.

Detection of OsHV-1 for surveillance and disease diagnosis is principally by real time polymerase chain reaction (qPCR) assay analysis of gill and mantle tissue. Several qPCR tests are available (Martenot *et al*. 2011; Jenkins *et al*. 2013; Lynch *et al*. 2013) and confirmation of the microvariants is by conventional PCR (cPCR) (Segarra *et al.* 2010). For diagnostic purposes the Australian case definition requires a positive Martenot qPCR test, a positive C2/C6 conventional PCR test (Segarra *et al*. 2010) and sequence analysis to confirm the systematic deletion of 12–15 base pairs in ORF 4 of the genome (encompassed by the C2/C6 primers) as found in the reference strain of OsHV-1 (GenBank # AY509253) (Department of Agriculture 2015). These assays are sensitive and facilitate quantification of viral copies in target tissue, but these tests do not provide evidence about the source of the DNA or its viability. Data from assays is difficult to interpret because the diagnostic sensitivity (DSe) and specificity (DSp) of the tests has not been established. OsHV-1 can occur in its hosts at low prevalence (Paul-Pont *et al.* 2014), thus potentially requiring large sample sizes for detection.

Surveillance in many Pacific Oyster farming areas is complicated by the presence of large populations of wild oysters. These wild oysters are typically intertidal, accessible only at low tide and sampling them is logistically challenging. Properly randomising sampling of these populations is difficult. Tissue testing is destructive and for farmed oysters has an associated cost to growers. Oysters specifically deployed in target areas (sentinels) can be tested, but in some cases deploying sentinels is difficult or impossible for safety or security reasons or because increasing the biomass of Pacific Oysters at a site is not permitted or not socially acceptable. Similar challenges for surveillance of other aquatic diseases have motivated the development of molecular surveillance methods to target environmental DNA (eDNA) of aquatic pathogens, including parasites, bacteria and viruses (Bass *et al.* 2015; Huver *et al.* 2015; Peters *et al.* 2018; Sana *et al.* 2018). Environmental monitoring for pathogens using eDNA methods has potential to provide low cost disease surveillance, with the additional advantage of being non-destructive and providing information about pathogen reservoirs and transmission pathways (Díaz-Ferguson and Moyer 2014; Bass *et al.* 2015; Stentiford *et al.* 2017; Peters *et al.* 2018). Environmental monitoring can also inform ballast water risk by testing ballast water or ballast water uptake or discharge areas (Gollasch *et al.* 2015).

Molecular methods targeting larval stages or eDNA are increasingly used for detection and monitoring of aquatic species, including endangered and invasive species, but these methods require validation before incorporation in management systems (Darling and Blum 2007; Díaz-Ferguson and Moyer 2014; Thomsen and Willerslev 2015; Goldberg *et al.* 2016). The South Australian Research and Development Institute (SARDI) has developed and refined plankton sampling and preservation methods, quality controls and qPCR assays for molecular marine pest surveillance (Ophel-Keller *et al.* 2007; Bott *et al.* 2010; Bott and Giblot-Ducray 2011a, b; Wiltshire and Deveney 2011; Bott and Giblot-Ducray 2012; Giblot-Ducray and Bott 2013; Deveney *et al.* 2017). These methods have undergone validation including assessment of laboratory (Ophel-Keller *et al.* 2007; Bott *et al.* 2010; Bott and Giblot-Ducray 2011a, b, 2012) and field performance (Deveney *et al.* 2017; Wiltshire *et al.* 2019a), assessment of diagnostic performance as sensitivity (DSe, likelihood of detection by an assay when target DNA is present in a sample) and specificity (DSp, likelihood that a detection is correct and not the result of a cross-reaction with non-target DNA) in plankton samples (Wiltshire *et al.* 2019b).

Seawater is a medium for horizontal transmission of OsHV-1 (Paul-Pont *et al.* 2013; Evans *et al.* 2017). The virus is typically associated with particulate matter in seawater (Evans *et al.* 2014; Evans *et al.* 2017) and OsHV-1 DNA has been detected in natural seawater samples, and on membrane filters used for filtration of infected seawater (Evans *et al.* 2017). OsHV-1 has been detected in Pacific Oyster larvae (Hwang *et al.* 2013). That OsHV-1 is found in plankton, either in or on larvae or adsorbed to particulates, suggested that the plankton sampling method used to detect marine pest eDNA could be combined with existing tests for OsHV-1 to detect this virus.

This project, therefore, aimed to assess if eDNA methods, based on the SARDI method used for molecular pest surveillance and existing OsHV-1 tests, could be used to detect OsHV-1 microvariant in plankton, and to provide validation data about diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of available OsHV-1 tests when applied to plankton samples. The diagnostic performance of the tests applied to oyster tissue was also assessed, and temporal variability of OsHV-1 occurrence in plankton and in oysters examined using samples from both SA and TAS. These data provide evidence about the suitability of eDNA for OsHV-1 surveillance and information to assist in eDNA survey design.

1.1.1. Objectives

The objectives of the project were to:

1. Assess eDNA detection of OsHV-1 in plankton samples as a tool for POMS surveillance and early detection

2. Understand diagnostic sensitivity (DSe) and specificity (DSp) of assays when used on eDNA for POMS detection

3. Understand diagnostic sensitivity (DSe) and specificity (DSp) of assays when used on Pacific Oyster tissue for POMS detection

4. Obtain preliminary data on temporal and spatial patterns in OsHV-1 occurrence in plankton and the relationship between OsHV-1 and oyster DNA occurrence in plankton samples

1.2. Methods

1.2.1. Sampling dates and locations

Plankton samples for OsHV-1 testing were collected from locations in SA and TAS between January 2017 and February 2019. In TAS 19 sets of samples were collected. Between January 2017 and August 2018, TAS plankton samples were collected from two oyster growing areas: Blackman Bay and Pipeclay Lagoon; and from the Derwent River, at Sullivan Cove and Prince of Wales Bay where wild oyster populations occur (Figure 1.1). In most sample sets, Blackman Bay samples included 1 – 2 samples collected from Dunalley at the western end of Blackman Bay. In the Derwent River, samples were collected from Prince of Wales Bay and Sullivan Cove. Between October 2018 and February 2019, TAS plankton samples were collected from growing areas: Pipeclay Lagoon and Upper Pittwater. In SA, 15 sets of samples were collected from the Port River estuary, Port Adelaide (Figure 1.2), where a population of wild Pacific Oysters has established. Oysters are not farmed in the Adelaide region, and the nearest farms are at Stansbury on the Yorke Peninsula, approximately 65 km across Gulf St Vincent from Adelaide. Between February 2017 and February 2018, samples were taken from four areas of the Port River adjacent to locations where Pacific Oyster occur: Upper Port, Inner Harbour, North Arm and Lipson Reach. Following the POMS mortality event in February 2018, plankton samples were also collected from Outer Harbor between March and July 2018, and in February 2019. The Port River was sampled from the original four locations between July 2018 and January 2019. Sampling dates and number of samples from each location are shown in Table 1.1.

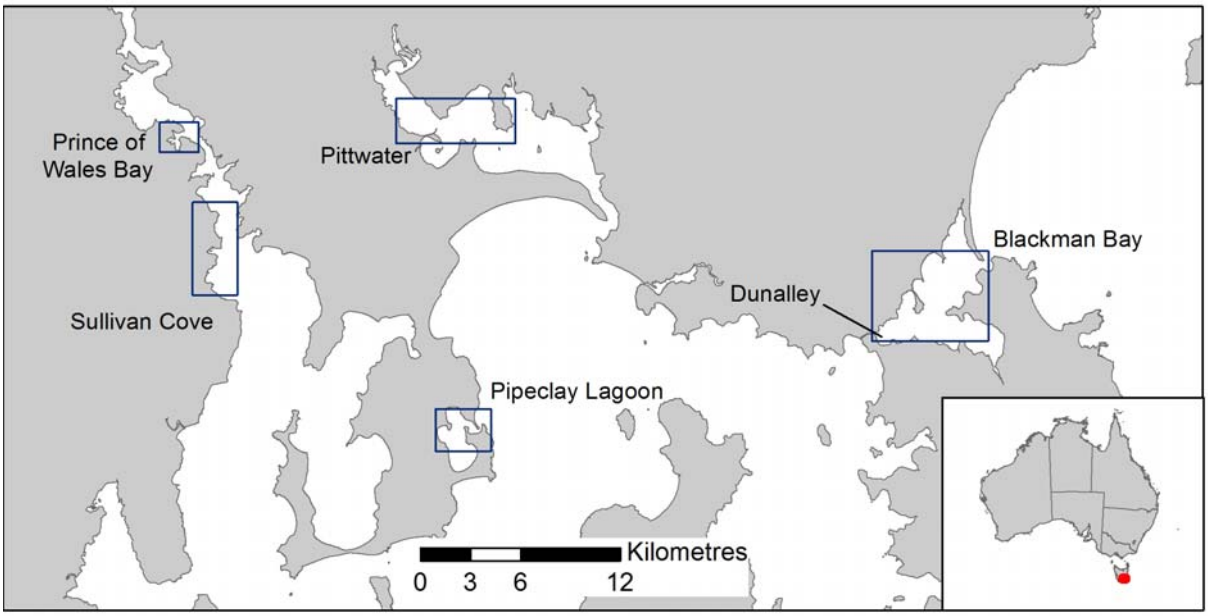


Figure 1.1. Sampling locations in Tasmania

Oysters were also collected from both SA and TAS for tissue sampling. In SA, 11 sets of wild oysters were collected adjacent to plankton sampling locations between February 2018 and January 2019 from Inner Harbour, North Arm and Lipson Reach. In TAS, five sets of oysters were collected from Pipeclay Lagoon between November 2018 and April 2019. Oysters were collected from farms and from wild oyster populations in the lagoon. Sampling dates and numbers of oysters sampled are shown in Table 1.2.

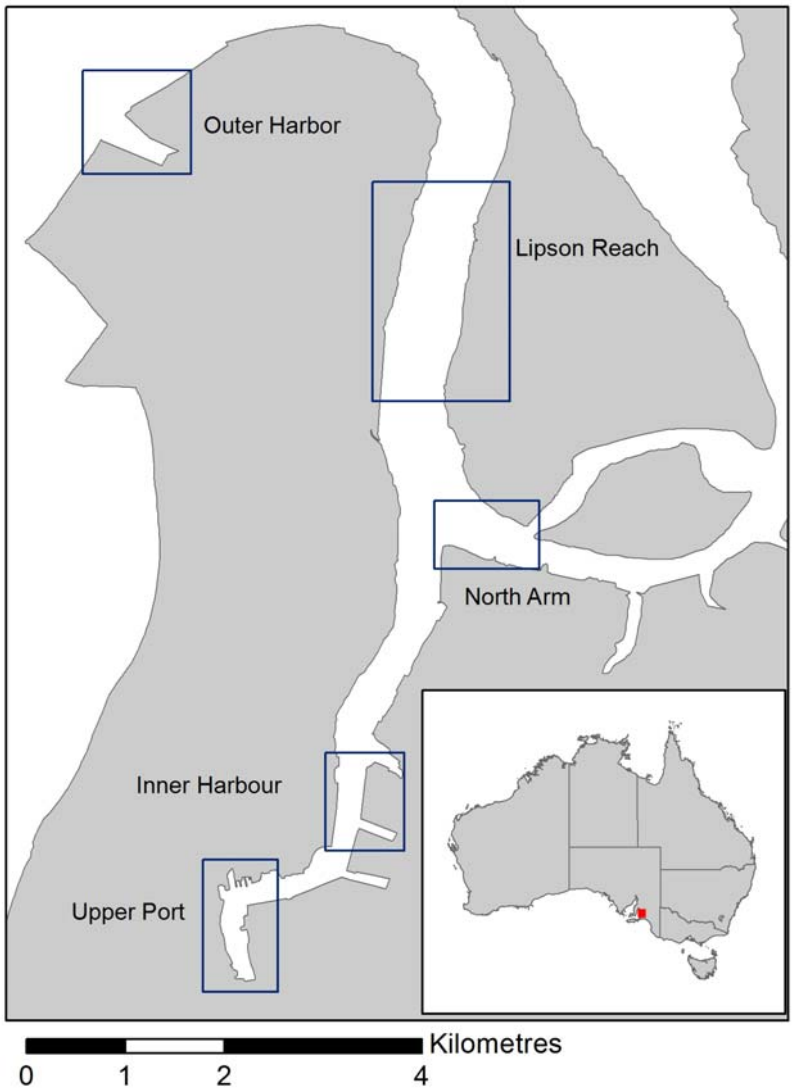


Figure 1.2. Sampling locations in South Australia

1.2.2. Sample collection and molecular analysis

Plankton samples were collected based on the methods developed by Giblot-Ducray and Bott (2013) and refined by Deveney *et al.* (2017). A conical mesh plankton net with mouth diameter 0.5 m, length 1.5 m and 50 µm mesh (Sea-Gear 150-50-50) fitted with a flowmeter (Sea-Gear MF315) was towed behind a vessel at a speed of ~1–1.5 m s^{-1} and depth of 0.5–1 m for a target distance of 100 m. After collection, plankton samples were concentrated down to a volume of ~40 mL by filtering through the mesh windows of the plankton net cod end and transferred to 120 mL tubes containing 80 mL sulfate based preservation buffer similar to Stanford University (2015). Plankton samples were kept cool in an insulated container immediately after collection and stored at ≤ 4 °C until returned to the South Australian Aquatic Sciences Centre (SAASC) for processing.

Plankton samples were filtered in the laboratory at SAASC using a manifold and sterile single-use filter cups with 0.45 µm filters (Thermo Scientific™ Nalgene™). Filters were transferred to 50 mL centrifuge tubes, frozen at -20 °C and freeze-dried until completely dehydrated prior to DNA extraction. DNA extraction and qPCR analysis were carried out by the SARDI Molecular Diagnostics Root Disease Testing Laboratory (RDTS). DNA was extracted from samples using 20 mL of DNA extraction buffer added to each sample before physical disruption (Ophel-Keller *et al.* 2008). The

efficiency and consistency of SARDI's method to extract environmental DNA is confirmed in comparison to commercial methods (Haling *et al.* 2011). Final DNA elution was in 160 µL elution buffer.

Table 1.1. Sampling dates, locations and number of plankton samples for oyster DNA and OsHV-1 testing collected in South Australia and Tasmania. See Figures 1.1-1.2 for locations

Collection date(s) / Location	Upper Port	Inner Harbour	North Arm	Lipson Reach	Outer Harbor	Total (Wild)	Total (Farm)
SA							
28/02/2017	5	5	5	5		20	
8/05/2017	5	5	5	5		20	
14/11/2017	5	5	5	5		20	
21/02/2018	5	5	5	5		20	
26/03/2018	3	3	3	3	3	15	
24/04/2018	3	3	3	3	3	15	
30/05/2018	3	3	3	3	3	15	
1/08/2018	3	3	3	3	3	15	
12/09/2018	3	3	3	3		12	
23/10/2018	3	3	3	3		12	
26/11/2018	3	3	3	3		12	
20/12/2018	3	3	3	3		12	
22/01/2019	3	3	3	3		12	
8-25/02/2019	3	8	8	3	3	25	
TAS	**Sullivan Cove**	**Prince of Wales Bay**	**Blackman Bay (Dunalley)**	**Pipeclay Lagoon**	**Pittwater**		
23-25/01/2017	7	3	5	5		10	10
23-26/02/2017	7	3	3	3		10	6
01-05/4/17	7	3	4 (1)	3		10	7
05-10/09/2017	6	3	3	3		9	6
17-26/10/2017	6	3	6	4		9	10
22-28/11/2017	6	3	4 (1)	3		9	7
11-28/12/2017	6	3	4 (1)	3		9	7
17-28/01/2018	6	3	5 (2)	3		9	8
22-27/02/2018	6	3	4 (1)	3		9	7
27-28/03/2018	6	3	4 (1)	3		9	7
27-30/04/2018	6	3	4 (1)	3		9	7
28-29/05/2018	6	3	4 (1)			9	4
25-27/06/2018	6	3	4 (1)	3		9	7
26-31/07/2018	6	3	4 (1)	3		9	7
24-30/08/2018	6	3	4 (1)	3		9	7
2-11/10/2018				4	4		8
8-22/11/2018				8	8		16
19-28/12/2018				5	5		10
13-17/02/2019				8	4		12

DNA extracts were tested in singleplex quantitative polymerase chain reaction (qPCR) performed on a QuantStudio7 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using a SARDI developed assay for Pacific Oyster (Bott and Giblot-Ducray 2012) and an exogenous organism that was added to samples to assess extraction efficiency and inhibition. For each PCR analysis batch, reference samples that do not cause inhibition had the inhibition control added, were extracted and tested by qPCR. A scaling factor was calculated for each plankton sample by comparing the yield of inhibition control DNA detected in that sample to the yield from the reference sample. A scale factor for a sample is calculated as the ratio of the control organism DNA yield in the reference samples to the yield in that plankton sample. The scale factor is used as a multiplier to correct the apparent DNA concentration as calculated from the C_T value of the target assay (here, oyster) for the effects of inhibition (Ophel-Keller *et al.* 2008). An aliquot of extracted DNA from each sample was sent to the Australian Animal Health Laboratory (AAHL) to be tested for OsHV-1.

Table 1.2. Sampling dates, locations and number of oyster samples for OsHV-1 testing collected in South Australia and Tasmania. See Figures 1.1-1.2 for locations

Date	Location				
SA	Inner Harbour	Lipson Reach	North Arm	Total (Wild)	Total (Farm)
21/02/2018	12	5	8	25	
2/03/2018	68	89	51	208	
24/04/2018	16	8	8	32	
28/05/2018	16	8	8	32	
26/06/2018	10	11	10	31	
14/08/2018	16	8	8	32	
12/09/2018	14	13	10	37	
23/10/2018	21	7	18	46	
26/11/2018	11	11	11	33	
20/12/2018	13	8	11	32	
22/01/2019	11	11	14	36	
TAS (Pipeclay lagoon)					
7-14/11/2018				160	160
12-14/12/2018				36	36
16-18/01/2019				32	36
13-15/02/2019				38	36
1-3/04/2019				37	37

Oysters collected in SA between February and May 2018 were kept cool in an insulated container, and delivered whole to VetLab for processing and analysis. All subsequent SA samples were stored at ≤ 4 °C until processing at the SARDI North Arm Store. Oysters collected in Tasmania were kept cool after collection in an insulated container, and processed in the field or at the Institute for Marine and Antarctic Studies, Taroona. After shucking, approximately 5 x 5 mm of mantle and gill were taken from each oyster and preserved in 70% ethanol. Ethanol preserved tissue samples were tested for OsHV-1 at AAHL. DNA was extracted with the QIAamp Mini kit, following the manufacturer's protocol from the tissue sample (total mass of approximately 25 mg) of each oyster. Extracted DNA quality and concentration was assessed using a Nanodrop® ND-2000 spectrophotometer (Thermo Fisher Scientific).

Table 1.3. OsHV-1 and T4 PCR assays; primer and probe sequences, cycling conditions and amplicon sizes

PCR	Primers/Probe	Sequence (5'-3')	Cycling	Reaction	Amplicon	Reference
OIE Martenot OsHV-1 qPCR[1]	BF B4 Probe B	GTC GCA TCT TTG GAT TTA ACA A ACT GGG ATC CGA CTG ACA AC **6FAM** TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC **TAMRA**	95°C 20s, 95°C 3s + 60°C 30s (45 cycles)	2 µl NA 23 µl MM[3]	102bp	Martenot *et al.* (2010)
EMAI OsHV-1 qPCR[1]	CRF CRR CR probe	CGT TTT ATC CAC CAC GAT TTT TAT T TAC ATC AAA CCC ACT TTT CCT ATG AT **6FAM** CAC TCA TGA AAA CAC CGC TAA GAT CAC TGC **TAMRA**	95°C 20s, 95°C 3s + 60°C 30s (45 cycles)	2 µl NA 23 µl MM[3]	88bp	Jenkins *et al.* (2013)
T4 FAST qPCR[1]	Phage F Phage R Phage Probe	CCA TCC ATA GAG AAA ATA TCA GAA CGA CGC TGG GAA AAG AGG AAT TAT TTA **VIC** AAC CAG TAA TTT CAT CTG CTT CTG ATG TGA GGC **QSY**	95°C 20s, 95°C 3s + 60°C 30s (45 cycles)	2 µl NA 23 µl MM[3]	101bp	Ninove *et al.* (2011)
Arzul OsHV-1 PCR[2]	C2 C6	CTC TTT ACC ATG AAG ATA CCC ACC GTG CAC GGC TTA CCA TTT TT	95°C 15 min, 94°C 30s + 50°C 60s + 72°C 60s (40 cycles) 72°C 7 min	2 µl NA 23 µl MM[4]	709bp	Renault *et al.* (2000) Arzul *et al.* (2001)
OsHV-1 C2-C3-C6 nPCR[2]	*Primary* C2 C6 *Nested* C3 C6	 As above As above GGCAAGATGAATGGCAAGAT As above	 95°C 15 min, 94°C 30s + 55°C 30s + 72°C 60s (40 cycles) 72°C 7 min As per primary reaction	 5 µl NA 45 µl MM[4] 5 µl NA 45 µl MM[4]	 709bp 574bp	In-house

qPCR1 = Performed at AAHL with 7500 Fast or QuantStudio Real-time PCR systems, at VetLab with QuantStudio 6 and Rotor-Gene Q. PCR[2] or nPCR[2] = Performed with Eppendorf and Veriti Thermal Cyclers, NA = nucleic acid, MM3 = TaqMan Fast Universal PCR master mix, MM4 = HotStarTaq PCR master mix

From each plankton nucleic acid extract tube, 18 µl was transferred to a 96 well PCR plate and spiked with 2 µl of DNA extracted from T4 Bacteriophage. As a positive control for T4, 18 µl nuclease free water was spiked with 2 µl T4 Bacteriophage extracted DNA. Nucleic acid extracts from plankton and oyster samples were tested with the OIE Martenot et al. (2010), and EMAI Jenkins *et al.* (2013) OsHV-1 qPCR assays in duplicate. Plankton extracts were also tested for T4 DNA using the FAST qPCR in singlicate. Oyster nucleic acid samples were assayed using the Taqman® Ribosomal RNA Control Reagents kit (#4308329, Applied Biosystems). Plasmid positive controls and no template negative controls were included in each PCR plate; all performed as expected. All wells with qPCR amplification curves that crossed a 0.1 fluorescence threshold were considered positive and a cycle threshold (C_T) was recorded. Samples with duplicate positive wells by qPCR were reported as positive and those with one well positive and one well negative were reported as indeterminate. The T4 qPCR C_T value for each plankton sample was compared to the T4 positive controls. All OsHV-1 positive or indeterminate plankton samples, and three positive oyster tissue samples per batch were tested with the OsHV-1 C1-C2-C6 and/or C2-C3-C6 nPCR assays in singlicate. OsHV-1 positive samples from January – September 2017 were also tested with the Arzul OsHV-1 PCR in singlicate. Assay details are provided in Table 1.3.

1.2.3. Mapping and statistical methods

Plankton or oyster tissue samples were regarded as positive for OsHV-1where a positive detection occurred in at least one replicate sample of either assay (i.e. where results were reported as either positive or indeterminate). For mapping, positive results are shown as either indeterminate (+) if one of the replicate tests was positive, or positive (++) if both replicates were positive. Maps were generated using ArcGIS 10.6 (Esri Inc).

Latent class models (LCMs) were used to estimate true prevalence of OsHV-1 in plankton samples and oyster tissue, and diagnostic sensitivity (DSe) and specificity (DSp) of the two tests as applied in each case. The models were fit in a Bayesian framework and allowed for covariance between the tests. For plankton samples, prevalence was modelled using sampling area and season as covariates with a logistic link. Sampling areas were defined as: SA-Wild, which comprised all SA samples since these did not include farm locations, TAS-Wild, samples from Derwent River, and TAS-Farm, samples from farming areas (although wild oysters also occur in these locations). Plankton samples were not always collected in the same month for each area, and there were insufficient data to resolve monthly patterns, but season was included to account for annual cycles in prevalence. The effect of oyster DNA in plankton samples on OsHV-1 prevalence was tested by running models including one of either oyster DNA yield or the oyster qPCR results as an additional covariate. The influence of inhibition as measured by the scale factor, on DSe of the tests when applied to plankton was also assessed using a logistic link. The natural logarithm of scale factor (lnSF) of each sample was used as a covariate, reflecting that the scale factor is a multiplicative effect; samples with no inhibition have scale factor = 1, hence lnSF=0 for these samples. To accommodate a potentially non-linear response to inhibition, the square of lnSF was also included as a covariate. For the LCM of oyster tissue results, prevalence was modelled by sampling set, with each sample set being a unique combination of population and sample time. Populations for this analysis were SA-Wild, TAS-Wild and TAS-Farm, but in this case, TAS Wild and Farm populations were oysters from the same area but from wild populations and farm stock respectively. Only results for samples tested with both assays could be used in the model therefore prevalence was estimated for the SA sample sets that were tested with only the Martenot assay using posterior predictions for DSe and DSp from the LCM and the 'truePrev' function from the R (R Core Team, 2019) package 'prevalence' (Devleesschauwer *et al.* 2014). For analyses of both plankton and oyster tissue results, the effect of covariates was assessed by examination of 95% credible intervals of the posterior predictions; covariate levels were considered to be different where the credible interval for the odds ratio between the covariates did not include 1. The Deviance Information Criteria (DIC) was also used to compare the plankton models with oyster DNA yield and oyster DNA presence to the base model. In all analyses Beta(16,4) priors were used for DSp of each test, reflecting 95% prior confidence that the true value is between 0.6 and 0.94, with most likely value 0.8. A normal(1.3863,1) prior was used for the intercept of DSe of each test (i.e. the DSe where there

is no inhibition), which reflects the equivalent prior distribution to DSp on the inverse-logit scale. Uniform priors were used for covariance, with bounds set based on minimum and maximum possible values, which depend on the test sensitivities and specificities (Gardner *et al.* 2000; Rahman *et al.* 2019). Diffuse normal(0,1) priors were used for all other parameters. Code for the LCMs was modified from that of Rahman *et al.* (2019), and is provided in the Appendix. LCM fitting was performed using Markov chain Monte Carlo (MCMC) simulation in JAGS v. 4.3.0 (Plummer, 2017) with three chains for 50,000 iterations, thinned at a rate of 50, following 2,000 iterations for adaptation and 10,000 iterations for burn-in. JAGS was run using the 'R2jags' package (Su & Yajima, 2015) in R. Convergence was assessed using the Gelman-Rubin convergence statistic, and confirmed by visual inspection of trace, density and autocorrelation plots generated using the 'MCMCplots' package (McKay Curtis 2015).

Posterior predictions of DSe from the plankton LCM were used to calculate the sample size (n) required to detect a target prevalence of between 0.01 and 0.5 with overall survey sensitivity (S_{SE}) of 0.8 and 0.95 as: $n = \log(1-S_{SE})/\log(1-Dse)$. These calculations were performed for each test individually and considering the case where both tests are applied with a sample being considered positive where a detection occurs in either test, i.e. the same case definition as used for results presented herein. Using this case definition, overall DSe (DSe_{COMB}) is given by: $DSe_{COMB} = 1- ((1-DSe_1)(1-DSe_2) + covp_{12})$, where DSe_1 and DSe_2 are the sensitivities of the two individual tests, and $covp_{12}$ is the covariance between results of the two tests in positive samples. The overall sensitivity of applying both tests to oyster tissue using the same case definition was also calculated using this formula. Under this case definition, overall specificity is given by: $DSp_{COMB} = DSp_1 x DSp_2 + covn_{12}$, where DSp_1 and DSp_2 are the sensitivities of the two individual tests, and $covn_{12}$ is the covariance between results of the two tests in negative samples.

1.3. Results

1.3.1. OsHV-1 and oyster DNA in plankton

Pacific Oyster and OsHV-1 DNA were detected in plankton samples from both SA and TAS. In SA, OsHV-1 was first detected in one of 20 plankton samples collected in November 2017, prior to the confirmed occurrence in wild oysters following POMS-related mortalities in February 2018 (see section 3.4). OsHV-1 was subsequently detected in plankton samples collected between February and May 2018, and in October and November 2018 and February 2019 (Table 1.4). The highest proportion of samples with OsHV-1 in SA occurred in March 2018, when 4 of 15 samples tested positive for OsHV-1. In TAS, OsHV-1 was detected in plankton from January to April 2017, and in all sets collected between November 2017 and February 2019 with the exception of December 2017 and July 2018 (Table 1.4). OsHV-1 was found most frequently in summer to early autumn (January – March 2017, January – April 2018, February 2019) in TAS samples, with the majority of (9/12) of samples in February 2019 testing positive for OsHV-1. Confirmatory tests performed on samples with OsHV-1 detections returned positive results for one SA sample (from November 2018) and a total of 7 TAS samples from 4 sample sets (January and April 2017, November 2018 and January 2019; Table 1.5) . There were insufficient data to formally analyse any patterns in confirmatory test detections.

Oyster DNA was detected in at least one sample from every sample set except for May 2017 samples from SA (Table 1.4). In both SA and TAS, Oyster DNA was detected most frequently between late spring and early autumn, with the highest oyster DNA yield in plankton samples collected between January and March each year. Oyster DNA was detected in all samples from February 2018 and October 2018 through February 2019 in SA, and in TAS samples collected in December 2018 and February 2019. This seasonal pattern of detection for oyster DNA coincides with the known spawning period for Pacific Oyster in temperate locations (Fabioux *et al.* 2005) and with the pattern observed in previous molecular surveillance for this species (Wiltshire *et al.* 2019a).

PCR inhibition, as measured by the scale factor, was evident in some sample sets (Table 1.4). In most cases, scale factors for samples with inhibition were below 5, but greater inhibition occurred in some cases. In SA samples from Dec 2018, 8 of 12 samples had scale factors > 5, with a maximum scale factor of 119, and 8/25 samples from February 2019 had scale factor > 5 with a maximum of 68. In TAS, 8/20 samples from January 2017 had scale factor > 5, with a maximum of 95, and 6/15 samples from June 2018 had scale factors > 5 with a maximum of 108. In the April 2018 TAS samples, two samples each had scale factor > 2000. PCR inhibition is likely to decrease likelihood of detection, but oyster DNA was still frequently detected in several sample sets where inhibition occurred, probably due to relatively high oyster DNA concentration in samples, thus permitting detection despite inhibition. Oyster DNA concentration, is, however, likely underestimated in these results. The scale factor may be used to adjust DNA yield for the effects of inhibition, but the relationship is unlikely to be linear, especially at higher scale factors. The average oyster DNA yield in samples shown in Table 3 has not been adjusted for effects of inhibition for this reason. Detection of OsHV-1, which is likely to occur at lower concentrations than oyster DNA in environmental samples, may have been reduced in sample sets with inhibition.

Table 1.4. Results of OsHV-1 and oyster DNA testing of plankton samples collected in South Australia (SA) and Tasmania (TAS). See Table 1.1 for specific sampling dates. Inhibition indicated by sample scale factor (SF) >2, number of samples with SF>5 shown in brackets. OsHV-1 detections classed as (+) detected in one of two replicates for either Jenkins or Martenot test, (++) detected in both replicates for either test.

Sample time	No. of Samples	Samples with inhibition	Samples with OsHV-1	% samples with OsHV-1	Oyster DNA detected	% samples with Oyster DNA	Average Oyster DNA conc
SA		**(SF >5)**	**+, ++**				
Feb 2017	20	1 (0)	0, 0	0.0%	16	80.0%	92.4
May 2017	20	0 (0)	0, 0	0.0%	0	0.0%	0.0
Nov 2017	20	9 (1)	1, 0	5.0%	19	95.0%	417.7
Feb 2018	20	6 (3)	1, 2	15.0%	20	100.0%	5210.7
Mar 2018	15	3 (2)	4, 0	26.7%	11	73.3%	122.7
Apr 2018	15	5 (1)	2, 0	13.3%	2	13.3%	35.8
May 2018	15	1 (0)	1, 0	6.7%	9	60.0%	23.1
Aug 2018	15	0 (0)	0, 0	0.0%	7	46.7%	95.8
Sep 2018	12	0 (0)	0, 0	0.0%	6	50.0%	2.9
Oct 2018	12	1 (0)	1, 0	8.3%	12	100.0%	45.9
Nov 2018	12	0 (0)	2, 0	16.7%	12	100.0%	54.4
Dec 2018	12	10 (8)	0, 0	0.0%	12	100.0%	327.9
Jan 2019	12	1 (0)	0, 0	0.0%	12	100.0%	324.3
Feb 2019	25	14 (8)	1, 0	4.0%	25	100.0%	258.9
TAS							
Jan 2017	20	20 (8)	2, 4	30.0%	18	90.0%	1378.9
Feb 2017	16	14 (1)	2, 0	12.5%	15	93.8%	35713.5
Apr 2017	16	13 (0)	6, 1	43.8%	15	93.8%	8428.3
Sep 2017	15	13 (0)	0, 0	0.0%	1	6.7%	92.1
Oct 2017	19	2 (0)	0, 0	0.0%	3	15.8%	0.5
Nov 2017	15	0 (0)	1, 1	13.3%	14	93.3%	359.5
Dec 2017	15	1 (0)	0, 0	0.0%	9	60.0%	3.7
Jan 2018	15	3 (0)	5, 1	40.0%	17	113.3%	2828.3
Feb 2018	15	0 (0)	4, 3	46.7%	16	106.7%	2658.2
Mar 2018	15	0 (0)	5, 3	53.3%	15	100.0%	2581.2
Apr 2018	15	4 (3)	2, 4	40.0%	14	93.3%	723.8
May 2018	12	0 (0)	0, 2	16.7%	2	16.7%	26.6
Jun 2018	15	7 (6)	1, 0	6.7%	3	20.0%	8.6
Jul 2018	15	0 (0)	0, 0	0.0%	2	13.3%	2.3
Aug 2018	15	1 (1)	1, 0	6.7%	4	26.7%	0.8
Oct 2018	8	2 (1)	1, 0	12.5%	1	12.5%	1.3
Nov 2018	16	1 (0)	0, 3	18.8%	5	31.3%	3.4
Dec 2018	10	0 (0)	1, 1	20.0%	10	100.0%	10.0
Feb 2019	12	1 (0)	2, 7	75.0%	12	100.0%	132.6

Table 1.5. Summary of samples with OsHV-1 detection by confirmatory tests. qPCR results for each sample shown as
(–) not detected, (+) detected in one of two replicates, (++) detected in both replicates. Confirmatory test results shown as (nt) not tested, (–) not detected, (✓) detected.

		qPCR results		Confirmatory test result		
Location	**Sample time**	**Jenkins result**	**Martenot result**	**Arzul cPCR**	**C1-C2-C6 nPCR**	**C2-C3-C6 nPCR**
SA	November 2018	+	–	nt	–	✓
TAS	January 2017	–	++	–	–	✓
		++	++	–	✓	✓
	April 2017	–	+	–	✓	–
	November 2018	–	++	nt	✓	–
	January 2019	–	++	nt	–	✓
		–	++	nt	✓	–
		+	++	nt	✓	✓

1.3.2. Maps of plankton testing results

Results for SA are shown in Figures 1.3 – 1.9, and for TAS in Figures 1.10 – 1.27. In each map, yield of oyster DNA is indicated by coloured symbols, with darker colour corresponding to higher yield (and, equivalently, lower C_T). OSHV-1 detections are indicated by orange (indeterminate) or red (positive) plus symbols.

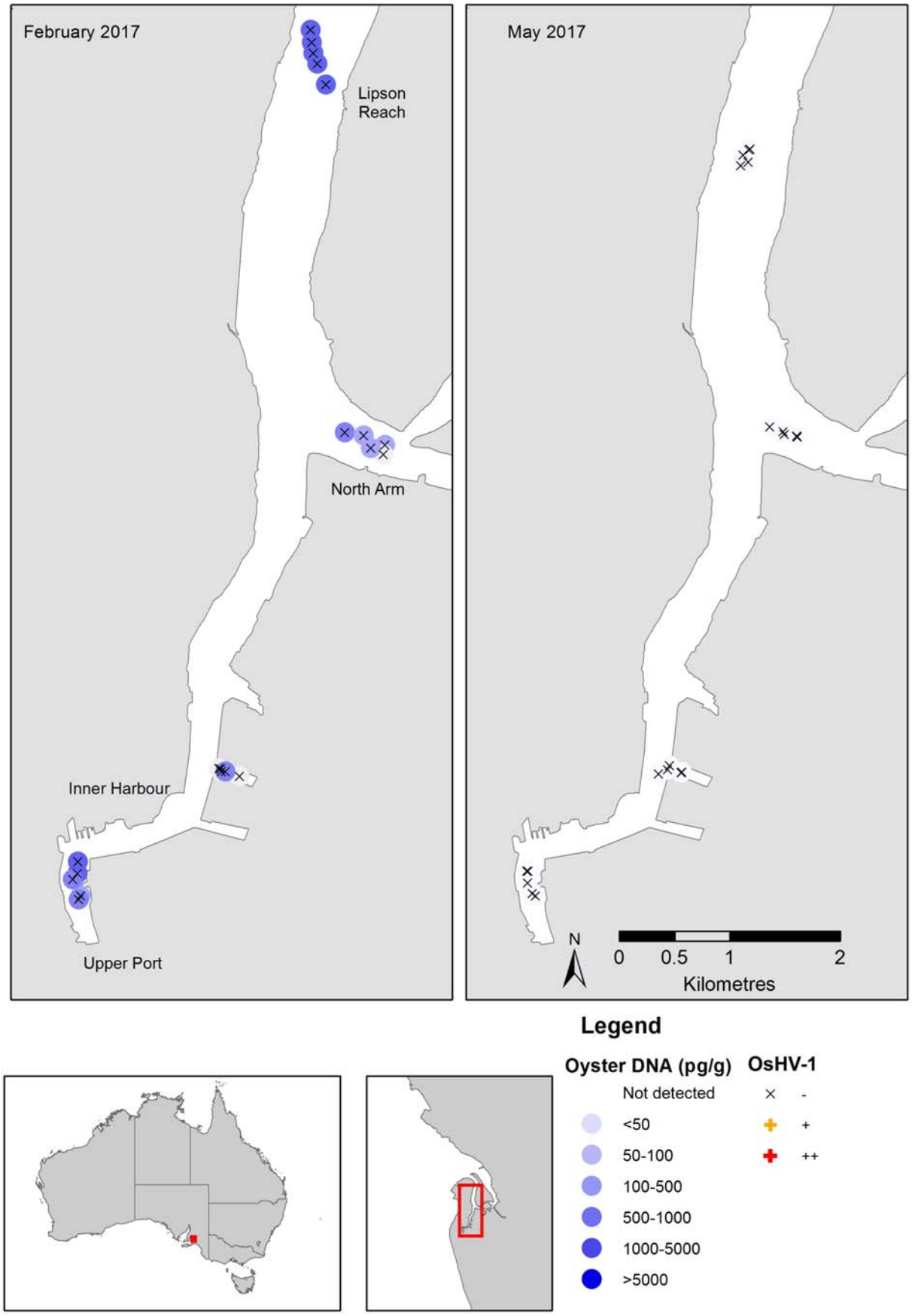


Figure 1.3. Map of plankton OsHV-1 and Oyster DNA testing results for SA samples from Feb and May 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

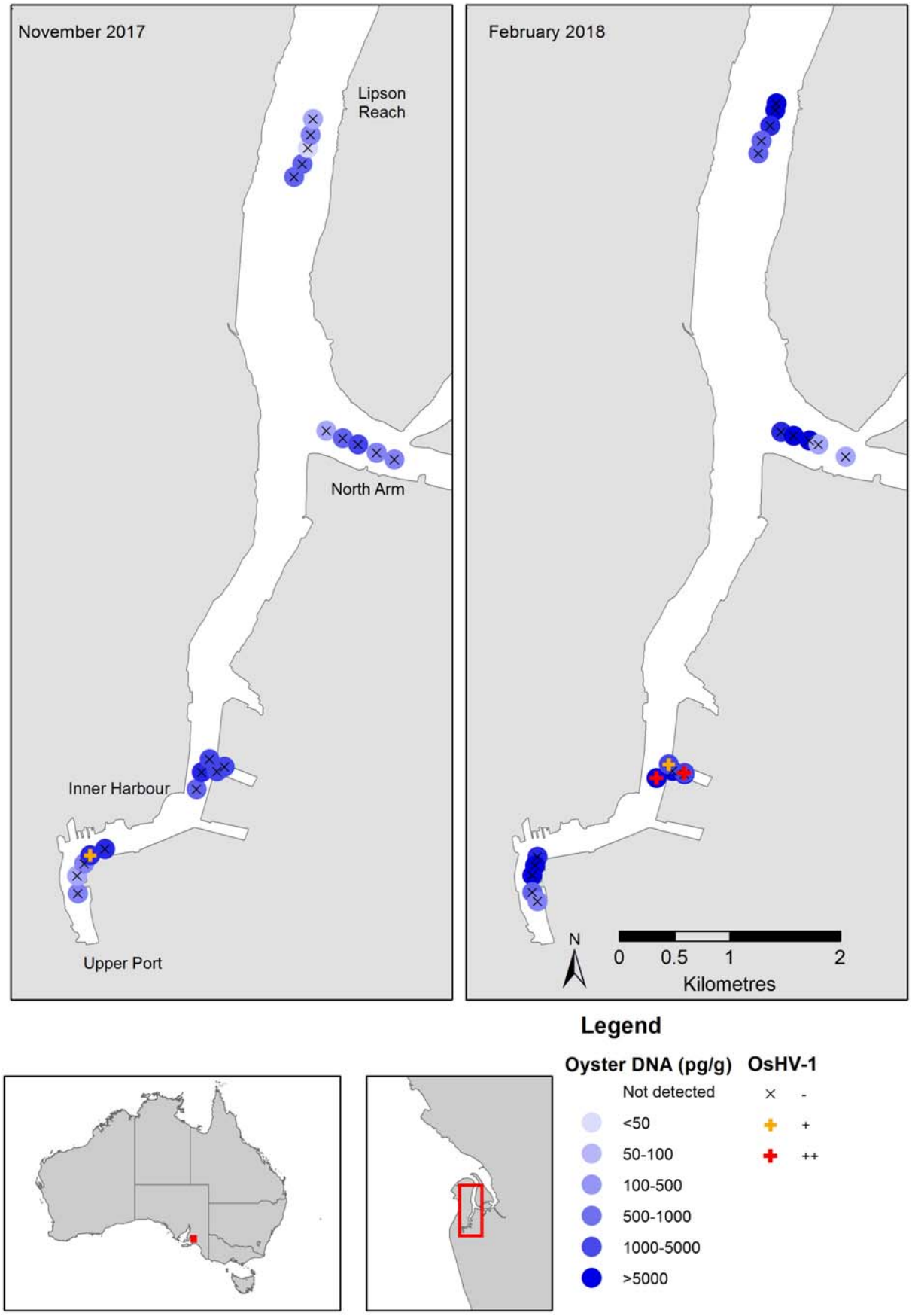


Figure 1.4. Map of plankton OsHV-1 and Oyster DNA testing results for SA samples from Nov 2017 and Feb 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

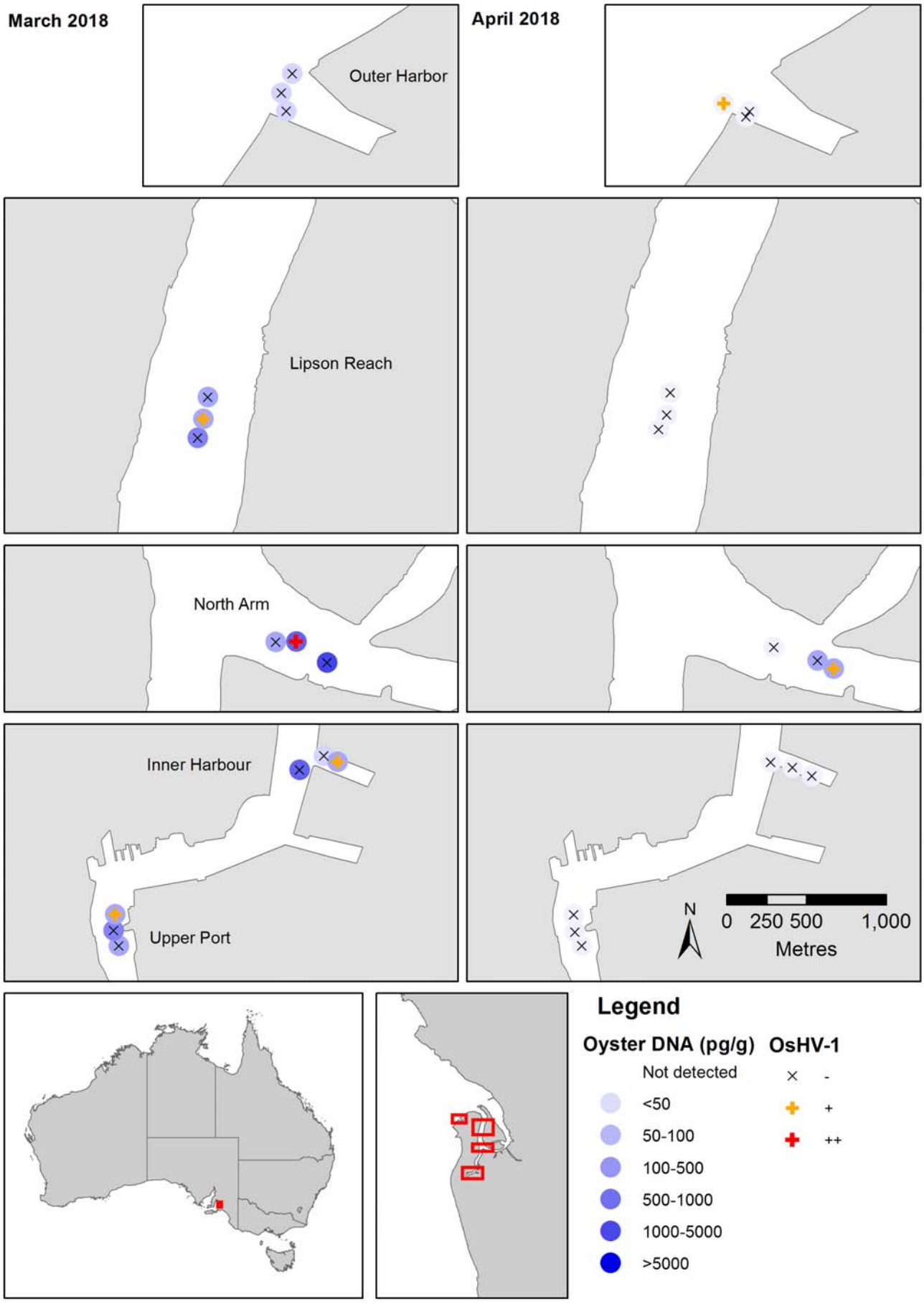

March 2018
April 2018
Outer Harbor
Lipson Reach
North Arm
Inner Harbour
Upper Port
N
0 250 500 1,000
Metres
Legend
Oyster DNA (pg/g)
Not detected
<50
50-100
100-500
500-1000
1000-5000
>5000
OsHV-1
-
+
++

Figure 1.5. Map of plankton OsHV-1 and Oyster DNA testing results for SA samples from Mar and Apr 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

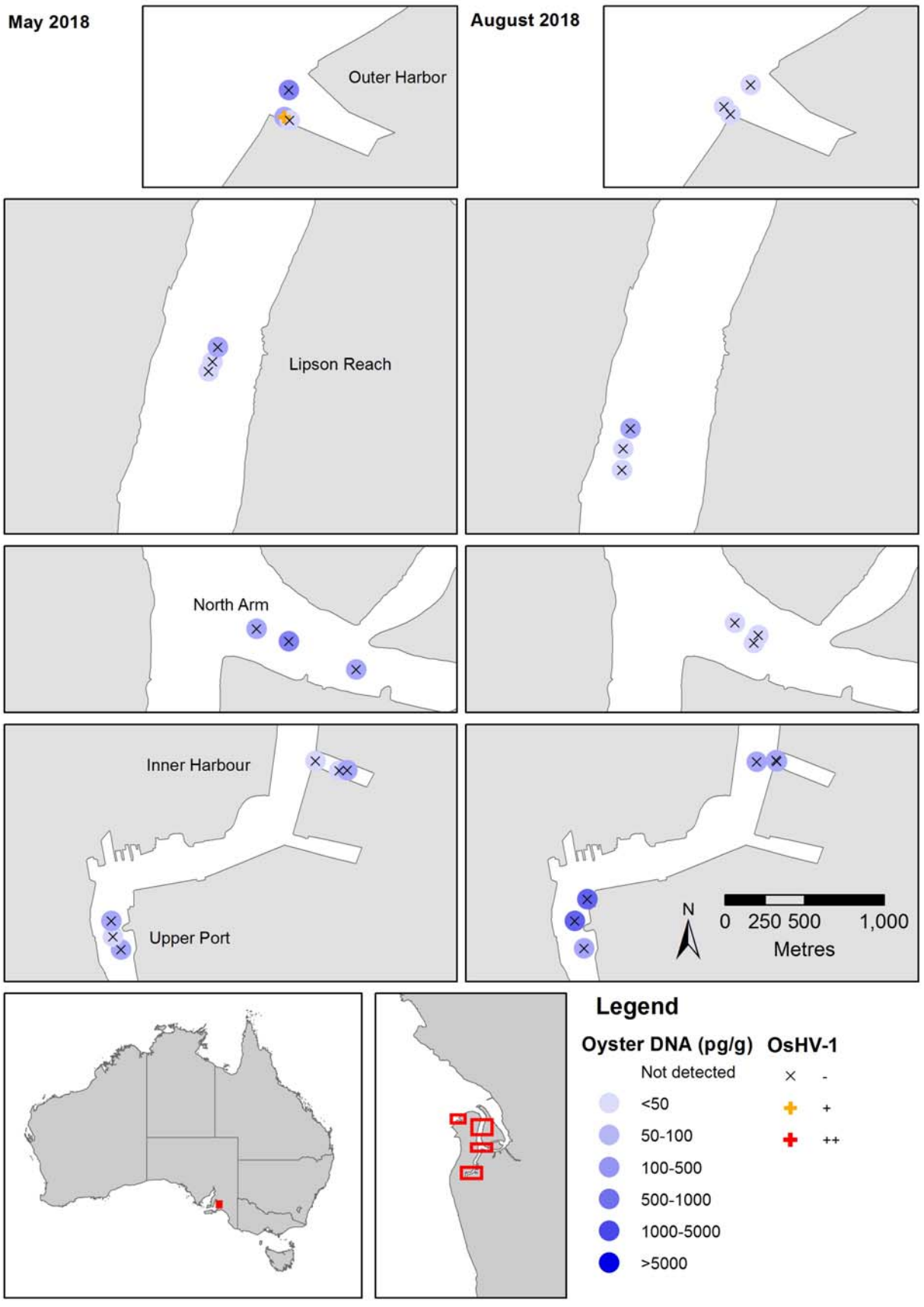


Figure 1.6. Map of plankton OsHV-1 and Oyster DNA testing results for SA samples from May and Aug 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

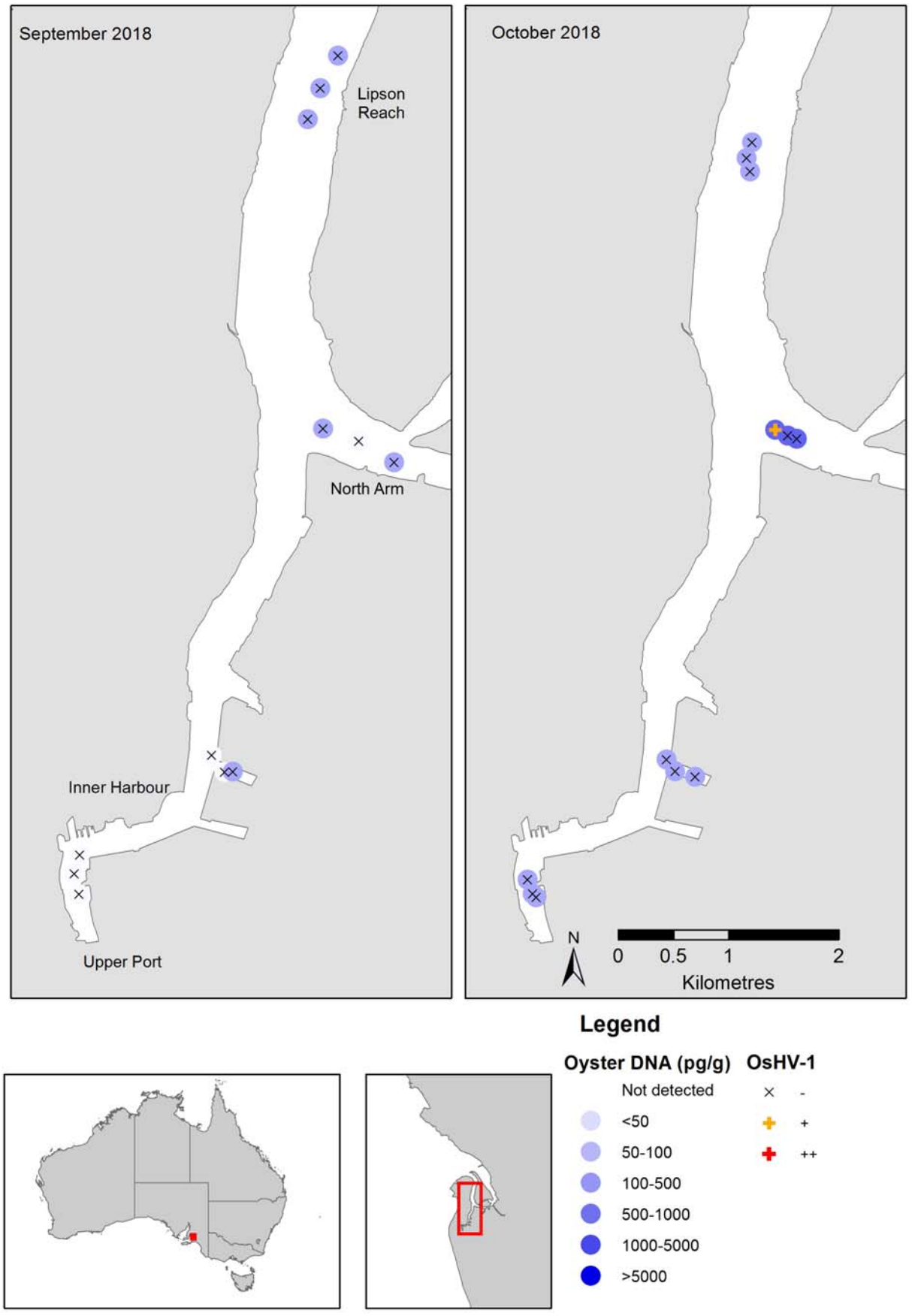


Figure 1.7. Map of plankton OsHV-1 and Oyster DNA testing results for SA samples from Sep and Oct 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

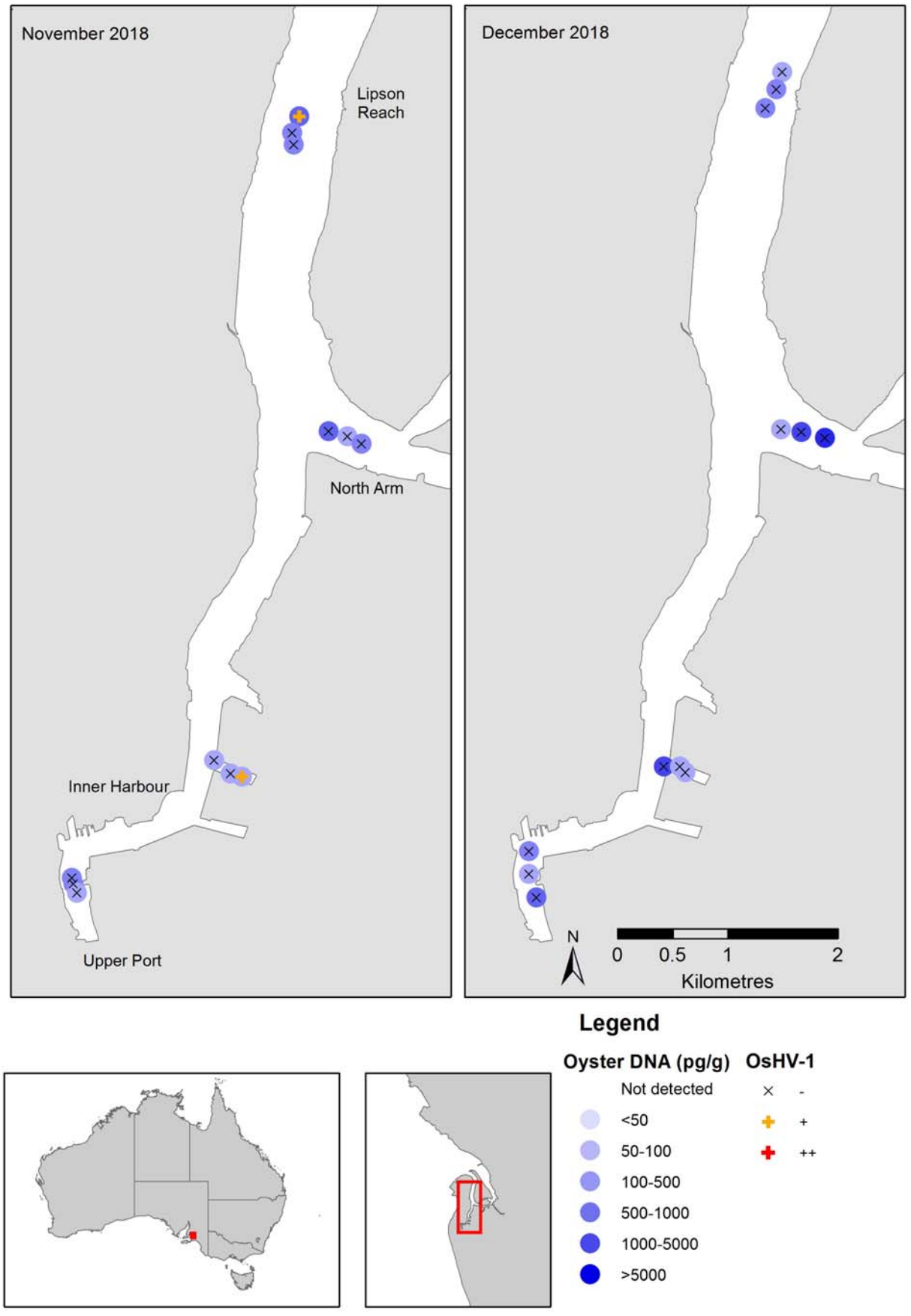


Figure 1.8. Map of plankton OsHV-1 and Oyster DNA testing results for SA samples from Nov and Dec 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

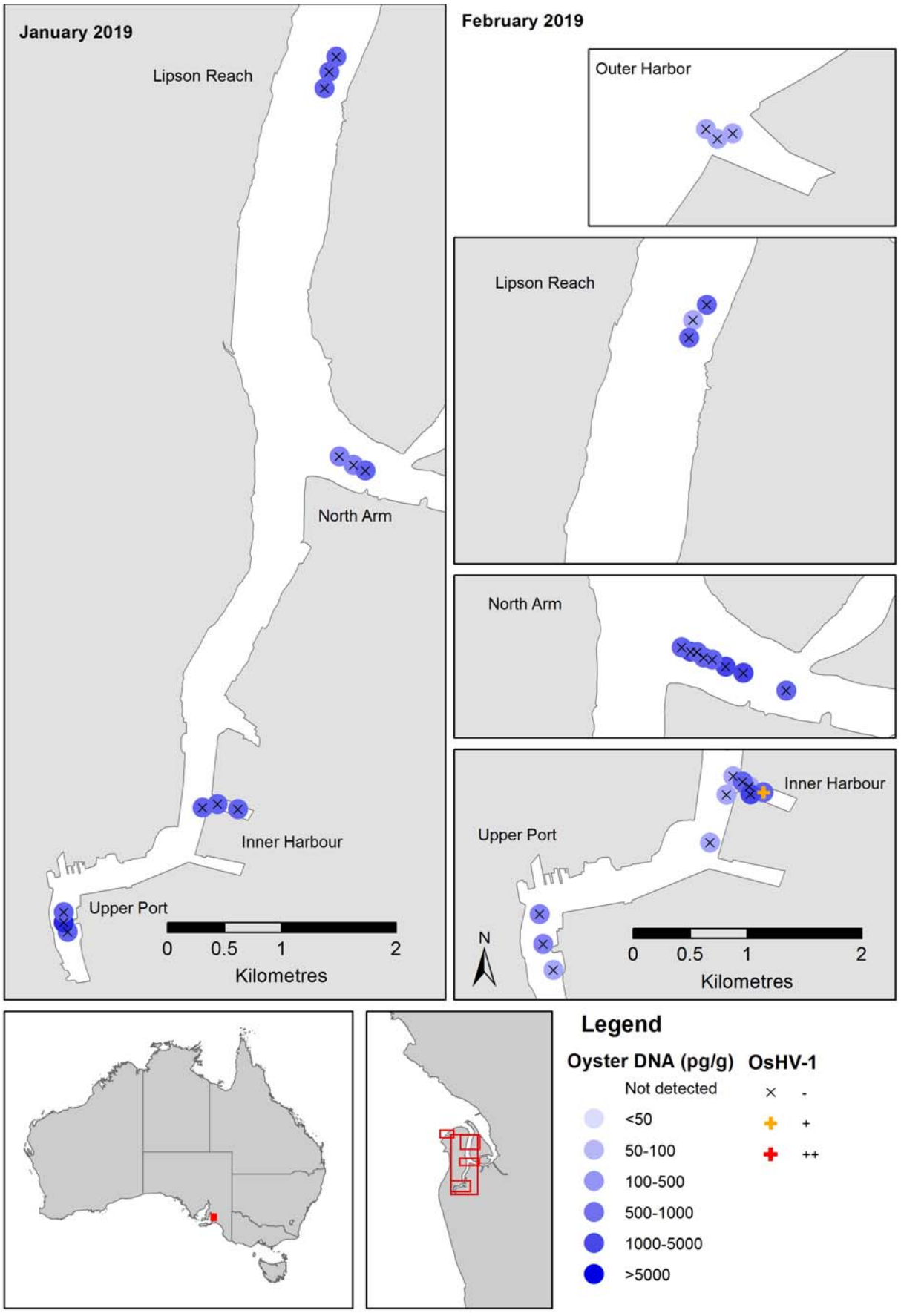


Figure 1.9. Map of plankton OsHV-1 and Oyster DNA testing results for SA samples from Jan and Feb 2019. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

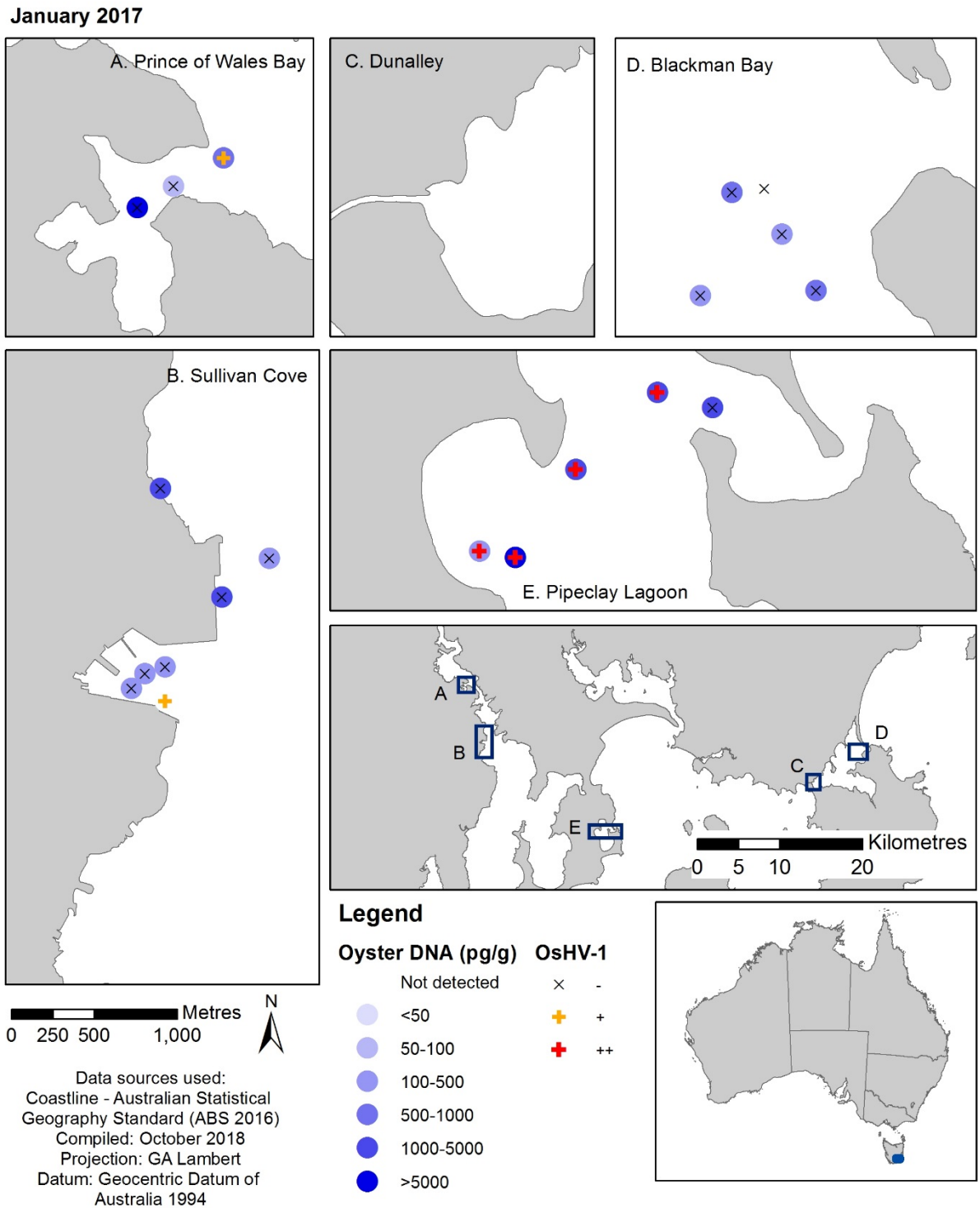


Figure 1.10. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Jan 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

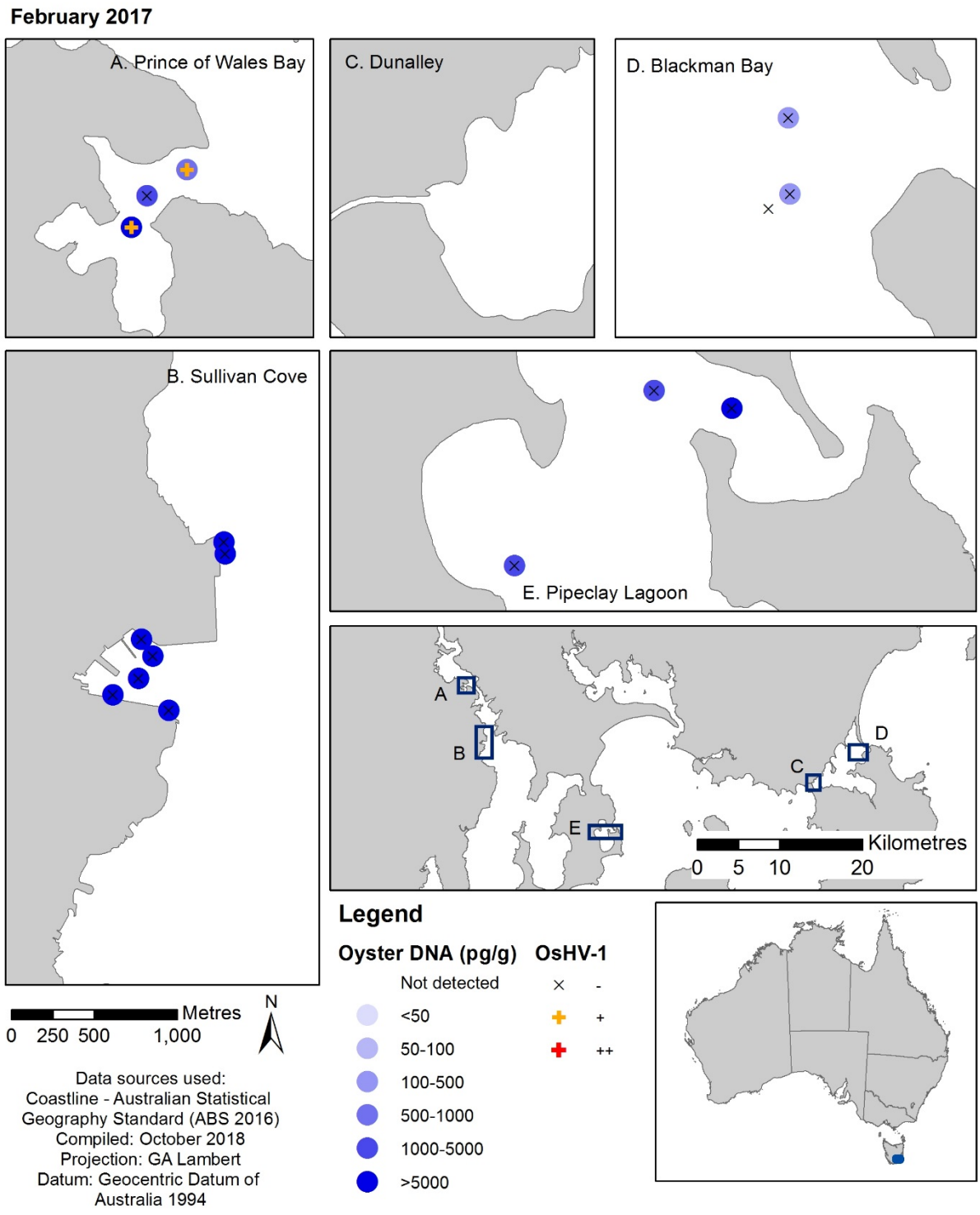


Figure 1.11. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Feb 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

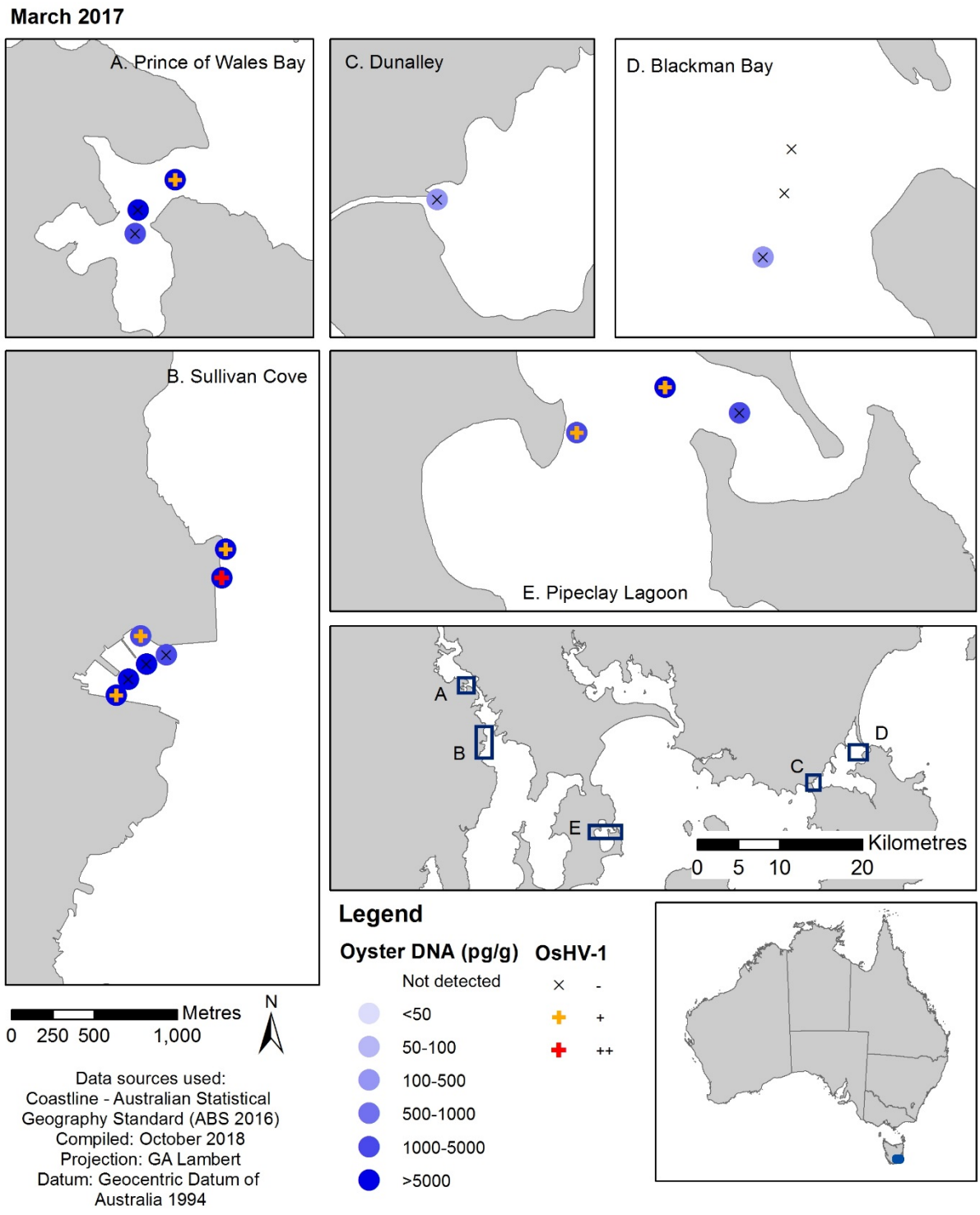


Figure 1.12. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Mar 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

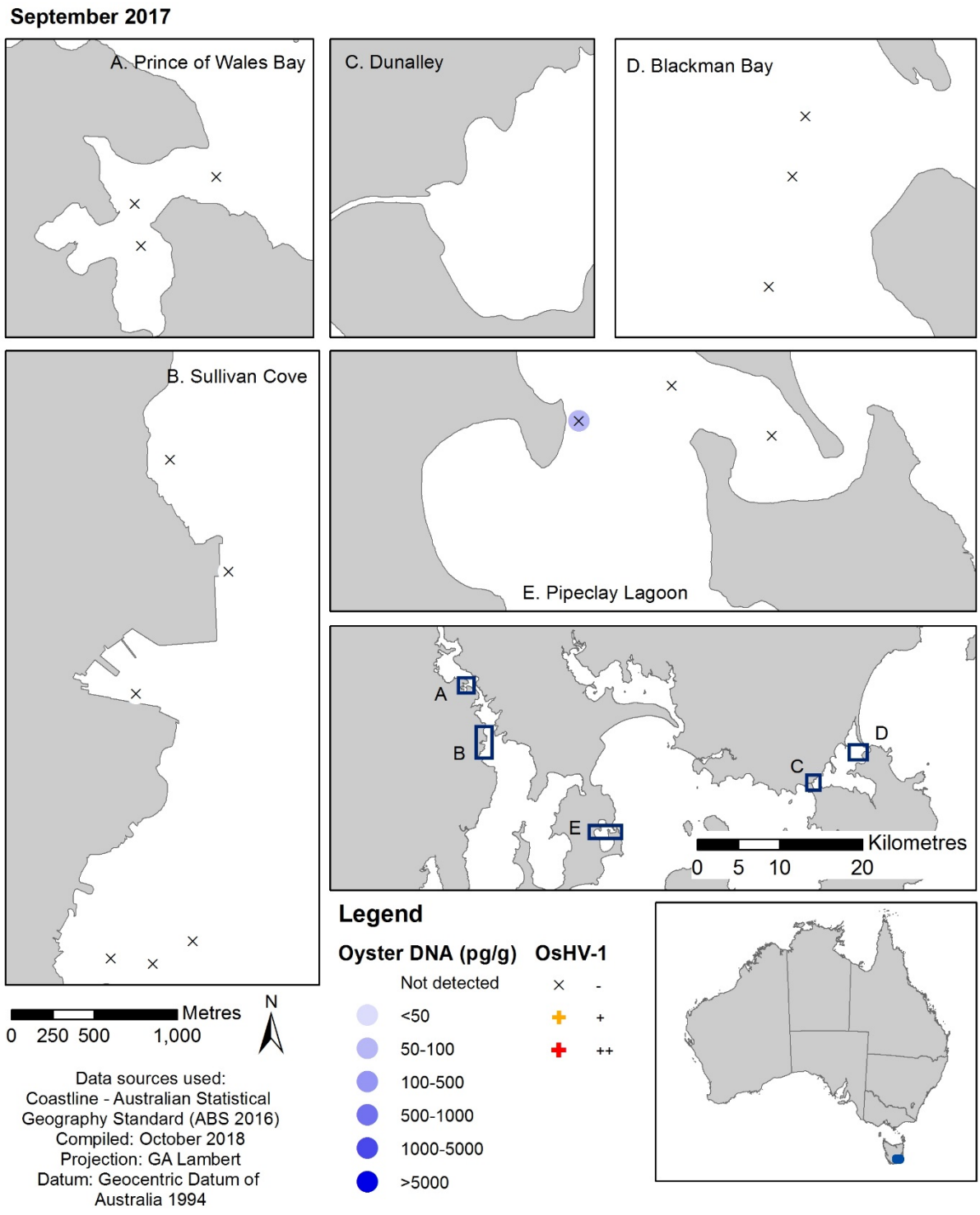


Figure 1.13. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Sep 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

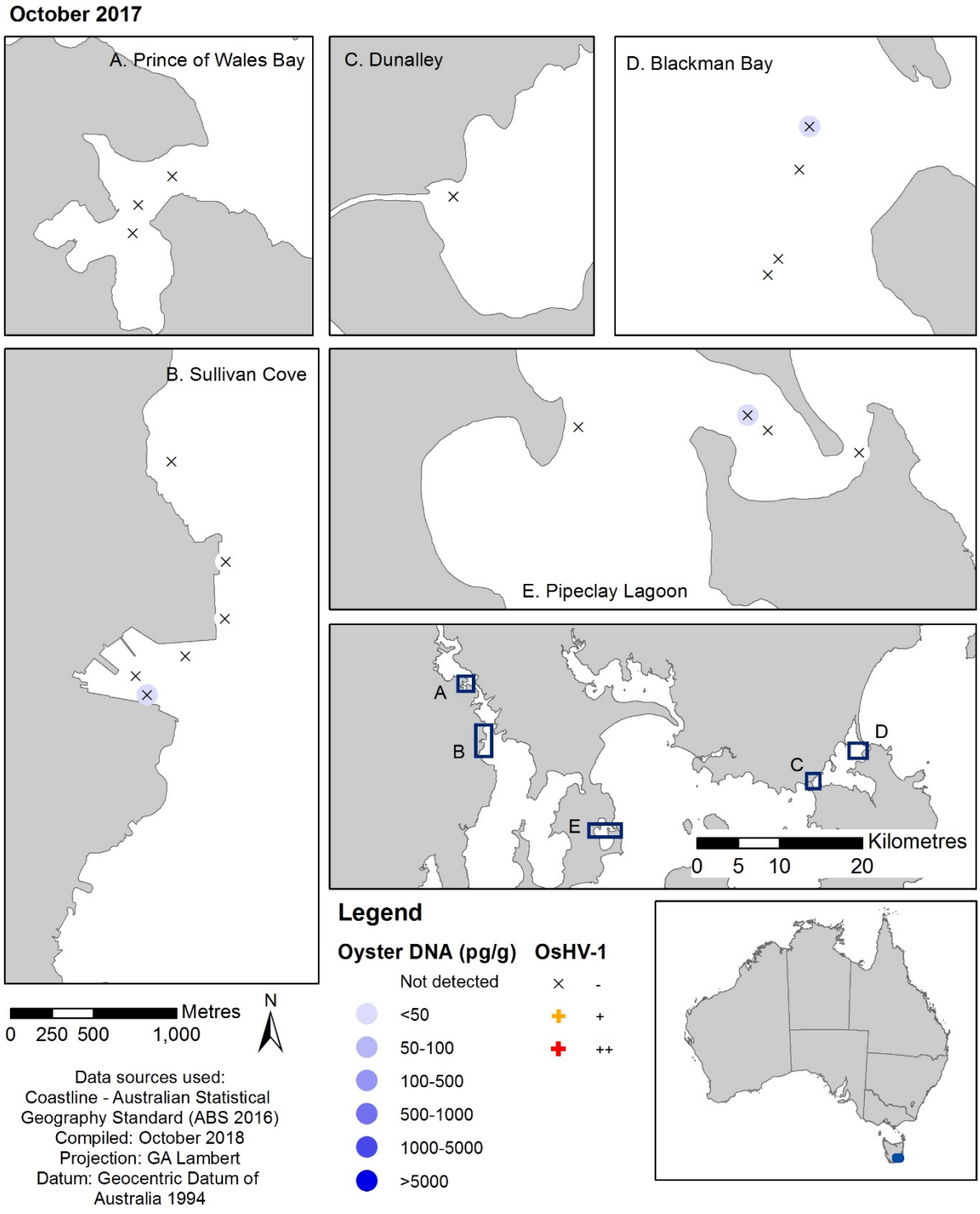


Figure 1.14. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Oct 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

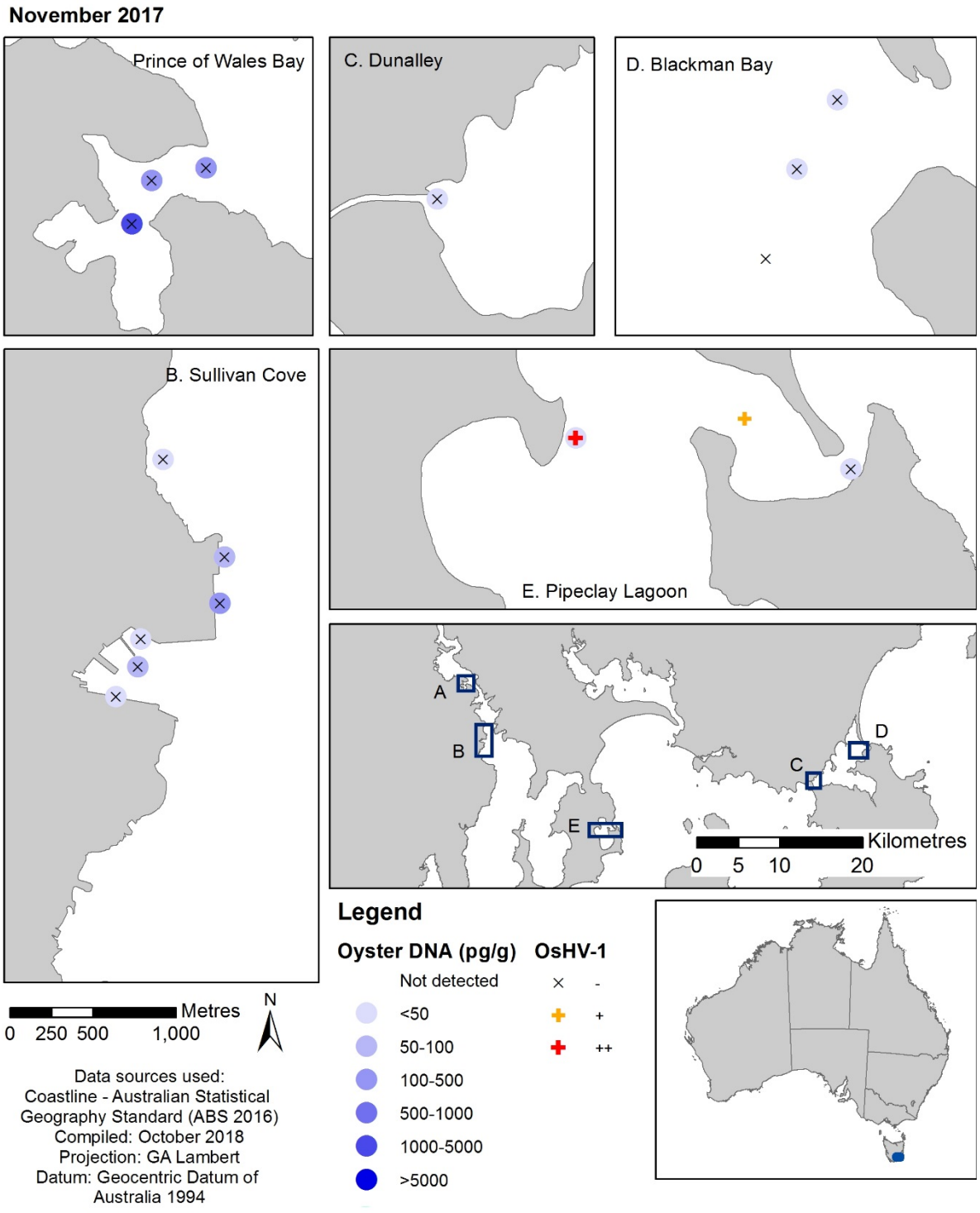


Figure 1.15. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Nov 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

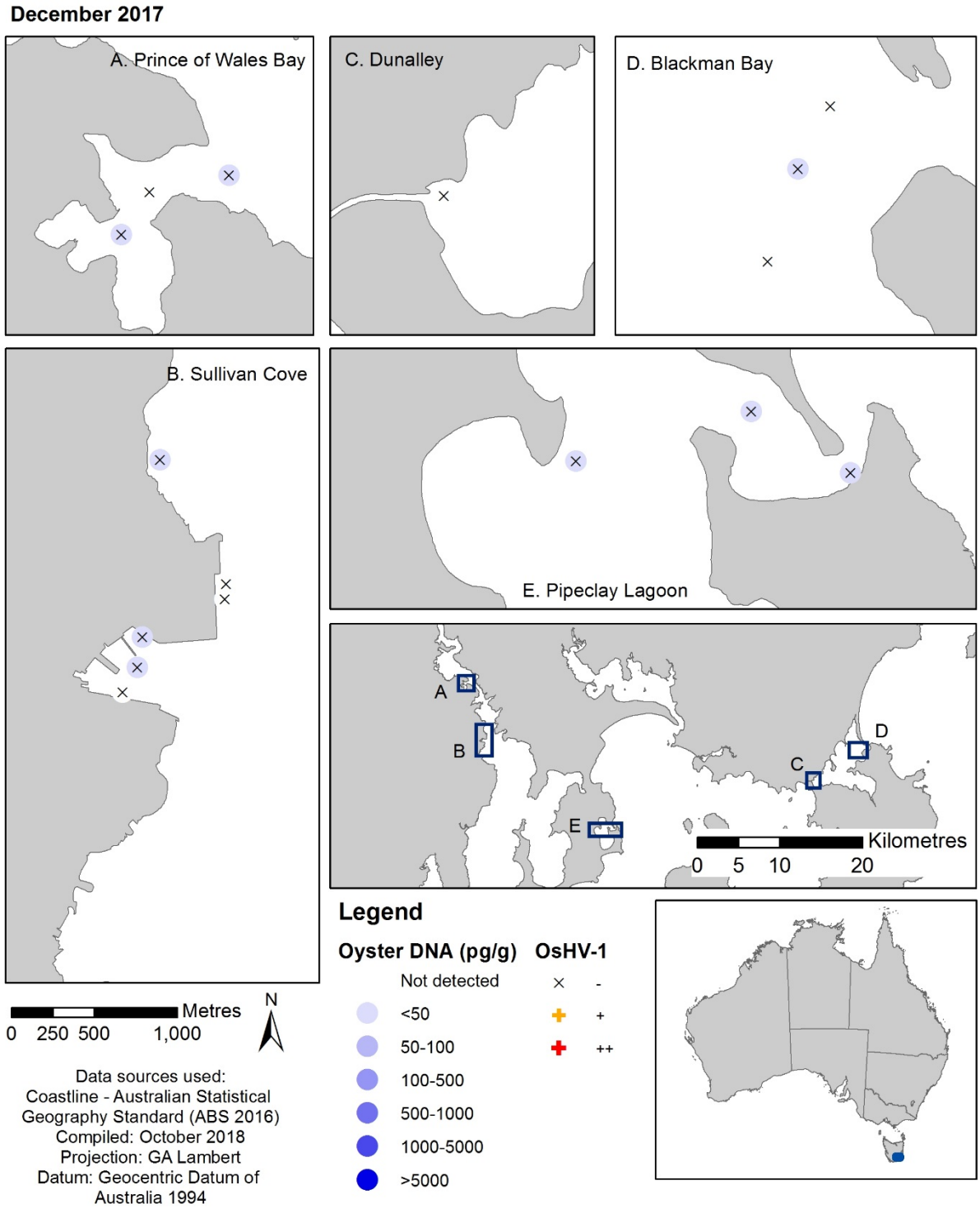


Figure 1.16. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Dec 2017. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

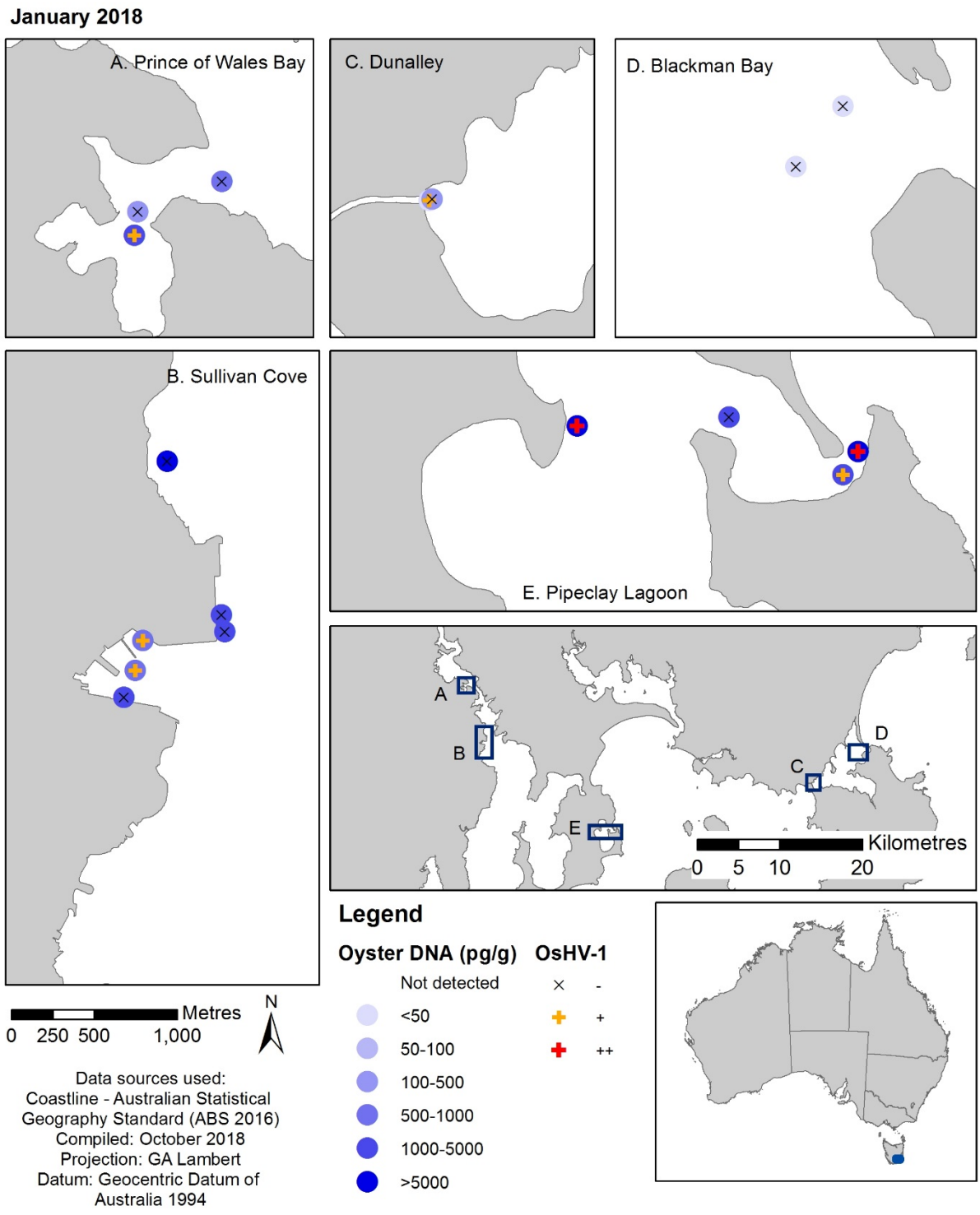


Figure 1.17. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Jan 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

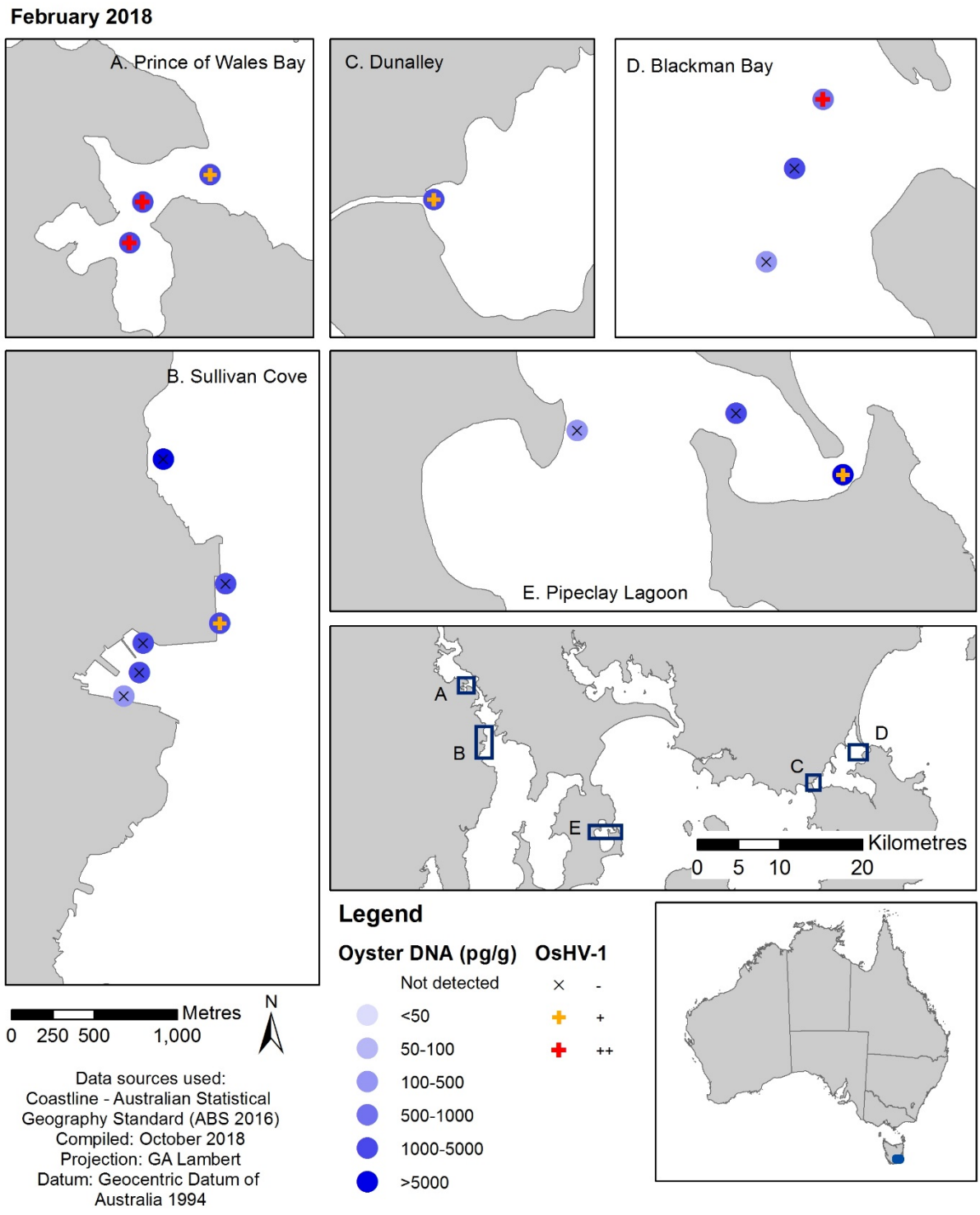


Figure 1.18. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Feb 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

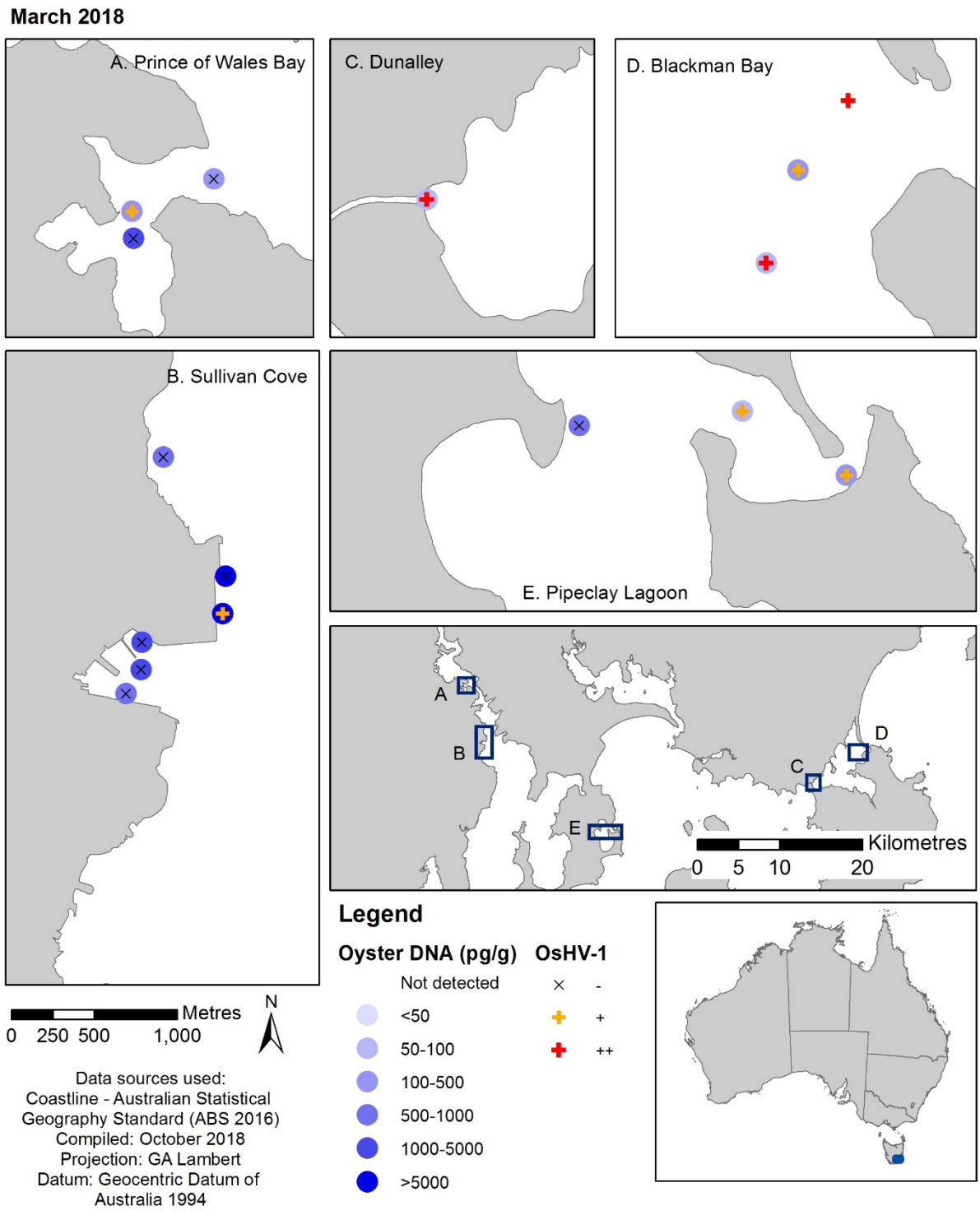


Figure 1.19. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Mar 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

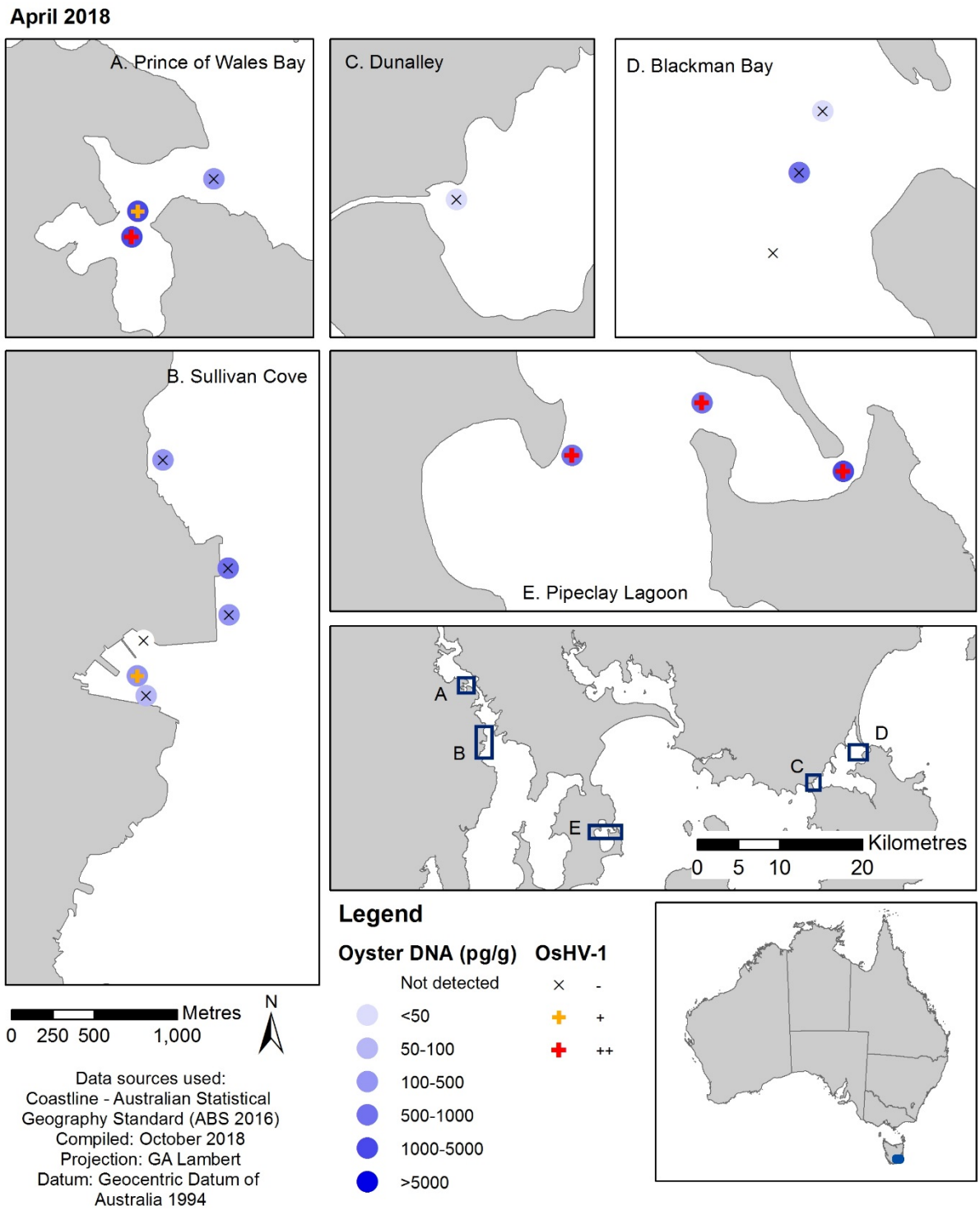


Figure 1.20. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Apr 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

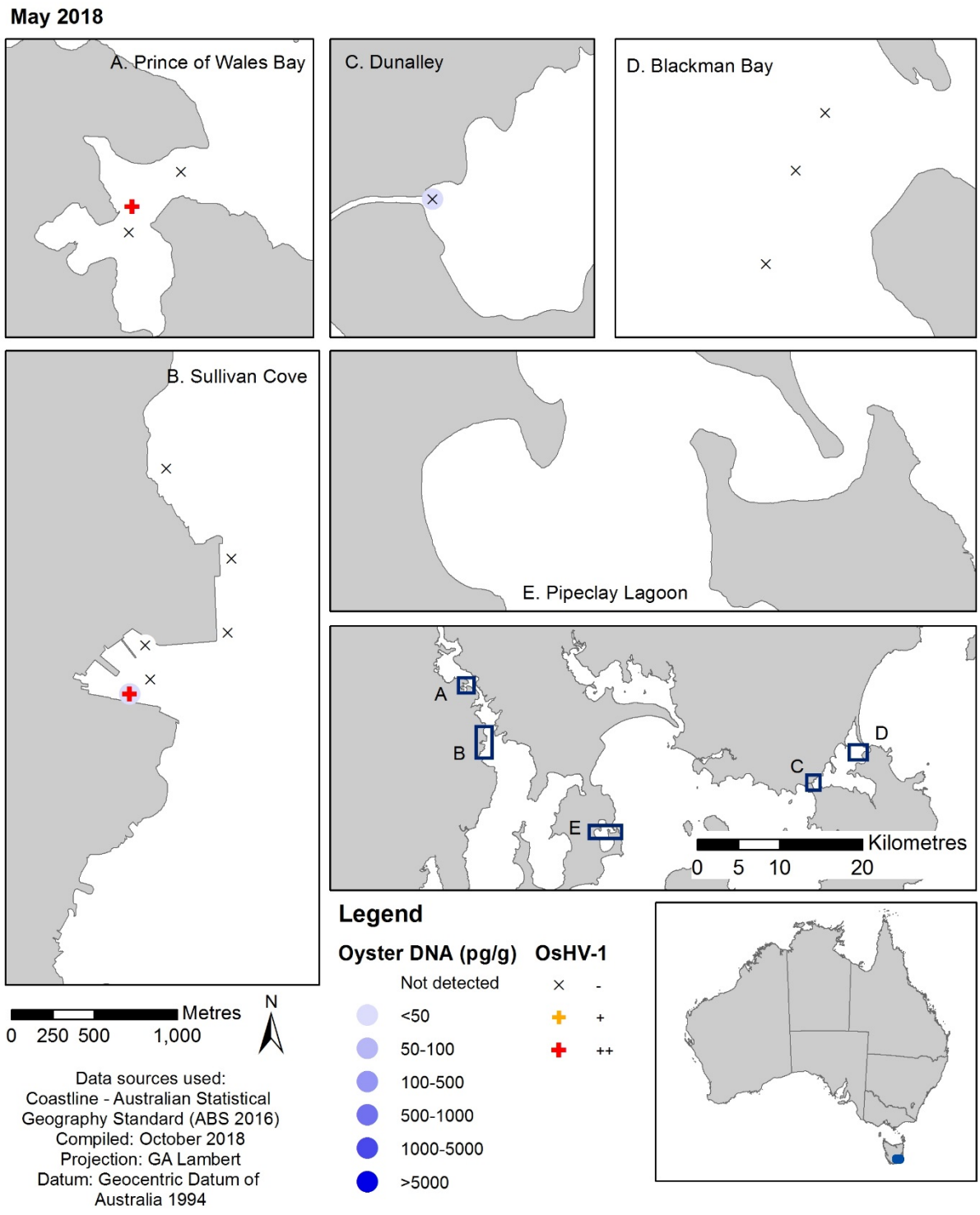


Figure 1.21. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from May 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

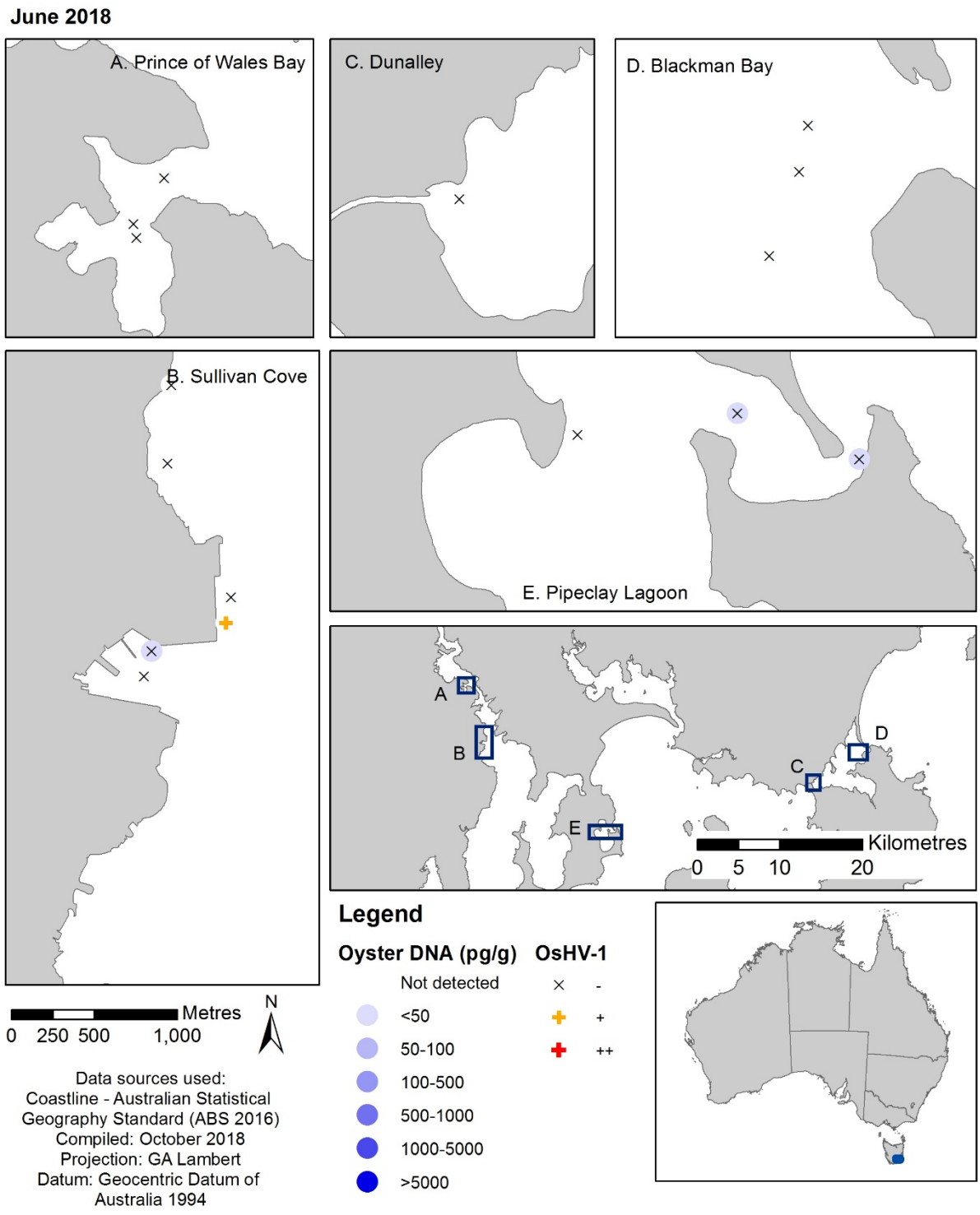


Figure 1.22. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Jun 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

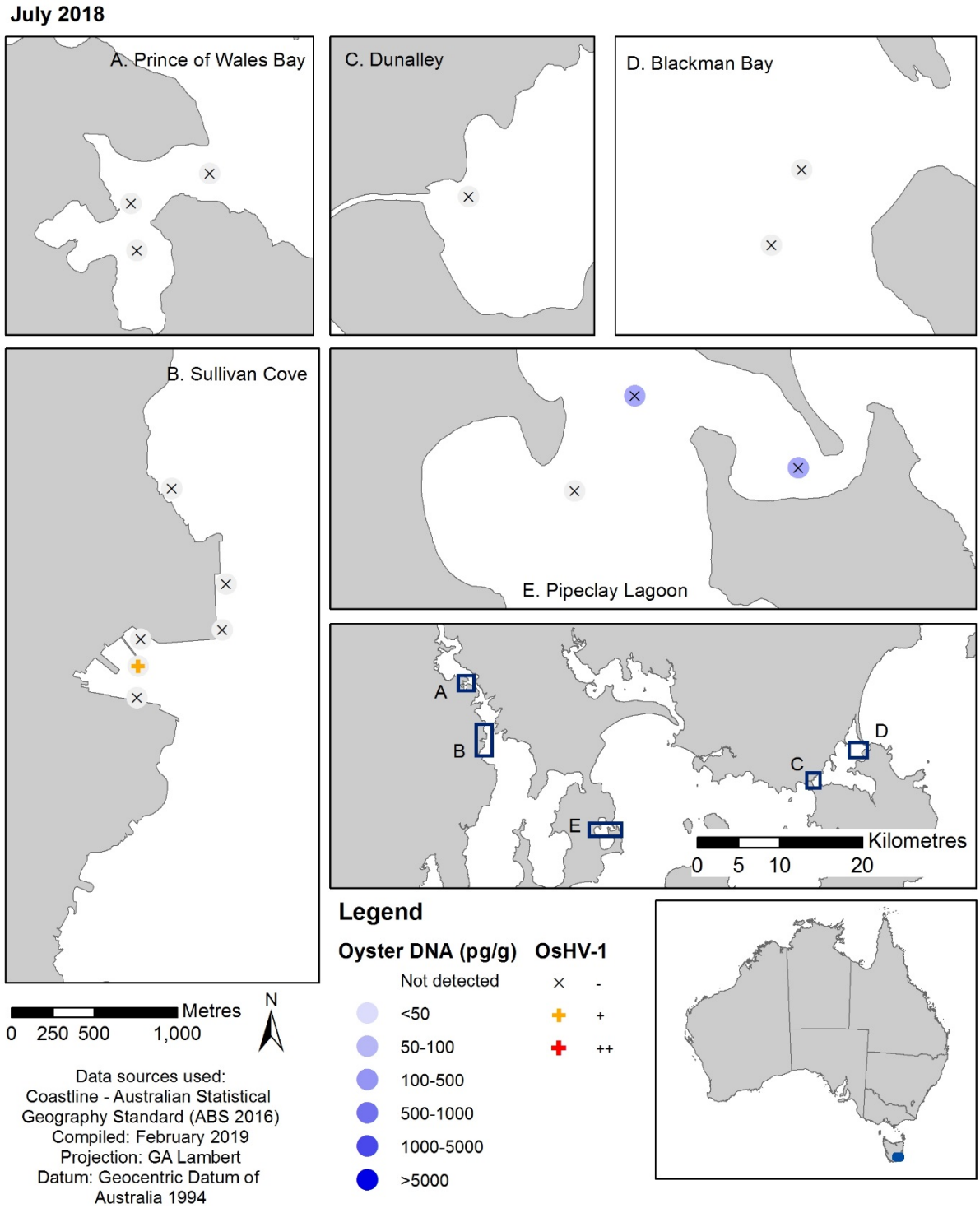


Figure 1.23. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Jul 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

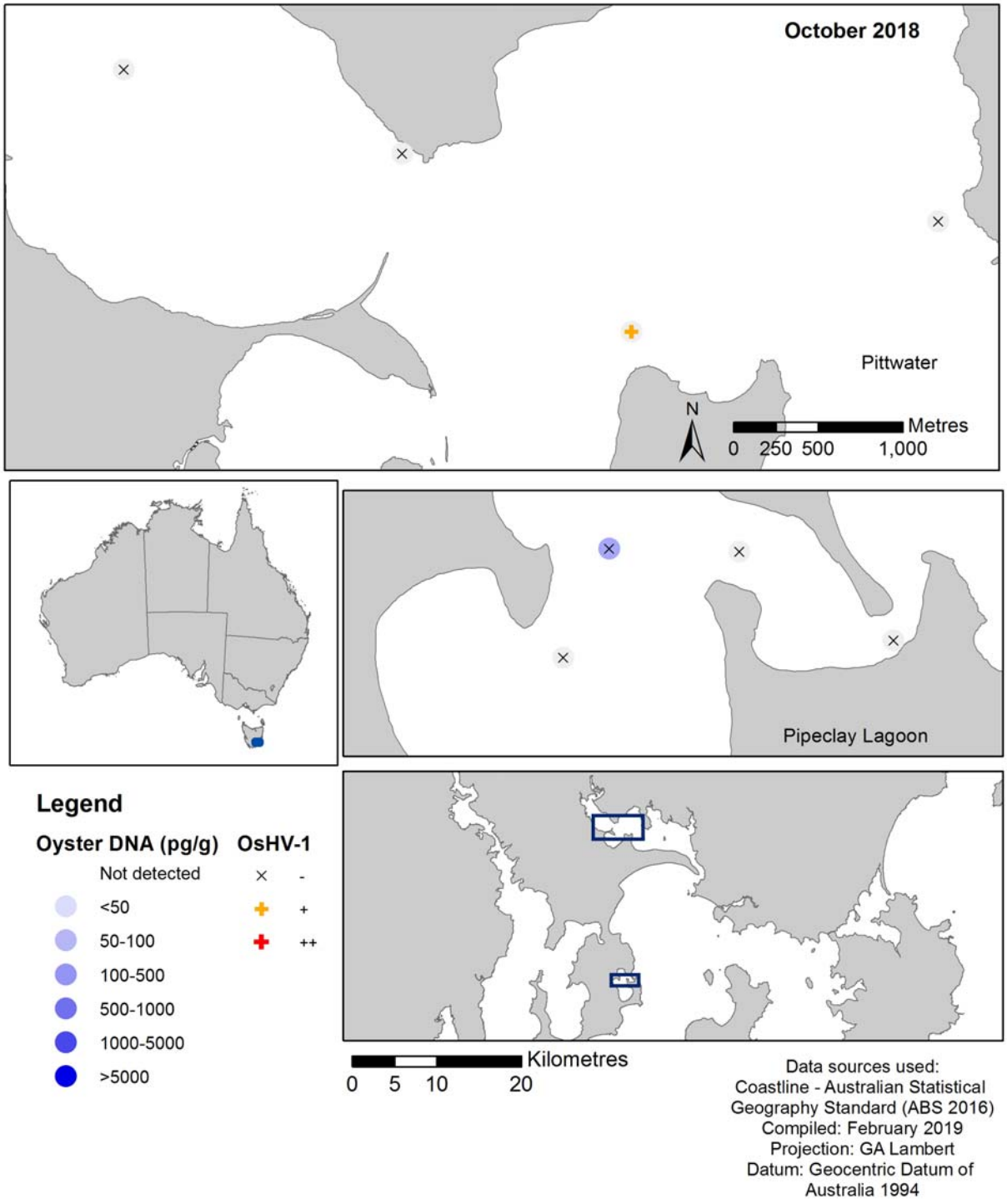


Figure 1.24. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Oct 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

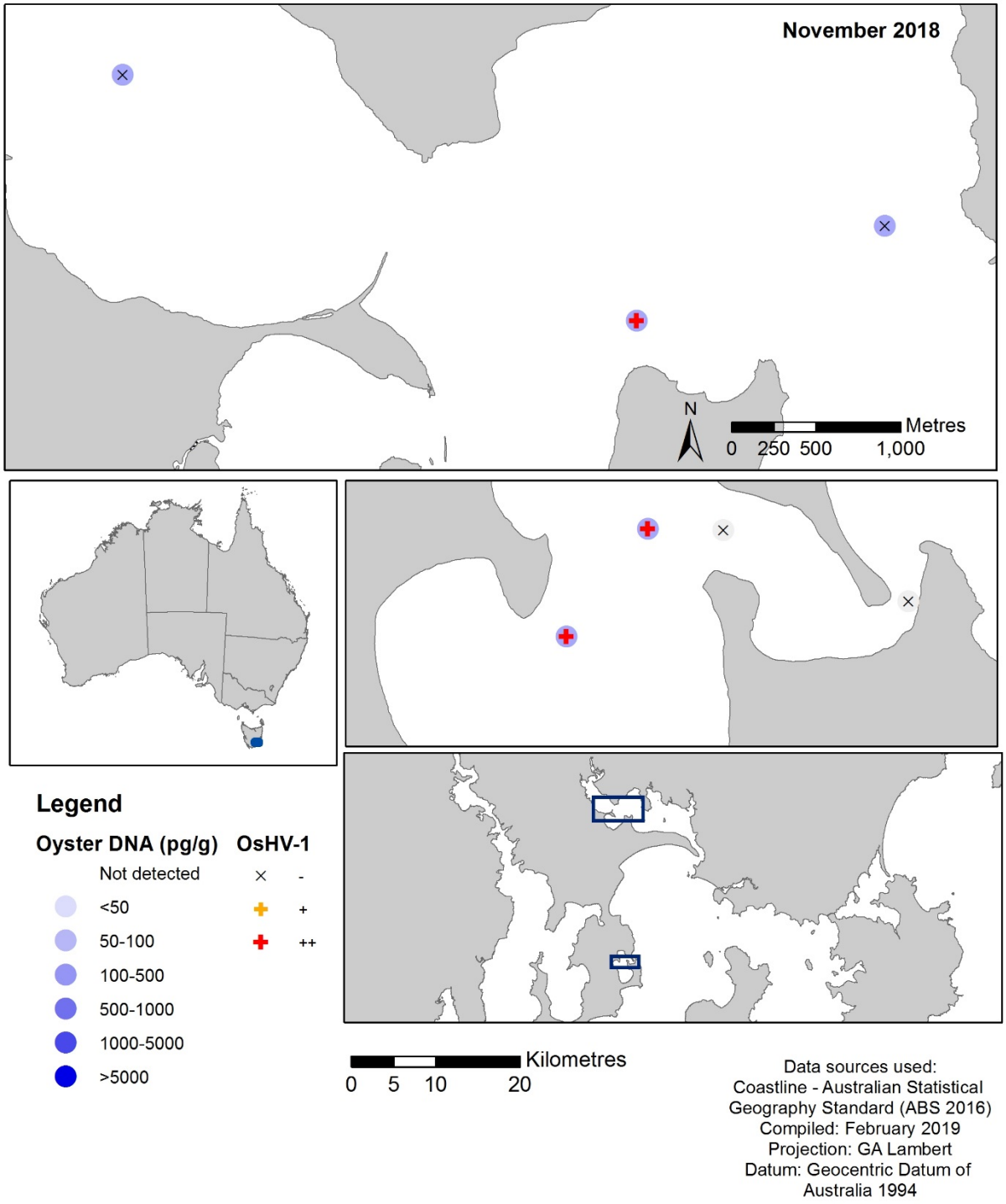


Figure 1.25. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Nov 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

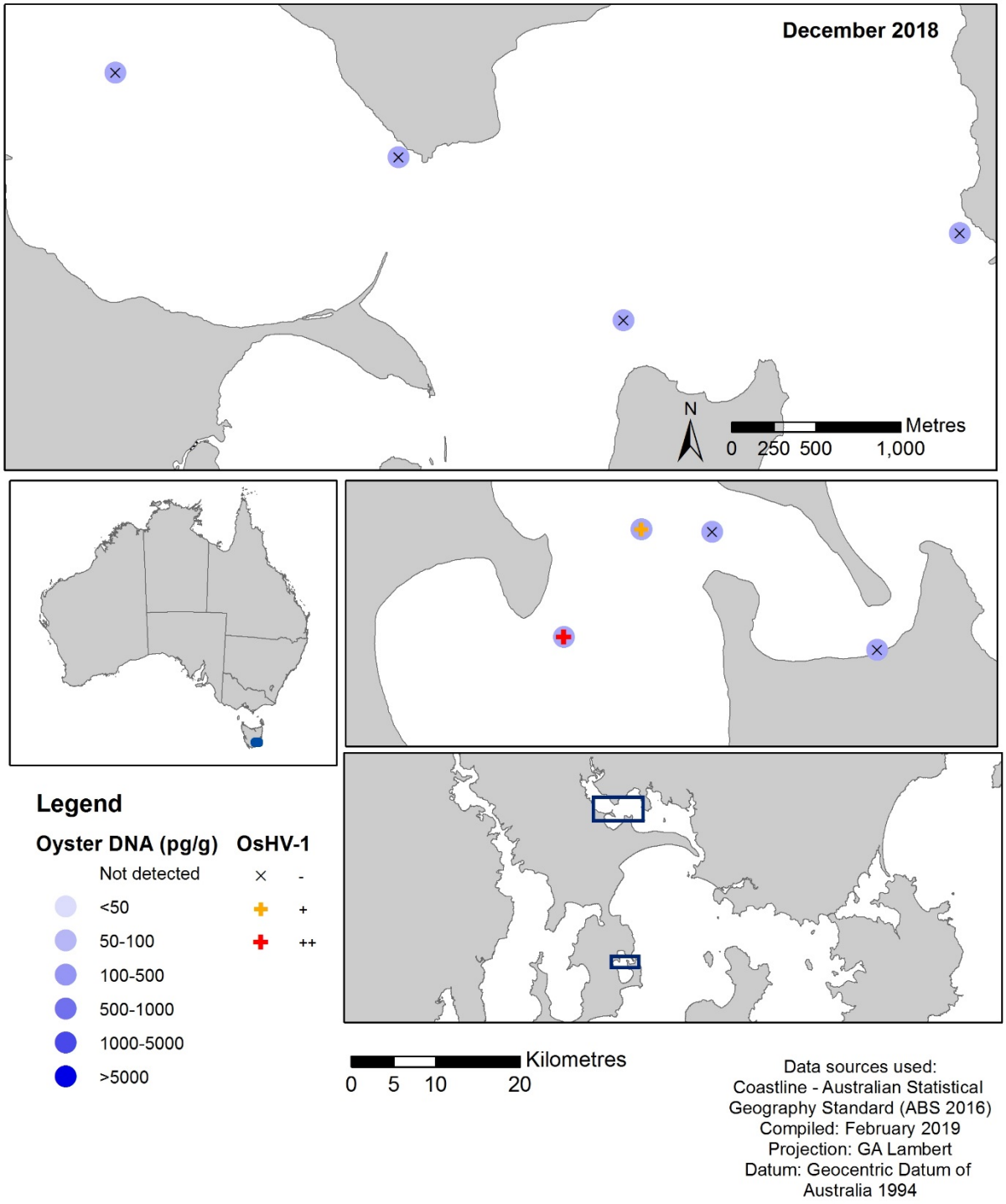


Figure 1.26. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Dec 2018. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

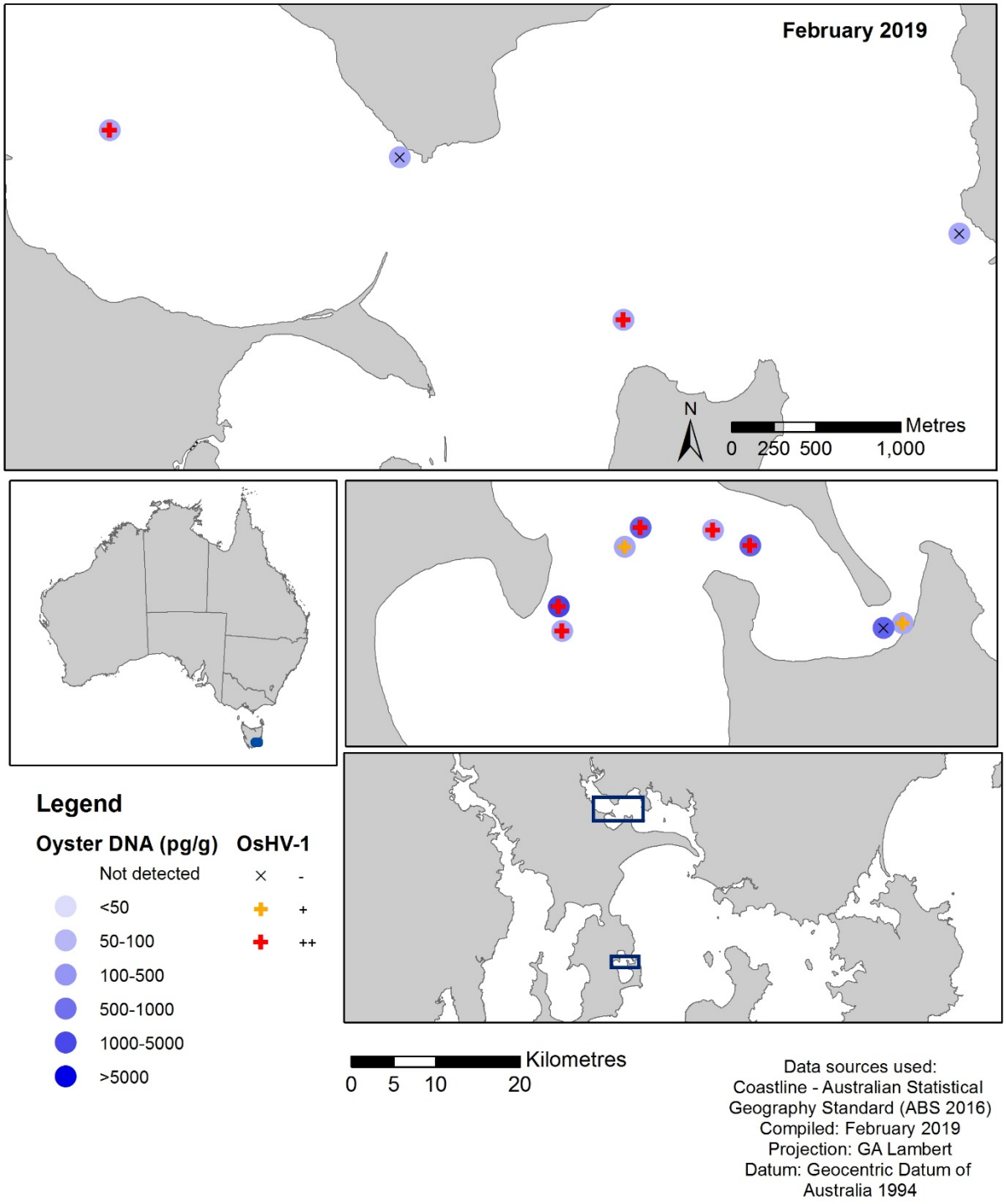


Figure 1.27. Map of plankton OsHV-1 and Oyster DNA testing results for TAS samples from Feb 2019. OsHV-1: (+) detected in 1 of 2 replicates for either test, (++) detected in both replicates for either test.

1.3.3. Diagnostic performance of assays for OsHV-1 applied to plankton

Both the Jenkins and Martenot assays detected OsHV-1 in plankton, but the Martenot assay obtained a greater number of detections (Table 1.6). The latent class models applied to plankton testing results showed that the Martenot assay had greater DSe than the Jenkins assay, while both assays showed high DSp (Table 1.7). There was slight positive covariance between test results for truly negative

samples, but no covariance evident in truly positive samples. Including oyster DNA yield or presence improved the fit of the model, as evidenced by a reduction in the Deviance Information Criteria (DIC) and the 95% confidence interval of the odds ratio for the effect of oyster DNA yield or presence not including 1. Including oyster DNA presence as a covariate led to a greater reduction in DIC than oyster DNA concentration. OsHV-1 occurrence in plankton was positively associated with oyster DNA yield and with oyster DNA presence, but some OsHV-1 detections occurred in samples without detectable oyster DNA (e.g. Figures 5, 10, 21 – 24). While inclusion of oyster DNA yield or presence as a covariate improved model fit, it should be noted that oyster DNA yield and likelihood of occurrence were both greatest around summer, which is also when OsHV-1 was most commonly detected. It is therefore unclear whether OsHV-1 occurrence in plankton is truly associated with occurrence of oyster DNA, or if this result is due primarily to the coincident seasonal occurrence of oyster DNA and OsHV-1. The inclusion of an oyster DNA effect marginally changed DSe and DSp estimates for both tests (Table 1.7). For the best model as assessed by DIC, DSe of the Martenot assay was 0.81 (95% credible interval 0.54 – 0.95) compared with 0.23 (95% credible interval 0.12– 0.38) for the Jenkins assay, while DSp of both assays was 0.96 (95% credible interval 0.92– 0.98 for Martenot assay, 0.93– 0.98 for Jenkins assay).

Table 1.6. Results of OsHV-1 testing of plankton by area, season and assay as used in latent class modelling.

Area	Season	Negative	Martenot only	Jenkins only	Both positive	Apparent prevalence
SA – wild	Summer	65	2	1	1	5.8%
	Autumn	38	3	2	2	15.6%
	Winter	15	0	0	0	0.0%
	Spring	52	1	2	1	7.1%
TAS – farm	Summer	39	16	0	5	35.0%
	Autumn	14	9	1	1	44.0%
	Winter	20	1	0	0	4.8%
	Spring	33	4	1	1	15.4%
TAS – wild	Summer	36	7	1	3	23.4%
	Autumn	25	8	1	3	32.4%
	Winter	25	1	0	0	3.8%
	Spring	27	0	0	0	0.0%

Table 1.7. Diagnostic performance (Dse, DSp) of two OsHV-1 assays applied to plankton samples as assessed by latent class modelling, and comparison of models including oyster DNA yield or oyster DNA presence as a predictor for OsHV-1 occurrence. OR = odds ratio for effect of oyster DNA on OsHV-1 occurrence.

Covariates: Parameter estimates	Area + Season	Area + Season + oyster DNA yield	Area + Season + oyster DNA presence
Jenkins assay			
DSe	0.22 (0.11 – 0.38)	0.23 (0.12 – 0.38)	0.23 (0.12 – 0.38)
DSp	0.95 (0.93 – 0.98)	0.96 (0.93 – 0.98)	0.96 (0.93 – 0.98)
Martenot assay			
DSe	0.82 (0.54 – 0.95)	0.80 (0.55 – 0.95)	0.81 (0.54 – 0.95)
DSp	0.95 (0.91 – 0.98)	0.95 (0.92 – 0.98)	0.96 (0.92 – 0.98)
Covariance in – samples	0.02 (0.00 – 0.05)	0.02 (0.00 – 0.04)	0.02 (0.00 – 0.04)
Covariance in + samples	0.00 (-0.04 – 0.04)	0.00 (-0.05 – 0.04)	0.00 (-0.04 – 0.05)
Oyster DNA effect (OR)	-	1.90 (1.29 – 3.01)	3.14 (1.37 – 7.83)
Model DIC	511	501.7	498.5

The models showed that DSe of both tests was adversely affected by PCR inhibition, as measured by scale factor, with the effect being greater for the Martenot test (Figure 1.28). Credible intervals for the effect of scale factor were wide, however, owing to the relatively low number of samples overall with high inhibition, and the uneven distribution of scale factor across sampling areas and seasons which makes effects of these factors difficult to separate.

Predicted true prevalence from the best model, i.e. including oyster DNA presence as a covariate, is shown in Figure 1.29. Prevalence was predicted to be higher in TAS – Farm areas than SA (odds ratio 4.76 95% credible interval: 2.27 – 10.55) but similar in areas with wild oysters for either state (odds ratio 1.89 95% credible interval: 0.75 – 4.41). Prevalence was similar in autumn to summer (odds ratio 2.04 95% CI: 0.97 – 4.31) but lower in winter (odds ratio 0.25 95% credible interval: 0.06 – 0.82) and spring (odds ratio 0.31 95% credible interval: 0.10 – 0.81). Credible intervals for prevalence predictions were wide due to the relatively low number of data points for each area/season combination and the relatively low DSe of the Jenkins test; prevalence will be most precisely estimated when all tests have high accuracy, thus providing high concordance between test results.

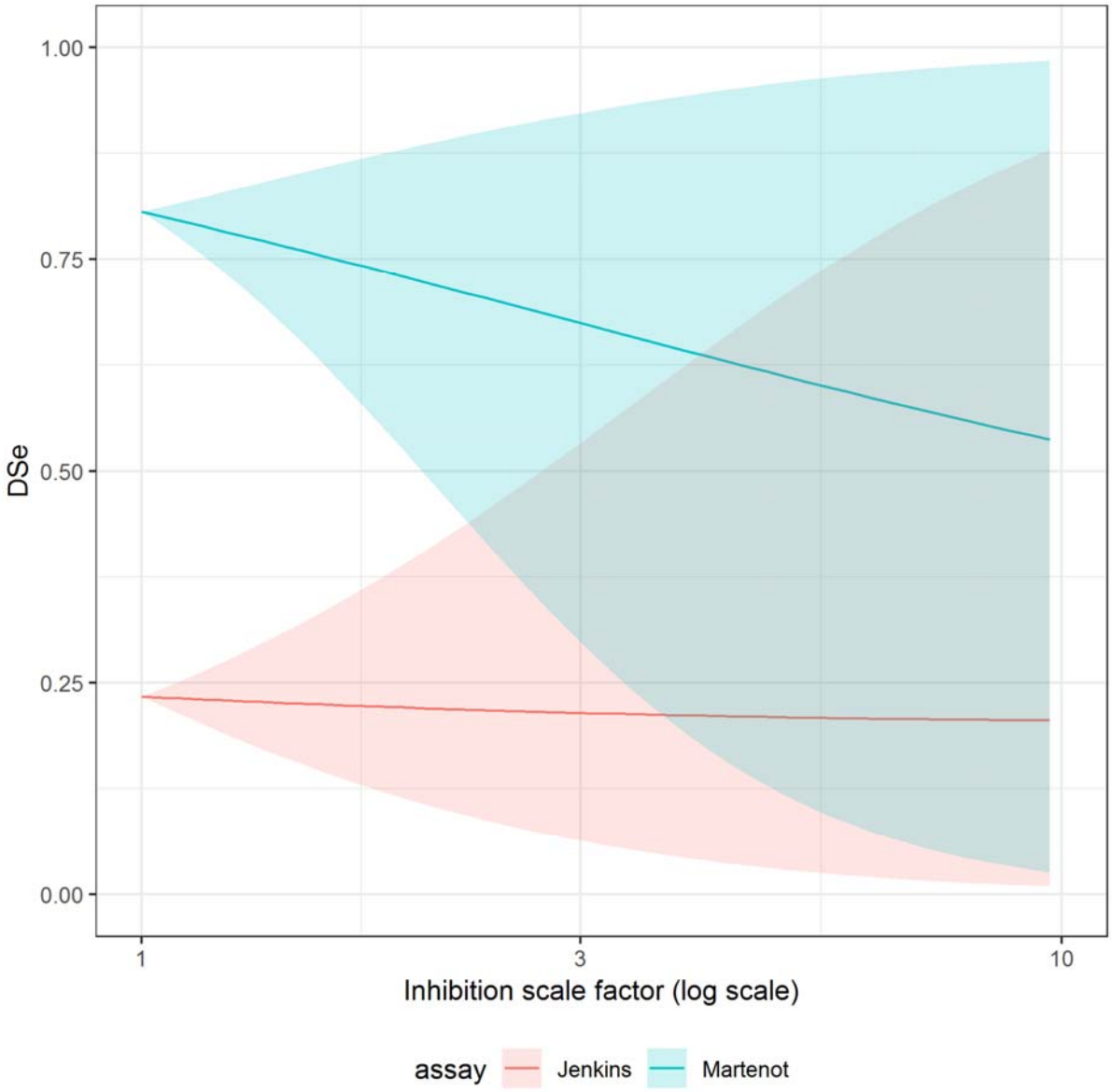


Figure 1.28. Predicted effect of PCR inhibition, as measured by scale factor, on diagnostic sensitivity (DSe) of assays for OsHV-1 applied to plankton samples. Lines show predicted mean and shaded area 95% credible interval.

Based on predicted DSe of the two assays from the best model, the overall DSe of applying both tests and considering a detection in either test as positive is 0.84 (95% credible interval 0.60 – 0.98) and the overall DSp is 0.94 (95% credible interval 0.90 – 0.97). The number of samples required for a survey sensitivity of 0.8 and 0.95 was predicted using the combined test sensitivity over a range of prevalence values (Figure 1.30). Here, prevalence is the proportion of plankton samples that are expected to

contain OsHV-1. The number of samples required decreases rapidly with increasing prevalence, but is higher for the Jenkins than either the Martenot assay or the combination of both assays. In the context of plankton samples, the interpretation of prevalence is somewhat different to the interpretation in animal disease. In animal disease, each sampling unit is typically an individual animal, hence prevalence is the proportion of infected animals. For plankton, however, a continuous medium is being sampled, hence, prevalence will vary depending on sample volume and capture efficiency of the plankton tow method used as well as with the concentration and patchiness of target DNA. Choosing a relevant value of prevalence for detection is therefore difficult. We extracted the number of required samples to detect a prevalence of 7%, this being the minimum mean predicted prevalence for the sampled areas over the summer-autumn period when OsHV-1 was most likely to occur (Table 1.8). If the Martenot assay is applied alone, 28 samples (95% credible interval 24 – 42) are required to detect this prevalence with a survey sensitivity (S_{SE}) of 0.8, or 52 (95% credible interval 44 – 78) for S_{SE} of 0.95. Due to the low DSe of the Jenkins test applied to plankton, there is only a marginal reduction in the number of samples required if both tests are applied as opposed to the Martenot test alone, while applying only the Jenkins test would mean required sample numbers of 98 for S_{SE} of 0.8 and 182 for S_{SE} of 0.95. These predictions are based on samples without PCR inhibition, and a larger number of samples would be needed for equivalent S_{SE} if inhibition was present.

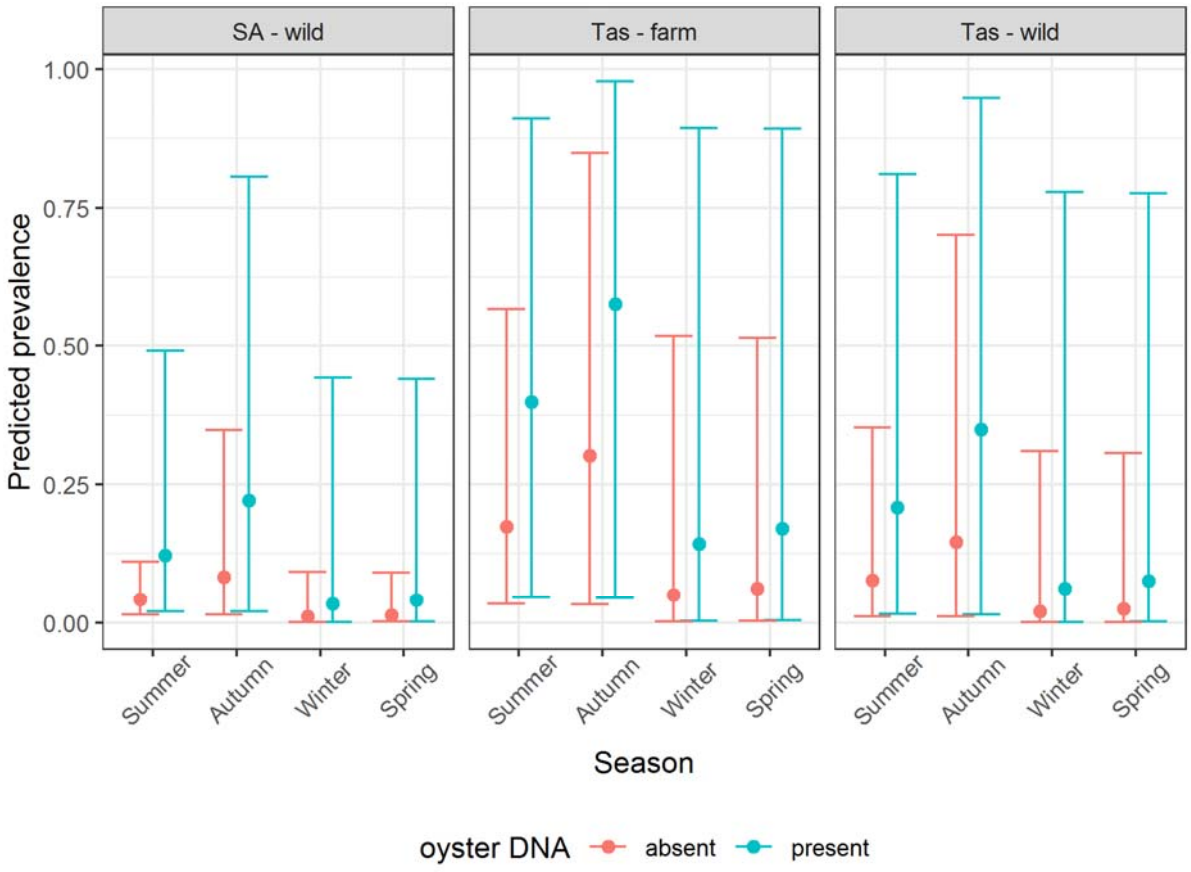


Figure 1.29. Predicted prevalence (proportion of plankton samples with OsHV-1) for samples with and without oyster DNA by area and season. Points show mean and error bars show 95% credible interval from latent class model.

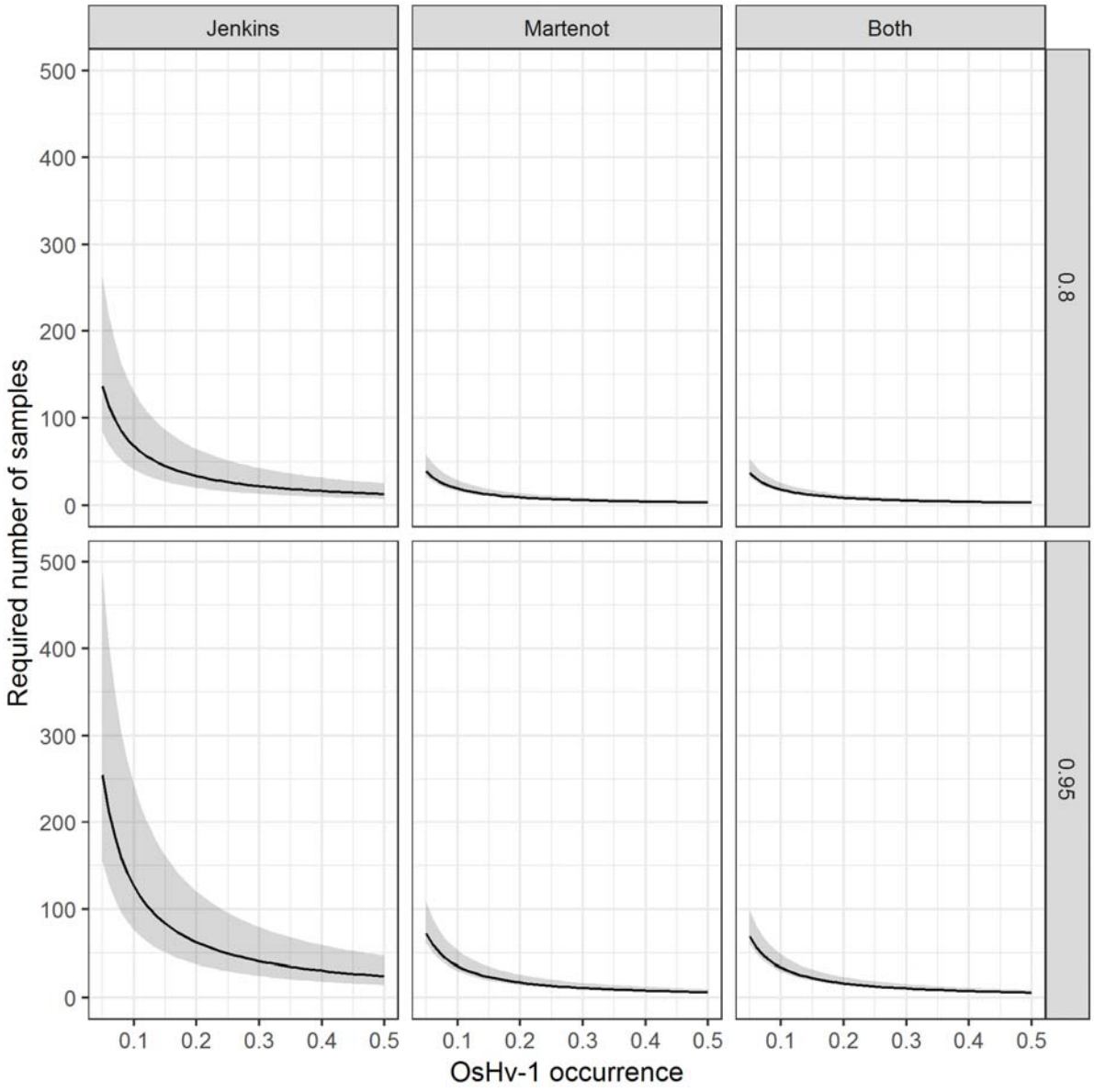


Figure 1.30. Predicted number of samples required to detect OsHV-1 for each assay and for both assays combined for survey sensitivities of 0.8 and 0.95 and a range of OsHV-1 prevalence (= proportion of samples containing OsHV-1 DNA). Lines show mean and shading shows 95% credible intervals.

Table 1.8. Predicted number of plankton samples required to detect OsHV-1 for each assay and for both assays combined for survey sensitivities (SSE) of 0.8 and 0.95 at OsHV-1 prevalence of 7%

Target SSE	Test applied	Number of samples Mean (95% credible interval)
0.8	Jenkins only	98 (60 – 187)
	Martenot only	28 (24 – 42)
	Both	27 (23 – 38)
0.95	Jenkins only	182 (111 – 348)
	Martenot only	52 (44 – 78)
	Both	49 (43 – 71)

1.3.4. OsHV-1 in oyster tissue

In SA, following a mass mortality event observed in wild Pacific Oysters in the Port River in February 2018, OsHV-1 was confirmed in oysters, with an apparent prevalence (number of positive samples out of total tested) of 64% (Table 1.9). OsHV-1 remained detectable in oyster tissue through all subsequent oyster sample sets collected in SA, but with prevalence decreasing from a maximum of 68.8% in March 2018 to a low of 13.0% in October 2018. Prevalence then increased through November – December 2018 to a maximum of 71.9% before declining to 19.4% in January 2019. No mass mortality event was observed concomitant with this increased prevalence in November – December 2018, however, and sampled oysters appeared healthy on collection.

In TAS, OsHV-1 was detected in wild oysters in each sample set between November 2018 and April 2019 with apparent prevalence of between 10.5 and 25% (Table 1.9). There were fewer detections in farmed oysters over the same period, and no OsHV-1 detection in December 2018; over other sample sets prevalence in farmed oysters was between 5.0 and 8.3%

Table 1.9. Results of OsHV-1 testing of oyster tissue samples collected in South Australia (SA) and Tasmania (TAS). See Table 2 for specific sampling dates. OsHV-1 detections classed as (+) detected in one of two replicates for either Jenkins or Martenot test, (++) detected in both replicates for either test. * samples tested using Martenot assay only

Location and date		Total (Wild)			Total (Farm)	
SA	Samples	OsHV-1 detect (+, ++)	Apparent prevalence	Samples	OsHV-1 detect (+, ++)	Apparent prevalence
Feb 2018*	25	1, 15	64.0%		.	.
Mar 2018*	208	28, 115	68.8%		.	.
Apr 2018*	32	0, 16	50.0%		.	.
May 2018*	32	0, 17	53.1%		.	.
Jun 2018	31	1, 11	38.7%		.	.
Aug 2018	32	5, 7	37.5%		.	.
Sep 2018	37	4, 7	29.7%		.	.
Oct 2018	46	2, 4	13.0%		.	.
Nov 2018	33	0, 13	39.4%		.	.
Dec 2018	32	7, 16	71.9%		.	.
Jan 2019	36	1, 6	19.4%		.	.
TAS						
Nov 2018	160	15, 16	19.4%	160	3, 5	5.0%
Dec 2018	36	3, 1	11.1%	36	0, 0	0.0%
Jan 2019	32	2, 6	25.0%	36	1, 1	5.6%
Feb 2019	38	1, 3	10.5%	36	2, 1	8.3%
Apr 2019	37	1, 6	18.9%	37	0, 2	5.4%

1.3.5. Diagnostic performance of assays for OsHV-1 applied to oyster tissue

Detection of OsHV-1 in oyster tissue samples occurred more frequently with the Martenot assay than the Jenkins assay in some sample sets, but the Jenkins assay returned more detections in other sets (Table 1.10). The latent class model showed that the Jenkins assay had higher DSe when applied to oyster tissue than to plankton, with DSe similar between the two assays for oyster tissue testing (Table 1.11). Mean predicted DSe was higher for the Martenot than Jenkins assay, but credible intervals overlapped. As with results in plankton, both assays had high DSp, and there was slight covariance between test results for negative samples, but not for positive samples. The overall DSe of applying

both tests and considering a detection in either test as positive, i.e. the case definition used in this report, is 0.92 (95% credible interval 0.75 – 1.0) and the overall DSp is 0.94 (95% credible interval 0.91 – 0.97).

Table 1.10. Results of OsHV-1 testing of oyster tissue by population, season and assay, with apparent prevalence (= proportion of samples containing OsHV-1 DNA) calculated considering any sample with a detection as positive, and true prevalence estimated from latent class modelling. *Samples tested using the Martenot assay only.

Population and sample time	Negative	Martenot only	Jenkins only	Both positive	Apparent prevalence	Estimated true prevalence
SA						
Feb 2018*	9	16	N/A	N/A	64.0%	0.73 (0.48 – 0.96)
Mar 2018*	65	143	N/A	N/A	68.8%	0.80 (0.67 – 0.97)
Apr 2018*	16	16	N/A	N/A	50.0%	0.58 (0.36 – 0.84)
May 2018*	15	17	N/A	N/A	53.1%	0.61 (0.39 – 0.87)
Jun 2018	19	0	2	10	38.7%	0.32 (0.09 – 0.68)
Aug 2018	20	4	1	7	37.5%	0.31 (0.09 – 0.67)
Sep 2018	26	3	0	8	29.7%	0.25 (0.07 – 0.59)
Oct 2018	40	2	1	3	13.0%	0.09 (0.01 – 0.34)
Nov 2018	20	3	0	10	39.4%	0.35 (0.10 – 0.70)
Dec 2018	9	7	1	15	71.9%	0.66 (0.31 – 0.90)
Jan 2019	29	2	0	5	19.4%	0.13 (0.08 – 0.22)
TAS – Farm						
Nov 2018	153	3	0	4	4.4%	0.03 (0.00 – 0.12)
Dec 2018	36	0	0	0	0.0%	0.04 (0.01 – 0.19)
Jan 2019	34	1	0	1	5.6%	0.05 (0.01 – 0.26)
Feb 2019	33	0	2	1	8.3%	0.05 (0.01 – 0.27)
Apr 2019	35	0	0	2	5.4%	0.05 (0.01 – 0.26)
TAS – Wild						
Nov 2018	129	3	11	17	19.4%	0.13 (0.03 – 0.37)
Dec 2018	35	1	0	0	2.8%	0.04 (0.01 – 0.23)
Jan 2019	26	3	1	2	18.8%	0.12 (0.02 – 0.44)
Feb 2019	35	0	0	3	7.9%	0.07 (0.01 – 0.29)
Apr 2019	31	1	3	2	16.2%	0.09 (0.01 – 0.35)

Table 1.11. Diagnostic performance (DSe, DSp) of two OsHV-1 assays applied to oyster tissue samples as assessed by latent class modelling

Covariates: Parameter estimates	Sample set
Jenkins assay	
DSe	0.72 (0.56 – 0.84)
DSp	0.96 (0.93 – 0.98)
Martenot assay	
DSe	0.85 (0.66 – 0.95)
DSp	0.96 (0.93 – 0.98)
Covariance in – samples	0.02 (0.01 – 0.04)
Covariance in + samples	0.03 (-0.03 – 0.12)

Due to the large number of prevalence values estimated by the latent class model (one for each of 17 sample sets), we did not compare all pairwise odds ratios for predicted prevalences. Rather, we selected October 2018 SA as the reference category for comparison, this sample set having the lowest

predicted prevalence of SA samples, and a similar or higher prevalence than TAS samples. Predicted prevalence was higher in SA in June, August, November and December 2018 than in October 2018, with odds ratios (OR) ranging from 6.3 (95% credible interval: 1.3 – 22) for August 2018, to 28 (95% credible interval: 5.7 – 98) for December 2018, the sample set with greatest prevalence that was included in the model. Note that SA samples from February to May 2018 were not included in the latent class model since these were tested with only the Martenot assay, and predicted prevalence for these sets was not included in this comparison. Prevalence in October 2018 SA samples was similar to that of the remaining SA sample sets and all TAS sample sets, with OR between 0.3 and 4.7, but with 95% credible intervals of the OR including 1 in all cases. For TAS samples, we also compared prevalence OR between wild and farm samples from each month. Prevalence was marginally higher in wild than farmed TAS oysters sampled each month, with OR being between 1.1 and 2.1, but 95% credible intervals of the OR included 1in each case.

The generally higher prevalence in SA than TAS may in part reflect the timing of sample collection, with initial SA samples being collected in 2018 during and immediately after a POMS mortality event. Following that event, which was associated with very high OsHV-1 prevalence, prevalence declined through winter and spring, before increasing again in summer of 2018-2019.

1.4. Discussion

Detection of pathogens from environmental samples has been proposed as an aquaculture health management tool (Stentiford *et al.* 2017) but this study is the first to provide practical demonstration of its utility and some validation. At some sites at risk of pathogen establishment, such as at ports in close proximity to farms, deploying sentinels may create additional real or perceived risk, and at such sites surveying without adding hosts may be beneficial. Environmental sampling is also more efficient and cost effective than sampling wild hosts which may be accessible only at low tide, rare and/or widely spaced, or in areas with limited access for safety and/or security reasons. The environmental approach is also beneficial for testing ballast water and understanding risk by sampling in ballast water uptake or discharge areas (Gollasch *et al.* 2015). Environmental sampling to collect the number of plankton samples (~30) required to detect a prevalence in plankton of <0.1 with survey sensitivity of at least 0.8 can typically be undertaken in <1 day, and samples can be processed rapidly (Wiltshire *et al.* 2019a) while sampling and processing molluscs often takes longer and is more logistically difficult. Confirmatory cPCR and nPCR tests worked with only moderate reliability with environmental samples, however, and understanding specificity of test results for environmental samples could be improved by developing confirmatory tests with enhanced sensitivity.

The principal disadvantage of environmental surveillance is that while it provides an indication of risk, environmental detection does not meet the case definition for infection (Department of Agriculture 2015) and as such is not suitable under the current approach for building evidence for making a self-declaration of freedom pursuant to OIE rules (OIE 2019a). eDNA detection tools indicate the presence of the pathogen but cannot indicate if virions are viable or infective, and there are few data to understand environmental detections of OsHV-1 as they relate to disease or prevalence or intensity in oysters. More intensive and coincident sampling is required to relate environmental pathogen load to prevalence and intensity in hosts, and to interpret risk in the context of the detections. Environmental surveillance, however, is a viable and useful method for area surveillance in commercial growing areas where the main aim is to understand when transmission commences to initiate harvests prior to outbreaks and associated mortality.

The peak release of OsHV-1 coincides with high levels of Pacific Oyster DNA in the environment and summer spawning of Pacific Oysters (Fabioux *et al.* 2005). The energetic cost of spawning may lead to reduced disease resistance in post-spawning oysters (Li *et al.* 2009), and stress is linked to OsHV-1 disease (Burge *et al.* 2006) but the relationship between spawning stress and OsHV-1 viral shedding/transmission has not been investigated. The consistent seasonal pattern of OsHV-1 disease (Whittington *et al.* 2019) and progression of subclinical infection to disease (de Kantzow *et al.* 2017) is likely to reflect a complex interaction of host and environmental factors (de Lorgeril *et al.* 2018). In

Port Adelaide, OsHV-1 detections peaked in plankton in autumn 2018 but this peak occurred during and immediately after the first outbreak and it is unclear if that will be typical for the site. In 2018-19 viral detections in plankton peaked during November with few detections over summer, indicating disease transmission was likely occurring earlier than in summer 2017-18, but a lack of sampling over December 2017 – January 2018 and subsequent to February 2019 means seasonal differences between the two years cannot be confirmed. Better understanding of environmental OsHV-1 transmission and infection would support programs that breed for resistance to the pathogen by facilitating infection experiments. Infection studies would provide a basis for selection of resistant stock and experiments to develop an understanding of the biological basis of resistance in non-susceptible lines. Such knowledge will also be vitally important if changes in oysters as a result of breeding programs create susceptibility to other pathogens or if renewed susceptibility emerges from changes in the host-pathogen system (Bishop and Woolliams 2014).

OsHV-1 detections in plankton generally occurred in samples that were also positive for Pacific Oyster DNA but it is unclear if virus is released in or with gametes, if larvae are infected, or virus is adhering to them. Pacific Oyster larvae can be infected with OsHV-1 (Hwang *et al.* 2013), but the oyster DNA detected in plankton samples is not necessarily from larvae, as other sources of oyster eDNA could also be detected. It is also unclear if diapedesis increases with metabolic rate in summer and virus is being shed directly from oysters with haemocytes and other cells. OsHV-1 is particle associated (Evans *et al.* 2014; Evans *et al.* 2017) but further investigations are required to understand the mechanism, and to which type of particles the virus typically attaches. The plankton net we used has 50 µm mesh and OsHV-1 virions are approximately 120 nm (Toldrà *et al.* 2018), therefore the virus we captured must be particle or larval associated or it would probably be lost through the net. *In situ* hybridisation of sections of mature pre-spawning oysters (Bueno *et al.* 2016), larvae and of centrifuge pelleted plankton could provide information on where in the environment OsHV-1 occurs and if vertical transmission is of concern for oyster biosecurity.

Understanding DSe and DSp for assays as applied to environmental samples facilitates designing surveys based on these techniques (OIE 2019b). The divergence in results between tests on plankton and oyster tissue imply that these assays, which were not designed for this use, may require further optimisation for environmental samples. Plankton sample numbers to achieve a target survey sensitivity are only marginally higher if only the Martenot test is used because the Jenkins test performed poorly on environmental samples. This indicates that the Martenot assay can be used alone on duplicate samples to achieve good survey sensitivity without the additional cost of testing all samples using multiple tests. It is not clear, however, how much test-to-test variability exists, and how much it is affected by sample DNA homogeneity or target concentration. Assessing this test-to-test variation meaningfully would require very large data set and, preferably, an additional independent test.

The DSe and DSp for the assays when applied to oyster tissue were substantially higher than when applied to plankton, particularly the Jenkins test. This indicates that the available detection systems for OsHV-1 in oyster tissue are excellent. The DSe and DSp we estimated can be used to design surveys and provide confidence about evidence of absence. Use of both tests provides improved DSe compared to a single test, which further highlights the difference in performance for the Jenkins test when applied to plankton and oyster tissue. It remains unclear, however, if the optimal method for assessing the status of an individual oyster is to take 4 DNA subsamples from a single extract and test them as 2x replicates of the Martenot test and 2x replicates of the Jenkins test as opposed to testing 4x subsamples using the Martenot test, which is marginally more sensitive. To resolve this question, an understanding of autocorrelation is required, estimating this would require more data and preferably another independent test applied for comparison.

Seasonal patterns of OsHV-1 prevalence, intensity and disease in SA are not firmly understood because the timing of the first POMS mortality event may not be typical and was probably driven more by establishment and initial spread of infection (Hatcher *et al.* 2012) rather than the factors that influence seasonality where OsHV-1 is established. In summer 2018-19 OsHV-1 prevalence peaked in

oysters in December 2018 and decreased in January. There was no clear mortality event in summer 2018-19; mortality was difficult to assess in wild oysters in this period because empty shells remained from earlier mortalities, including knock down activities and the 2017-18 POMS event. Sentinels deployed between January and April 2019 incurred up to 50% mortality (SARDI unpublished data) but it is likely that surveillance over several summers would be required to understand the temporal expression of disease (Whittington *et al.* 2019).

In Tasmania, prevalence of OsHV-1 in oysters was low. It is unclear if this is because an increasing proportion of stock in Tasmania are from resistant family lines (Matthew Cunningham, Australian Seafood Industries, personal communication, August 2019) which do not develop disease and can be refractory to infection (de Lorgeril *et al.* 2018; Martenot *et al.* 2019), or if it is due to immune priming (Green and Speck 2018). Our surveillance data support that the disease appears later in the season in Tasmania than it does in mainland Australia (Ugalde *et al.* 2018), with disease outbreaks most likely in January or February.

Overall, the oyster surveillance data supports that OsHV-1 infections remain in a proportion of oysters year-round, and that as the water temperature rises in spring the prevalence and viral load increase until mortalities commence in summer. There is strong evidence that prevalence and viral load reflect complicated influences of extrinsic factors and innate aspects of the host-pathogen system which vary between localities and populations of hosts.

1.5. References

Arzul I, Renault T, Lipart C, Davison AJ (2001) Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *Journal of general virology* 82, 865-870.

Bass D, Stentiford GD, Littlewood DTJ, Hartikainen H (2015) Diverse Applications of Environmental DNA Methods in Parasitology. *Trends in Parasitology* 31, 499-513.

Bishop S, Woolliams JA (2014) Genomics and disease resistance Studies in livestock. *Livestock Science* 166.

Bott NJ, Giblot-Ducray D (2011a) Molecular tools for detection of marine pests: Development of diagnostic PCR assays for the detection of significant marine pests: *Carcinus maenas, Ciona intestinalis* and *Undaria pinnatifida*. Report prepared for Adelaide & Mt Lofty Ranges Natural Resources Management Board. South Australian Research and Development Institute (Aquatic Sciences), SARDI Publication No. F2011/000503-1. SARDI Research Report Series No. 587, Adelaide.

Bott NJ, Giblot-Ducray D (2011b) Molecular tools for detection of marine pests: *Musculista senhousia, Corbula gibba* and *Perna canaliculus* quantitative PCR assays. Report prepared for Biosecurity SA. . South Australian Research and Development Institute (Aquatic Sciences), SARDI Publication No. F2010/000991-1. SARDI Research Report Series No. 522, Adelaide.

Bott NJ, Giblot-Ducray D (2012) Development of a qPCR assay for the Pacific Oyster, *Crassostrea gigas.* Report prepared for Biosecurity SA. . South Australian Research and Development Institute (Aquatic Sciences), SARDI Publication No. F2012/000210-1. SARDI Research Report Series No. 641, Adelaide.

Bott NJ, Giblot-Ducray D, Deveney MR (2010) Molecular tools for detection of marine pests: Development of putative diagnostic PCR assays for the detection of significant marine pests: *Asterias amurensis, Carcinus maenas, Undaria pinnatifida* and *Ciona intestinalis*. South Australian Research and Development Institute (Aquatic Sciences), SARDI Publication No. F2010/000669-1. SARDI Research Report Series No. 509, Adelaide.

Bueno R, Perrott M, Dunowska M, Brosnahan C, Johnston C (2016) In situ hybridization and histopathological observations during ostreid herpesvirus-1-associated mortalities in Pacific Oysters *Crassostrea gigas*. *Diseases of Aquatic Organisms* 122, 43-55.

Burge C, J Griffin F, Friedman C (2006) Mortality and herpesvirus infections of the Pacific Oyster *Crassostrea gigas* in Tomales Bay, California, USA. *Diseases of aquatic organisms* 72, 31-43.

Darling J, Blum M (2007) DNA-Based Methods for Monitoring Invasive Species: A Review and Prospectus. *Biological Invasions* 9, 751-765.

de Kantzow MC, Hick PM, Dhand NK, Whittington RJ (2017) Risk factors for mortality during the first occurrence of Pacific Oyster Mortality Syndrome due to Ostreid herpesvirus – 1 in Tasmania, 2016. *Aquaculture* 468, 328-336.

de Lorgeril J, Lucasson A, Petton B, Toulza E, Montagnani C, Clerissi C, Vidal-Dupiol J, Chaparro C, Galinier R, Escoubas J-M, Haffner P, Dégremont L, Charrière GM, Lafont M, Delort A, Vergnes A, Chiarello M, Faury N, Rubio T, Leroy MA, Pérignon A, Régler D, Morga B, Alunno-Bruscia M, Boudry P, Le Roux F, Destoumieux-Garzón D, Gueguen Y, Mitta G (2018) Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific Oysters. *Nature Communications* 9, 4215.

Department of Agriculture (2015) AQUAVETPLAN Disease Strategy: Infection with ostreid herpesvirus-1 microvariant (Version [1]). In 'Australian Aquatic Veterinary Emergency Plan (AQUAVETPLAN)'. (Australian Government Department of Agriculture: Canberra, ACT)

Deveney MR, Giblot-Ducray D, Wiltshire KH (2017) Marine pests testing methods:contributing to establishment of an Australian Testing Centre for Marine Pests. SARDI Aquatic Sciences, SARDI Publication No. F2017/000273-1. SARDI Research Report Series No. 963, Adelaide.

Devleesschauwer B, Torgerson PR, Charlier J, Levecke B, Praet N, Roelandt S, Smit S, Dorny P, Berkvens D, Speybroeck N (2014) prevalence: Tools for prevalence assessment studies. R package version 0.4.0.

Díaz-Ferguson EE, Moyer GR (2014) History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments *Revista de Biología Tropical* 62, 1273-1284.

Evans O, Hick P, Alford B, Whittington RJ (2017) Transmission of Ostreid herpesvirus-1 microvariant in seawater: Detection of viral DNA in seawater, filter retentates, filter membranes and sentinel *Crassostrea gigas* spat in upwellers. *Aquaculture* 473, 456-467.

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

Fabioux C, Huvet A, Le Souchu P, Le Pennec M, Pouvreau S (2005) Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. *Aquaculture* 250, 458-470.

Gardner IA, Stryhn H, Lind P, Collins MT (2000) Conditional dependence between tests affects the diagnosis and surveillance of animal diseases. *Preventive Veterinary Medicine* 45, 107-122.

Giblot-Ducray D, Bott NJ (2013) Development of a plankton sampling protocol for molecular testing of marine pests. Report prepared for Biosecurity SA. . South Australian Research and Development Institute (Aquatic Sciences), SARDI Publication No. F2013/000281-1. SARDI Research Report Series No. 734, Adelaide.

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF, McKee A, Oyler-McCance SJ, Cornman RS (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution* 7, 1299-1307.

Gollasch S, Minchin D, David M (2015) The Transfer of Harmful Aquatic Organisms and Pathogens with Ballast Water and Their Impacts. In 'Global Maritime Transport and Ballast Water Management: Issues and Solutions'. (Eds M David and S Gollasch) pp. 35-58. (Springer Netherlands: Dordrecht)

Green JT, Speck P (2018) Antiviral Defense and Innate Immune Memory in the Oyster. *Viruses* 10.

Hatcher MJ, Dick JTA, Dunn AM (2012) Disease emergence and invasions. *Functional Ecology* 26, 1275-1287.

Huver JR, Koprivnikar J, Johnson PTJ, Whyard S (2015) Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. *Ecological Applications* 25, 991-1002.

Hwang JY, Park JJ, Yu HJ, Hur YB, Arzul I, Couraleau Y, Park MA (2013) Ostreid herpesvirus 1 infection in farmed Pacific Oyster larvae *Crassostrea gigas* (Thunberg) in Korea. *Journal of Fish Diseases* 36, 969-972.

Jenkins C, Hick P, Gabor M, Spiers Z, Fell S, Gu X, Read A, Go J, Dove M, O'Connor W, D Kirkland P, Frances J (2013) Identification and characterisation of an Ostreid herpesvirus-1 microvariant (OsHV-1 μVar) in *Crassostrea gigas* (Pacific Oysters) in Australia. *Diseases of aquatic organisms* 105, 109-26.

Li Y, Qin J, Li X, Benkendorff K (2009) Spawning-dependent stress responses in Pacific Oysters *Crassostrea gigas*. *Aquaculture* 293, 164-171.

Martenot C, Faury N, Morga B, Degremont L, Lamy J-B, Houssin M, Renault T (2019) Exploring First Interactions Between Ostreid Herpesvirus 1 (OsHV-1) and Its Host, *Crassostrea gigas*: Effects of Specific Antiviral Antibodies and Dextran Sulfate. 10.

Martenot C, Oden E, Travaillé E, Malas JP, Houssin M (2010) Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific Oyster *Crassostrea gigas*. *Journal of Virological Methods* 170, 86-89.

Ninove L, Nougairede A, Gazin C, Thirion L, Delogu I, Zandotti C, Charrel RN, De Lamballerie X (2011) RNA and DNA Bacteriophages as Molecular Diagnosis Controls in Clinical Virology: A Comprehensive Study of More than 45,000 Routine PCR Tests. *PLOS ONE* 6, e16142.

NSW DPI (2016) Pacific Oyster Mortality Syndrome (POMS) Accessed: 7 Aug 2019. URL: https://www.dpi.nsw.gov.au/fishing/pests-diseases/animal-health/aquaculture/poms

OIE (2019a) Infection with ostreid herpesvirus 1 microvariants. In 'Manual of Diagnostic Tests for Aquatic Animals'. (World Organisation for Animal Health: Paris)

OIE (2019b) Principles and methods of validation of diagnostic assays for infectious diseases. In 'Manual of Diagnostic Tests for Aquatic Animals'. (World Organisation for Animal Health: Paris)

Ophel-Keller K, Loo M, Bott N, McKay A (2007) Development of gene probes for introduced marine pest species (*Musculista senhousia, Corbula gibba, Sabella spallanzanii*). A report for Natural Heritage Trust. South Australian Research and Development Institute, Adelaide.

Paul-Pont I, Dhand NK, Whittington RJ (2013) Spatial distribution of mortality in Pacific Oysters *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission. *Diseases of Aquatic Organisms* 105, 127-138.

Paul-Pont I, Evans O, Dhand NK, Rubio A, Coad P, Whittington RJ (2014) Descriptive epidemiology of mass mortality due to Ostreid herpesvirus-1 (OsHV-1) in commercially farmed Pacific Oysters (*Crassostrea gigas*) in the Hawkesbury River estuary, Australia. *Aquaculture* 422-423, 146-159.

Peters L, Spatharis S, Dario MA, Dwyer T, Roca IJT, Kintner A, Kanstad-Hanssen Ø, Llewellyn MS, Praebel K (2018) Environmental DNA: A New Low-Cost Monitoring Tool for Pathogens in Salmonid Aquaculture. 9.

Rahman AKMA, Smit S, Devleesschauwer B, Kostoulas P, Abatih E, Saegerman C, Shamsuddin M, Berkvens D, Dhand NK, Ward MP (2019) Bayesian evaluation of three serological tests for the diagnosis of bovine brucellosis in Bangladesh. *Epidemiology and Infection* 147, e73.

Renault T, Arzul I, Lipart C (2004) Development and use of an internal standard for oyster herpesvirus 1 detection by PCR. *Journal of Virological Methods* 121, 17-23.

Sana S, Williams C, Hardouin EA, Blake A, Davison P, Pegg J, Paley R, Zhang T, Andreou D (2018) Phylogenetic and environmental DNA insights into emerging aquatic parasites: implications for risk management. *International Journal for Parasitology* 48, 473-481.

Segarra A, Pépin JF, Arzul I, Morga B, Faury N, Renault T (2010) Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific Oysters, *Crassostrea gigas*, in France in 2008. *Virus Research* 153, 92-99.

Stentiford GD, Sritunyalucksana K, Flegel TW, Williams BAP, Withyachumnarnkul B, Itsathitphaisarn O, Bass D (2017) New Paradigms to Help Solve the Global Aquaculture Disease Crisis. *PLOS Pathogens* 13, e1006160.

Thomsen PF, Willerslev E (2015) Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* 183, 4-18.

Toldrà A, Andree KB, Bertomeu E, Roque A, Carrasco N, Gairín I, Furones MD, Campàs M (2018) Rapid capture and detection of ostreid herpesvirus-1 from Pacific Oyster *Crassostrea gigas* and seawater using magnetic beads. *PLOS ONE* 13, e0205207.

Ugalde SC, Preston J, Ogier E, Crawford C (2018) Analysis of farm management strategies following herpesvirus (OsHV-1) disease outbreaks in Pacific Oysters in Tasmania, Australia. *Aquaculture* 495, 179-186.

Whittington RJ, Liu O, Hick PM, Dhand N, Rubio A (2019) Long-term temporal and spatial patterns of Ostreid herpesvirus 1 (OsHV-1) infection and mortality in sentinel Pacific Oyster spat (*Crassostrea gigas*) inform farm management. *Aquaculture* 513, 734395.

Wiltshire KH, Deveney MR (2011) Final monitoring report for the 2010-2011 marine pest survey of the Port of Adelaide, South Australia. South Australian Research and Development Institute (Aquatic Sciences), SARDI Publication No. F2011/000386-1. SARDI Research Report Seres No. 575, Adelaide.

Wiltshire KH, Giblot-Ducray D, Prowse T, Deveney MR (2019a) Validation of molecular surveillance: parallel traditional and molecular surveys for priority marine pests in the ports of Gladstone, Brisbane, Melbourne and Hobart. Report to Department of Agriculture and Water Resources. South Australian Research and Development Institute (Aquatic Sciences), Adelaide, SARDI Publication No. F2019/000040-1. SARDI Research Report Series No. 1019. .

Wiltshire KH, Prowse T, Buss JJ, Giblot-Ducray D, Lieu YN, Deveney MR (2019b) Diagnostic performance of molecular tests used to detection priority marine pests in plankton samples. South Australian Research and Development Institute (Aquatic Sciences), Adelaide.

1.6. Appendix

1.6.1. JAGS code for latent class models

```
model{
  ## Priors
  ## Sensitivities and specificities
  Sp1 ~ dbeta(16,4)
  Sp2 ~ dbeta(16,4)
  for (j in 1:2){
   alpha[j] ~ dnorm(1.3863,1) # Intercept: Expected Se with no inhibition
  }
  for(j in 1:4){
   beta[j] ~ dnorm(0,1) # Slope: inhibition (log scale factor) effect
  }
  ## Prevalence
  for (k in 1:K) {
    b_prev[k] ~ dnorm(0,1) #Prevalence covariate parameters
  }
  ## Correlation
  max.lp12 <- max(lp12[])
  min.up12 <- min(up12[])
  covp12 ~ dunif(max.lp12,min.up12)

  ln12 <- max(-(1-Sp1)*(1-Sp2), -Sp1*Sp2)
  un12 <- min(Sp1*(1-Sp2),(1-Sp1)*Sp2)
  covn12 ~ dunif(ln12,un12)

  ## Probabilities for each different result
  for (i in 1:n) {

   y[i,1:4] ~ dmulti(p[i,1:4],1)

   logit(Se1[i]) <- alpha[1] + beta[1]*lnSF[i] + beta[2]*lnSF[i]*lnSF[i]
   logit(Se2[i]) <- alpha[2] + beta[3]*lnSF[i] + beta[4]*lnSF[i]*lnSF[i]
   logit(pi[i]) <- inprod(b_prev[],X[i,])

   p[i,1] <- pi[i]*((1-Se1[i])*(1-Se2[i])+covp12) + (1-pi[i])*((Sp1)*(Sp2)+covn12) ## 0, 0
   p[i,2] <- pi[i]*((1-Se1[i])*(Se2[i])-covp12) + (1-pi[i])*((Sp1)*(1-Sp2)-covn12) ## 0, 1
   p[i,3] <- pi[i]*((Se1[i])*(1-Se2[i])-covp12) + (1-pi[i])*((1-Sp1)*(Sp2)-covn12) ## 1, 0
   p[i,4] <- pi[i]*((Se1[i])*(Se2[i])+covp12) + (1-pi[i])*((1-Sp1)*(1-Sp2)+covn12) ## 1, 1

   ## Set limits on covariances

   lp12[i] <- max(-(1-Se1[i])*(1-Se2[i]), -Se1[i]*Se1[i])
   up12[i] <- min(Se1[i]*(1-Se2[i]),(1-Se1[i])*Se2[i])


  }
}
```

Chapter 2 Development of a rapid method for POMS detection and enumeration

Prof James G. Mitchell and Dr James S. Paterson

Summary

The Ostreid herpesvirus (OsHV-1) infects the Pacific Oyster, *Crassostrea gigas* and causes Pacific Oyster Mortality Syndrome (POMS), a disease characterised by high, rapid mortality. This project aimed to detect and enumerate OsHV-1 using flow cytometry, by Flinders University in collaboration with the South Australian Research Development Institute (SARDI) Aquatic Sciences. This is the first time that OsHV-1 has been detected and enumerated using flow cytometry with virus specific fluorescent probes. The project successfully developed an OsHV-1 test that is rapid, inexpensive and quantitative that can be used on Pacific Oyster farms or hatcheries to assist in monitoring OsHV-1 and to improve preventative measures where the virus is present.

Detection of OsHV-1 is predominantly by quantitative polymerase chain reaction (qPCR) assays. The sensitivity of qPCR facilitates amplification of OsHV-1 DNA from low concentrations of the virus; qPCR can, however, be time consuming and expensive for Pacific Oyster farms and hatcheries to implement in routine monitoring for the virus. This project aimed to develop a rapid, low cost and quantitative test for OsHV-1. Further aims were to establish this test in strategically located testing centres to support an industry wide monitoring program. This could allow continued monitoring for OsHV-1 during critical times at lower cost and with faster response capacity.

The flow cytometry test for OsHV-1 was developed using an off the shelf entry level flow cytometer and targeted seawater and/or oyster tissue test matrices. The Merck Muse flow cytometer (Muse) was used as the development platform for this test after successful testing of seawater and using specific tests for control viruses using adjustments to factory settings and the selection of suitable fluorescent probes.

Analytical sensitivity of the Muse cytometer was determined by analysing seawater samples for viral and bacterial abundance. This was achieved and both groups of microorganisms could be detected and enumerated. To further test the reliability of the output from the Muse, a comparison of cytometers was conducted with seawater samples. There were no significant differences in viral abundance in results from three cytometers, while measured bacterial abundance was greater on the Muse cytometer.

Further testing of the Muse cytometer was conducted by analysing pure cultures of two viruses of similar size to OsHV-1: *Dengue virus* and *Influenza A virus*. These viruses were successfully detected and enumerated using generic nucleic acid fluorescent stains. Samples of *Dengue virus* were then used for initial testing of the capacity of the Molecular Beacons and primer specific fluorescent probes we developed. *Dengue virus* was successfully detected and enumerated using Molecular Beacons on the Muse cytometer.

An OsHV-1 specific assay was designed and detection and enumeration of OsHV-1 was successfully achieved on the Muse cytometer using the assay. Initial analyses were conducted on OsHV-1 samples fixed in glutaraldehyde. To determine the validity of the developed test in a field setting, fresh samples of oyster tissue infected with OsHV-1 were then analysed in a PC-2 laboratory. OsHV-1 in these samples was successfully detected and enumerated.

Oyster tissue samples were then tested to validate the developed test on oyster tissues obtained from a SARDI Aquatic Sciences infection study. After mastication of the oyster tissue, samples were analysed on the Muse flow cytometer and detection of the virus ranged in concentration between oyster individuals. Concentrations of OsHV-1 ranged from 7.00 10^2 per ml^{-1} to 2.93×10^7 per ml^{-1}. The lower analytical detection limit of the test, furthermore, was 14 OsHV-1 particles per ml^{-1}.

The project developed technology for rapid detection and quantitation of OsHV-1. Government agencies and growers now have a rapid method for detecting OsHV-1 to 14 OsHV-1 particles per mL in seawater. The portability and ruggedness of the Muse flow cytometer means that operation and OsHV-1 detection can occur in the field. Testing costs approximately $2 per sample and preparation time is approximately 1-hour, with sequential samples able to be tested approximately 3 minutes apart. Changes in OsHV-1 concentration can therefore be tracked over the course of a day. This project has also laid the groundwork for the development of rapid detection methods for other aquaculture pathogens.

Following on from this project priorities include: running a series of workshops for growers and hatchery operators to demonstrate the method; a trial program using a lend cytometer; provision of an acquisition, training, set up and calibration service for those that wish to adopt the assay; discussing implementing the assay at a field relevant laboratory; and consideration of developing this technique for other aquaculture pathogens.

2.1. Introduction

Ostreid herpesvirus 1 (OsHV-1) causes disease outbreaks in the Pacific Oyster, *Crassostrea gigas* characterised by rapid onset of high mortality. OsHV-1 mortalities were first observed in France in 2008 and OsHV-1 related mortalities have subsequently been observed in Europe, USA, New Zealand and Australia (Burge, *et al*., 2006; Segara, *et al*., 2010; Keeling, *et al*., 2014; de Kantzow, *et al*., 2017). In Australia the virus and disease were first observed in November 2010 in an outbreak that caused almost 100% of farmed and wild oysters in the Georges River in New South Wales. OsHV-1 related mortality events have occurred in Tasmania since 2016 with substantial impacts on *C. gigas* production and domestic market access for Tasmanian produced oyster spat. OsHV-1 was detected in feral Pacific Oysters in the Port River, South Australia in November 2017 but the South Australian industry remains free of the virus and the disease it causes.

Detection of OsHV-1 is predominantly by quantitative polymerase chain reaction (qPCR) assays. The sensitivity of qPCR allows amplification of OsHV-1 DNA even from samples containing low concentrations of the virus. qPCR is, however, time consuming and expensive for farms and hatcheries to implement as routine monitoring tools. Samples of *C. gigas* are supplied as tissue, and extraction methods can lose up to 70% of viral DNA from tissue samples. Another method used to detect OsHV-1 is *in situ* hybridisation (ISH) where fixed histological sections of *C. gigas* tissue are treated with a fluorescent probe to identify the virus by microscopy. This technique is time consuming and requires a suitable laboratory and qualified staff. A quicker, inexpensive method to detect OsHV-1 is essential to the future farming and hatchery production of *C. gigas* globally, so that farmers and hatcheries can monitor OsHV-1 in close to real time facilitating very rapid responses to detection of the virus.

Aquatic microbes have been routinely enumerated by flow cytometry for more than 20 years (Li, *et al*., 1995; Marie, *et al*., 1997; Lebaron, *et al*., 2002; Gasol, *et al*., 2009; Paterson, *et al*., 2013) and marine viruses have been enumerated using flow cytometry extensively since 1999 (Marie, *et al*., 1999; Brussaard, 2004; Seymour, *et al*., 2008; Brussaard, *et al*., 2010). The technique is rapid, quantitative and inexpensive compared to other methods. Flow cytometry often uses nucleic acid fluorescent stains that targ*et* all viruses within a sample. Using this method it is impossible to determine the presence or abundance of a specific virus. Organism specific fluorescent probes, however, provide the potential to test for only one or a group of target organisms. Thi approach uses a fluorescent labelled DNA probe for detection and enumeration of a specific target in an environmental sample containing many different species of bacteria and viruses. We aimed to adapt this approach to testing for OsHV-1.

The aim of this project was to develop a rapid, low cost, quantitative test for OsHV-1. This test will support an industry wide monitoring and rapid response testing program. This will allow continued surveillance for OsHV-1 in relevant areas at critical times, particularly in summer, without the need for sending samples to laboratories for qPCR analysis.

2.1.1. Objectives

The objectives of the original project were as follows:

1. Create a POMS specific fluorophore probe
2. Obtain a flow cytometry signal from the fluorophore probe
3. Create standard protocols for POMS detection
4. Create standard protocols for POMS enumeration
5. Establish guidelines for interpretation of data

6. Work with industry/government to identify appropriate commercialisation models

2.2. Methods

2.2.1. Enumeration of viruses and heterotrophic bacteria in seawater

Viruses and heterotrophic bacteria were enumerated using a Muse flow cytometer (Merck Millipore). Seawater was collected in triplicate 1 mL samples from Glenelg Beach, South Australia and fixed with glutaraldehyde (0.5% final concentration), incubated on ice for 15 minutes, then quick frozen in liquid nitrogen and stored at -80°C prior to analysis (Marie, *et al*., 1997; Brussaard, 2004). Prior to analysis, samples were thawed and diluted 1:10 with 0.02 µm filtered TE buffer (10 mM Tris, 1 mM EDTA), then stained with SYBR-I Green solution (1:20,000 final dilution; Molecular Probes) and finally incubated in the dark at 80ºC for 10 min before analysis (Marie, *et al*., 1997; Lebaron, *et al*., 2001; Brussaard, 2004). Yellow and orange fluorescence and forward-angle light scatter (FSC) were recorded for each sample. To further optimise the enumeration of viruses and heterotrophic bacteria on the Muse flow cytometer, samples were stained with SYTO Orange 81 (Molecular Probes) fluorescent stain for optimal fluorescence excitation and emission for the Merck Muse flow cytometer. Samples were prepared and analysed using the same method as described above for samples run with SYBR-I Green.

2.2.2. Detection and enumeration of two viral species

Two viruses available as pure viral suspensions purified from cell culture, *Dengue virus* (DENV) and *Influenza A virus (H3N2)* were obtained to test the detection and enumeration sensitivity of the Muse cytometer. Both viruses were supplied at viral titres of approximately 10^7 per mL. Samples were supplied fixed in glutaraldehyde (0.5% final concentration), snap frozen in liquid nitrogen and stored at -80°C until analysis. Samples of both viruses were analysed with the Muse cytometer using the same method as described for the non-specific analysis of seawater samples.

2.2.3. Development of a POMS specific fluorophore

Fluorescent probes specific for OsHV-1 and *Dengue virus* were constructed using the Sigma-Aldrich Australia online tool. Molecular Beacons were developed for each virus that allowed sequence specific and sensitive detection. For OsHV-1, the primers C2F (CTCTTTACCATGAAGATACCCACC) and C6R (GTGCACGGCTTACCATTTTT) were used (Arzul, *et al*., 2001), with the attachment of a HEX fluorophore and BHQ-1 quencher. For *Dengue virus*, the primers DENV5.1F (GCAGATCTCTGATGAATAACCAAC) and DENV3.2R (TTGTCAGCTGTTGTACAGTCG) with the attachment of a HEX fluorophore and a BHQ-1 quencher were used. The BHQ-1 molecule was used to quench the fluorescence of the HEX fluorophore until the attachment of the primer sequence occurred in the reaction.

2.2.4. Source of OsHV-1 virus

Initial OsHV-1 samples were obtained from the Animal Health Laboratory, Department of Primary Industries, Parks, Water and Environment, Tasmania, Australia. Virus was purified by filtration from infected oyster tissue and suspended in filtered autoclaved seawater. These samples were snap frozen in liquid nitrogen and transported at -80°C. Further oyster tissue samples were obtained from the South Australian Research Development Institute (SARDI) from an infection study and field samples that were snap frozen and shipped to Flinders University at -80°C. Tissue samples of approximately 2 mm x 3 mm were cut from the oyster and placed in a microfuge tube with 500 µl of filtered and sterilised TE Buffer (10 mM Tris, 1 mM EDTA). The sample was then mechanically macerated for 5 min then centrifuged for 1 min at 6000 rpm. The supernatant containing OsHV-1 was separated and filtered through a 0.22 µm syringe filter and collected in a sterilized microfuge tube, snap frozen in liquid nitrogen and stored at -80°C until analysis.

2.2.5. Sample preparation for *Dengue virus* and OsHV-1 detection

Samples for *Dengue virus* and OsHV-1 were diluted to assist detection and not saturate the cytometer. 25 µl of each virus extract was diluted in 225 µl of 0.02 µm filtered TE buffer (10 mM Tris, 1 mM EDTA). For each sample, 0.5 µl each of forward and reverse primer probe (200 nM final concentration) was added and the samples were placed on a heat block at 80°C for 10 minutes. Samples were then moved to a secondary heat block for incubation at 60°C for 60 minutes. After incubation samples were removed from the heat block and stored in the dark at room temperature until analysis.

2.2.6. Flow cytometric analysis of *Dengue virus* and OsHV-1 using Molecular Beacons

Samples were analysed on a Merck Muse® cytometer (Muse cytometer). Prior to each session 5.3×10^4 per mL calibration beads were prepared and run in triplicate for quality control and calibration of the volumetric sensor. After preparation and incubation, samples were loaded into the cytometer and analysed for 2 minutes or 50,000 events. After analysis of each sample the cytometer was rinsed with MilliQ water to eliminate sample contamination and signal crossover.

2.2.7. Data analysis

Raw flow cytometry (FCS) files were exported from the Muse cytometer and analysed in FlowJo™ software (BD Life Sciences https://www.flowjo.com/) and viral detections were discriminated based on differences in forward scatter (FSC) and yellow fluorescence. Concentrations were calculated using the raw FCS files combined with the calibrated analysed volume of each sample recorded by the Muse cytometer.

2.3. Results

2.3.1. The Merck Muse flow cytometer

The Merck Muse flow cytometer is a compact and portable entry level machine that is sold off the shelf primarily for the analysis of blood cells (Figure 2.1). Successful adjustments to factory settings and the selection of suitable fluorescent dyes, however, enabled detection and enumeration of heterotrophic bacteria and viruses. The machine is small (27 cm × 21 cm × 21 cm) and portable, allowing users to transport the machine and analyse samples at multiple locations or in the field if required. The creation of saved settings for any specific test, such as OsHV-1 detection, enables a straightforward, rapid pre-analysis preparation.

Figure 2.1 Overview of the Merck Muse Cell Analyser, highlighting (A) physical bench top size of the machine, (B) the sample loading arm and (C) the touch screen interface.

2.3.2. Enumeration of viruses and heterotrophic bacteria in seawater

Viruses and heterotrophic bacteria were initially enumerated from seawater on the Muse cytometer using a generic nucleic acid fluorescent stain, SYBR-I Green. Analysed samples exhibited clear separation of viral and bacterial populations based on yellow fluorescence (Figure 2.2). Viral abundance ranged from $2.5 \times 10^7.mL^{-1}$ to $1.3 \times 10^8.mL^{-1}$ and bacterial abundance ranged from $6.8 \times 10^7 .mL^{-1}$ to $9.3 \times 10^7.mL^{-1}$. Seawater samples were then compared on two other flow cytometers, BD Canto II and Beckman Coulter CytoFlex S, at the Flinders Medical Centre for comparison to the results obtained on the Muse cytometer (Figure 2.3). Viral abundance was greater on the CytoFlex S than the Canto II and Muse cytometers (Figure 2.4), while bacterial abundance was greater on the Muse cytometer (Figure 2.4).

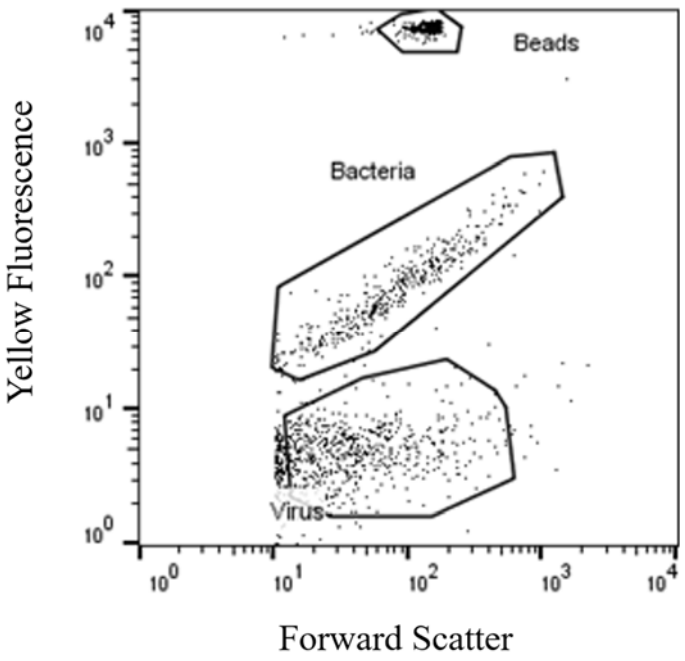


Figure 2.2 Representative cytogram from the Muse cytometer of a seawater sample stained with SYBR-I Green. Beads population represents 1 µm green fluorescent beads added as a size and concentration standard. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

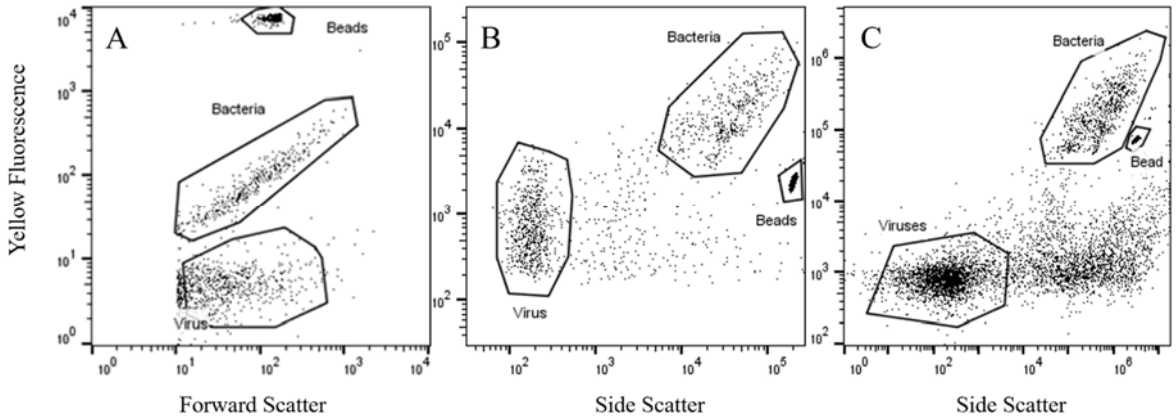


Figure 2.3 Representative cytograms from (A) Merck Muse, (B) BD Canto II and (C) Beckman Coulter CytoFlex S cytometers from identical seawater samples. Populations represent viruses and heterotrophic bacteria, and 1 µm green fluorescent beads were added as a size and concentration standard. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

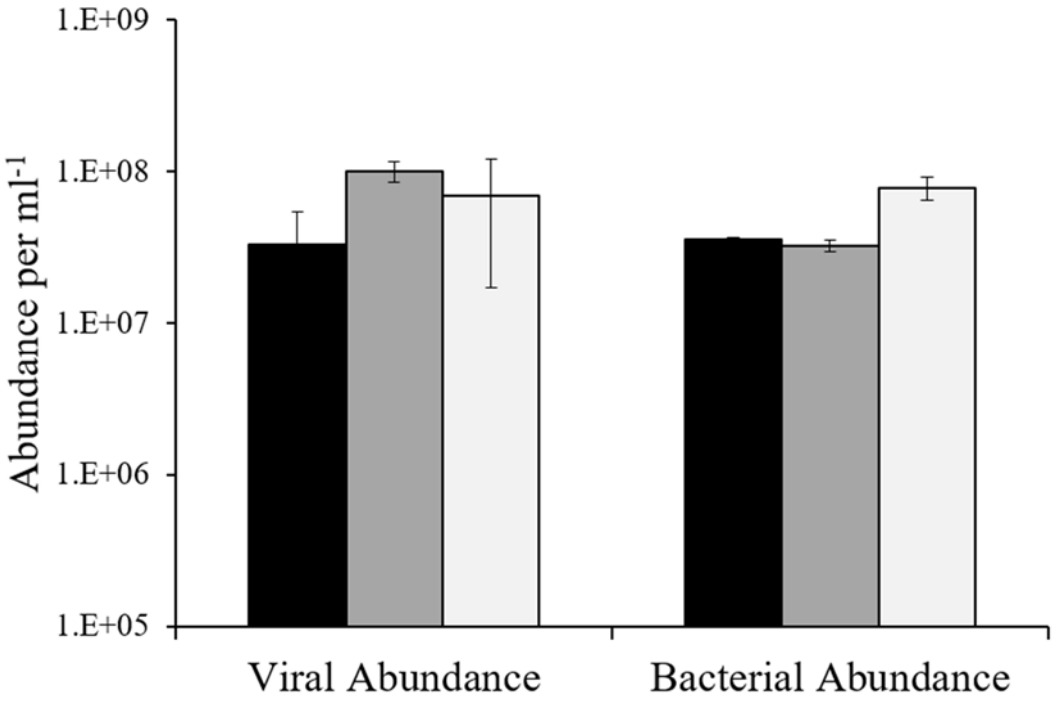


Figure 2.4 Abundance of viruses and heterotrophic bacteria from seawater analysed on the Canto II (black bars), CytoFlex S (dark grey bars) and Muse (light grey bars) flow cytometers. Error bars represent standard deviation of the mean.

2.3.3. Detection and enumeration of Dengue and Influenza A

Detection and enumeration of *Dengue virus* and *Influenza A virus* was successfully achieved on the Muse cytometer using the fluorescent nucleic acid stain SYTO Orange 81. Clear cytometric populations of each virus were present (Figure 2.5). Both viruses were supplied at viral titres of approximately 10^7.mL^{-1}. The enumeration of these samples on the Muse cytometer obtained concentrations of 2.4×10^6.mL^{-1} for *Dengue virus* and 2.5×10^6.mL^{-1} for *Influenza A virus*.

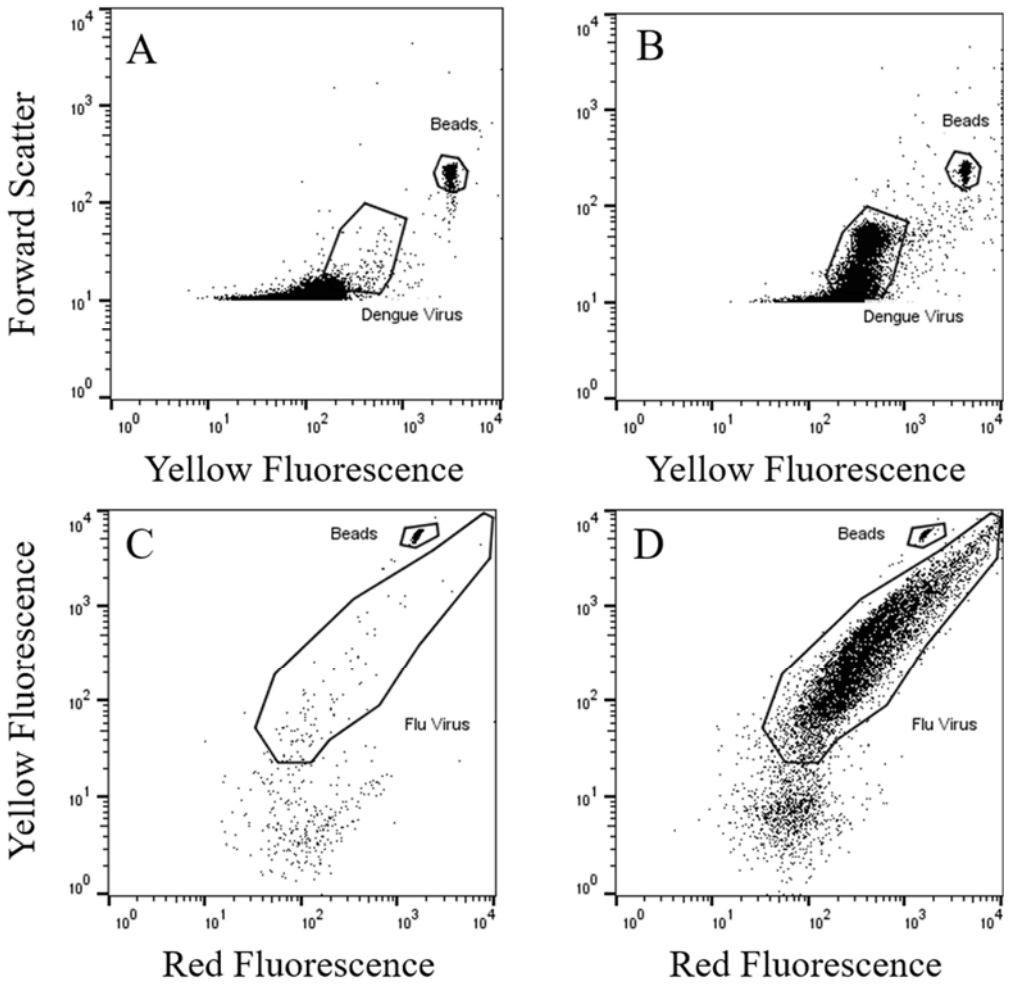


Figure 2.5 Cytograms representing *Dengue virus* and *Influenza A virus* analysed on the Muse cytometer using the nucleic acid fluorescent stain SYTO Orange 81. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

2.3.4. Detection and enumeration of *Dengue virus* using primer probes

Dengue virus was used as an initial test of the capability of the Molecular Beacons developed for the project. The detection and enumeration of *Dengue virus* using Molecular Beacons was successfully achieved on the Muse cytometer. One clear population was observed which was used to enumerate the samples analysed (Figure 2.6). Using the Molecular Beacons, the abundance of *Dengue virus* ranged between 1.4×10^6 and 2.0×10^6.mL^{-1}.

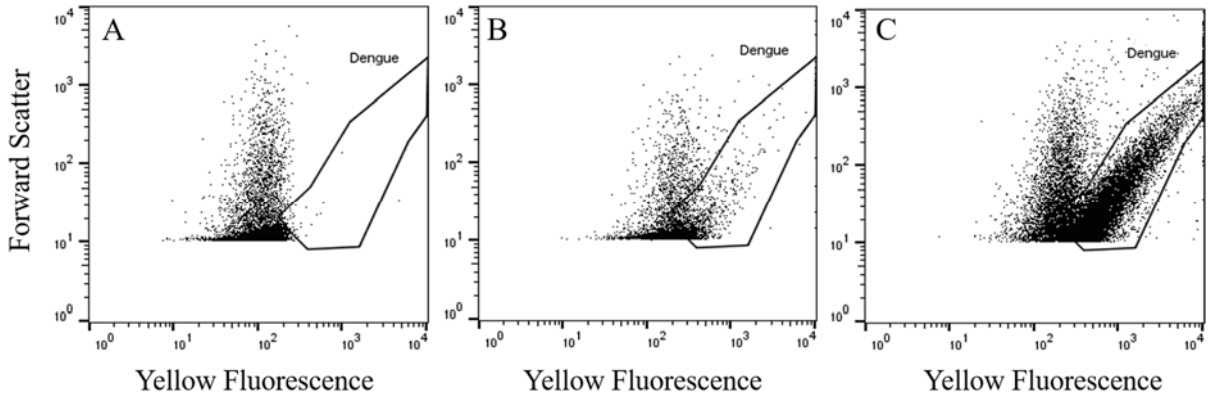


Figure 2.6 Representative cytograms of *Dengue virus* analysed on the Muse cytometer using the Molecular Beacons primer probe. Samples of (A) blank with probes, (B) *Dengue virus* without probes and (C) *Dengue virus* with probes were used to calculate the concentration of the virus. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

2.3.5. Detection and enumeration of OsHV-1

Detection and enumeration of OsHV-1 was achieved on the Muse cytometer using the C2–C6 primer probe specific Molecular Beacons we developed. Analysis of samples supplied by the Animal Health Laboratory in Tasmania exhibited a distinct population separate from background machine noise (Figure 2.7). The concentration of OsHV-1 recorded in the samples ranged from 6.3×10^6 to 1.0×10^7.mL^{-1}. Further modification of the Muse cytometer settings was required to encapsulate the OsHV-1 population on the cytogram.

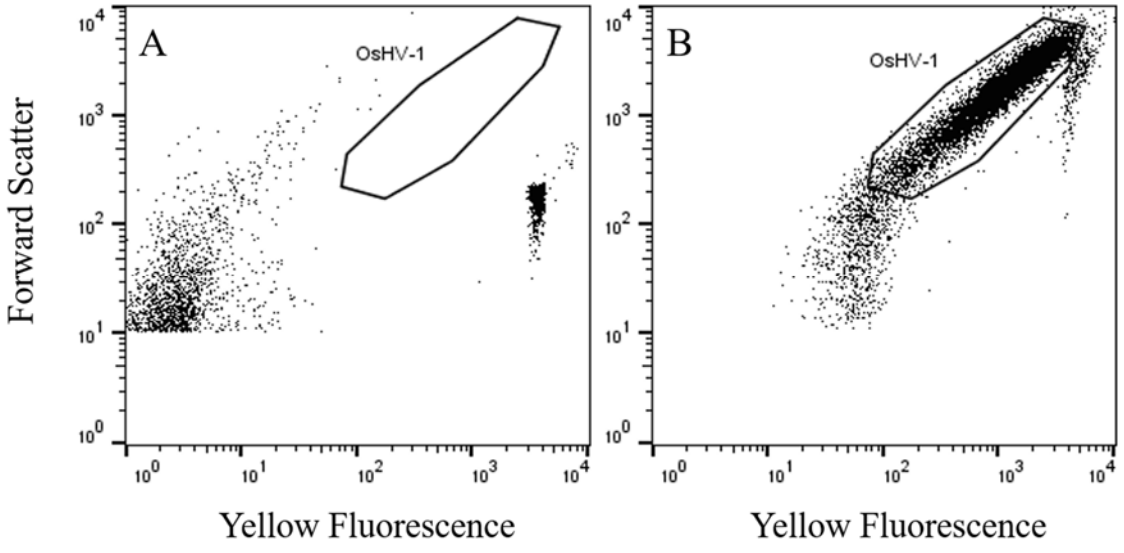


Figure 2.7 Cytograms of OsHV-1 samples obtained from the Animal Health Laboratory, Tasmania exhibiting (A) absence of viral signal in the blank with probes and (B) presence of viral signal with OsHV-1 and probes. Samples were analysed on the Muse cytometer using OsHV-1 specific Molecular Beacons. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

To confirm that the developed OsHV-1 test can be transferred to other flow cytometers a second Merck Muse cytometer was purchased and located in a PC-2 specific laboratory. This cytometer was also used for the analysis of OsHV-1 samples that were not fixed with glutaraldehyde. The detection and enumeration of OsHV-1 in samples supplied by the Animal Health Laboratory in Tasmania on a secondary cytometer that were not fixed with glutaraldehyde was successfully achieved (Figure 2.8). The concentration of OsHV-1 in the samples run on the PC-2 Muse cytometer ranged from 1.01 to $1.21 \times 10^7.mL^{-1}$.

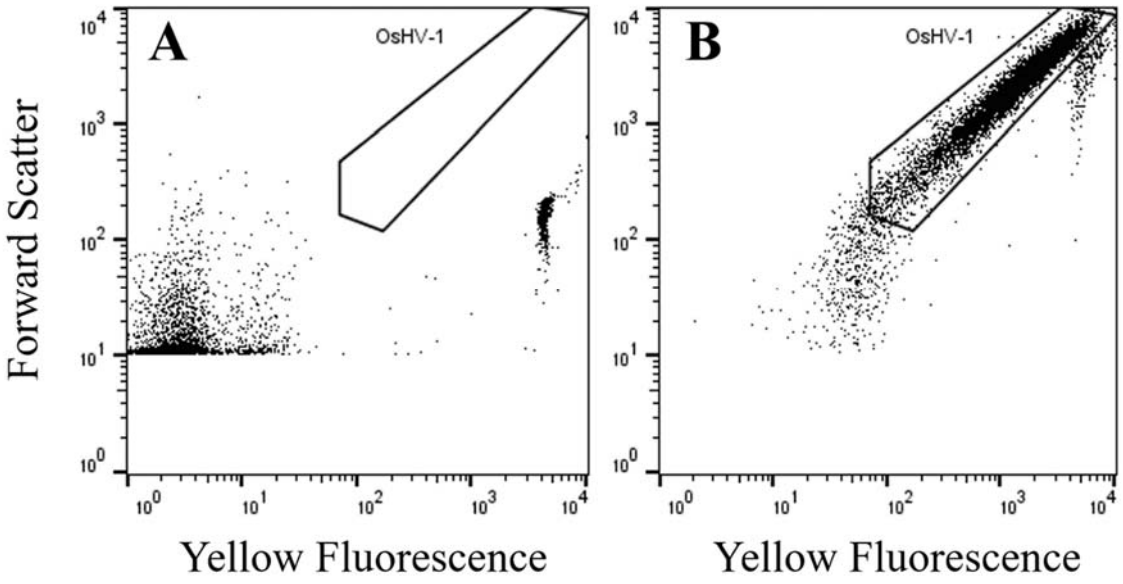


Figure 2.8 Cytograms of OsHV-1 samples obtained from the Animal Health Laboratory, Tasmania showing (A) no viral signal in the blank with probes and (B) OsHV-1 positive signal from a sample incubated with probes. Samples that were not fixed with glutaraldehyde were analysed on a secondary Muse cytometer housed in a PC-2 laboratory using OsHV-1 specific Molecular Beacons. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

Infected oyster samples supplied by SARDI Aquatic Sciences were successfully analysed on the Muse cytometer (Figure 2.9). Tissue of each oyster was mechanically macerated, filtered and samples were incubated with the OsHV-1 probe then analysed on the Muse cytometer. The lower detection limit for this test was $1.4 \times 10^1.mL^{-1}$. The concentration of virus from oyster 562 was $2.86 \times 10^7.mL^{-1}$ and from Oyster 566 was $2.93 \times 10^7.mL^{-1}$.

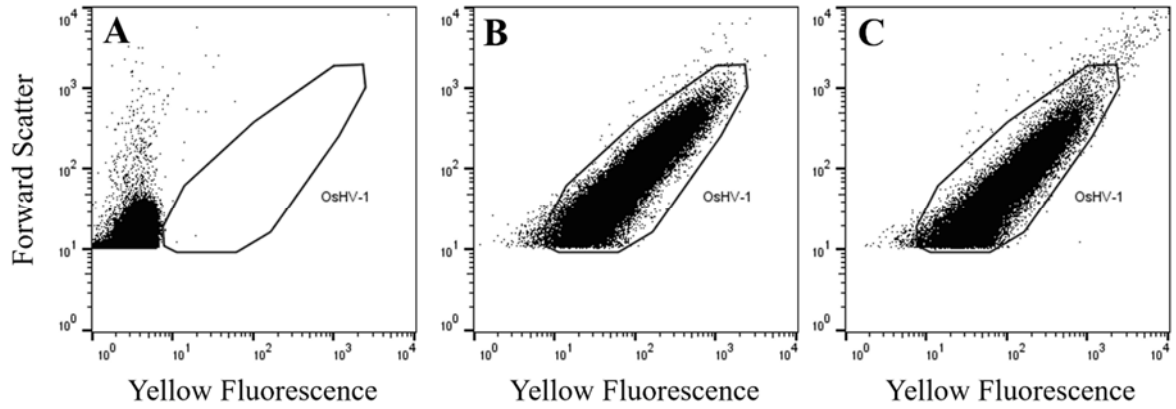


Figure 2.9 Cytograms of OsHV-1 from extracted oyster tissue obtained from SARDI Aquatic Sciences showing (A) blank samples containing probes, (B) Oyster 566 sample confirming presence of OsHV-1 at a concentration of 2.93×10^7.mL^{-1} and (C) Oyster 562 sample confirming presence of OsHV-1 at a concentration of 2.86×10^7.mL^{-1}. Samples were analysed on the Muse cytometer using OsHV-1 specific Molecular Beacons. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

Further analysis of oyster samples supplied by SARDI Aquatic Sciences exhibited varying levels of OsHV-1 infection (Figure 2.10). Following the mechanical maceration of the oyster tissue and the incubation with the OsHV-1 probe, samples were analysed on the Muse cytometer. The concentrations of OsHV-1 from Oyster 545 was 7.00×10^2.mL^{-1}and from Oyster 533 was 7.01×10^2.mL^{-1}.

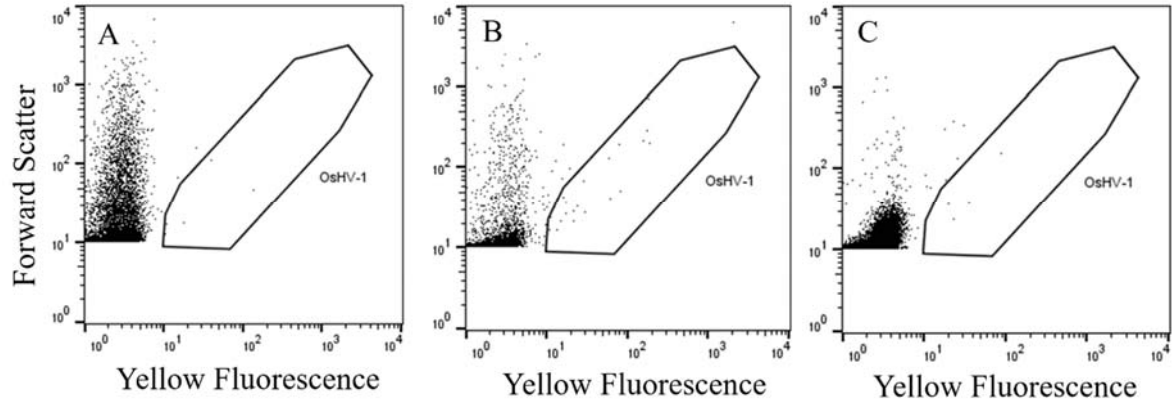


Figure 2.10 Cytograms of OsHV-1 from extracted oyster tissue obtained from SARDI Aquatic Sciences showing (A) blank samples containing probes, (B) Oyster 545 sample confirming presence of OsHV-1 at a concentration of 7.00×10^2.mL^{-1} and (C) Oyster 533 sample confirming presence of OsHV-1 at a concentration of 7.01×10^2.mL^{-1}. Samples were analysed on the Muse cytometer using OsHV-1 specific Molecular Beacons. Yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size.

2.4. Discussion

This research aimed to test if recent developments in flow cytometry sensitivity and portability could be adapted to develop an OsHV-1 flow cytometry detection system for seawater and oyster tissue. This was achieved. The POMS probe was created using standard molecular biology and flow cytometry methods and calibrated against Australian-sourced viral samples and a probe for the dengue virus. Confirmation of successful probe creation was obtaining the cytometry signal from the probe; we detected OsHV-1 over a concentration range from 1.4×10^1 to 2.9×10^7.mL^{-1}. This was achieved with

quenched probes designed to be specific to OsHV-1. Our analytical limit of detection was $1.4 \times 10^{1}.mL^{-1}$ and easily detected OsHV-1 even after 100 fold dilution of field samples (Figure 2.9).

The viral load detected in infected oysters indicates that the release of virus from infected farming regions or areas with dense populations of feral Pacific Oysters is likely to be in the vicinity of 10^{10}-10^{15} viruses per day over the period of an intense outbreak. This confirms the observations of Segarra et al. (2010) that overwhelming viraemia is associated with large outbreaks of OsHV-1.

2.5. Guidelines for interpretation of results

The flow cytometry signal is visualised using cytograms, where yellow fluorescence on the x-axis indicates the amount of DNA and the forward scatter on the y-axis indicates particle size. Figure 2.7 shows the undiluted OsHV-1 probe providing signal (Figure 2.7B) and an absence of signal in the control (Figure 2.7A).

The overfull effect of measurement points in Figure 2.7 is misleading because the solid black represents up to 100 measurement points on top of each other. The points to the lower left outside the box in Figure 2.7B represent partially stained, possibly fragmented OsHV-1 virions that contain less DNA than a complete OsHV-1 virion. The measurement points to the upper right of the box and dropping downwards indicate complete OsHV-1 genomes that are smaller because they may be missing some capsid protein, which causes less scattering and moves the points down the cytogram. Overall, the OsHV-1 probe signal behaves as expected (Figure 2.7).

We developed a series of checks for interpretation of the flow cytometry results based on a test with 15 undergraduate students:

- Check the axes to make sure that yellow fluorescence is on the x-axis and forward scatter is on the y-axis (Figure 2.7).
- Compare samples to the positive and negative controls and check to ensure that a majority of measurements fall within the OsHV-1 gate.
- If the detections fall outside of the OsHV-1 gate, check the settings on the Muse.
- If the detections obscure all or part of the gate, dilute the sample and re-test.
- The negative controls should show no, or few dots and the positive controls should show numerous dots, with the density depending on the control used.
- Check that there is not too much signal in the samples or the positive control. Excess signal is illustrated in Figure 2.7B and is indicated by the ‘running’ of dots out of the top and bottom of the OsHV-1 box. For proper interpretation the signal needs to be quantified. This is achieved by subtracting the negative control from the sample. This should be done automatically on an Excel spreadsheet, but it should be checked that this has been done. The sample OsHV-1 concentration is the target concentration minus the negative control concentration. A negative number indicates more signal in the negative control than in the sample. A large negative number may indicate that the negative control has been contaminated or that the control has been switched for a test sample. A negative number close to zero may mean there is no OsHV-1 in the sample and both sample and control are show only noise. Random fluctuations may lead to the control noise being larger than the sample noise, thus producing a negative number.
- Samples should be repeated in at least triplicate, and samples should be taken from different tubes. The results of the replicates should be averaged and compared. It is prudent to perform statistical tests when comparing samples. The simplest test is to calculate the 95% confidence intervals and compare if the ranges overlap. If they do not overlap, then the samples are

significantly different. It is worth noting, however, that there is often a difference between statistically significant and biologically significant. If one sample has 100,000 OsHV-1 viruses.mL^{-1} and another has 500,000 OsHV-1 viruses.mL^{-1} they may be statistically significantly different. If only 1,000 OsHV-1 viruses.mL^{-1} are required to infect and kill an oyster, however, the viral concentrations will both kill the oysters and there is little biological difference.

- Sets of samples should be taken over time to assess temporal variation. If one of the replicates is a factor of 10 higher or lower than the other two samples, a mistake may have been made preparing one of the samples. The most frequent mistake is finding one of the replicates is close to zero while the others are significant. A zero value in this situation indicates that any of the critical steps in sample preparation may have been missed.

2.6. References

Arzul, I., Renault, T., Lipart, C., Davison, A.J., 2001. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. Journal of General Virology 82, 865-870.

Brussaard, C.P.D., 2004. Optimisation of procedures for counting viruses by flow cytometry. Applied and Environmental Microbiology 70, 1506-1513.

Brussaard, C.P.D., Payet, J.P., Winter, C., Weinbauer, M.G., 2010. Quantification of aquatic viruses by flow cytometry. Manual of Aquatic Viral Ecology 11, 102-109.

Burge, C.A., Griffin, F.J.,Friedman, C.S., 2006. Mortality and herpesvirus infections of the Pacific Oyster Crassostrea gigas in Tomales Bay, California, USA. Diseases of Aquatic Organisms 72(1), 31-43.

de Kantzow, M.C., Hick, P.M., Dhand, N.K., Whittington, R.J.J.A., 2017. Risk factors for mortality during the first occurrence of Pacific Oyster Mortality Syndrome due to Ostreid Herpesvirus–1 in Tasmania, 2016. Aquaculture 468(1), 328-336.

Gasol, J.M., Zweifel, U.L., Peters, F., Fuhrman, J.A., Hagström, A., 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Applied and Environmental Microbiology 65, 4475-4483.

Keeling, S., Brosnahan, C., Williams, R., Gias, E., Hannah, M., Bueno, R., McDonald, W., Johnston, C., 2014. New Zealand juvenile oyster mortality associated with Ostreid Herpesvirus 1 an opportunistic longitudinal study. Diseases of Aquatic Organisms 109(3), 231-239.

Lebaron, P., Servais, P., Agogué, H., Courties, C., Joux, F., 2001. Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? Applied and Environmental Microbiology 67, 1775-1782.

Lebaron, P., Servais, P., Baudox, A.C., Bourrain, M., Courties, C., Parthuisot, N., 2002. Variations in bacterial specific activity with cell size and nucleic acid content assessed by flow cytometry. Aquatic Microbial Ecology 28, 131-140.

Li, W.K.W., Jellett, J.F., Dickie, P.M., 1995. DNA distributions in planktonic bacteria stained with TOTO or TO-pro. Limnology and Oceanography 40, 1485-1495.

Marie, D., Partensky, F., Jacquet, S., Vaulot, D., 1997. Enumeration of cell cycle analysis of natural populations of marine picoplankton by flow cytometry using a novel nucleic acid dye. Applied and Environmental Microbiology 63, 186-193.

Marie, D., Brussaard, C.P.D., Thyrhaug, R., Bratbak, G., Vaulot, D., 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. Applied and Environmental Microbiology 65, 45-52.

Paterson, J.S., Nayar, S., Mitchell, J.G., Seuront, L., 2013. Population-specific shifts in viral and microbial abudnance within a cryptic upwelling. Journal of Marine Systems 113-114, 52-61.

Segarra, A., Pépin, J.F., Arzul, I., Morga, B., Faury, N., Renault, T., 2010. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific Oysters, Crassostrea gigas, in France in 2008. Virus Research 153, 92–99.

Seymour, J.R., Seuront, L., Doubell, M.J., Mitchell, J.G., 2008. Mesoscale and microscale spatial variability of bacteria and viruses during a *Phaeocystis globosa* bloom in the Eastern English Channel. Estuarine, Coastal and Shelf Science 80, 589-597.

Chapter 3 South Australian Oyster Mortality Syndrome (SAMS) investigation in Pacific Oysters (*Crassostrea gigas*)

Dr Stephen B. Pyecroft and Dr Johanna Mahadevan

Summary

South Australian Oyster Mortality Syndrome (SAMS) is considered to cause high but sporadic mortality of Pacific Oysters (*Crassostrea gigas*) within South Australian waters. The use of the term SAMS implies that all mortalities have a common cause but this is not as yet proven.

SAMS was defined in a South Australian oyster industry funded workshop (Madin *et al*, 2013) and this project attempts to develop the understanding of SAMS and its relevance to the industry four years after this workshop.

The study described in the Chapter sampled material from mortality events and applied techniques commonly used in oyster diagnostics (i.e. histopathology, microbiology), combined with other, less commonly used techniques (i.e. histochemistry, immunohistochemistry, haemocyte analysis, haemolymph biochemistry) and other techniques under development for understanding mortality in Pacific Oysters.

Combining this 'oyster centric" information with available environmental data guided interpretation of findings from pathological investigations during mortality events of Pacific Oysters. This project aimed to provide a focused approach to further developing the case definition for SAMS: >10% mortality of 20-60 mm oysters (<12 months old) during their first winter (Madin *et al,* 2013), and help direct mitigation strategies to reduce or remove the problem. Trigger based response strategies could be developed for management of impacts of mortality events on farms if causal factors were identified. Samples from SAMS cases and indicative findings from traditional techniques were used to guide application of less conventional technology.

The time frames for mortality investigations were limited to late November 2017 to August 2019 (including one whole winter and a portion of the 2019 winter periods). This is highlighted as the current case definition for SAMS includes a statement about early grow-out stock experiencing their first winter at sea.

The availability of Pacific Oyster spat and juveniles was reduced due to changed hatchery supply as a result of the outbreak of OsHV-1 in the Tasmanian Pacific Oyster industry, traditional suppliers of juvenile Pacific Oyster to South Australia producers. The focus on routine grow-out and stock assessment was affected by the focus away from SAMS industry effects, as a result of issues with stock supply. Four case investigations were undertaken during the project period. One of the four pathological investigations was from early 2018 the remaining three cases were from 2019 despite repeat contact and industry interaction by the project investigators. Three of the reported submissions did not meet the current SAMS case definitions but the findings are indicative of the causes of mortality in Pacific Oysters in South Australia.

3.1. Introduction

South Australian Mortality Syndrome (SAMS) is thought to have begun as unexplained high mortality in Pacific Oysters in 1998 in Smoky Bay, South Australia (McGowan, 2013). Its occurrence causes high but sporadic mortality and is thought to have occurred in Smoky Bay, Denial Bay, Coffin Bay, Cowell and Stansbury (McGowan, 2013).

In 2013 a workshop on SAMS evaluated losses associated with the syndrome from two perspective: the estimated changes in overall mortality based on observations made at grading and the loss of oysters to market relative to numbers of spat introducted to oyster farms (Madin, *et al.* 2013). Both methods predicted an overall loss of approximately 10% of production attributable to SAMS, which equates to a loss of $AU3-3.5 million per year in farm income (Madin, *et al.* 2013). The SAMS workshop attempted to describe and characterise these mortalities. Based on consistent findings described by producers attending the workshop a broad working case definition was developed for SAMS: a rapid >10% mortality of 20-60 mm oysters (<12 months old) during their first winter (Madin, *et al.* 2013).

Since 2013 there was no further systematic work on SAMS cases, despite the workshop recommendations of: improved surveillance; centralised information management; increased laboratory analysis; to workshop husbandry practices; analyse data from existing sources; and seek input from a wider range of experts. One Pacific Oyster producer/The University of Adelaide project which proposed to establish a definition of a 'healthy South Australian Pacific Oyster'. Ongoing passive surveillance and mortality investigation by PIRSA was also conducted for exclusion of OsHV-1 related Pacific Oyster Mortality Syndrome (POMS).

Due to the paucity of reporting, surveillance and diagnostic investigations into SAMS from 2013 to 2017 there is little understanding of the current prevalence of SAMS occurring in South Australian leases. A clinical disease picture relies on findings observed in individual animals as well as its characterisation on a farm level (Madin, *et al.* 2013). In 2013 the workshop noted there were reports of mortalities with different patterns to the case definition and that the case definition could not be regarded as the only mortality pattern that occurs in South Australian oyster farms (Madin, *et al.* 2013).

The diagnostic component of this project aimed to improve and add to the case definition of SAMS or identify a causative agent by investigating further commonalities in oyster mortalities that resemble the SAMS case definition. To achieve this, it was imperative to have an understanding of the prevalence of SAMS based on industry perception. A survey of SA oyster producers was undertaken to understand the currency of four main issues. The survey aims were:

- To gain an overview of the producers' understanding of SAMS
- To understand the perception of producers about the significance of SAMS to their production
- To understand the current perceived occurrence of SAMS
- To inform producers of this research project and encourage diagnostic submissions from oyster mortality events.

3.1.1. Objectives

The overall project aims were to investigate the causative agent of SAMS, provide improved understanding of causes, a more refined case definition, to improve diagnostic technologies and provide recommendations about improved husbandry to maximise survival.

3.2. Methods

3.2.1. Industry survey

A group of 13 questions was created for this survey. An online cloud-based service SurveyMonkey™ was used to create and promote the South Australian Oyster Mortality Survey (Appendix 1). As a University of Adelaide requirement, the final survey was submitted for human ethics approval prior to survey distribution and was deemed to meet the requirements of the National Statement of Ethical Conduct in Human Research (*Approval number H-2018-066*).

The survey was promoted through the South Australian Oyster Growers Association (SAOGA) to ensure its distribution to all sections of the Pacific Oyster industry, particularly grow-out producers in South Australia. All participants in the survey were provided with an online link as well as options for postal surveys if requested, and were given 60 days to respond. Online access through the portal was discontinued 90 days post initial distribution.

3.2.2. Pathology investigations

A deliverable for the projects within the un-explained oyster mortality sections of the Future Oysters CRC-P was to investigate and develop alternative oyster health monitoring and diagnostic assays. During research investigations within the School of Animal and Veterinary Sciences, University of Adelaide for defining a healthy ac, the potential for using haemolymph samples as health indicators was indicated as proof of concept. Through this current funded project the modality was further investigated as a transitional diagnostic test.

A draft manuscript (Appendix 2) summarises the research investigations and the outcomes of using Pacific Oyster haemolymph as a diagnostic indicator of tissue injury and pathology

To further understand SAMS and unexplained mortality events in farmed Pacific Oysters in the South Australia oyster industry it was necessary to investigate mortality events and have submissions of moribund Pacific Oyster from farmers as they occurred. The survey results during this project and other industry engagement undertaken by this project, indicated that the SA industry was willing in principle to be involved in the submissions for disease investigations. In the context of further investigating SAMS it was imperative that under the current case definition, samples be received from mortality events that occurred in winter. The timing of this project meant that opportunities to receive samples was limited. Sample submission was promoted for any mortality event above acceptable levels on a farm and/or industry level. Of four submissions only the submission in August 2019 met the SAMS case definition. All but one submission was in conjunction with a PIRSA state response to rule out OsHV-1 infection. Currently OsHV-1 presence is limited to Port Adelaide and investigations of mortalities are part of ongoing state wide surveillance and management of this regional infection.

Samples from all submissions were processed using the following methods. There was some variation in what methods were applied to each sample submission dependent upon the size of the submitted oysters and the access to various microbiological identification methods.

Gross and necropsy examination and morphometrics- Individual oysters were shucked by initially removing the cap shell at the articulation of the shell hinge ligament and then by severance of the adductor muscle at the cap shell attachment. Each oyster was examined under dissecting light microscope for gross tissue changes, shell liquor was collected for wet light microscopy examination, haemolymph removed from the pericardial sac by sterile needle and syringe from oysters greater than 30mm shell length and then the oyster meats removed from the shell by severance of the adductor muscle at the cup shell attachment. Meats were examined utilising methods described by Adelson (2016) for density and condition (where deemed appropriate), and then all fixed and processed as described below for histopathological examination.

Histology – Standard methods (Howard *et al*, 2004) for tissue collection, fixation in Seawater formalin, processing and embedding into paraffin wax blocks for microtome thin sections (3um) preparation and staining with H&E, were undertaken. All slide preparations were permanently cover slipped before examination under light microscope.

Microbiology – Primary samples (haemolymph or liquor from within the shell space (oysters less than 30mm shell length)) were plated onto Sheep Blood Agar plus 2% salt, Zorbells Marine agar (ZMA) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS). Growth was recorded after 24 and then 48 hours incubation of samples at 25ºC within a temperature controlled incubator.

Isolate identification was undertaken combining morphological description of growth on the various media, gram staining properties, biochemical profile (MicroSys™) and Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS). Where possible validation of the available MALDI-TOF database was undertaken by using data from biochemical profiles obtained for the same isolate.

3.3. Results

3.3.1. Survey responses

A total of 14 oyster produces responded to the survey of which 79% confirmed an interest in participating in the project by contributing samples from mortality events. The survey responses, excluding question 13 revealing contact information, are contained in Appendix 2.

There are 336 oyster licenses for growing Pacific Oysters within South Australian waters and 115 associated farmers (PIRSA, 2017; EconSearch, 2018). The 14 responses thus represent an 8.2% response rate, providing insight into the current significance and possible occurrence of SAMS, and the overall perception of the definition of SAMS (Figure 3.1).

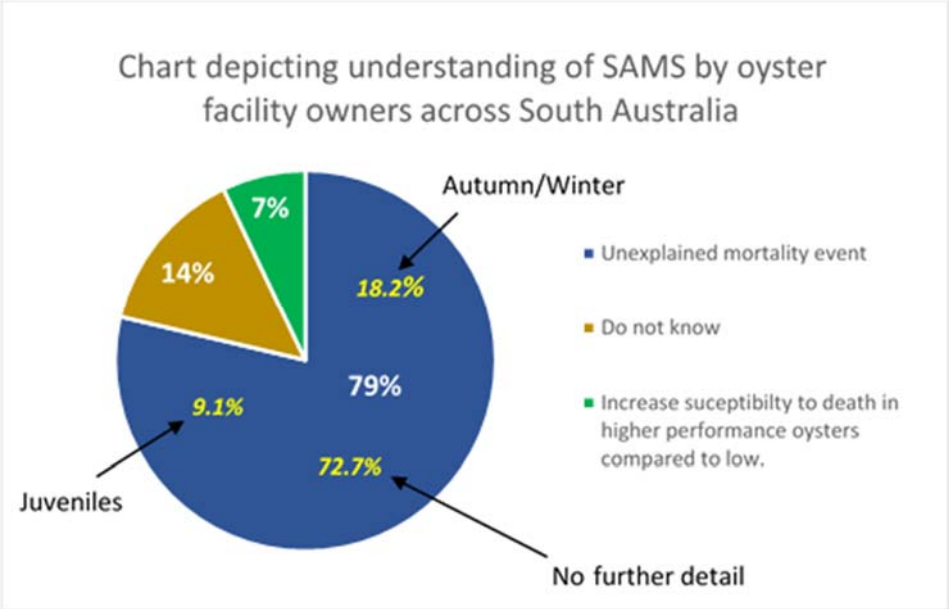


Figure 3.1. Pie chart depicting the understanding of SAMS by participants (n=14) of the Oyster SAMS survey.

The graphical representation of results shows the majority of participants (79%) believe SAMS to be an unexplained mortality event. Of those, only 9% associate the syndrome with juveniles while 18% associate it with a seasonal occurrence in Autumn/Winter. The remaining 72% provided no further detail. The minority of participants were of the opinion that SAMS is an increase susceptibility to death in higher performance oysters compared to low performance animals, while 14% of the participants had no understanding of SAMS. This is interesting information considering that in 2013 a working definition of the syndrome was created. The investigators infer that this may be due to the lack of continued momentum from the 2013 SAMS Workshop with no further SAMS specific surveillance or research undertaken.

Figure 3.2 shows that 50% of respondents believe SAMS to be currently present on their lease(s), while approximately 29% who were uncertain. Based on contact details available, these leases appear to be distributed evenly through coastal oyster regions of South Australia; Smoky Bay, Stansbury, Port Lincoln and Cowell.

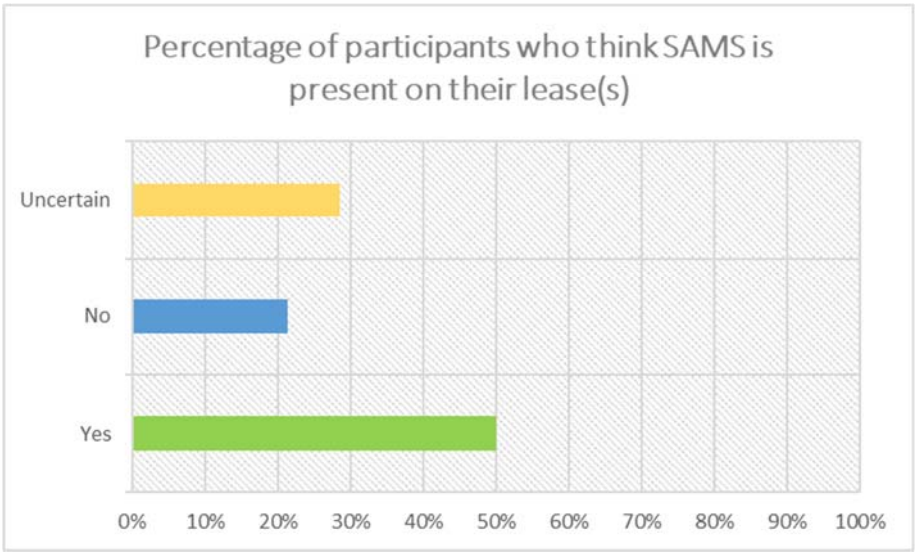


Figure 3.2. Chart showing the percentage of survey participants who believe SAMS is present on their lease(s).

The survey does not give a clear indication that increased mortality occurred over 2016 and 2017. This was attributed to the various responses that did not provide details for mortalities experienced over the two years. However, as an overview, the data revealed that the mortalities experienced by the majority of producers in both years drastically exceeded their acceptable mortality rates (Figure 3.5). It is important to note that this data does not differentiate between sizes of oysters and represents an overall mortality experienced by producers. Only 35% of participants had mortalities assessed diagnostically to understand mortality events during 2016-17 (Figure 3.6).

The extent of these mortalities when coupled with the significant spat supply issues experienced in 2016 will further impact the supply of saleable size oysters in the future for the South Australian Oyster industry (PIRSA, 2017). Since the 2016 outbreak of Pacific Oyster Mortality Syndrome (POMS) occurred in Tasmania, a ban was implemented on oyster imports from that state (PIRSA, 2017). This ban had a significant impact on the South Australian oyster industry because at the time 80% of spat were imported from certified Tasmanian hatcheries (PIRSA, 2017). A report overviewing the economic contribution of Aquaculture in SA for years 2016 and 2017 inferred a decline in sales of mature oysters between 29% and 68% in 2017/18 due to this spat shortage, however sales were expected to recommence from March 2019 pending the timeline of when producers were impacted by the spat shortage (EconSearch, 2018). The survey also showed that only 35% of participants had diagnostic assessments undertaken to investigate their mortality events during 2016-17 (Figure 3.6).

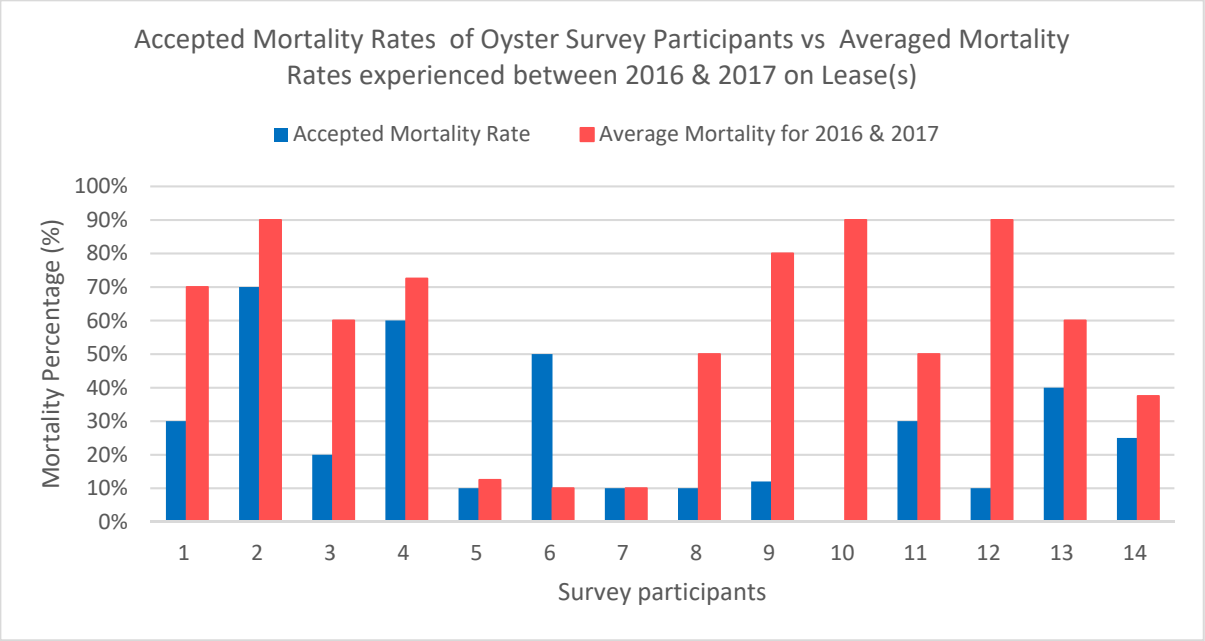


Figure 3.3. Diagram comparing the accepted mortality rate to the average recorded mortality rate between 2016 & 2017 for each oyster survey respondent.

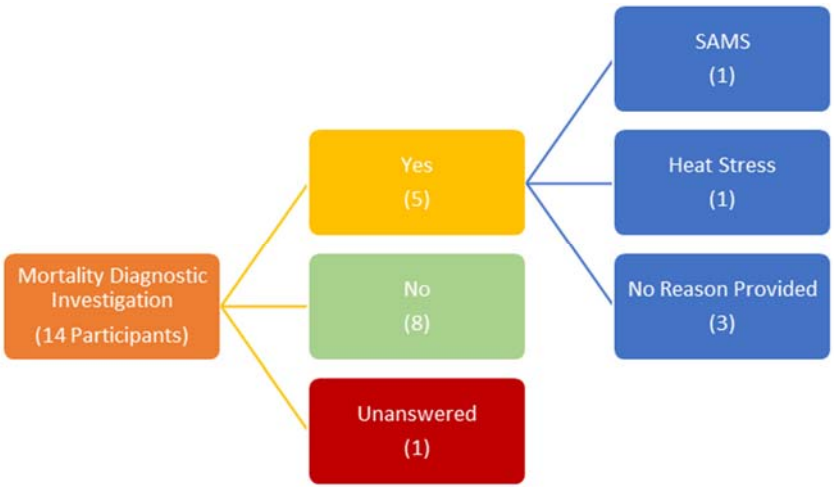
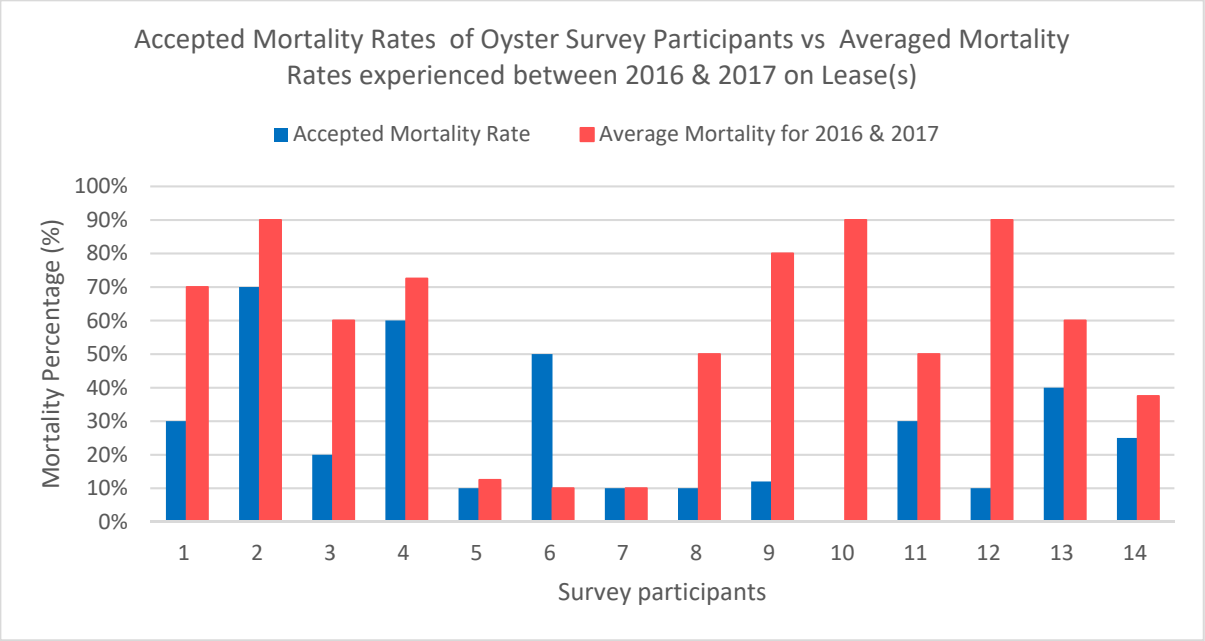


Figure 3.4. Diagram summarizing oyster survey participant responses to performing diagnostic investigation on oyster losses on leases.

Referring samples for testing and diagnostic laboratory testing was one of the six recommendations made at the 2013 SAMS workshop (Madin, *et al.* 2013). Having 35% of participants engage in diagnostic assessment is a positive outcome which needs to be promoted further. In 2016, an honours thesis proposed to establish a definition of a "healthy" South Australian Pacific Oyster. The methods used to enable this included gross morphological, histological and microbiological assessment of pacific oysters that varied in age and genetic lineage. There were no incidences of SAMS reported during the time of this project and a true representation of non-affected oysters was established (Adelson, 2016). The work outlined in this Chapter also serves as an example of the benefits of sample submission and can now be utilised as an essential tool for diagnostic comparison purposes when assessing further disease outbreaks (McGowan, 2013).



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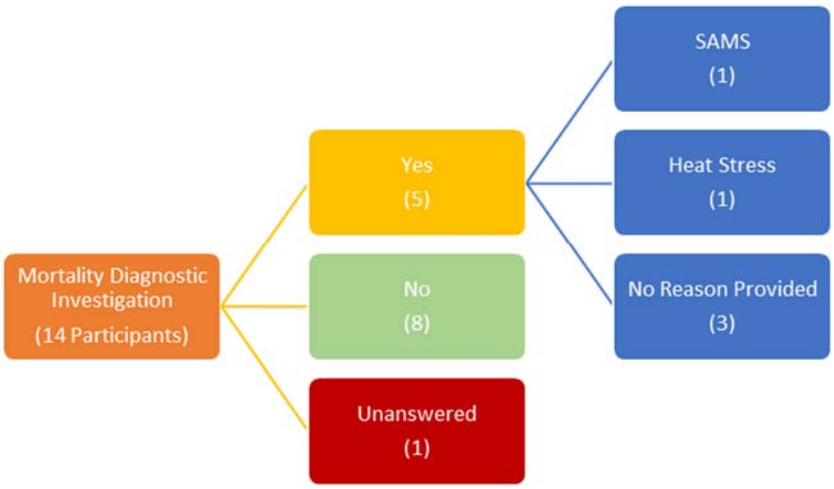

Figure 3.6. Diagram summarizing oyster survey participant responses to performing diagnostic investigation on oyster losses on leases.

3.3.2. Pathology reports

Case 1

AQUATIC ANIMAL PATHOLOGY REPORT
UA Veterinary Diagnostic Laboratory reference number: 18-0985
Species: Pacific Oyster (*Crassostrea gigas*)
Time and date of death: 8/5/2018
Time and date of post mortem examination: 8/5/2018
Carcass condition & preservation: Five separate cohorts of oyster of varying age and size each in a zip lock bag were received. Four of those cohorts were placed in a large esky and one cohort arrived in a separate small esky.

HISTORY:

Samples were submitted for examination mid May 2018 after an investigation by PIRSA following a farmer reporting greater than normal mortality (>10% mortality) at grading in Smoky Bay and subsequently at Coffin Bay. One farm in each of these growing areas was affected. A stock stand still was ordered and an investigation was co-ordinated by PIRSA utilising the services of the state veterinary diagnostic laboratory service (Vetlab) and the South Australian Shellfish Quality Assurance Program (SASQAP) under its auspices. The Initial sampling was undertaken to rule out the presence of OsHV-1 infection (POMS) in the affected stock and to identify any possible other infectious agents or obvious causalities. All stock tested negative by Australian and New Zealand Standard Diagnostic Protocols for the presence of OsHV-1.

Relevant information from the Situation Reports (SitRep) from the PIRSA coordinated investigation are as follows:

Unusually high mortality of oysters on 1 farm: approximately 30% mortality over 7 days

Location: Smoky Bay Biosecurity Zone
Oyster size: ~60+mm (~15 months since sourced from the hatcheries)
Oyster history: affected stock includes batches from 3 different hatcheries. Previous mortalities on this farm investigated including February 2018 with negative results for the POMS virus. This bay tested negative for the POMS virus on 6 April 2018.
No other unusually high mortality reports in the surrounding area.
On 30 April 2018, a second farm in Smoky Bay also reported a mortality of 13% for 1 batch and provided samples.
That farmer reported grading his stock (50+mm size) a couple weeks before on a 35 degree day and stock left out over night before being placed back out onto the farm. Other batches of stock on his farm are fine.

On the 29th April 2018 an unusually high mortality of oysters on 1 farm: approximately 50% mortality at grading (1 batch only) from Coffin Bay Biosecurity Zone was reported by one farmer.
Oyster size: ~12+mm.
Oyster history: affected stock from one hatchery, originally sourced at 4mm size. Other batches, including larger stock originally sourced from same hatchery at 8mm are ok.
Coffin Bay mortalities were investigated in February 2018 on 2 farms and were negative for the POMS virus. Coffin Bay tested negative to the POMS virus during the proof of freedom surveillance in March / April 2018 (results received 6 April).
No other unusually high mortality reports in the surrounding area.

During this investigation SASQAP reported that algae counts had been very low in the areas for over 2 months, which may indicate low food availability. Water temperatures were ~18-20 degrees Celsius.

Samples were made available by 8th May, 2018 for this project once POMS had been ruled out as a cause of the mortalities and arrived via SASQAP from affected farms. Histories were scant but oysters originated from Smoky Bay (Cohort 2-5) and Port Lincoln (Cohort 1).

Samples arrived with ice blocks melted and samples at ambient temperature.

Relevant further history also includes the following. Earlier in the year a state wide investigation of oyster mortalities in a number of separate farm leases within three of the states' growing areas (i.e. Coffin Bay, Smoky Bay and Cowell) starting in late January 2018 and extending to mid-February 2018 was undertaken also by PIRSA as a POMS response.

Oysters affected ranged in size from 2 to 25mm and there were variable histories of stock loss dependant on farm affected and growing area. Loss was reported in stock recently received from

hatcheries and from juvenile stock that had been on one farm for 12 months. Not all cohorts on each farm were affected similarly, with most reports indicating recently arrived stock being the most affected. Stock from the same hatchery distributed to various growing areas around the state only experienced mortalities in the areas under investigation.

Air temperatures in affected areas were approximately 40ºC and water temperatures ranged from 22-25ºC in the affected areas for the January and February period. Results from SASQAP indicated no harmful algal species being present during the period just prior to and during the mortality investigations, in all growing areas and that cell counts fluctuated throughout the period but did not drop below critical levels for adequate nutrition of oysters for prolonged periods during the investigation period. No samples were submitted to this project for examination from this February 2018 response investigation

NECROPSY FINDINGS:
The following tables for each cohort are a summary of morphometric measurements of the animals' submitted for examination. This protocols was developed by Adelson (2016). Cohort 1 is from a single lease and Cohorts 2-5 are also from a single lease.

COHORT 1:
These oysters were in the small esky. Origin Port Lincoln. Haemolymph was collected from oyster 1, 2 and 3 for microbiological analysis. Animals were last handled on 12/4/2018 and were from a 2000 screen spat on 28/5/2017 (n=12) (Figure 3.5, Table 3.1).

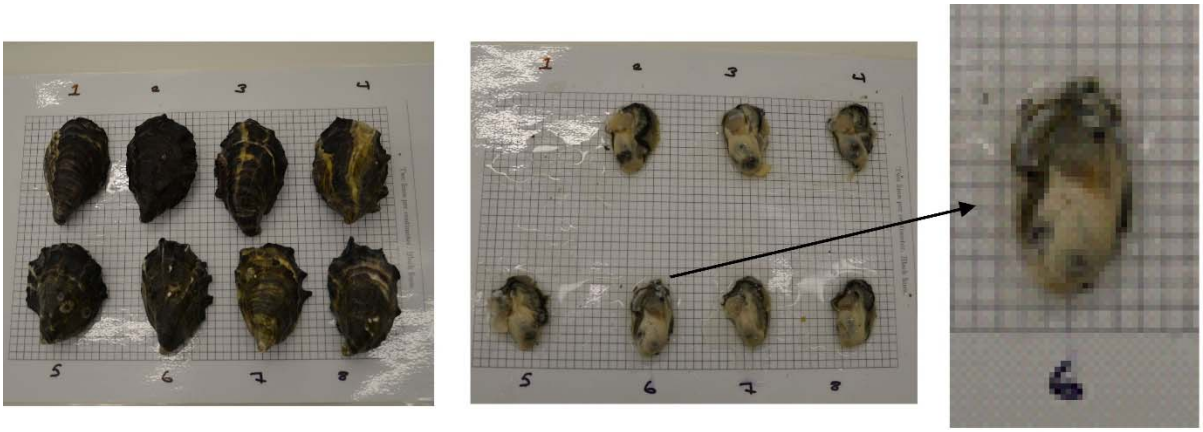

Figure 3.7. Gross examination of shell shape and meat condition. Oyster 6 condition score of 1.

Table 3.1. Data recorded from examinations of oysters in cohort 1.

No.	Comments	Total Length (cm)	Shell Depth (cm)	Condition Score	Total Weight (g)	Meat Weight (g)	Meat Displaced Volume (ml)	Density (g/cm^3)
1	Poor Condition	7.2	2	1	22.3	5.8	10	0.6
2		7.1	2	0	27.6	6.3	7	0.9
3		7.7	2.3	0	27.4	7.3	5	1.5

4		7.3	2	0	27.4	4.3	5	0.9
5		6.4	3.1	1	30.7	5.5	8	0.7
6		6.8	2.6	1	33.5	6.6	8	0.8
7		6.7	2.3	1	34	4.7	4	1.2
8		6.6	2	2	31.2	4.9	5	1.0

COHORT 2:
These animals were in a large esky. Origin Smoky Bay. Haemolymph was collected from oyster 1, 2 and 3 for microbiological analysis. Zip lock bag was labelled – Kelladie. Animals were last handled 18/4/2018 hand graded as placed from 6mm to 12mm mesh bags. Original stocked 14/2/2018. (n=18) (Figure 3.6, Table 3.2).

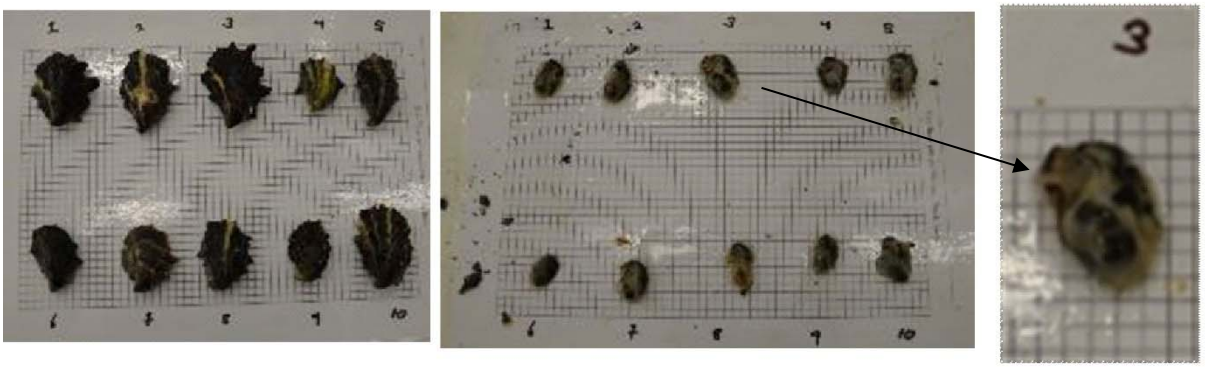

Figure 3.8. Gross examination of shell shape and meat condition. Oyster 3 condition score of 0.

Table 3.2. Data recorded from examinations of oysters in cohort 2.

No.	Comments	Total Length (cm)	Shell Depth (cm)	Condition Score	Total Weight (g)	Meat Weight (g)	Meat Displaced Volume (ml)	Density (g/cm^3)
1		4.5	1.7	0	11.2	1.8	1	1.8
2		5.5	1.7	0	9.8	1.6	1	1.6
3		5.6	1.8	0	10.2	2.1	2	1.1
4	Abnormal Purple Appearance	3.5	1.6	0	4.9	1.2	0.5	2.4
5		4.5	1.4	0	9.8	1.8	2	0.9
6	Abnormal - oyster appears dark	4	1.2	0	5.8	0.7	0.5	1.4
7		3.8	1.1	0	6.3	1.3	1	1.3
8		4.3	1.1	0	10.1	1.3	1	1.3
9		3.9	0.8	0-1	4.8	0.9	1	0.9
10		5.2	1.6	1	9.9	1.8	2	0.9

COHORT 3:
These oysters were in the large esky. Origin Smoky Bay. Haemolymph was collected from oyster 1, 2 and 3 for microbiological analysis. Zip lock bag was labelled – Kelladie. Animals last handled on 20/4/2018. From 17mm spat 2/11/2016. Biofouling marked with barnacles and algae present. (n=13) (Figure 3.7, Table 3.3).

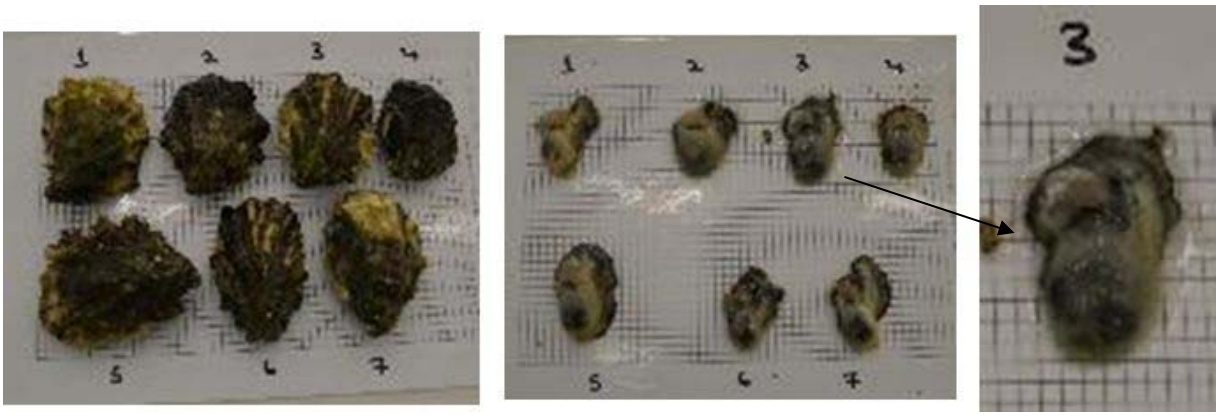

Figure 3.9. Gross examination of shell shape and meat condition. Oyster 3 condition score of 1.

Table 3.3. Data recorded from examinations of oysters in cohort 3.

No.	Comments	Total Length (cm)	Shell Depth (cm)	Condition Score	Total Weight (g)	Meat Weight (g)	Meat Displaced Volume (ml)	Density (g/cm^3)
1		8	3.3	2	46.1	7.4	8	0.9
2		7.4	3	2	48.9	8.7	10	0.9
3		7.4	3.5	1	49	8	8	1.0
4		6.8	2.8	1	36.5	6.5	6	1.1
5		8.4	4.8	1	73.3	10.2	10	1.0
6		7.2	3	1	40.7	5.8	4	1.5
7		8.2	3.4	1	51.6	7.7	8	1.0

COHORT 4:
These oysters were in a large esky. Origin Smoky Bay. Animals were last graded by hand on 27/4/2018 from 6mm to 12 mm mesh bags. Original stocking 6/4/2017. Haemolymph was collected from oyster 1, 2 and 3 for microbiological analysis. Zip lock bag was labelled – Camerons. (n=19) (Figure 3.8, Table 3.4).

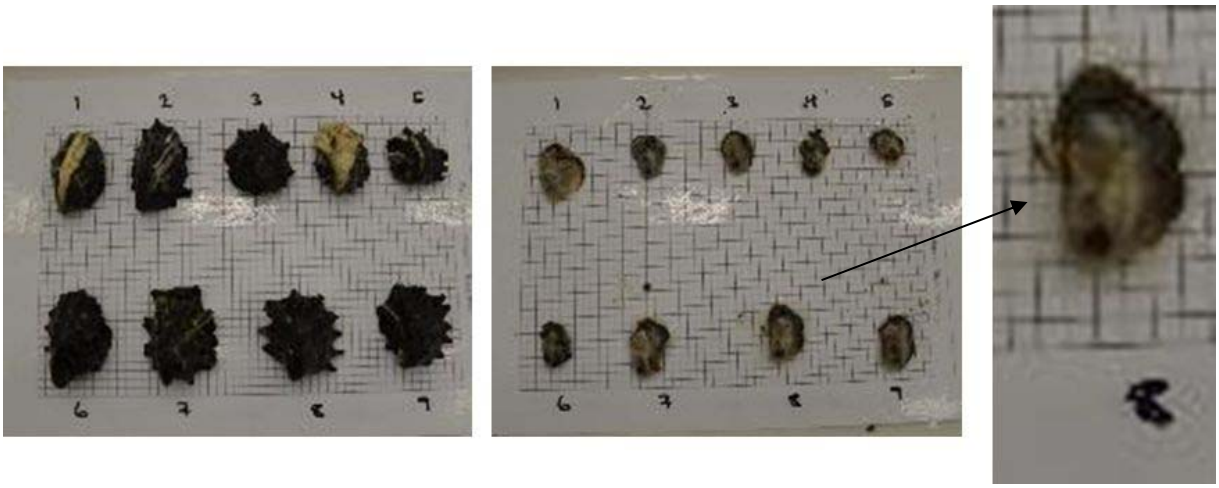

Figure 3.10. Gross examination of shell shape and meat condition. Oyster 8 condition score of 2.

Table 3.4. Data recorded from examinations of oysters in cohort 4.

No.	Comments	Total Length (cm)	Shell Depth (cm)	Condition Score	Total Weight (g)	Meat Weight (g)	Meat Displaced Volume (ml)	Density (g/cm^3)
1		5.3	2	2	16.1	3.2	2.5	1.3
2	Soft Shelled	5.4	2.2	1	9	1.8	1.5	1.2
3		4.4	1.5	0	8.4	1.7	1.5	1.1
4	Float on volume test	4.5	1.8	0	8	1	0.5	2.0
5		3.3	1.4	1	7.6	1.3	1	1.3
6		5.3	2.6	0	9.9	1.7	2	0.9
7		5.4	2.6	1	15.2	2.6	2	1.3
8		4.5	2.3	2	14.5	2.5	2	1.3
9		4.4	1.7	1	14.2	2.3	2	1.2

COHORT 5:
These oysters were in a large esky. Origin Smoky Bay. Haemolymph collection and meat displaced volume measure was not undertaken for this cohort due to individual oyster size. Animals were graded underwater on 19/4/2018. Out of spat trays to 6mm baskets 20/1/2018. (n=18). Zip lock bag was labelled – SARDI (Figure 3.9, Table 3.5).

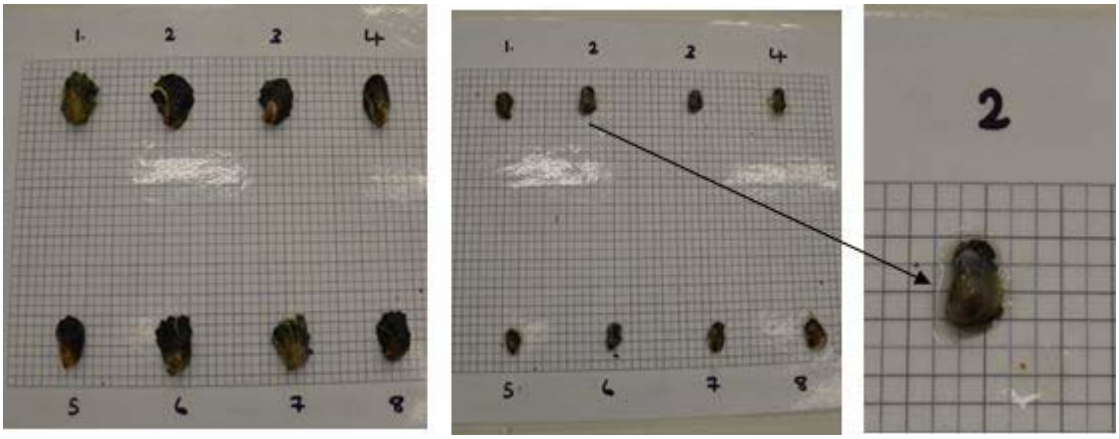

Figure 3.11. Gross examination of shell shape and meat condition. Oyster 2 condition score of 1.

Table 3.5. Data recorded from examinations of oysters in cohort 5.

No.	Comments	Total Length (cm)	Shell Depth (cm)	Condition Score	Total Weight (g)	Meat Weight (g)
1	Dark oyster - suspect dead	2.5	1	0	1.6	0.2
2		2.6	0.8	1	1.8	0.3
3		2.4	0.7	0	1.3	0.2
4	Dark oyster - suspect dead	2.9	0.9	1	2	0.3
5		2.3	1	1	1.2	0.2
6		2.7	0.8	0	1.9	0.2
7	Soft shelled	2.3	0.8	1	1.4	0.2
8		2.2	0.8	1	1.6	0.3

MICROBIOLOGICAL RESULTS

15 significant isolates grown from the haemolymph of various oysters sampled onto Sheep blood agar plus salt and TCBS media and then identified utilising the MicroSys™ identification system of biochemical typing. These identifications were undertaken by the Animal Health Laboratories, Mt Pleasant, DPIPWE, Tasmania.

1 *Vibrio* sp. (unidentifiable to species level)
2 *Vibrio* sp. (unidentifiable to species level)
3 *Vibrio* sp. (unidentifiable to species level)
4 *Vibrio* sp. (unidentifiable to species level)
5 *Vibrio* sp. (Phenon 8)
6. *Vibrio* sp. (unidentifiable to species level)
7 *Vibrio* sp. (Phenon 8)
8 *Vibrio* sp. (unidentifiable to species level)
9 *Vibrio* sp. (unidentifiable to species level)
10 *Vibrio* sp. (unidentifiable to species level)
11 *Vibrio* sp. (Phenon 8)
12 *Vibrio* sp. (unidentifiable to species level)
13 *Vibrio* sp. (unidentifiable to species level)
14 *Vibrio* sp. (unidentifiable to species level)
15 *Vibrio* sp. (unidentifiable to species level)

HISTOLOGY FINDINGS:

Detailed histological findings are listed in Appendix 3. A summary of significant histopathological findings are presented below.

Cohort 1.
Three fish were examined from this group.
In the mantle there was shell gland dense epithelium. Multifocal areas of epithelial erosions and associated connective tissue oedema were also present. There are zonal areas of the hepatopancreas (HP) with unidentified round cells within the lumen (sloughed enterocytes or amoeba?). The HP generally appears degenerative in all fish. There were multifocal aggregations of haemocytes on the

myocardial wall. Leydig tissue is reduced in amount across the group with increased density some of which is vacuolated. There were haemocyte aggregations present in vacuolated areas. Low to no stomach or intestinal luminal contents.

Cohort 2.
Three fish were examined from this group.

The epithelium of the mantle was hyperplastic with rare focal sub-epithelial oedema. Low to no stomach or intestinal luminal contents. Mild to moderate focal haemocyte migration through the intestine and stomach wall epithelium and there was variable Leydig tissue depletion with occasional focal density.

Cohort 3.
Three fish were examined from this group.

There was hyperplasia of the shell glands of the distal mantle. Multifocal haemocyte aggregations on the shell surface of the mantle within the connective tissue. Focal erosion and oedema of the epithelium around the viscera. Moderate numbers of Brown Cells throughout the meat.

There is atrophy/loss of fibres within the heart with marked dense peripheral aggregation of haemocytes around the endocardium. The pericardium appears thickened with vacuolated epithelium. There was intra-lesional bacteria associated with haemocyte aggregations. There are two types present: Filamentous bacillary form and stumpy bacillary form that appear to be sporulating. Moderate numbers of brown cells throughout the mantles connective tissue.

A focal erosion if the epithelial lining of the gills which is more common on the proximal ends of the gills compared to distal. Variable vacuolisation in the connective tissue of the gills. Focal haemocyte aggregations in the proximal extent of the gills associated with dilated haemolymph channels. Majority of the haemocytes are large and granulated.

There is moderate transmigration of haemocytes across the stomach epithelium. There is no stomach contents apart from haemocytes and a granular pink matrix. One bacterial plaque is noted. There is a focus of long bacterial invasion into the style with associative necrosis.

In one oyster there were two focal areas where the normal intestinal epithelium is lost and there is an aggregation of GIT content material with copious bacterial elements within. There is no significant haemocyte reaction around them. The hepatopancreatic tissue against it is undergoing coagulative necrosis.

There is copious filamentous to bacillary bacteria in the Leydig tissue. There is multifocal areas of haemocyte aggregation some associated with bacteria.

Cohort 4
Three fish were examined.

Generalised erosion of the epithelium of the mantle and the body of the oyster. The shell gland appears dense and active but there is marked loss of epithelium. Hepatopancreas is generally healthy with small numbers of dilated or generalised epithelial metaplastic change and autolytic degeneration.

Heart shows thin myocardium with multifocal aggregations of haemocytes in the myocardial tissue itself to no change at all.

Mild transmigration of haemocytes numbers across the stomach and intestinal wall.

Cohort 5
Eight fish examined from this group.

There was either no significant change noted or the fish were advanced in autolysis.

CASE 1 SUMMARY

The key in the interpretation of this submission is to note that the oysters submitted have variable geographic origins. Environmental influences at each grow out area were different prior to submission of the animals for diagnostic purposes. Cohorts 2-4 have variable origins and grow-out history despite being submitted from a single lease holding in Smoky Bay. The common clinical sign across all submitted cohorts is one of increased mortalities (above farm set triggers for reporting) at the time just prior to when the animals were submitted for examination. There is a single reporting point for these submissions, however it cannot be determined from the submitted history whether there is a single point influence that has produced increased mortalities in the oyster stocks.

The significant findings in the oyster stock from the Port Lincoln submission were a lack of ingesta in the intestinal lumen and stomach, and evidence of Leydig tissue atrophy and degeneration indicating utilisation of lipid and glycogen from this tissue. The presence of unidentified amoeba within the hepatopancreatic tubular lumens is noted. This may indicate high bacterial counts within the ingesta or other conditions which favour amoeba presence. This has been noted in the past from oysters during health surveillance and disease diagnostic investigation but the significance is yet to be determined. Increased numbers may indicate immunosuppression within the host. Whilst active shell glands were noted, the erosion and ulceration of the mantle and body epithelium may indicate sub-optimal water quality. The presence or sub-epithelial oedema is indicative of osmo-regulatory failure and/or low haemolymph protein levels. There was no indication of reduced salinity levels and the lease from which the stock was taken is situated such that coastal runoff from rain events should have minimal effects. Generally the animals were in poor condition as indicated from gross appearance, morphometrics and histopathological changes.

The four oyster cohorts from Smoky Bay that were submitted presented a range of pathologies. Cohorts 2 and 4 presented with mild focal epithelial erosion and low feed intake. Condition scores were low and there was evidence of Leydig tissue atrophy. The variable amounts or intestinal and stomach wall migration of haemocytes indicated increased turnover of inflammatory cells as well as possible luminal antigens producing an opsonisation of cells. Cohort 5 presented as small oysters undergoing varying degrees of death with autolysis possibly induced by poor sample handling.

The epithelial changes observed in the other cohorts was also observed within samples from cohort 3. All cohorts showed active gland cells within the mantle indicating active shell growth. There were changes indicative of ante mortem bacterial infection in cohort 3 animals. Intralesional bacteria associated with haemocyte aggregations and changes in the gill tissue indicated active response to infection. All animals in this cohort were of poor condition. There were significant myocardial changes observed, with the presence of Brown Cells indicating possible debilitation.

The microbiological results from this submission indicate mixed *Vibrio* spp presence rather than primary infection by previously known pathogenic bacteria. The fact that all isolates were unidentifiable to species levels is significant. Samples were not plated to media specialised for the growth of Flavobacteriaceae and so the filamentous bacteria observed in one of the Cohort 3 animals was not able to be identified.

Generally all animals submitted were of poor condition and showed signs of poor nutritional status. Any bacterial isolates were more likely secondary rather than primary. There was no pathological evidence suggestive of viral infective agents. It is worthy of note that this investigation does not meet the current case definition of SAMS as a result of the animals being investigated at the commencement of winter and the animals are of varying age groups, some going into the first winter on farm and others having experienced at least one winter on farm before experiencing increased mortalities.

Case 2

AQUATIC ANIMAL PATHOLOGY REPORT
UA Veterinary Diagnostic Laboratory reference number: 19-00236
Species: Pacific Oyster
Culture Type: Lease grown
Time and date of death: 14/2/2019
Time and date of post mortem examination: 14/2/2019
Carcass condition & preservation: Four separate cohorts of oyster varying in age and size each in a respective zip lock bag were submitted for examination. The samples had already been sampled ex farm by Vetlab and then extra animals sent on to this project 7 days from farm collection. Note: Bag labelled A (Cohort A) was empty shells.

HISTORY:
Cohorts A & B were collected from a single farm and the other two cohorts were submitted from two other separate farms in the Cowell growing area of South Australia. Samples were submitted due to the on farm and state mortality trigger levels being exceeded (i.e. >20% mortality). Samples tested negative for the presence of OsHV-1 (POMS) qPCR by Vetlab. All stock was approximately 20mm in shell length.

No detailed history was provided with stock but some information was provided on the bags in which the oysters were submitted.

Cohort A – 57.5% dead no growth, 42.5% dead with growth (assumption by project staff was that the animals had been stocked for a period long enough that growth could be observed in normal healthy stock., time frame not provided).

Cohort B – All animals alive on collection 6/2/2019

Cohort C – 60% mortalities in submitted stock cohort. No history of whether stock recently introduced to farm or whether had been on farm for a period of growth prior to mortalities observed.

Cohort D – 75% alive, 16.7% dead from recently introduced stock, 8.3% dead from previously introduced stock (n=277 animals examined). Percentages are of the stock from this on farm cohort, a portion of what was submitted for examination. No history of origin of stock or period on farm.

MICROBIOLOGICAL RESULTS.
Samples submitted for culture and identification were the liquor from body cavity between shells.

Cohort B:
There was mixed bacterial growths from this cohort however all growth on plates was mild to nil and not significant enough to warrant further identification.

Cohort C:
Significant population of bacterial growth was noted from this cohort. MALDI-TOF was used to identify the significant isolated colonies. The following identifications were obtained from heavy growth colonies.

Identification: *Vibrio pomeroyi* (2.13)* & *Vibrio gigantis* (1.86)*

Cohort D:
Several significant population of bacterial growth were noted from this cohort. MALDI-TOF was used to identify organism which displayed heavy growth.

Identification:
- Yellow colony on TCBS: *Vibrio pomeroyi* (2.01)* & *Vibrio gigantis* (2.02)*

- Green colony on TCBS: *Vibrio alginolyticus* (2.09)* *Vibrio natriegens* (2.03)*

* MALDI-TOF score for matching algorithm. A score above 2 is deemed a significant identification match.

HISTOLOGY FINDINGS:
The full details of histopathological findings from this case can be found in Appendix 4.
A number of animals were autolysed and non-diagnostic.

A summary of the significant findings is given below.

Cohort B – 13 sections of separate oysters were examined. There was mild focal erosion of the external epithelium of the mantle and body with occasional associated sub-epithelial oedema.

There were variable amounts of digesta within the lumen of the stomach and intestine. All samples had variable sized styles, indicating intake and processing of food prior to submission. There were *Uronema*-like ciliated organisms within the tissues of some animals. A number of animals had marked thinning of the myocardial strands with variable focal aggregation of haemocytes. Leydig tissue was scant or dense with associated haemocyte aggregations. There were hyalinised changes in the epithelium of kidney tissue in a number of animals.

Cohort C - 12 sections of separate oysters were examined. The significant findings across most animals in this cohort are little to no intestinal or stomach ingesta, haemocytosis in the Leydig tissue, and autolysis of tissues.

Cohort D - 12 sections of separate oysters were examined. There is a notable hyperplasia of all types of haemocytes (haemocytosis) across all tissues. There was a low amount of digesta in the intestinal tract and in animals that had ingesta, it was monomorphic in nature (one type of algae). There was a generalised hepatopancreatic tubular necrosis across all oysters (autolytic change or tubular toxicosis? (toxic algae)) () Figure 3.10, 3.12). The change was noted distinctively as all other tissues were well preserved. In most animals examined there were rudimentary undifferentiated gonadal crypts but in a couple of the fish there were well differentiated testes containing spermatozoa (Figure 3.10, 3.11). The heart appeared thin in two oysters (Figure 3.12).

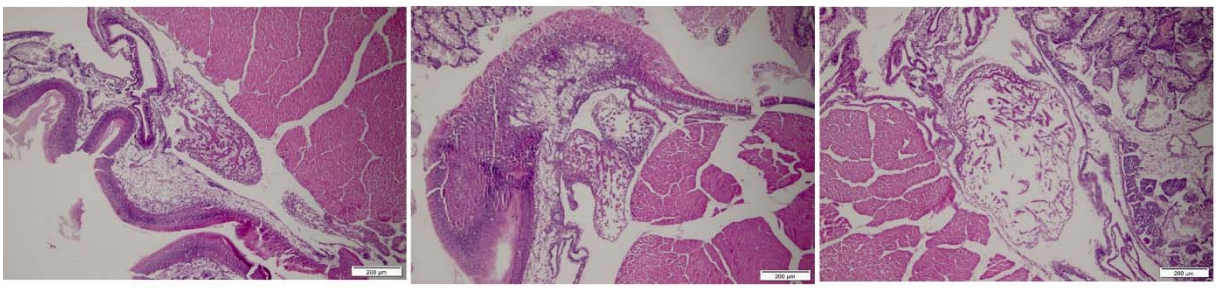

Figure 3.12. Myocardium of Pacific Oysters from Cowell investigation. Left-dense myocardium, centre- loss of focal density, right-marked thinning of myocardium.

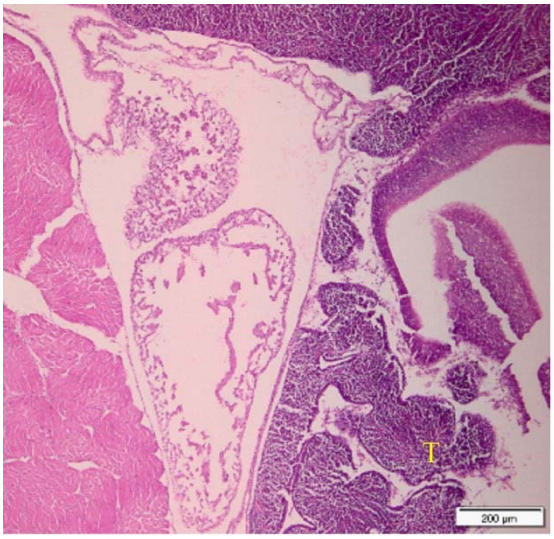

Figure 3.13. Mature male gonad in juvenile oyster of approximately 20mm shell length (T –testes).

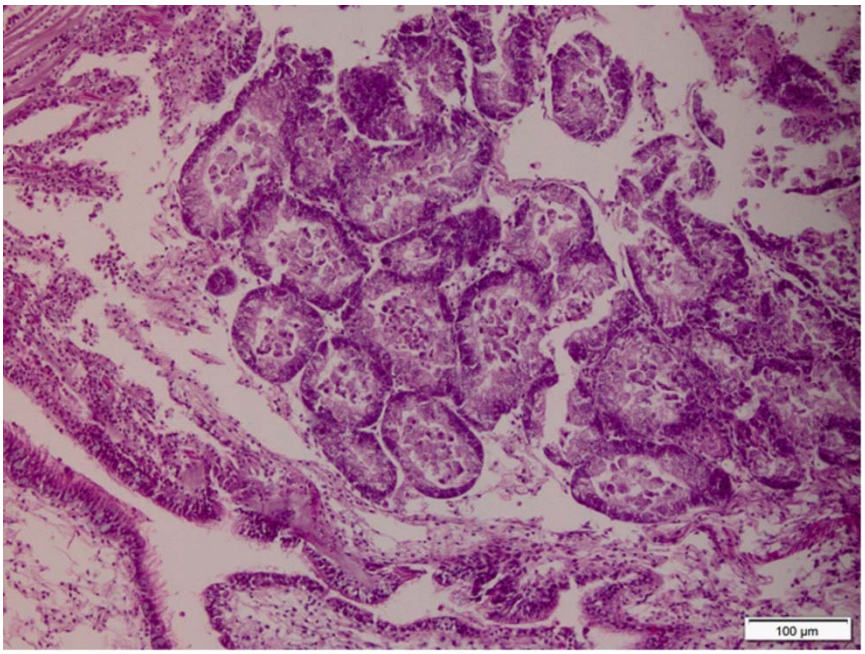

Figure 3.14. Necrotic hepato-pancreatic tubule epithelium.

CASE 2 SUMMARY

Unlike the previous case the oysters submitted were all from the same growing area although from 3 separate farms with their own stock management systems. Variable mortality rates were noted in the juvenile stock. Samples had arrive 7 days post collection from farm and so some changes observed may have to be interpreted in this context. Time out of water leading to prolonged closure of shell and developing anaerobic conditions within the shell cavity may lead to pathological changes. When interpreting subtle changes, particularly observed on histological examination, this can impede causal diagnosis.

The microbiological isolates deemed significant enough to process to the level of species identification for this case, were representative of those known to be associated with Pacific Oysters worldwide (Carson *et al*, 2009; Romalde *et al*, 2014; Travers *et al*, 2015). The identified species, with the exception of *V. alginolyticus*, have not been reported as a causal agent of morbidity or mortality in Pacific Oysters. *V. alginolyticus* and *V. natriegens* are within the *V. harveyi* Clade as determined by molecular taxonomy (Travers *et al*, 2015), a group that contains a number of bacterial species that are commonly associated with and causal to, significant mortalities in molluscs, crustacea and teleost fish, particularly in hatchery settings.

Members within the clade have been found to possess a number of virulence factors (i.e. adhesion molecules for epithelium and haemocytes, extra cellular polysaccharides, lytic proteins and siderophores, and iron acquisition). The presence of these factors in the isolates from this case, was not investigated. The presence of the bacteria species is worthy of note but not casually diagnostic.

The significant histopathlogies noted in animals from this submission are the necrotising pathologies particularly within the hepatopancreas, the marked generalised haemocytosis in and around organs, renal epithelium changes, and the variable change observed within the myocardium of the oyster from one farm.

The necrosis and sloughing of the hepatopancreatic tubular epithelium is reminiscent of algal, petroleum and other aromatic organic hydrocarbons as well as bacterial toxins. These bacterial toxins could be a primary pathogenic event or secondary to immunosuppression and over growth of endemic bacterial populations. Given the isolation of *V. alginolyticus* in this case there is a possibility of bacterial pathogenesis in these mortalities. The animals were juvenile, not long on farm and may have contained an endemic bacterial population from previous environments that was able to cause mortalities as the animals established into a new environment.

The presence of ciliated protozoa within the tissues indicated possible carriage of the organisms from other more heavily stocked environments or are an indication of opportunistic invasion as a result of immunosuppression.

The precocious male gonad development observed is worthy of note as it may indicate exogenous hormonal influence (again causality is unable to be established) or may be just a genetic feature of the batches propagated for release to farm.

The presence of low to no ingesta within the animals examined is consistent with a time out of water and the presence of a monomorphic algal population within one animal may indicate low or even selective growth of phytoplankton in the vicinity of the animals when collected. The algae was not identified and so implications of causation of hepatopancreatic epithelial necrosis cannot be established. A predominance of a toxic species within the gut and hepatopancreatic lumen is the usual case when such diagnosis of algal toxicosis is made.

Generalised haemocytosis is common in systemic viral and bacterial infections in Pacific Oysters. There were no other specific pathological changes that would suggest a primary viral cause of the mortalities and given the isolation of potentially pathogenic bacteria from some of the animals, mortality is consistent with bacterial septicaemia.

The thinning of the myocardia and renal hyalinisation are noted but cannot be interpreted. They may indicate a degree of toxicosis given the lack of inflammatory reaction associated with the changes.

It should again be noted that the sampled oysters by size and timing do not fit the SAMS case definition. The case is a significant mortality event with one farm quoting 60% mortality in on farm stock, but it was not a case of SAMS.

Case 3

AQUATIC ANIMAL PATHOLOGY REPORT
UA Veterinary Diagnostic Laboratory reference number: 19-00995
Species: Pacific Oyster
Culture Type: Lease grown
Time and date of death: 22.5.2019
Time and date of post mortem examination: 24.5.2019
Carcass condition & preservation: Two sample cohorts were submitted for examination. Live spat (n=70) and dead shell (n=80). Sent in zip lock bags that were held in esky keeping samples cool.

History:
A PIRSA lead oyster mortality investigation was launched on May 22nd, 2019 when a single producer reported mortality rates in stock ranging from 30-70% on leases on the western beach areas of Smoky Bay. The stock was from one sourced hatchery, 5-10mm (spat) in size (at submission) and had been on the lease in intertidal conditions for 6 months. Some initial growth from 2mm had been seen, which slowed prior to the mortality event. SASQAP had reported very low algal counts in the bay and no harmful phytoplankton had been observed in routine surveys of the bay near the affected area. Water temperatures had dropped to 15-16^{o}C on or about 12th May after being 2-3^{o}C warmer. Samples were submitted for rule out OsHV-1 by PCR protocol and all samples submitted were negative for the presence of the OsHV-1

On farm, the owner had observed empty shells and shells that still contained dead and decaying oyster material that smelled, indicating ongoing mortalities at time of sampling.

Necropsy findings:
Moderate to high levels (variable) of mixed ecto-commensal organisms associated with the live oysters submitted.

Wet prep examination: Shell liquor was examined under light microscope to reveal sessile ciliate protozoa, free swimming flagellated protozoa. Small rotifers and amphipod crustaceans of mixed morphologies were observed moving in the seawater around the whole oysters. Variable cap closure responsiveness was observed in animals re-submerged in seawater.

MICROBIOLOGICAL FINDINGS.
Samples were obtained from the shell liquor of animals from the live animal cohort and plated as described previously for isolation and identification of significant bacterial isolates.

Below is a list of bacterial isolates that showed strong growth from some oysters, however it is important to note that these isolates were not cultured consistently in every animal. Identification was made using MALDI-TOF by the University of Adelaide.

1. *Vibrio fortis* (1.85)
2. *Vibrio tasmaniensis* (1.82)
3. *Vibrio pomeroyi* (1.97)
4. *Vibrio gigantis* (1.89)
5. *Vibrio chagasii* (1.71)
6. *Vibrio kanaloae* (1.73)

* MALDI-TOF score for matching algorithm. A score above 2 is deemed a significant identification match.

HISTOLOGY FINDINGS:

15 oysters were prepared and examined for histopathology. The full details of histopathological findings are given in Appendix 5.

The following is a summary of the significant pathologies observed.

There was variable mild to focally extensive erosion of the mantle with associated sub-epithelial oedema. Some of these affected areas were also associated with haemocyte aggregation and acute inflammatory change. Intralesional filamentous and mixed population bacteria were occasionally seen.

Mild infiltration of the mantle by Brown Cells was also noted in many of the samples and one focal area of attached sessile protozoa was also observed.

The gills of many of the oysters had epithelial erosion, generalised oedema of connective tissue with the presences of Brown Cells and focal haemocyte aggregations. Trichodina-like ciliated protozoa were mild to moderate in numbers between the gill tissues most samples examined (Figure 3.13). All situated within the areas between the gill tissues. Occasional focal necrosis of gill tissue was present.

Commonly the Leydig tissue was depleted, with mild focal aggregates of haemocytes randomly distributed and containing moderate numbers of Brown cells.

Within the hepatopancreas of many of the samples examined there was variable atrophy of the tubular epithelial lining that ranged from atrophy to epithelial metaplasia. In some animals, 60 to 70% of the epithelium was transformed giving the tubules a dilated appearance.

A number of animals examined displayed myocardial pathology. These ranged from marked focal haemocyte aggregation within the myocardium with associated large numbers of Brown cells present within the ventricle and myocardial necrosis, to mild thickening of the endocardium and complete atrophy of the myocardium.

Occasional myopathy and myositis with intralesional bacteria of the adductor muscle was observed

There were variable amounts of digesta in the stomach and intestine, with stomach ingesta composed of protein plaques and filamentous algae as well as mixed zooplankton and mollusc larvae. Focally extensive areas of haemocyte aggregations surrounding various parts of the gastrointestinal tract were also noted.

CASE 3 SUMMARY

This case was one of spat mortalities post stocking from a hatchery. There was evidence of growth post stocking in all cohorts on lease but with variable mortality rates measured across similar aged stock. The mortality was ongoing at sampling as evidenced by necrotic meats still present in some sampled stock. Whether the initial mortalities occurred close to the time of sampling for this investigation or whether ongoing at a low level for a longer period, is hard to ascertain.

There was a significant number of mixed ectocommensal organisms in the shell liquor and associated with the live animals examined. This mixed population was dominated by *Trichodina*-like organisms indicating a high level of suspended bacterial flora in the liquor and possibly the seawater associated with the animals prior to harvest for submission (Figure 3.13). Classification of the ciliated protozoa (i.e. *Trichodina*-like) is made because no specific examination was made of the organisms to type to genus and species level. Their morphology is consistent with *Trichodina* spp and we have therefore classified them as *Trichodina*-like. *Trichodina* are commonly associated with disease in teleost fish, crustacea and molluscs, but it is yet to be proven that their presence is primarily causal to any of the conditions with which they are associated. High numbers of organisms present in confined anatomical positions (e.g. gill space) are assumed to be irritant to the host by the physical nature and movement of the parasite itself. If this is the case, then it may help explain the focal erosion of epithelial surfaces of the mantle and gill observed in the histopathology in this case. These erosions may also be explained

by the excretion of extracellular products from the bacteria cultured in many of the shell liquor fluids sampled.

The bacteria isolated and identified from the animals sampled in this submission have been reported as commonly associated with molluscs and particularly oysters (Travers *et al,* 2015; Romalde *et al,* 2014). It should be noted however that the MALDI-TOF score was below the optimum level of 2 for each of the isolates.

Vibrio tasmaniensis, V. kanaloae,V. pomeroyi,V. gigantis,V.chagasii are members of the *V. splendidus* clade and have been variably associated with *C. gigas* mortalities and disease. *Vibrio tasmaniensis* and its genetically closely associated species *V. kanaloae,* have been associated with abductor muscle lesions in *C.gigas* and are indicated in producing excretory toxic proteins (Travers *et al,* 2015). Adductor myositis and myopathy were noted in some of the oysters examined from this submission. *Vibrio fortis* is a member of the *V.harveyi* clade, other members of which have been associated with oyster disease. *Vibrio fortis* is not explicitly implicated in pathology but has been associated with gastroenteritis in aquatic organisms and producing extracellular substances found in biofilms.

The significant numbers of animals in this case which showed haemocyte hypercellularity and haemocyte aggregations within tissue would suggest that an antigenic stimulation was present, and in particular a bacterial antigen effect. Haemocyte aggregation and focal cellulitis was also observed in a number of animals in this submission, indicating ante mortem disease. Intralesional bacteria can be primary or form part of the post mortem flora of oysters. The presence of filamentous bacterial forms in occasional lesions may indicate post mortem proliferation.

The significant histopathologies observed in this case are consistent with stored energy utilisation (Leydig tissue degeneration and atrophy) and antigenic response characterised by Brown cell accumulations, haemocyte aggregations, focal inflammation and necrosis with intralesional bacterial elements in various tissues. This would be interpreted as signs of a bacterial septicaemia. The lack of pure growth of a single species of bacteria ruled out a primary bacterial infection, but indicate more of a secondary obligate pathogen. The generalised oedema of tissues is indicative of osmoregulatory imbalance, either as a result of suboptimal salinity levels on lease or subsequently during transport. It should be noted the animals were in transport to the laboratory, before examination, for 7 days.

The hepatopancreas was variably atrophic and degenerate in the samples submitted. There were also specific findings of suppurative hepatopancreatitis. The normal interpretation of this finding in *C. gigas* would be that the animals were in a poor nutritional condition prior to sampling (atrophy and apparent dilation of hepatopancreatic tubule lumens). This may not be the case with these animals due to findings of dense ingesta within E-gut structures of some animals. The contents of the ingesta were suggestive of adequate mixed filtrates pre sample collection. Interestingly, mollusc larvae and amoebae were noted within the ingesta, suggesting turbid water conditions. These hepatopancreatic changes indicate that the animals had adequate filtrate available, but the nutritional quality may not be optimal for maintenance of normal functional morphology of the hepatopancreas and normal energy levels within the oyster.

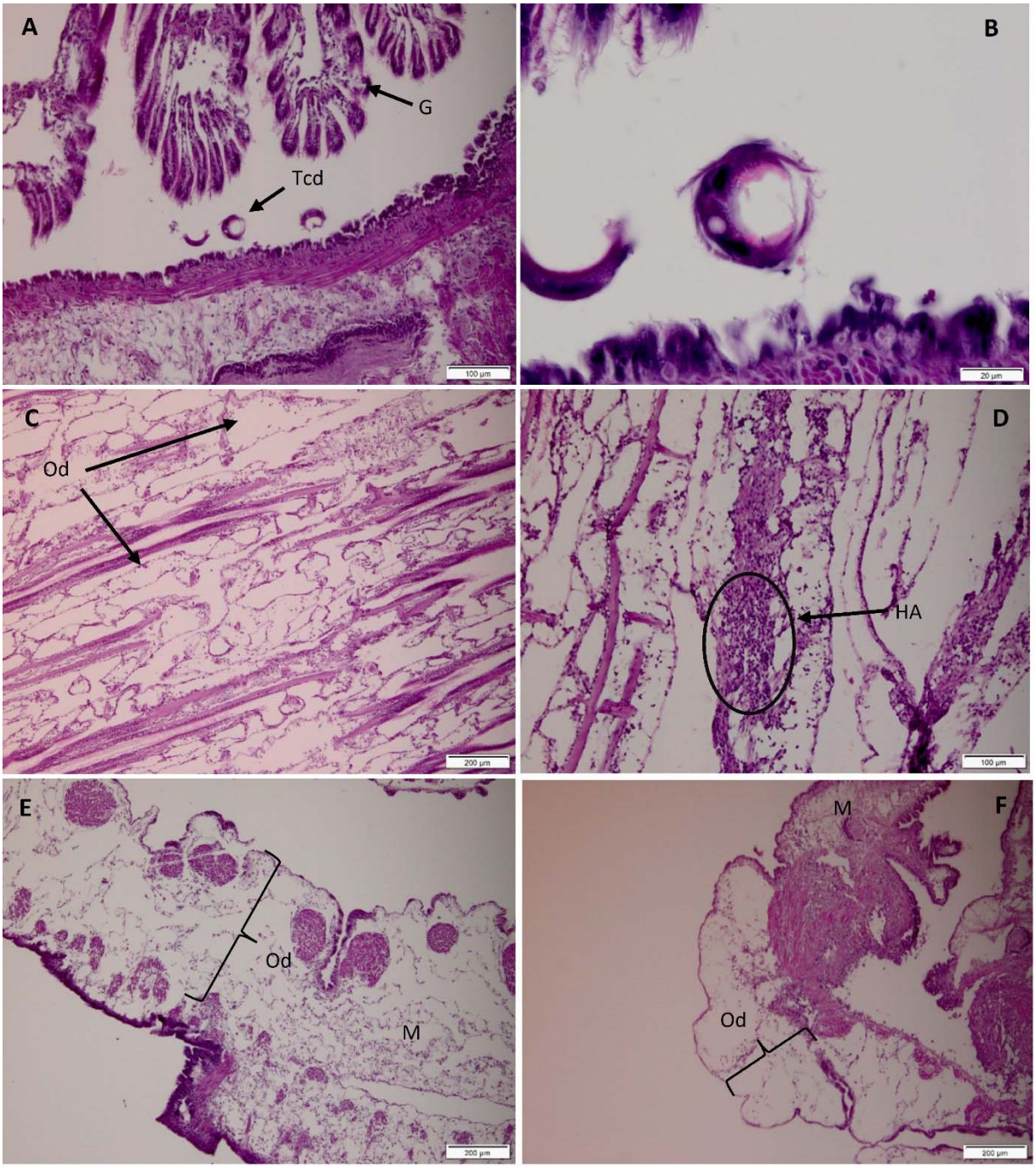

Figure 3.15. A: Trichodina-like ciliated protozoa [Tcd] observed in the gills [G], B: Magnification of Trichodina-like protozoan, C: Generalised oedema [Od] of the gill connective tissue, D: Epithelial erosion of the gills with generalised oedema of the connective tissue and focal haemocyte aggregations [HA], E: Sub-epithelial oedema of the mantle [M], F: Focally extensive erosion and sub-epithelial oedema observed in the mantle.

The myocardial changes observed in a number of the animals are unexplained. The myocardial atrophy and degeneration could be associated with poor nutritional status. The generalised tissue oedema and hypervolaemia may also contribute to expansion of the cardiac space and subsequent myocardial atrophy and degeneration. However there is pathology that would suggest that cardiovascular function was compromised in some of the animals.

Finally the case investigation does not meet the current SAMS case definition, due to the size of oysters examined and the timing of the event.

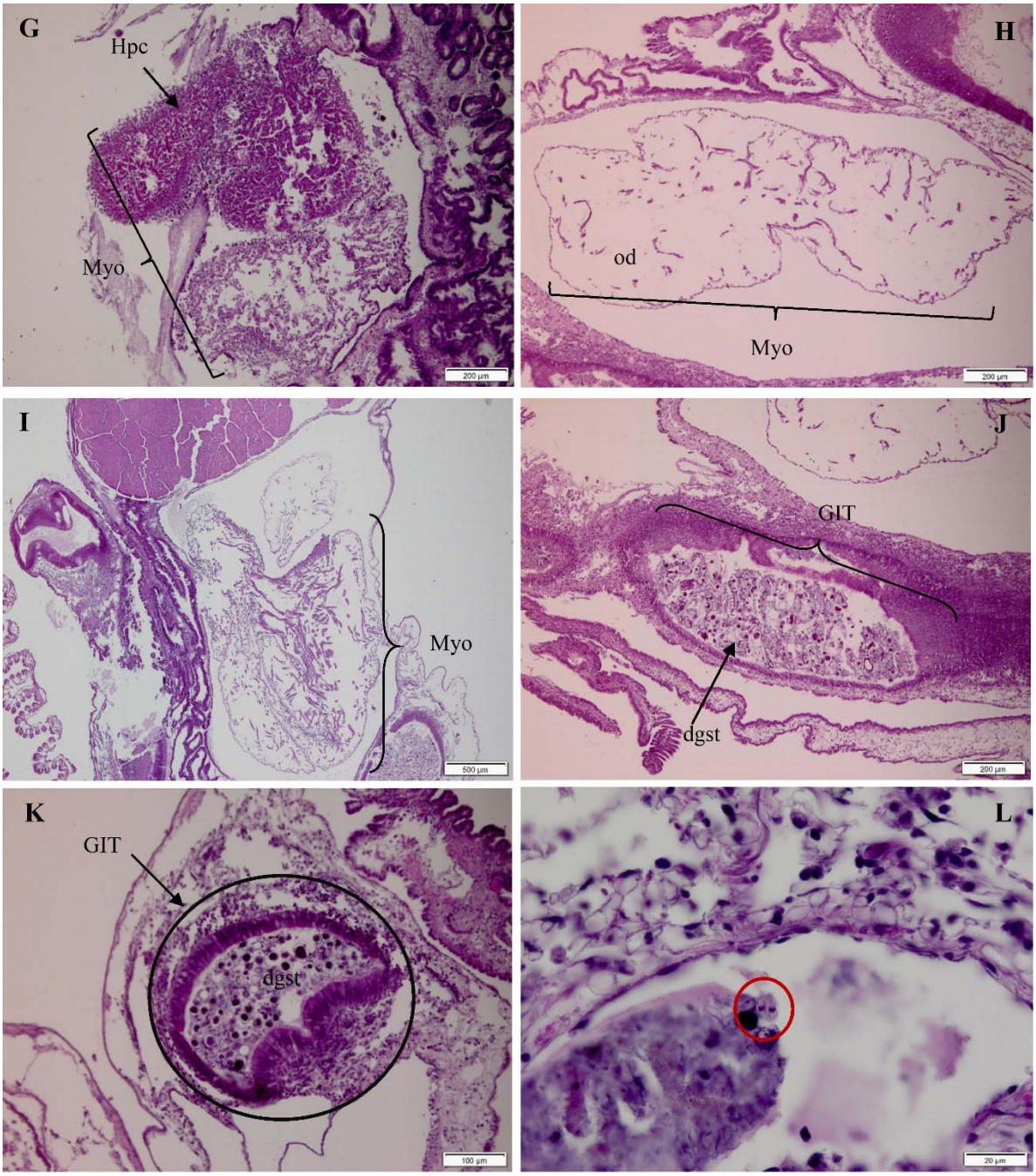

Figure 3.16. G: Thickening of the myocardium [Myo] with hyper-cellularity [hpc] and focal haemocyte aggregations [HA] , H: Myocardial atrophy and oedema [od], I: Regional myocardial degeneration and atrophy within the ventricle and atrium, J: Transmigration of haemocytes within the gastrointestinal tract [GIT] wall with moderate amounts of digesta [Dsgt] (protein plaques and filamentous algae) present within the lumen, K: Magnified image of the oyster E-Gut with moderate mixed population of digestive content within the lumen, L: Magnified image of GIT luminal contents showing an unidentified protozoal body (Myxosporidial?Ameoba?) [Red circle].

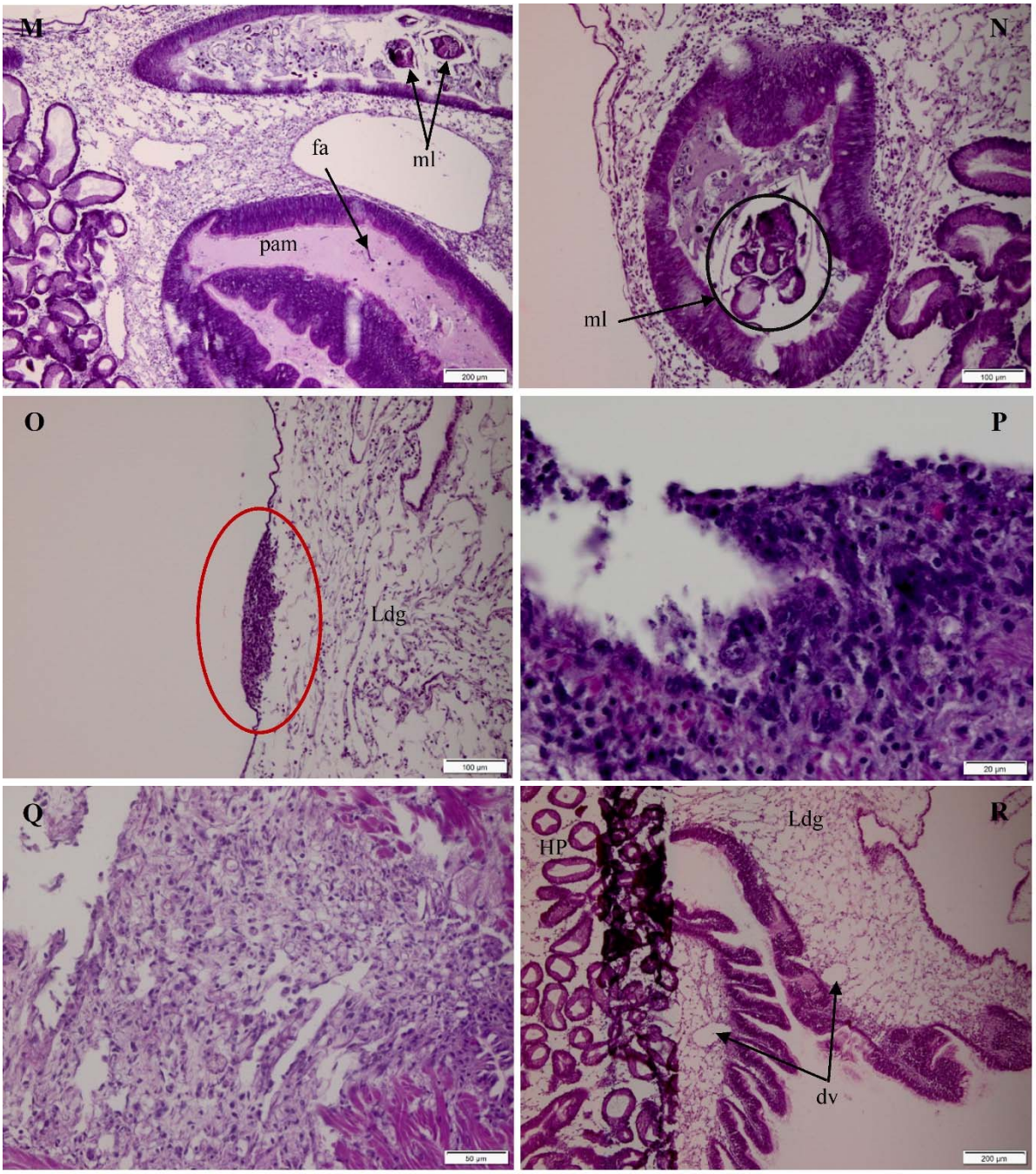

Figure 3.17. M: Variable constituents of digesta [dgst] within the intestine [GIT] composed of pink amorphous material [pam], filamentous algae [fa] as well as mixed zooplankton including mollusc larvae [ml], N: Variable digesta within the intestine with presence of mollusc larvae, O: Depleted appearance of Leydig tissue [Ldg] with a focal zone of inflammatory foci and intra-lesional bacteria [red circle], P: Higher magnification of zone of inflammation with haemocyte aggregations [HA] and bacteria, Q: Magnified image of Leydig tissue showing similar haemocyte aggregations and bacterial presence, R: Depleted Leydig tissue surrounding the hepatopancreas (HP) with evidence of dilated vasculature [dv] observed.

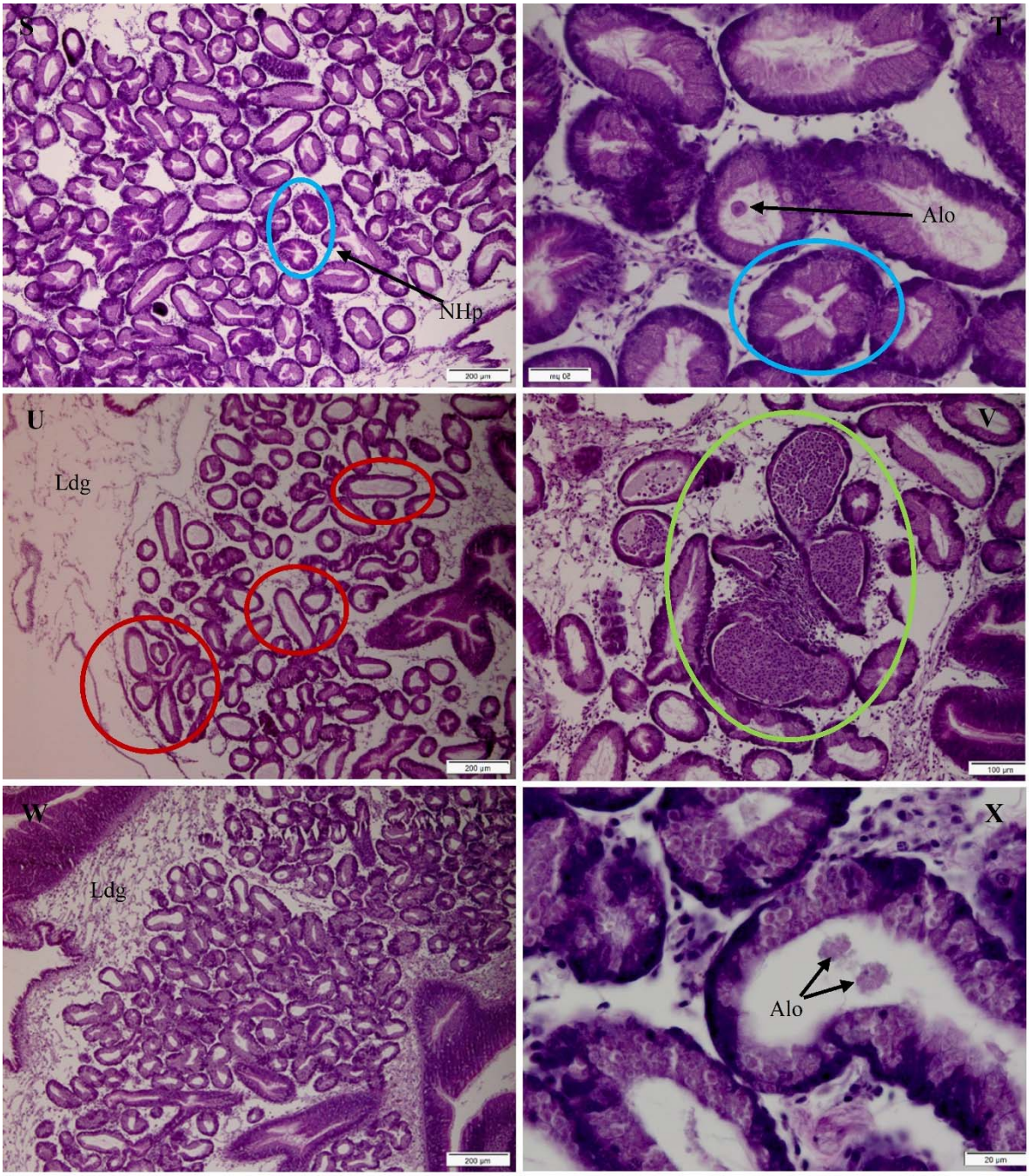

Figure 3.18. S: Low percentage of hepatopancreatic [HP] tubular epithelial atrophy with presence of amoeba-like organisms [Alo]. Normal tubular appearance [NHp], T: Magnification of image S focussing on the amoeba-like organism. Normal tubular appearance is highlighted [blue circle] U: Atrophy of epithelial cells lining the hepatopancreatic tubules giving a dilated appearance to the tubules [dilated tubules demarcated in red circle], note also the depletion of the surrounding Leydig tissue [Lgd]. Changes to epithelial lining range from atrophy to metaplasia V: Focal hepatopancreatitis [green circle] surrounded by dilated tubules, W: Moderate percentage of atrophy and dilation of hepatopancreatic tubules, note the normal surrounding leydig tissue [Ldg], X: Magnified image showing amoeba-like organisms within the hepatopancreatic tubular lumens. Tubular epithelium appears traNSFormed by marked granulation of cytoplasmic contents.

Case 4

AQUATIC ANIMAL PATHOLOGY REPORT
UA Veterinary Diagnostic Laboratory reference number: 19-01607
Species: Pacific Oyster
Culture Type: Lease grown
Time and date of death: Not specified but oyster mortalities above farm trigger points noted from Late July- Mid August 2019.
Time and date of post mortem examination: 9th August 2019
Smoky Bay (South Australia) Pacific Oyster mortalities

History:

On July 31st, 2019 five of the approximately 20 oyster farmers in Smoky Bay, South Australia reported increased to high unexplained mortalities in their grow-out Pacific Oysters. Leases were situated on the northern beach side of the bay. The mortalities were reported to PIRSA who initiated a low level investigation of the mortalities. The presence of OsHV-1 was ruled by PCR testing of a sample from each farm. All tested negative for the presence of the virus. SASQAP water testing indicated very low algal cell counts (<20,000 cells/L seawater) around leases in the bay and no toxic algae had been reported in routine sampling. Water temperatures had been measured in the range of 10-12^{o}C with minimum air temperatures 0-2^{o}C (June to early August 2019).

Duplicate samples from the original submission for OsHV-1 rule out were not sent to the University of Adelaide but a second group of samples from 3 affected farms in Smoky Bay were submitted for analysis on the 9th August 2019. Each farm submitted a number of cohorts of oysters from their lease sites within the 'affected' area.

A Smoky Bay farmers meeting was held on the 9th August to discuss the mortalities. It was reported that approximately 12 farms had experienced higher than normal mortalities during the end of June and July 2019. Discussions failed to identify any husbandry techniques or activities that could be deemed as risk factors to instigate the mortalities (e.g. grading or handling of stock).It was noted that much of the *Posidonia australis* sea grass had shed leaves and so had contributed to an organic load in the bay. Modelling of ocean currents in the bay for July 1-7, 2019 had shown very little movement of particles and hence minimal water flows around the bay. All affected leases were in the north of the bay. A local land-based crop farmer had been reported as spraying a MCPA herbicide plus Zn, Mg and Cu liquid fertiliser on three consecutive days (18-20 June, 2019). The framer reportedly used appropriate spray drift mitigation strategies, and investigations by PIRSA compliance with water sampling had assessed the risk level of contact of chemical with the oyster leases as 'virtually zero'.

Gross pathology:

Three farms submitted samples – Farm 1 (four cohorts), Farm 2 (three cohorts), Farm 3 (five cohorts).

Oysters were randomly selected from each cohort for detailed gross, microbiological and histopathological examination. Haemolymph biochemistry was not undertaken due to the size of the animals and the lack of volume available for analysis. A minimum of 200µl is required for full analysis.

Table 3.6. Gross pathology observed in oysters originating from Farm 1

Cohort	Animal number	Shell length (mm) + SD for cohort	Shell pathology	Meat pathology	Wet preparation of liquor	Comments for cohort
1	1	35 (7.6)	nil	Pale meat tissue	NSF	All shells of the oysters in this cohort had pronounced thin protruding new shell growth cap and cups shell (Frill). All cap shell edges were over hung by cup shell edge. Faecal material contained fibrous material, small algal particles
1	2		Brown ring lesion edge of the cup shell	Pale meat tissue	TLC, nematodes and cell debris present in liquor	
1	3		Small yellow shell	Swollen pericardium and retracted meat edge (Mantle)	TLC, flagellated and ciliated protozoans, vacuolated effete haemocytes	
1	4		nil	Swollen pericardium	nil	
1	5		Brown ring lesion on cup shell	Adductor muscle markedly different coloration	TLC present	
2	1	31.5 (4.7)	Brown organic material around mantle	Swollen pericardium	TLC present	All shells of the oysters in this cohort had pronounced thin protruding new shell growth cap and cups shell (Frill). All cap shell edges were over hung by cup shell edge. Faecal material contained fibrous material, small algal particles
2	2		nil	nil	Low numbers TLC	
2	3		nil	Swollen pericardium	TLC present	
2	4		nil	nil	TLC present	
2	5		Ridged shell growth	Swollen pericardium and dark coloured meat		
3	1	24.5 (5.1)	Marked frill shell growth	Brown material in mantle edge. Multifocal white streaking in gill tissue.	TLC present	Shells were small and round in shape. Bizarre shell shapes present with new shell growth.
3	2		nil	Inflammatory proliferation (granular	Filamentous plant material.	

				haemocytes) around area of adductor insertion on cap shell. Brown material in mantle. Swollen pericardium		
3	3		nil	nil	TLC and filamentous plant material	
3	4		nil	Pale gill colour and swollen pericardium	NSF	
3	5		nil	Pale hepatopancreas and swollen pericardium.	TLC present. Haemocyte clots.	
4	1	33.4 (7.9)	nil	Swollen pericardium	TLC and flagellated protozoa, haemocyte clumps	Obvious new shell growth in two distinct rings.
4	2		nil	Adductor muscle changes in colour. Chalky deposits within the meat	TLC present	
4	3		nil	Watery heart	TLC and faecal material with filamentous plant material	
4	4		nil	Swollen pericardium	TLC and ciliated epithelium in clumps	
4	5		nil	Very swollen pericardium	NSF	

NSF – no significant findings; TLC –Trichodina-like ciliates

Table 3.7. Gross pathology observed in oysters originating from Farm 2

Cohort	Animal number	Shell length (mm) + SD for cohort	Shell pathology	Meat pathology	Wet preparation of liquor	Comments for cohort
1	1	30.8 (8.3)	nil	Swollen pericardium and small meat volume	TLC	Variably sized shells with shapes more 'teardrop' than irregular all have thin frill of new shell growth
1	2		nil	Swollen pericardium with very thin and small meat volume	TLC and fat globules and ciliated epithelium	
1	3		nil	Swollen pericardium, very thin meat and marked two toned (brown) colouration of adductor muscle	TLC present with clumped haemocytes	
2	1	31.1 (3.6)	nil	Pale meat colour and small meat volume	TLC present	Marked orange shell streaks and new frill growth
2	2		Brown ring staining in mantle edge cup shell	Small dark coloured meat	NSF	
2	3		nil	Small pale meat	Low numbers of TLC present with clumped haemocytes	
3	1	37.6 (5.6)	nil	Solid dark coloured meat with prominent pericardium	Cellular debris only	Slipper shell shapes in cup shell. Frill growth present all shells and even growth lines.
3	2		White shell matt appearance cap shell only	Small meat volume. Swollen pericardium	Low numbers of TLC and haemocyte clumps	
3	3		nil	Small meat volume. Swollen pericardium	Amoeba and ciliated protozoa and haemocyte clumps	

NSF – no significant findings; TLC –Trichodina-like ciliates

Table 3.8. Gross pathology observed in oysters originating from Farm 3

Cohort	Animal number	Shell length (mm) + SD for cohort	Shell pathology	Meat pathology	Wet preparation of liquor	Comments for cohort
1	1	39.2 (9.1)	nil	Watery small meat	TLC and cell debris	14 oysters submitted 6 DOA. Rough shell growth with marked frill.
1	2		Brown ring on mantle edge	Watery small meat	TLC present	
1	3		nil	Watery small meat	Anaerobic smell on shucking. Filamentous bacteria and cell debris	
2	1	46.6 (9.3)	nil	Small firm meat	TLC	Variable weight and hollow sounding shells despite animals being alive 2 DOA of 19
2	2		Brown ring around mantle edge	Watery meat with swollen pericardium	TLC and cell debris	
2	3		Brown staining cup shell	Watery meat	NSF	
3	1	36.1 (4.1)	nil	Small volume watery meat	TLC and filamentous bacteria	Good shell growth across all fish. 6 DOA of 20
3	2		nil	Small watery meat	TLC and filamentous bacteria and ciliated protozoa with haemocyte debris	
3	3		nil	Small watery meat	TLC	
4	1	49.9 (6.0)	nil	Small compact meat	Anaerobic smell. TLC, ciliated protozoa, Rotifers.	Shells rounded in shape with copious frill shell growth 2-3

4	2		nil	Small solid meat. Swollen pericardium	Filamentous bacteria and ciliated protozoa	marked ridges of shell growth. All animal alive
4	3		nil	Small meat	TLC	
5	1	33.0 (9.0)	nil	Pale coloured solid meat	TLC with cell debris	Good frill growth but shells thin and friable
5	2		nil	Solid small meat	TLC and Rotifer	
5	3		nil	Small meat	TLC	

NSF – no significant findings; TLC –Trichodina-like ciliates; DOA – dead on arrival

The significant gross pathologies noted across the three farms were: brown staining at the edge of the mantle, swollen pericardia, the presence of protozoa (e.g. TLC) and other protozoal commensals within the liquor as well as occasional filamentous bacteria, and adductor muscle lesions associated with haemocyte aggregations. Most cohorts showed evidence of shell growth, as evidenced by mild to copious amounts of frilled shell. Where the meats were found to be watery or small, there is an indication of poor nutrition close to submission. A small number of animals had focal myositis of the adductor muscle.

Figure 3.19. Farm 1- Three Selected cohort samples indicating growth profiles.

Figure 3.20. Farm 2- Three Selected cohort samples indicating growth profiles.

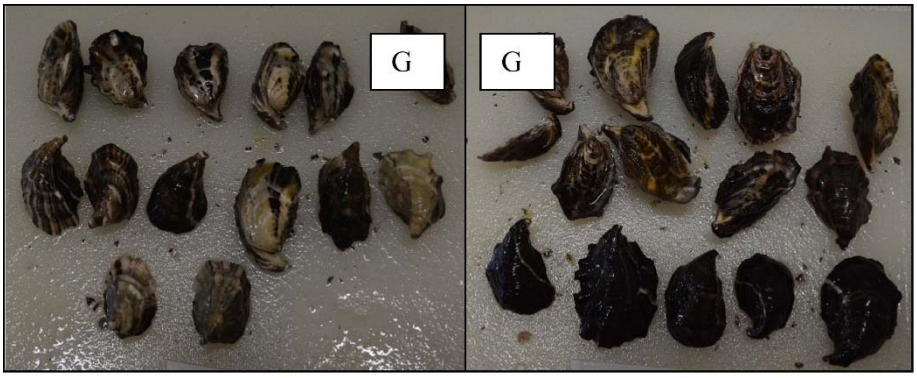

Figure 3.21. Farm 3- Three Selected cohort samples indicating growth profiles.

Figure 3.22. Variable pericardial enlargement [red square] observed in oysters from Farm 1 and 2.

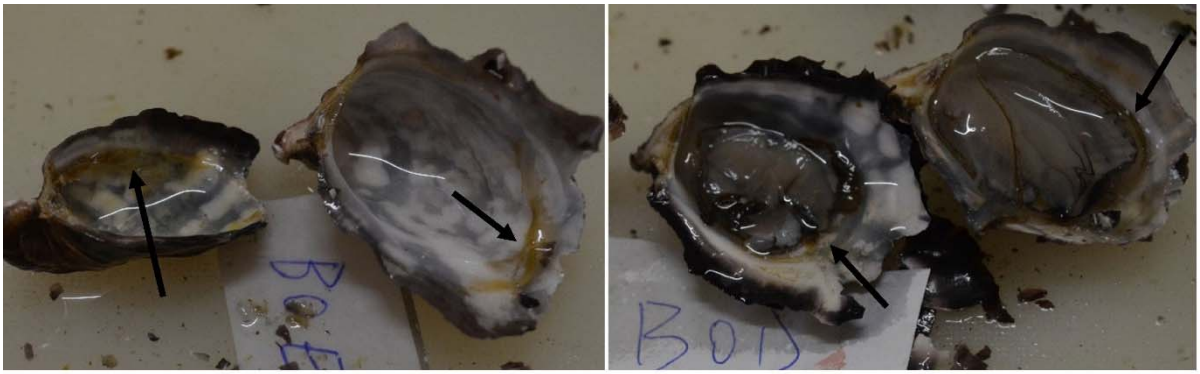

Figure 3.23. Brown ring gross lesions observed [Black arrows].These lesions were noted on samples origination from Farm A and C.

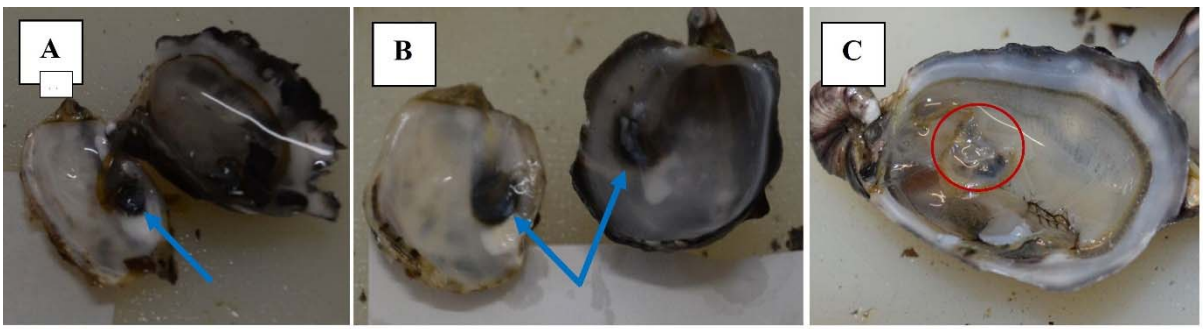

Figure 3.24. Oyster samples with mild adductor muscle lesions are visible on image A and B [blue arrows]. This finding was noted across all farms. Image C represents a chronic inflammatory lesion in the Leydig tissue from Farm C resulting in a calcified 'pearl' formation [red circle].

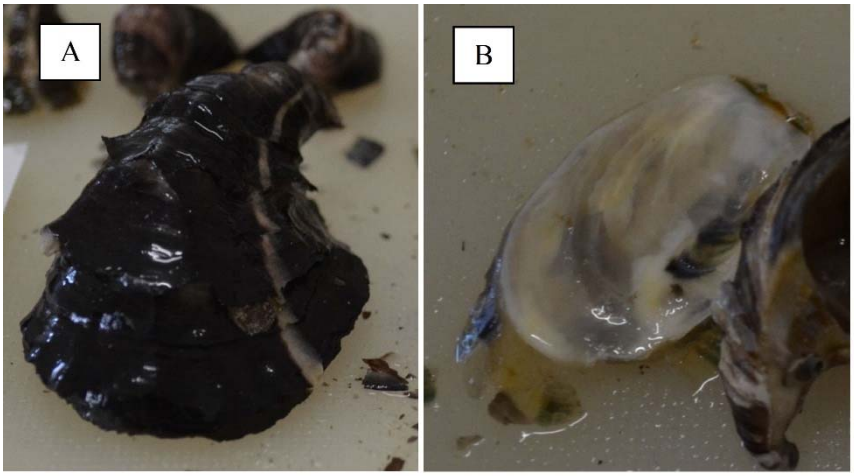

Figure 3.25. Shell changes above were noted from Farm C. A. Stepped frill growth of shell. B. Is a shell blister in the cup shell [blue circle].

MICROBIOLOGICAL FINDINGS.

Samples of haemolymph from the fish examined grossly were taken and plated onto SBA+2% salt and TCBS and incubated at 25ºC for 24 hours and plate growths recorded. Approximately 20% of all oysters sampled from the three submitting farms had no significant growth of bacteria present. The remaining 80% had moderate to high mixed bacterial growth on both media plates. Whilst some samples had a predominant form of bacterial colony there were no samples that had a monomorphic population of bacterial colonies, which would indicate a primary pathogenic bacteria. The predominant colonies in the heaviest growths on the plates were selected for identification. Identification by utilisation of MicroSys™ (biochemical analysis) was undertaken by staff of Berrimah Veterinary laboratory, Northern Territory, Australia. MALDI-TOF analysis was undertaken at the University of Adelaide to assess congruence between the methods and to gain some guidance in development of a standard protocol for the identification of bacterial isolates associated with Pacific Oyster mortalities. Table 3.9 compares the result for the isolates. There appears to be a significant difference between the results from each method however the difference may not be as great as first appears. The confidence rankings of the MALDI-TOF analysis are in the low confidence level (i.e. 1.70-1.99) and so the identifications are not exactly similar to type strains of the named bacteria, however the organisms (*V. lentus, V.tasmensis and V. pelagius*) are closely related to or within the Vibrio splendidus clade.

Table 3.9. Results from bacterial identification using two methods for the same isolate from Pacific Oyster haemolymph.

COLONIES	MALDI-TOF	MICROSYS™
Col 1 – AOOB3	No ID	*Vibrio splendidus*
Col 2 – AOOB2	No ID	*Vibrio splendidus*
Col 3 – AOOA3	Insufficient sample	No ID
Col 4 – AOOB3	*Vibrio lentus* (1.73)	*Vibrio splendidus*
Col 5 – AOOB3	No ID	*Vibrio splendidus*
Col 6 – BOA3	No ID	No ID
Col 7 – BOA1	*Vibrio tasmaniensis* (2.02)	*Vibrio splendidus*
Col 8 – BOA2	*Vibrio tasmaniensis* (1.88)	*Vibrio splendidus*
Col 9 – BOA2	*Vibrio tasmaniensis* (1.81)	*Vibrio splendidus*
Col 10 - BOA2	*Vibrio tasmaniensis* (1.75)	*Vibrio splendidus*
Col 11 – EO1A1	*Vibrio pomeroyi* (1.92)	*Vibrio splendidus*
Col 12 – EO1C3	*Vibrio lentus* (1.79)	*Vibrio splendidus*
Col 13 – EO1D1	*Vibrio tasmaniensis* (1.87)	*Vibrio splendidus*
Col 14 – EO1C3	No ID	No ID
Col 15 - EO1D4	*Halomonas aquamarina* (1.85)	No ID
Col 16 - EOID2	*Vibrio pelagius* (1.86)	Probable Phenon 45

HISTOLOGY FINDINGS:
The full description of histological findings can be found in Appendix 6 and the following is a summary of the significant findings from each submitting farm, by cohort.

Farm 1.

Cohort 1 –

There was mild to focally extensive dilation of connective tissue and oedema of the mantle in all oysters examined. There was focal erosion of the epithelium with underlying oedema, accompanied by focal areas of haemocyte aggregation within and around dense Leydig tissue. Surrounding the oedematous areas, the underlying epithelium is transformed showing squamous metaplasia. There were also areas in some fish that were thickened and spongiotic, with focal areas with migration of effete cells through the mantle epithelium. One of the 5 animals had normal appearance in the hepatopancreas. The rest had from 30-50% of HP tubules papering dilated and atrophic. The Leydig tissue in all animals was normal and densely packed. There was marked diffuse vacuolisation of the atrial and to a lesser extent the ventricular myocardium. The vacuolisation appears to be associated with mural macrophages and within the vacuoles are well defined granular foci. There were focal areas of myocardial necrosis. Occasional aggregation of brown cells were also noted. The large peri-adductor muscle ganglion in all animals exhibited marked peripheral neuronal proliferation with some also having mild inflammatory infiltrates and neuronal vacuolation. Mild focal myopathy of the adductor muscle was also apparent in this group. The GIT in these animals had minimal to low content and no other significant changes.

Cohort 2 –

Histopathological changes were limited to HP atrophy and tubular dilations, ganglionic change similar to Cohort 1, mild atrial macrophage vacuolation and gill epithelium erosion and vacuolation. Leydig tissue in all fish was depleted and degenerate

Cohort 3 –

There was mild to focally extensive epithelial erosion and associated sub-epithelial oedema in all fish from this cohort. There were similar hyper-cellular changes in the ganglion similar to the other cohorts and Leydig was dense in all fish.

Cohort 4 –

All fish had similar severe changes related to the mantle as described for cohort 1. Vacuolation of the ganglia was also present with hypercellularity. GIT contents included filamentous plant material and was scant in volumes in the stomach but compact within the intestine. The adductor muscle of one animal had degenerative changes with fibre contraction and hyalinisation.

Across all cohorts there was moderate to extensive numbers of TLC within the gill spaces and associated with external epithelium.

Farm 2

Cohort 1 –

Mantle histopathology included focally extensive oedema and associated erosion of the epithelium. There is extensive sub-epithelial oedema within the connective tissue particularly around the HP tubules. There was also generalise sub-epithelial oedema of the gills and focal haemocyte aggregations within the supportive connective tissue. The distal palps commonly showed epithelial vacuolisation. There was also vacuolisation of the oesophagus, stomach and intestine wall and moderate amounts of stomach and intestinal contents present in all fish, which was of mixed morphology. Haemocytes plaques were also present within the stomach lumen in some animals, and moderate amounts of transmigrational haemocyte across the intestinal wall. The Leydig tissue was sparse with focal haemocyte aggregations and loss of structure. The HP showed marked atrophy of the epithelium in all oysters, with dilatation of tubules noted. There was extensive myocyte vacuolisation with brown cell aggregation, and intracellular macrophage inclusions on both ventricle and atrium in some animals. Focal adductor muscle degeneration was observed associated with ganglionic nerve vacuolation as well as peri-nuclear vacuolisation. Gills in two fish showed extensive sub-epithelial vacuolation and oedema.

Cohort 2 –

All fish showed extensive sub-epithelial oedema and proliferation of shell glands within the mantle. The gills had generalised oedema of the connective tissue. There were low stomach contents and the Leydig tissue was depleted. The HP had 40-50% atrophic tubules.

Cohort 3 –

Similar external epithelial changes and associated oedema as other cohorts, were observed in this cohort. The Leydig tissue was atrophic, HP moderately to severely atrophic with one animal having significant squamous metaplasia of the lining epithelium. The vacuolation of the atrial and ventricular mural macrophages was observed, but was mild to moderate with no vacuolar contents. Ganglionic nerves were moderately vacuolated and hyper-cellular. There were little to no stomach and intestinal contents and any present contained filamentous plant material.

Farm 3

Cohort 1 –

Mild pathologies were observed within animals from this cohort. The stomach and intestine had moderate to high amounts of ingesta being made up of mixed plants and algal components. There was moderate hyperplasia of mantle shell gland tissue, mild heart macrophage vacuolation, depleted Leydig tissue, dilation of the HP tubules in order of 30-90% of tubules affected.

Cohort 2 –

There was mild to extensive focal oedema of the mantle epithelium with extensive bullae formation and haemolymph vessel dilation in some animals. Occasional foci of shell gland proliferation was noted. The adductor muscle had focal hyalinisation and degenerative change present in some animals. Mild to no change was seen in the ganglion. In two animals, the myocardium was thin and atrophic, and without structure (degeneration) (Figure 3.14). Others possessed mild myocardial vacuolation. Mild to moderate HP tubular degeneration was noted with amebae like organisms present within the lumen of some tubules.

Cohort 3 –

All animals in this cohort had histopathologies consistent with cachexia i.e. depletion of Leydig tissue, dilation of HP tubules, lack of GIT lumen contents, degenerative adductor muscle fibres. There was additional cardiac vacuolation and thinned myocardium.

Cohort 4 –

There was focal proliferation of shell glands in the mantle and multifocal areas of sub-epithelial oedema surrounding the body of the oyster with presence of occasional sub-epithelial bullae. One animal had a focal sub-epithelial aggregation of active haemocytes and fibrosis (chronic inflammatory reaction) a corresponding haemocyte vessel appeared thickened from mural haemocyte aggregates and hyalinisation. In the gills there was marked haemolymph channels dilation with scant number of haemocytes present. The GIT had little contents and the Leydig tissue contained multifocal areas of haemocytes aggregations surrounding the vasculature. The tissue was depleted and regionally depleted in all animals. Marked to moderate HP tubule dilation was present with amoeba noted.

Cohort 5 –

The changes observed in the animals in this cohort were similar to those seen in cohort 4.

CASE 4 SUMMARY

This case was of a number of farm leases in a single grow out area (Smoky Bay) being affected with high mortality rates (up to 80%) reported in a period that meet the current case definition of SAMS. The leases affected were in the northern beach area of the bay, and samples received for the project investigation were received 7 days after those collected for the PIRSA response investigation undertaken to rule out the presence of POMS. During the investigation it was indicated by industry that up to 20 leases in the bay may have experienced unexplained elevated mortality.

Samples were received from three producers for this project investigation. A number of cohorts from each lease were submitted for investigation and the selection criteria by which the farmer chose the cohorts was not clearly defined. The bay wide influences that were important in this investigation where the possible contamination of the leases close to a farm where the spray application of MCPA herbicide plus Zn, Mg and Cu liquid fertiliser had occurred over a period in mid-June 2019 and the anecdotal report of *Posidonia australis* sea grass shedding leaves and so contributing to an organic load in the bay. A PIRSA investigation of the chemical application had ruled out the possibility of contact of the chemical to the leases either by aerosol drift or through run off, ruling out possible effects on the oysters. The potential organic load increases in the bay were not investigated.

The significant gross findings from the oysters submitted indicated a period of active growth in all leases as evidenced by growth rings and frilled shell. The size of stock submitted indicated that all were juveniles and so meat condition scores were low as a result of minimal to rudimentary gonad development. The condition of the stock was observably low, and some animals were well below acceptable condition being watery in consistency and meats being shrunken and small. Algal cell counts in the bay for the period during the investigation indicated very low to low levels of available

feed. It was also noted in the majority of stock that GIT luminal contents were low indicating low food filtration rates. These findings suggest that there were periods of adequate feed availability prior to the time of investigation due to the evidence of shell growth. Confusing these findings were some cohorts of stock that had normal Leydig tissue and hepatopancreatic morphology that was consistent with good nutrition. Some stock, therefore, were either in an area with better food availability or and they had been stocked at lower density to take advantage of available feeds. There may also have been some other undocumented management strategy applied to these animals which produced better condition and growth indicators.

GIT contents in some cohorts were unusual in their morphology, indicating filtration of larger organic materials rather than single celled organisms. The true nature of the ingesta inclusions was not further investigated to ascertain the identity of the material.

Another significant finding of gross pathology was the expansion of the pericardial sac in a number of animals from each farm. The pathogenesis of this presentation has not been identified for Pacific Oysters but it can be related to oyster storage out of water, submersion of oysters in low salinity water (i.e. after rainfall) or randomly presented in oysters with variable culture histories. The Bureau of Meteorology rainfall data for the period June to August 2019 for the bay area were as follows: June 49.8mm, July 29.7mm and August 19.0mm. All figures were average too low for the historically recorded data suggesting that low salinity emersion may not be the aetiology of the expanded pericardial sacs in the examined animals. Histologically examined hearts showed a range of myocardial vacuolation, macrophage inclusions and myopathy and myositis, indicating that the cardiac swellings are associated with myocardial pathology. The sign may be an indication of myocardial dysfunction and haemolymph congestion due to reduced functional capacity of the heart (i.e, congestive heart failure). The myocardial changes observed in this case are unusual and are not reported in the literature.

The extensive sub-epithelial oedema and haemolymph vessel dilation observed in many of the animals in this case may also be a result of reduced cardiac function or again may be due to osmotic influences, although the latter is not supported by the rainfall data. Unless the animals were exposed to freshwater during the submission process, the cause is unexplained.

The microbiological findings are interesting but not definitive. No one organism was isolated from all oysters, ruling out a single pathogenic bacterial isolate as the cause of the mortality event. The findings of mixed morphology, and low to heavy growth of bacteria from many of the animals sampled, is indicative of overwhelming bacterial flora proliferation as a result of declining environmental water quality, host immunosuppression and/or a terminal event as the animals died of other pathology. The situation of mixed populations of *Vibrio* spp. being associated with mortality events in Pacific Oysters has been reported worldwide (Carson *et al*, ; Romalde *et al*, 2013; Travers *et al*, 2015; Solomieu *et al*, 2015;Go, *et al*, 2017) and no clear explanation has been given as to their specific role in the cause of Pacific Oyster mortalities. The identification of the isolates examined from this case highlights the challenges of undertaking bacterial identification in aquatic animal disease investigations. There still remains debate as to the most appropriate way to identify these bacteria. Despite the specific issues with the identification of isolates in this case, some insight in the pathologies observed can be made. The bacteria isolated are from the Splendidus CLADE of Vibrios some of which have been studied and found to possess a number of pathogenic mechanisms. *Vibrio lentus* has been shown to excrete proteinases and cause epithelial erosions and systemic infections in octopus (*Octopus vulgarus*) and turbot (*Scophthalmus maximus*). Many of the animals examined in this case had mantle and body epithelial erosions and ulcers. *Vibrio tasmanensis* has been shown to have virulence factors (i.e. OmpU) which allows interaction between the bacteria and oyster muscle producing significant adductor muscle lesions. Many of the oysters examined in this case had subtle to overt adductor muscle necrosis and myositis. Adductor muscle lesions lead to poor shell closer and or failure, which reduces filtration effectiveness and hence feeding, and predisposes the animal to predation and provides access to the tissues to other biota. The majority of animals examined had mixed zooplankton and ectocommensal protozoa within the shell cavity and oyster tissues. The predominance of TLC

found in this case indicates a combination of this physiological compromise with declining water quality conditions around the oysters at the time of their collection. The TLC were in sufficient numbers in some of animals examined too also contribute to any epithelial tissue damage. *Vibrio splendidus* is commonly associated with mass mortalities of larval and juvenile Pacific Oysters and has been associated with mortality events similar to those associated with SAMS.

Histological changes observed in the animals from this case support the association of bacteria agents, are: significant generalised and focal haemocyte proliferation and aggregations in various tissues; mural macrophage proliferation and changes observed in the myocardium; and the focal suppurative pathologies in the adductor muscles and Leydig tissue in some animals. Air temperatures for June and July were average, with August being below average. Colder than normal weather in August may have played a role in immunosuppression of the oysters allowing infection by opportunistic pathogens.

The unusual neural changes seen in the major peri-adductor muscle ganglion are worthy of note. The changes indicate neuronal degeneration and mild neuritis with, in some animals, a neuronal body proliferation. This is an undescribed pathology and worthy of further investigation.

This case met some of the criteria for the current SAMS case definition and so adds some insight as to the details of cases that come under this definition. It does not allow a definitive casual diagnosis for SAMS but directs where further research could be targeted for understanding the nature of un-explained mortality events in Pacific Oysters in South Australia.

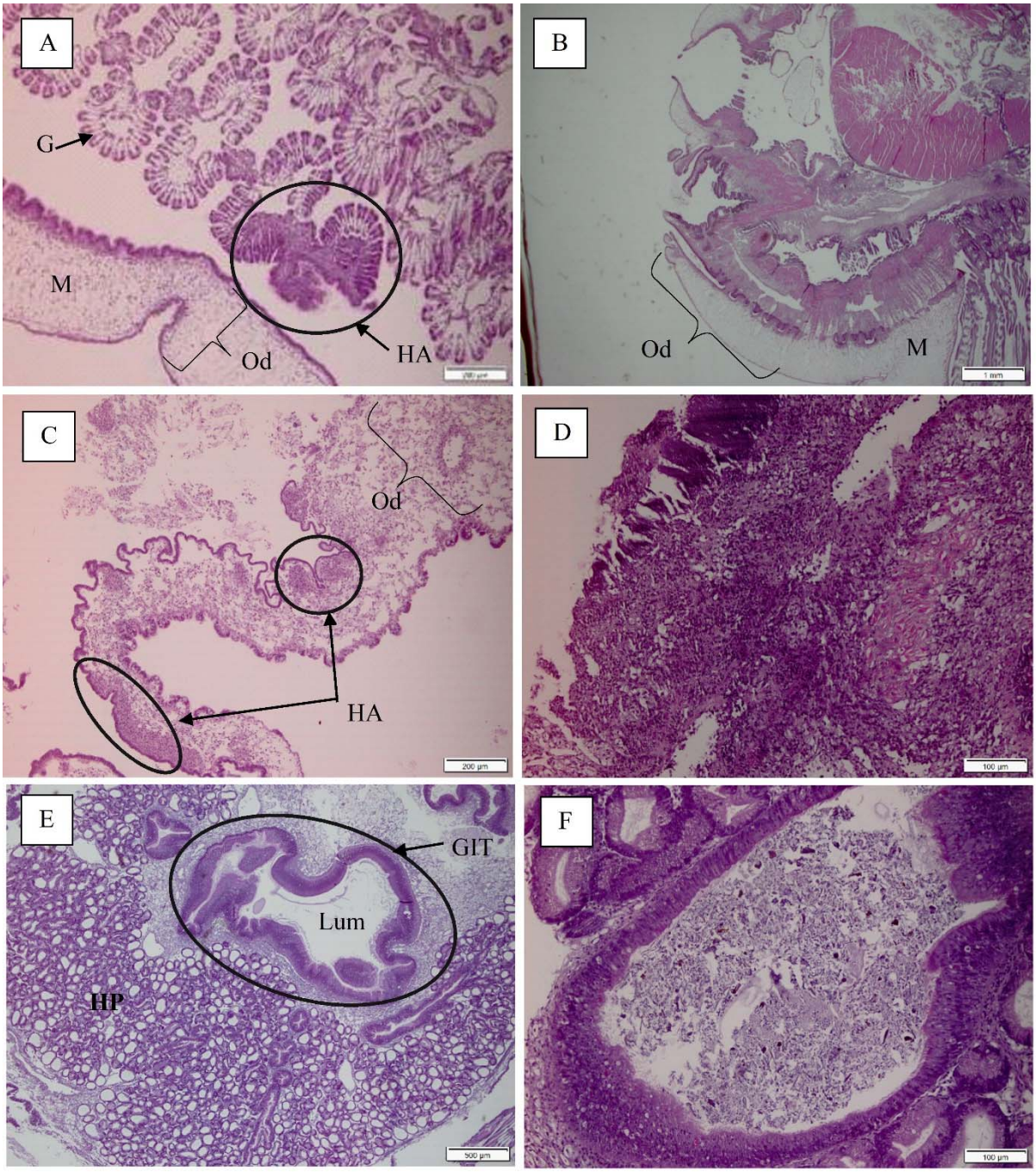

Figure 3.26. A: Haemocyte aggregations [HA] of the gills (G) with sub-epithelial mantle (M) oedema [Od], B: Generalised sub-epithelial oedema of the mantle, C: Multifocal haemocyte aggregations and oedema of the mantle, D: Image focusing on focal region of mantle necrosis, note the hyper cellularity and degeneration of tissue architecture, E: Marked hepatopancreatic [HP] tubular atrophy and luminal dilation as well as a gut section [GIT] with mild to no content present within the lumen [Lum], F: Magnified image of intestine with moderate to marked mixed gut content present within its lumen.

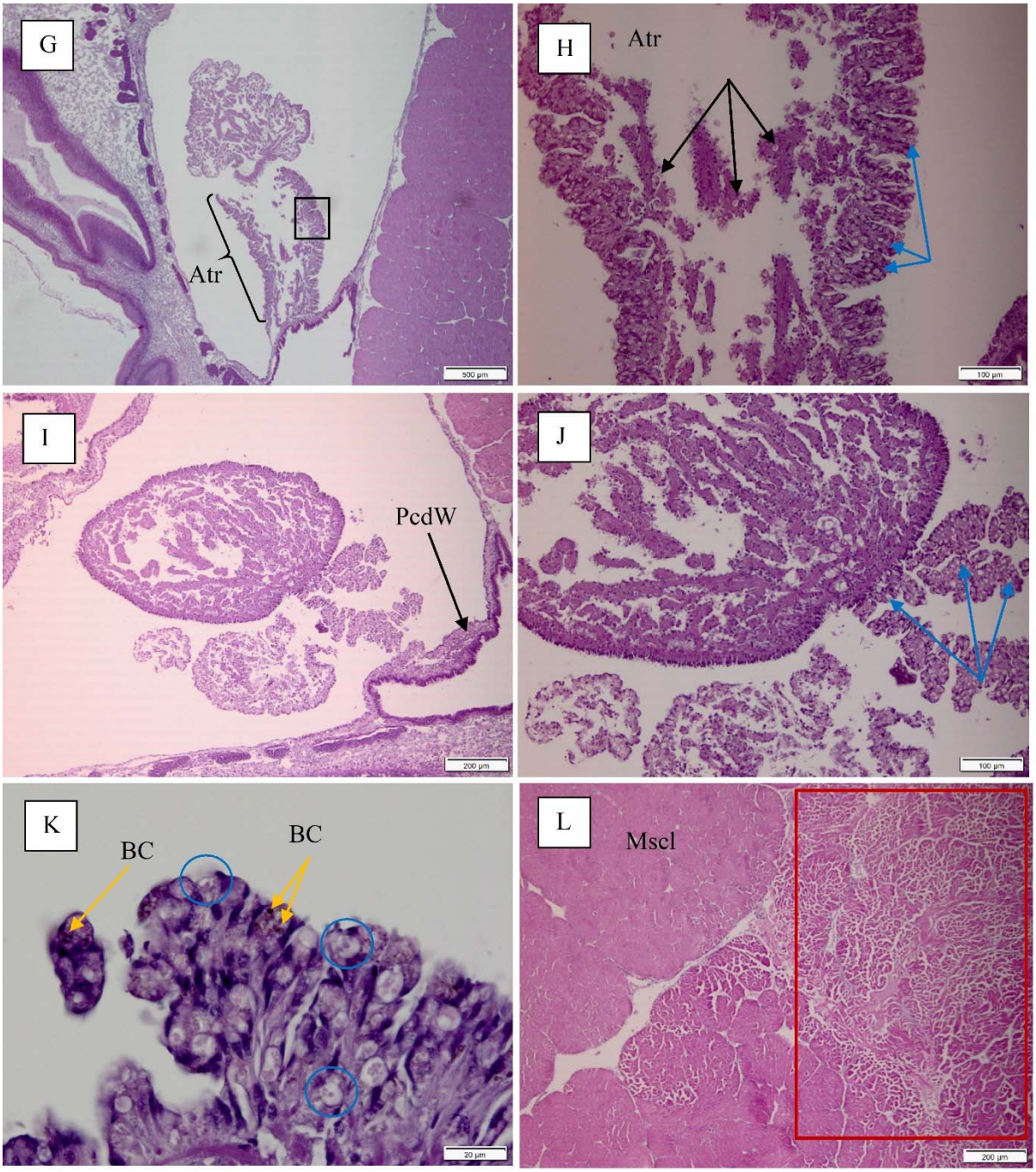

Figure 3.27. G: Thickening [black square] of the atrial region [Atr] of the myocardium with mild vacuolisation of myocytes, H: A higher magnification of the atrial wall of image G showing myocyte vacuolisation [black arrows] and hypertrophy as well as vacuolisation of the mural macrophages [blue arrows]. Note that the vacuoles contain debris, I: Similar myocardial changes observed but these changes are more generalised and involving the pericardial wall [PcdW], J: Higher magnification of image I showing vacuolated macrophages [blue circle] in the myocardium, K: Magnified image of macrophage inclusions and brown cells [BC – yellow arrows], L: Marked zonal hyalinising degeneration [red square] with mild to no haemocyte response, note the area of normal muscle [Mscl]

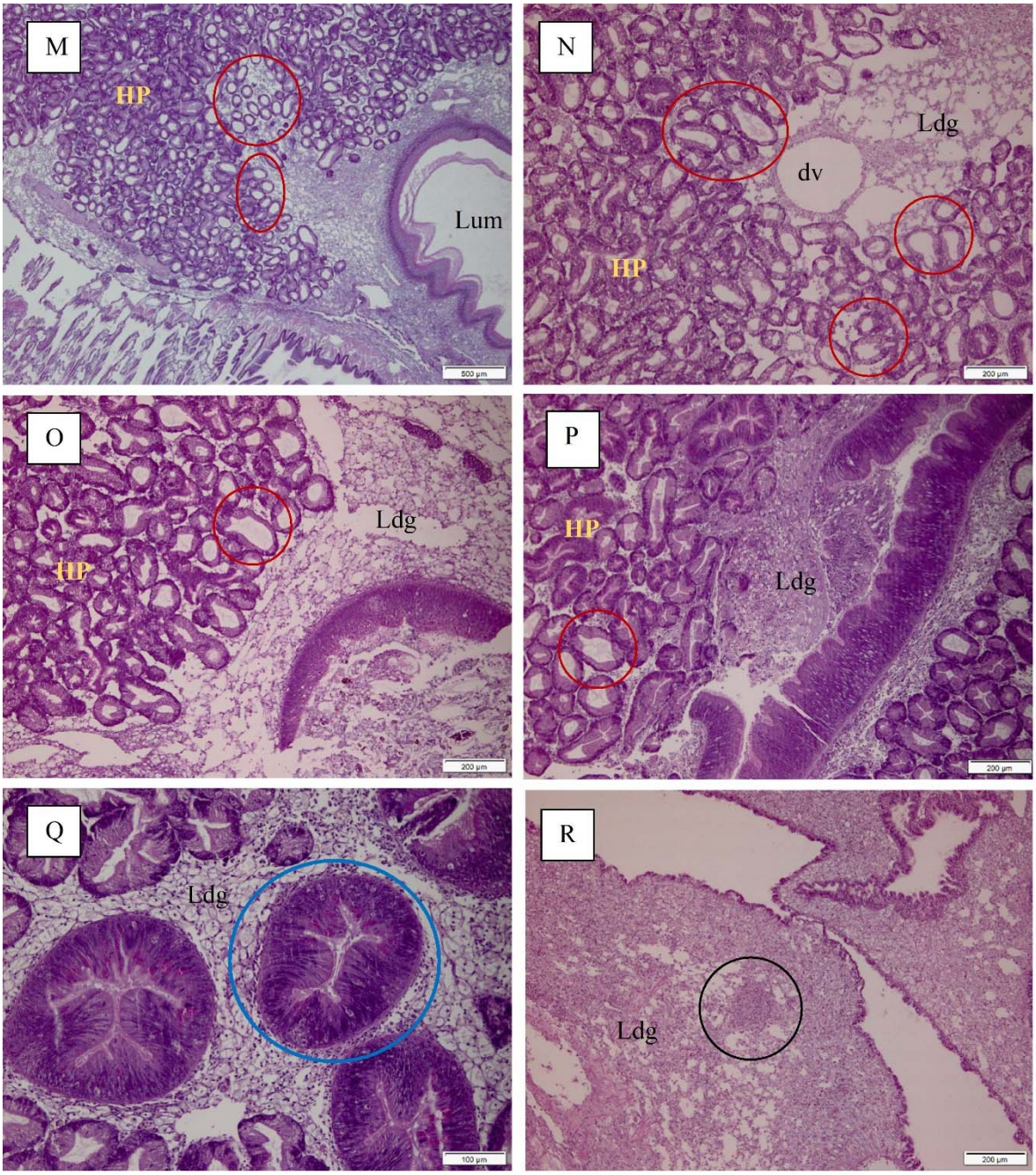

Figure 3.28. M: Marked or generalised atrophy and dilation [red circles] of the hepatopancreatic [HP]tubules. Note the minimal intestinal content within the gut lumen [Lum], N: Magnified image of generalised hepatopancreatic tubular atrophy and dilation [red circles] as well as dilated vasculature [dv], O: Moderate percentage of tubular atrophy and degeneration [red circles], note the depleted nature of the leydig tissue [Ldg], P: Mild percentage of hepatopancreatic tubular atrophy and dilation [red circles] with dense surrounding leydig tissue, Q: Normal appearance of hepatopancreatic tubules [blue circle] and leydig tissue, R: Focal area of inflammation observed within the leydig tissue [black circle] suggestive of non-specific haemocyte aggregating response to possible bacterial infection.

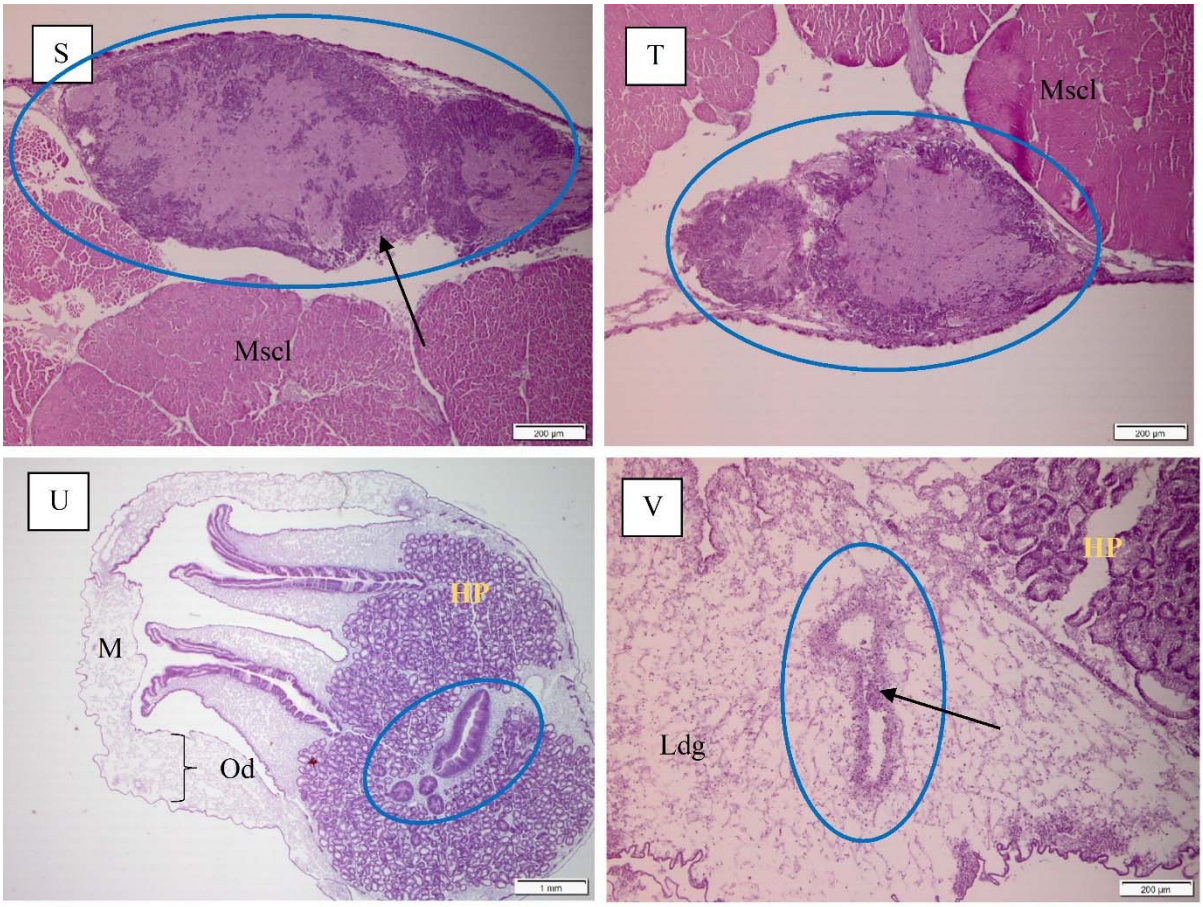

Figure 3.29. S: Mild focal vacuolation of ganglia [blue circle] with hypercellularity of neurons and mild architectural disruption. Mild infiltration of haemocytes (ganglioneuritis) [black arrow]. Adductor muscle degeneration [Mscl], T: Marked random neuronal vacuolation and hypercellularity [bluecircle], U: Image of whole oyster with generalised marked oedema [Od] of the mantle [M], with variable gut content present within stomach and intestinal lumen [blue circle] as well as a high percentage of atrophy and dilation of the hepatopancreatic tubules [HP], V: Vasculitis of the vessel [red circle] in the leydig tissue [Ldg] where vessel is dilated and surrounded by aggregating haemocytes [black arrow]

3.4. Discussion

This project has allowed the focused investigation of a number of unexplained mortality events of cultured Pacific Oysters in South Australia. All but one of the events investigated within this project were also subject to investigation by PIRSA Biosecurity under the remit of exotic or emerging emergency animal disease management. Only one case met the SAMS case definition but all cases aided in understanding of Pacific Oyster mortality events in SA.

At the commencement of the project we undertook an industry survey to assess the importance of SAMS to the industry. The industry survey indicated that the majority of the correspondents had a limited understanding of the case definition of SAMS. All correspondents had experienced un-explained mortalities in their cultured stock and believed it was a significant economic concern for their business. The other significant findings were that despite experiencing higher levels of un-explained mortalities than they had set as trigger points for their farms only very few would have mortalities investigated by submission of samples to a diagnostic laboratory. They met their reporting requirements to PIRSA as set by their lease compliance but no further investigation was undertaken.

Given the emergence of OsHV-1 (POMS) in the Australian oyster industry and particularly within the Tasmanian Industry, a supplier of spat to South Australia, it was important to understand the priority of SAMS within the various production areas within the state. The time gap between the Ausvet commissioned workshop and report (July 2013) and the commencement of this project had meant that four years had lapsed since any focused industry engagement with research on the topic. During that period however the School of Animal & Veterinary Science, University of Adelaide had undertaken a series of internally research projects with in-kind support of individual SA oyster industry members. These health surveillance based projects attempted to help define a "normal oyster" within the context of limited production systems and production areas within the state. Resources limited the scope of these projects but laid some of the ground work for the development of the SAMS component of the Future CRC projects investigation un-explained mortalities in cultured Pacific Oyster stocks.

The strategic recommendations for the SAMS workshop held in July 2013 were to:

- Obtain a better understanding of what defines a 'normal' oyster in the South Australian production environments
- Better understand the nutritional requirements of Pacific Oysters
- Improve data farm and environmental data management with an industry coordinated focus
- Improve information extension.

From a diagnostic perspective, and what has been re-iterated from the results of the work in this project, a coordinated approach to mortality investigation is the key factor for understanding the events and then developing mitigation strategies to limit the occurrence and impact of events when they happen.

In an attempt to better understand the normal Pacific Oyster and to develop new assays for diagnostic investigations, the biochemistry of oyster haemolymph was studied. Serum biochemistry is an important tool in health monitoring in higher vertebrates and so its application for oyster health was investigated. The study revealed some parallels with higher vertebrate applications, and for the first time studied the enzymatic composition of the various tissues in a Pacific Oyster and tested the transitional possibilities for the use of haemolymph in investigating organ pathology. The diagnostic assay relies upon a minimum volume of haemolymph for analysis, and so application in smaller oysters (<20ml in length) is limited, but with further development the assay could be a useful tool in routine health assessment of Pacific Oysters.

The four case investigations undertaken were of oysters submitted from three geographically removed production areas within the state (i.e. Smoky Bay, Port Lincoln, and Cowell). The commonality of the cases was high un-explained mortalities of various age groups of Pacific Oysters stocked on grow out production farms. During the period of study, there was anecdotal evidence that more growing areas had been affected by un-explained mortalities. Cases were submitted in different periods of the year and not just the window for SAMS, so environmental conditions were different in regards to seasonal temperatures, water movement and feed availability. Stock was of different age categories, sourced from different hatcheries, and subject to different farm methods for propagation, grading and management. Samples were submitted in various ways and over various periods from farm to laboratory receipt. Some samples had been previously handled by diagnostic laboratories and others had been sent straight to the PI from farm. Transport time from farm to PI was also variable. These uncontrolled variables are highlighted as they have a significant impact on the overall interpretation of the pathology observed within the project. If every case had met the SAMS case definition many of the variables would still be affecting the overall interpretation of pathology results from a causal interpretation.

Tthere were a number of common findings for the cases and a number of unique pathologies which require further investigation which was outside the scope of time and resource for this project.

Many of the animals presented with signs of poor condition, low GIT ingesta contents, and hepatopancreatic atrophy. All these histological signs are indicative of poor nutrition either as a result of animals not taking in adequate food or insufficient food being present in the environment.

A mixed flora of *Vibrio* spp was isolated from all cases at moderate to high levels. Their role in primary pathogenicity or as secondary opportunistic pathogens is discussed within each case report, but there was no clear indication from any of the cases that bacterial pathogens were the primary cause of mortality. In each case and individual oyster, they played a significant role in morbidity and mortality but were not a key feature of SAMS. The pathologies such as tissue haemocyte aggregation, adductor myositis, epithelial erosion and possibly the cardiac myositis and myopathy are indicative of pathogenic bacterial infection. The continued surveillance of the bacterial flora and characterisation of the bacteria present in Pacific Oysters is an important adjunct to understanding the normal Pacific Oyster.

Protozoal flora associated with moribund and dead oysters is not unusual, however there was a predominance of *Trichodina*-like ciliates in the cases from Smoky Bay. These organisms proliferate in conditions of high organic load and warm temperatures. These conditions also facilitate proliferation of bacterial flora which are the primary food source for *Trichodina*. These conditions will also cause immune stress to the host oyster. The predominance of infestation in the cases from Smoky Bay begs the question of whether the water quality in the areas around the oysters lends itself to *Trichodina*-like ciliates proliferation naturally or as result of the organic load presented by dieing oysters or whether there is a population of TLC that are more pathogenic to the oysters themselves. High numbers of *Trichodina*-like ciliates will cause irritation of epithelial structures so they will add to pathology if present.

The key interesting pathologies observed were the acute necrotising hepatopancreatic lesions in the oysters in Case 2 from Cowell, the heart lesions, in particular the lesions in the oysters from Smoky Bay, and the neuro-ganglionic pathologies again in the oysters from Smoky Bay. The lesions of the HP are consistent with necrotising lesions associated with algal toxicosis and petroleum contamination of oysters. No cause was identified in this case.

The cardiac lesions may be explained by the presence of significant bacterial infection, however the histopathology is unique in morphology and requires further investigation. The neurological pathologies are also unique and may indicate toxicosis, developmental abnormalities or as yet un-identified viral agents, none of which could be investigated within the scope of this project.

Mortality events in cultured Pacific Oysters are documented from around the world and a consistent theme comes from them. A coordinated approach to diagnostics is required to first understand the various factors that impact oyster health and then allow an informed diagnostic outcome which can be used for possible future mitigation and management. These principles were highlighted in the workshop report for SAMS in 2013 (Madin *et al*, 2013). The key for progressing our understanding of un-explained mortality events in Pacific Oysters is to follow the recommendations from this report. Given the diversity of mortality events experienced within this project it would be advisable to broaden the scope of investigations to include all un-explained mortalities in commercial settings and not limit an industry approach to just cases that fit into the current case definition of SAMS.

There were a number of logistical and operational issues that were highlighted by this project. These are in the context of reporting, investigating and dispatching samples for diagnostic work. There are PIRSA specified protocols for the SA oyster industry for reporting of mortality events in Pacific Oysters and they should be followed. Despatching samples to diagnostic laboratories in a timely and prescribed manner is a key issue in diagnostic rigour and removing any artefactual influences on pathology observed in the received samples. The submission of samples for this project had variability that should be improved on, in future diagnostic cases. Case coordination for diagnostic outcomes is also a key to interpreting what can be a very multi-factorial investigation. A ‘one stop shop’ approach where all information is coordinated under a case coordinator would add rigour to diagnostic outcomes. This approach is well established for other species in veterinary diagnostics. The gathering

of relevant data for all cases is also paramount for interpretation of findings. The development of software (i.e. MiShell) for management and environmental data should be encouraged and resourced.

Understanding of what constitutes a study unit in any investigation is also key to outcomes. Whether this is at bay, lease or rack level, it should be defined so that relevant supportive information is gathered and applied.

Finally, what this work has affirmed is that without a complete data set of information associated with any mortality event, an accurate or even probable diagnosis is not possible.

3.5. References

Adelson, H., 2016, Crassostrea Gigas Surveillance in South Australia – Histopathology and Health of the Pacific Oyster, Bachelor of Science (Animal Science) with honours, The University of Adelaide, Australia.

Battison, A 2006, 'Tissue distribution and hemolymph activity of six enzymes in the American lobster (Homarus Americanus): Potential markers of tissue injury', *Journal of Shellfish Research, 25*(2), 553-560.

Bernard, F 1974, 'Particle sorting and labial palp function in the Pacific Oyster crassostrea gigas (thunberg, 1795)', *The Biological Bulletin, 146*(1), pp. 1-10.

Carson, J., Higgins, M., Wilson, T.K., Gudkovs, N., Bryant, T.N., 2006, Aquatic Animal Health Subprogram, Vibrios of Aquatic Animals: Development of a National Standard Diagnostic Technology, FRDC Project No. 2001/628

Chávez-Villalba, J, Pommier, J, Andriamiseza, Pouvreau,S, Barret, J, Cochard, J & Le Pennec, M 2002, 'Broodstock conditioning of the oyster Crassostrea gigas: Origin and temperature effect', *Aquaculture,* 214(1), pp. 115-130.

Courtney, P, Breen, F, Cameron, B, Crawford, C, Cunningham, M, Goc, J and Poke, J 2017, 'Super Oyster: The oyster that could save Tasmania's industry', *Landline*. RMIT Publishing, Melbourne (Vic.).

Department of Primary Industries and Regions 2016, 'Help protect SA's oyster industry', *Government of South Australia*, viewed 1 October 2018, http://www.pir.sa.gov.au/fishing/publications/fishfacts_e-newsletter/fish_facts_july_2016/help_protect_sas_oyster_industry

Department of Primary Industries and Regions 2018, 'Pacific Oyster Mortality Syndrome', *Government of South Australia*, viewed 1 October, 2018, http://www.pir.sa.gov.au/aquaculture/aquatic_animal_health/pacific_oyster_mortality_syndrome

eClinpath 2013, 'Chemistry', Cornell University, viewed 11 October 2018, http://www.eclinpath.com/chemistry/

Eble, A and Scro, R 1996, 'Chapter 2 – General Anatomy', in V Kennedy, R Newell and A Eble (eds.), *The Estern Oyster: Crassostrea virginica*, 2nd Edn, pp 19-30.

Fougerouse, A, Rousseau, M, and Lucas, J 2008, 'Chapter 3 - Soft Tissue Anatomy, Shell Structure and Biomineralization', in P Southgate and J Lucas (eds), *The Pearl Oyster*, Elsevier, pp. 77-102.

Geffré, A, Concordet, D, Braun, J and Trumel, C 2011, 'Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel', *Veterinary Clinical Pathology*, 40(1), pp. 107-112.

Go, J, Deutscher, A, Spiers, Z, Dahle, K, Kirkland, P, & Jenkins, C 2017, 'Mass mortalities of unknown aetiology in Pacific Oysters Crassostrea gigas in Port Stephens, New South Wales, Australia', *Diseases of Aquatic Organisms*, 125(3), pp. 227-242.

Gustafson, L, Stoskopf, M, Showers, W, Cope, G, Eads, C, Linnehan, R, and Levine, J 2005, 'Reference ranges for hemolymph chemistries from Elliptio complanata of North Carolina', *Diseases of Aquatic Organisms, 65*(2), 167-76.

Hoffmann, W and Solter, P 2008, 'Chapter 12 - Diagnostic Enzymology of Domestic Animals', in J Jerry Kaneko, J Harvey and M Bruss (ed.), *Clinical Biochemistry of Domestic Animals,* 6th Edn, Elsevier Academic Press, USA, pp. 351-378.

Howard, D, Lewis, E, Keller, B and Smith, C 2004, 'Histological techniques for marine bivalve mollusks and crustaceans', *NOAA Technical Memorandum NOS NCCOS* 5, pp. 218

Jefferson, R 2018 'Aquaculture Production Report 2016-2017'. *NSW Department of Primary Industries*, viewed 18 October 2018, https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0009/750726/Aquaculture-Production-Report-2016-2017.pdf

Li, Y, Qin, J G, Li, X, & Benkendorff, K 2009, 'Monthly variation of condition index, energy reserves and antibacterial activity in Pacific Oysters, Crassostrea gigas, in Stansbury (South Australia)', *Aquaculture*, 286(1), pp. 64-71.

Olafsen, J Flectcher, T and Grant, P 1992, 'Agglutinin activity in Pacific Oyster (Crassostrea gigas) hemolymph following in vivo Vibro anguillarum challenge', *Developmental and comparative immunology*, 16, pp. 123-138.

Patrick, S, Faury, N & Goulletquer, P 2006, 'Seasonal changes in carbohydrate metabolism and its relationship with summer mortality of Pacific Oyster Crassostrea gigas (Thunberg) in Marennes–Oléron bay (France)', *Aquaculture*, 252(2), pp. 328-338.

Romalde, J.L., Dieguez, A.L., Lasa, A., Balboa, S., 2013, New Vibrio species associated to molluscan microbiota: A review, Fronteirs in Microbiology, 4, 413; 1-11

Solomieu, V.B., enault, T., Travers, MA, 2015, Mass mortality in bivalves and the intricate case of the Pacific Oyster, *Crassostrea gigas*, Journal of Invertebrate Pathology, 131; 2-10

Travers, MA., Miller, K., Roque, A., Friedman, C.S., 2015, Bacterial diseases of marine bivalves, Journal of Invertebrate Pathology, 131, 11-31.

3.6. Appendices

3.6.1. Appendix 1 – South Australian Oyster Mortality Survey

South Australian Oyster Mortality Survey

This research project revolves in obtaining a better understanding to the South Australian Mortality Syndrome (SAMS). Over the last 10 years the South Australian Oyster industry has doubled its output and with this growth there has been an increase in mortalities observed by the industry. Mortality events surrounding juvenile oysters during the late autumn to early winter period have been noted to be a significant impediment to the industry and has been labelled as 'South Australian Mortality Syndrome' (SAMS). Currently there is no causative agent identified with little knowledge on management of this syndrome.
The set goals of this project is as follows:
- Investigate causative agent
- Improve understanding of causes
- Provide a complete case definition
- Improve diagnostic technologies
- Improve husbandry to maximise survival.

This project is being primarily investigated by Dr. Stephen Pyecroft and is funded by the Future Oyster – CRC as well as the FRDC industry.
You are being invited as you are owners or management officers of South Australian Oyster facilities to answer an optional survey where questions are aimed to inform researchers involved in the Future-CRC as to the importance of SAMS to the Pacific Oyster Industry. Pending the participants interest in the survey they can choose to provide us oyster samples.

The survey should take no longer than 10 minutes to complete with no foreseeable risks associated.This project presents itself with benefits that could improve husbandry and management strategies to allow better survival rates in juvenile oysters. There is also the potential to discover a causative agent allowing the syndrome to be conclusively be defined.

Participation in this project is completely voluntary. If you agree to participate, you can withdraw from the study at any time.Responses to the survey will occur spontaneously through the online portal. These results will be kept private and confidential. Throughout this project timeline there will be specific points where reports are required to assess findings and progress. These reports are termed as milestone reports. In order to maintain confidentiality through reports published, all identifiers will be removed and replaced with an accession number. As accession number represents number identification tag used in veterinary diagnostic laboratories. (eg. 18-0039).

If participants conclude the survey and are willing to provide oyster samples for assessment they shall be contacted by the investigators of this project or through the South Australian Oyster Industry. Reponses to surveys will be kept electronically and retained for a minimum of 5 years. The primary investigator and co-investigator are the only two people who will have access to these folders. The same use of accession numbers will be applied to any publications that may come from this project.
The study has been approved by the Human Research Ethics Committee at the University of Adelaide (approval number H-2018-066). This research project will be conducted according to the

NHMRC National Statement on Ethical Conduct in Human Research (2007). If you wish to speak with an independent person regarding concerns or a complaint, the University's policy on research in involving human participants or your rights as a participant, please contact the Human Research Ethics Committee on:
Phone: +61 8 8313 6021
Email: hrec@adelaide.edu.au

If however, you have questions or problems associated with he practical aspect of your participation int he project, or wish to raise a concern to complaint about the project, then you should consult to Investigators of this project. Please do not hesitate to contact us if you also have any queries in regards to general oyster health and management. The co-investigator Dr. Johanna Mahadevan can be contacted on:
Email: johanna.mahadevan@adelaide.edu.au

1. What is your understanding of SAMS?

2. What impact do you think SAMS is having on the industry?

* 3. Do you think SAMS is an entity on your lease(s)

- Yes
- No - Please skip to question 5
- Uncertain - Please skip to question 5

4. If **YES** above, what impact is SAMS having on your lease(s)?

* 5. What is the accepted percentage level of mortality per year class on your lease(s)?

* 6. What was the percentage of mortality at your lease(s) for 2016 and 2017 respectively?

* 7. Have you experienced any periodic increase in mortality over the last 2 years (e.g. 2016 - 2017)?

- Yes
- No - please skip to question 9
- Uncertain - please skip to question 9

* 8. Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?

- [ ] Yes
- [ ] No - please skip to question 11
- [ ] Uncertain - please skip to question 11

9. If **YES** above, what time of year did you experience these increases in mortalities?

10. Were there any diagnosises as South Australian Mortality Syndrome (SAMS)?

- ( ) Yes
- ( ) No
- ( ) Uncertain

11. Would you participate in a surveillance program by contributing oyster samples that will aid in providing better understanding and management protocols of SAMS by the oyster industry?

- ( ) Yes
- ( ) No

12. If you have answered 'Yes' above, please provide us with your contact information

Name	
Address	
Address 2	
City/Town	
ZIP/Postal Code	
Email Address	
Phone Number	

Responses

Participant 1:

Q1 What is your understanding of SAMS?
Respondent skipped this question.

Q2 What impact do you think SAMS is having on the industry?
Respondent skipped this question.

Q3 Do you think SAMS is an entity on your lease(s)?
No – please skip to question 5.

Q4 If YES above, what impact is SAMS having on your lease(s)?
Respondent skipped this question

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
30%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
70%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
No – please skip to question 9

Q8 If YES above, what time of year did you experience these increases in mortalities?
Respondent skipped this question.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
No - Respondent skipped this question.

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Respondent skipped this question.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes.

Participant 2:

Q1 What is your understanding of SAMS?
South Australian Mortality Syndrome usually during winter or after growth bursts.

Q2 What impact do you think SAMS is having on the industry?
Loss of production causing loss of jobs, wealth, inability to trade.

Q3 Do you think SAMS is an entity on your lease(s)?
Yes

Q4 If YES above, what impact is SAMS having on your lease(s)?
Dead oysters affecting live oysters to live lack of production.

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
30 – 70%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
30 – 90%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
February

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
Yes2

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
No

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Unknown – testing for POMS only

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes.

Participant 3:

Q1 What is your understanding of SAMS?
Unexplained oyster death

Q2 What impact do you think SAMS is having on the industry?
High spat cost, lost revenue due to poor spat availability

Q3 Do you think SAMS is an entity on your lease(s)?
Yes

Q4 If YES above, what impact is SAMS having on your lease(s)?
Loss of production due to poor spat availability and high mortality

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
20%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
60%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
All year

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?

Yes

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Uncertain

Q11 What was the diagnosis from submitted samples if NOT SAMS?
No reason given

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
No

Participant 4:

Q1 What is your understanding of SAMS?
I think SAMS is largely due to high performance oysters being more susceptible to being killed than the slower growing poorer performing oysters we used to get.

Q2 What impact do you think SAMS is having on the industry?
It has cost growers a lot of money but ultimately it is a husbandry and breeding selection issue

Q3 Do you think SAMS is an entity on your lease(s)?
Yes

Q4 If YES above, what impact is SAMS having on your lease(s)?
See answer to question 2

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
The average over the last 8 years has been about 60% mortality rate

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
60% 2016 and 85% 2017. The 2017 result was largely due to receiving very small immature spat.

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
Respondent skipped this question.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Uncertain

Q11 What was the diagnosis from submitted samples if NOT SAMS?
I believe it is genetic and husbandry related

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
No

Participant 5:

Q1 What is your understanding of SAMS?
Unexplained mortality event.

Q2 What impact do you think SAMS is having on the industry?
Slight isolated incidents, not a major concern.

Q3 Do you think SAMS is an entity on your lease(s)?
Yes

Q4 If YES above, what impact is SAMS having on your lease(s)?
2-5% of mortality in each basket

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
10%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
15% in 2016 and 10% in 2017

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
No – please skip to question 9

Q8 If YES above, what time of year did you experience these increases in mortalities?
June

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
No - Respondent skipped this question.

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Respondent skipped this question.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes.

Participant 6:

Q1 What is your understanding of SAMS?
First time hearing of it.

Q2 What impact do you think SAMS is having on the industry?
Unknown.

Q3 Do you think SAMS is an entity on your lease(s)?
No – please skip to question 5.

Q4 If YES above, what impact is SAMS having on your lease(s)?
Respondent skipped this question

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
Less then 5%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
Less then 1%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
No – please skip to question 9

Q8 If YES above, what time of year did you experience these increases in mortalities?
Respondent skipped this question.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Respondent skipped this question.

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Respondent skipped this question.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Respondent skipped this question.

Participant 7:

Q1 What is your understanding of SAMS?
Unexplained mortality of oysters through Autumn winter

Q2 What impact do you think SAMS is having on the industry?
Varies from year to year. In a bad year it is significant

Q3 Do you think SAMS is an entity on your lease(s)?
No – please skip to question 5.

Q4 If YES above, what impact is SAMS having on your lease(s)?
I believe SAMS is all to with metabolism. We have bred a more dynamic oyster than we had 10 – 15 years ago. In the years when we have a calm autumn, oyster growth is fast and they can't be slowed down, and then we have a rapid temperature drop, we experience high mortality. I analyse it like a sprinter trying to run a marathon. If left to their own devices they simply metabolise too quickly or burn out or don't have the resilience and hardness to tolerate other stresses.

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
5% - 10% when buying 5-6mm spat.

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?

Much higher but all from spat purchased sub 5mm

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
Feb 16

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
Yes

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
No

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Heat stress

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes

Participant 8:

Q1 What is your understanding of SAMS?
A name given to the unexplained mortality we have been experiencing over the last decade.

Q2 What impact do you think SAMS is having on the industry?
Call it SAMS if you like The high mortality has given us very little chance of recovering from the spat shortage caused by POMS in Tasmania.

Q3 Do you think SAMS is an entity on your lease(s)?
Yes

Q4 If YES above, what impact is SAMS having on your lease(s)?
We are experiencing mortality in spat which we've paid high prices for and an extra 10% on top for ASI levy dying at rates of up to 70% before we even get them into 6mm baskets

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
Would be happy with 10% which is what we used to cull. Now it is over 50% which is not actually acceptable.

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
Over 50%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
Respondent skipped this question.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Respondent skipped this question.

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Respondent skipped this question.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes.

Participant 9:

Q1 What is your understanding of SAMS?
Unexplained death in oysters.

Q2 What impact do you think SAMS is having on the industry?
Not really sure on industry but it is having major impact on my operation.

Q3 Do you think SAMS is an entity on your lease(s)?
Uncertain – please skip to question 5.

Q4 If YES above, what impact is SAMS having on your lease(s)?
Respondent skipped this question

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
5% - 12%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
30% - 50% in some lines and up to 80% in others.

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Uncertain – please skip to question 9

Q8 If YES above, what time of year did you experience these increases in mortalities?
February through to May so far after handling.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
Yes

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Yes

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Respondent skipped this question.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes

Participant 10:

Q1 What is your understanding of SAMS?
An acronym for unexplained oyster mortalities.

Q2 What impact do you think SAMS is having on the industry?
Mortality rates of around 30%

Q3 Do you think SAMS is an entity on your lease(s)?
Uncertain– please skip to question 5.

Q4 If YES above, what impact is SAMS having on your lease(s)?
Respondent skipped this question

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
No mortalities would be good. Tasmania has a lot better outcome than SA

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
Around 30% excluding 2mm spat which in some cases was up to 90%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
All year round

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
No

Q11 What was the diagnosis from submitted samples if NOT SAMS?
No

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes

Participant 11:

Q1 What is your understanding of SAMS?
Unexplained mortality in juvenile oysters

Q2 What impact do you think SAMS is having on the industry?
Financial impact and wasted resources

Q3 Do you think SAMS is an entity on your lease(s)?
Yes

Q4 If YES above, what impact is SAMS having on your lease(s)?
Reduction of sales by 30%

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
30%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
50%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
Respondent skipped this question.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Respondent skipped this question.

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Respondent skipped this question.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes

Participant 12:

Q1 What is your understanding of SAMS?
Oysters dying for unknown reason, but last 18 months mortality of up to 90% seems to be something other than what we previously knew as SAMS.

Q2 What impact do you think SAMS is having on the industry?
Might as well had POMS losing 90% of seed between 2-8mm, to me SAMS only ever affected 40-60mm stock and was never high enough % to worry about. I think we were dealing with poor SA seas as SAMS had never affected my seed before 2016.

Q3 Do you think SAMS is an entity on your lease(s)?
Uncertain – Please skip to question 5

Q4 If YES above, what impact is SAMS having on your lease(s)?
Respondent skipped this question.

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
Less than 10%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
90% or more

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
No – please skip to question 9

Q8 If YES above, what time of year did you experience these increases in mortalities?
Every batch of seed 16/17 had 90% or more mortality. I haven't changed the way I have always operated my seed. I believe the seed was too young, grown too quickly, fed too much and couldn't handle the change of environment when going to sea.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
Yes

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
No

Q11 What was the diagnosis from submitted samples if NOT SAMS?
How can we call it SAMs when we don't know what SAMS is. They just died, don't what of, so I guess we call it SAMS.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes

Participant 13:

Q1 What is your understanding of SAMS?
In basic terms, it's an unexplained mortality event within South Australian Waters

Q2 What impact do you think SAMS is having on the industry?
These unexplained mortality events are having a massive impact, when taking into consideration the current spat situation.

Q3 Do you think SAMS is an entity on your lease(s)?
Uncertain – please skip to question 5.

Q4 If YES above, what impact is SAMS having on your lease(s)?
Respondent skipped this question.

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
10% - 20% up to juvenile, 40% maturity.

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?

20% - 40% up to juvenile, 50% - 60% to maturity.

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
Respondent skipped this question.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?
No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Uncertain

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Weakened animal condition, in conjunction with "environmental factors"

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
Yes

Participant 14:

Q1 What is your understanding of SAMS?
Unexplained mortality in oysters

Q2 What impact do you think SAMS is having on the industry?
It's impact is seasonal (one year bad next almost none) so it's difficult to get the right amount of stock.

Q3 Do you think SAMS is an entity on your lease(s)?
Yes

Q4 If YES above, what impact is SAMS having on your lease(s)?
It kills a percentage of oysters each year.

Q5 What is the accepted percentage level of mortality per year class on your lease(s)?
25%

Q6 What is the percentage of mortality at your lease(s) for 2016 & 2017 respectively?
2016 25% 2017 50%

Q7 Have you experienced any period increase in mortality over the last 2 years (2016-2017)?
Yes

Q8 If YES above, what time of year did you experience these increases in mortalities?
Respondent skipped this question.

Q9 Have you had any diagnostics performed on affected oysters during losses on your lease(s) for 2016 or 2017?

No – please skip to question 12

Q10 Was there any diagnosis of South Australian Mortality Syndrome (SAMS)?
Respondent skipped this question.

Q11 What was the diagnosis from submitted samples if NOT SAMS?
Respondent skipped this question.

Q12 Would you participate in a surveillance program by contributing oyster samples that will aid in providing a better understanding and management protocols of SAMS by the oyster industry?
No

3.6.2. Appendix 2 – Clinical Pathology and Haemolymph reference range development

Towards the development of a biochemical reference range for Pacific Oysters (*Crassostrea gigas*) haemolymph

Annaliese C. Heijkoop[1], Johanna J. Mahadevan[1] and Stephen B. Pyecroft[2]

[1]School of Animal & Veterinary Sciences, Faculty of Science, the University of Adelaide, Roseworthy Campus, Roseworthy, SA 5371, AUSTRALIA
[2]Department of Veterinary Pathology & Public Health, School of Animal & Veterinary Sciences, Faculty of Science, The University of Adelaide, Roseworthy Campus, Roseworthy, SA 5371, AUSTRALIA

Abstract: In 2017, research undertaking advanced aquatic disease surveillance for known and undefined pacific oyster (*Crassostrea gigas*) pathogens was initiated. To undertake this, methods of disease monitoring and surveillance for this species were expanded to obtain new information that could aid in improving the definition of South Australian Mortality Syndrome (SAMS). The goal of this study was to develop a biochemical haemolymph reference range for *Crassostrea gigas* that may be of use in future assessment and surveillance of oyster heath as well as investigate biochemical enzyme distribution in various oyster organ tissues to develop insight on respective tissue function and assist in accurate interpretation of biochemical findings. Two cohorts of adult Pacific Oysters were obtained from two South Australian oyster leases. Haemolymph was sampled (n=80) directly from the heart or pericardial cavity. Tissue samples were taken from seven specific oyster organs that had been collected from five oysters per cohort (n=10) and homogenised in phosphate buffered saline (PBS). Haemolymph and tissue samples were centrifuged and the resulting supernatants were analysed for 35 clinical analyte parameters. In addition to this, haemolymph samples (n=20) were collected three days post insult from additional oysters that were manually stressed and were analysed for the same 35 analyte parameters. The results of the tissue analysis suggest that organ activity is similar to that of terrestrial mammals with analytes such as ALP, ALT, GGT, amylase, cholesterol and glucose being found predominantly in the hepatopancreas. Findings from the treated animals indicated variable changes in

haemolymph values indicating a usefulness of the tool. This study concludes with the provision of a reference range resource for *C. gigas* haemolymph as well as a preliminary understanding of tissue enzyme levels, which aid in the interpretation of haemolymph biochemistry for monitoring oyster health.

1. **INTRODUCTION:** The Pacific Oyster (*Crassostrea gigas*) is an important aquaculture species that's subsequent cultivation has generated a multimillion-dollar industry with substantial economic impact globally (Li et al. 2009). Farmed in New South Wales, South Australian and Tasmanian waters of Australia the industry is worth $4.6, $26 and $32 million dollars respectively (Jefferson, 2018, Courtney *et al.* 2017, Department of Primary Industries and Regions 2016).

   However, in recent decades increasing occurrences of uncontrolled disease outbreaks such as pacific oyster mortality syndrome (POMS) and unexplained mass summer mortalities have occurred throughout Asia, Europe, North America and Australasia (Go et al. 2017). Diseases such as POMS can have up to 100% mortality and has spread rapidly since its first appearance in France in 2008. Sweeping through New Zealand, New South Wales (2010), Tasmania (2016) and now wild populations in Port River, South Australia in less than a decade (Courtney et al. 2017; Department of Primary Industries and Regions, 2018). Significant losses to disease pose growing concerns within the industry and consequently the ability to assess the health status of these animals is becoming a vital necessity.

   Growth rate, mortality and quality index (dry meat weight/dry shell weight x 1000) are commonly monitored non-invasive 'field parameters' in bivalve health (Patrick *et al.* 2006). Other more specific methods of health assessment rely on destructive methods to analyse physiological and biochemical changes within the tissues (Pouvreau *et al.* 2006). As bivalves are encompassed by a shell, it is common practice to remove the animal from the shell to analyse anatomical structures and soft tissues (Chavez-Villalba *et al.* 2002). Samples of haemolymph for haematology and tissue fixation for histological examination are also prepared (Howard *et al.* 2004). This process can be highly labour intensive as numerous samples are usually required to make a significant diagnosis. While histological and biochemical techniques can yield critical information, these techniques are not only time consuming, but also highly invasive and result in loss of stock and respective economic output. As science advances, the development of alternative less invasive quantitative methods are being considered (Pouvreau *et al.* 2006). Evaluation of haemolymph enzyme activity may be a useful non-lethal measure for health and disease surveillance in *C. gigas*, similar to blood samples used in human and veterinary medicine (Battison, 2006; Gustafson *et al.* 2005).

   Clinical chemistry is used to diagnose, monitor and screen for disease through analysis of enzyme activity in serum, plasma, urine and other bodily fluids (eClinpath, 2013). The enzyme activity detected in serum is dependent on a number of factors including; organ specificity, mechanism of enzyme release, enzyme clearance rate and enzyme synthesis (Hoffmann and Solter, 2008). Enzymes can be tissue specific or distributed among several tissues and have varying concentrations in organs which can be reflected in blood serum levels. These blood serum levels can be used to calculate reference intervals (RI) which act as a baseline range for comparing interindividual variation (Battison, 2006). Furthermore, RI can be used to identify organ injury by marked increases in serum enzyme activity due to compromised cellular integrity (Hoffmann and Solter, 2008).

   Haemolymph is the circulatory fluid equivalent to blood in bivalves and other mollusks with open circulatory systems. Collection of haemolymph can be a simple and non-lethal procedure, ensuring that health assessment does not lead to loss of stock (Gustafson *et al.* 2005). Previous studies on

C. gigas have not mentioned issues with sampling or clotting of haemolymph indicating this method to be a viable sampling technique (*Go et al. 2017; Olafsen et al. 1992)*. Haemolymph has also been found to be a useful diagnostic tool in the identification of pathogens and health status in other shellfish species (Gustafson *et al.* 2005; Battison, 2006). Current knowledge of biochemical composition and physiological pathways for molluscs is relatively specific and limited (Patrick et al. 2006) and Pacific Oyster tissue and haemolymph metabolite levels have not been thoroughly investigated in the literature. There is currently no known haemolymph reference range for Pacific Oysters and no such studies have been published with respect to their tissue enzyme and metabolite levels.

The aim of this research project was to investigate if analysis of haemolymph enzyme activity could also be applied in the pacific oyster, *C. gigas.* In order to evaluate the viability of this concept three factors needed to be assessed, (1) the establishment of a reference range from a population of healthy animals; (2) determining the distribution of selected enzymes in various tissues; and (3) assessing if haemolymph enzyme activity could be altered by environmental stressors and organ damage (Battison, 2006; Gustafson *et al.* 2005). This research may allow those working within the oyster industry insight into oyster function. Additionally, the development of the reference range for haemolymph may be a useful tool for monitoring oyster health.

2. MATERIALS AND METHODS

2.1 Animal Origin:

Adult Pacific Oysters (*C. gigas)* aged 20-36 months, sized 70-100mm, grown in intertidal waters were obtained from two commercial oyster leases in South Australia. Oysters supplied by Turners Oysters, Cowell (n=50) and SA Premium Oysters, Smokey Bay (n=50), were transported on ice from their farmed leases. On delivery, oysters were kept in a cool room at approximately 4°C, and not held longer than four days under these conditions in accordance with University policy. Twenty oysters, ten from each cohort, used in the stress trial were initially kept in a cool room (4°C) for 2 days, then moved into a salt water flow through tank system (17-18°C) for a duration of 7 days before the stress trial began.

2.2 Biochemical Analysis:

All samples for biochemical analysis were analysed using the Beckman Coulter AU480 Chemistry Analyser within one day of collection. A full biochemistry panel for 35 analytes was used, selection was based on analytes offered for analysis by the Chemistry Analyser.
Analytes measured included Sodium, Potassium, Chloride, Bicarbonate, Glucose, Urea, Creatinine, Calcium, Inorganic Phosphate, Albumin, Total Protein (TP), Total Bilirubin C, Alkaline Phosphatase (ALP), Aspartate aminotraNSFerase (AST), Alanine aminotraNSFerase (ALT), Creatinine Kinase (CK), Cholesterol, Amylase (AMY), Lipase, Total Bile Acids (TBA), Magnesium, Gamma glutamyl traNSFerase (GGT), Iron, Triglyceride, Glutamate dehydrogenase (GLDH), D-3 Hydroxybutyrate, LIP, ICT, HEM, Uric acid, Lactate, Globulin, Ca:P ratio, Na:K ratio and Anion Gap.

2.3 Tissue Enzyme Distribution Study:

Ten adult Pacific Oysters *(C. gigas)*, the first five from each cohort were used to analyse tissue enzyme distribution. Oysters were opened using a hinge approach where the adductor muscle was carefully cut as close to the upper shell as possible using a disposable scalpel blade. The upper shell was then completely removed. Tissue samples of heart, muscle, labial palps, mantle, gastrointestinal tract (GIT), leydig and hepatopancreas (HP) were dissected from each oyster. Tissues were immediately place in pre-weighed tubes containing 0.5mL of chilled PBS. Empty tube

weight, tube weight with PBS and tube weight with PBS and tissue sample were all recorded in order to calculate enzyme concentration per gram of tissue. These samples were then refrigerated at 4°C until further processing and analysis, all samples were analysed within one day of collection.

Tissues (≤2g) in PBS were then minced with clean micro dissecting scissors for 1 minute (min) and then homogenised with sterile disposable plastic homogeniser for 1 min to ensure no large particles of tissue remained. This homogenised solution was immediately vortexed and then centrifuged at 10,000rpm for 5 min to obtain tissue supernatant. The supernatant was then traNSFerred into analysis tubes and run through the chemistry analyser within an hour of processing.

Supernatant for muscle, labial palps, mantle, GIT and leydig required a 1 (50μL) in 10 (450μL) dilution for successful analysis. Half of the supernatants per organ tissue were diluted with distilled water (dH_2O), while the other half were diluted using PBS. To calculate the concentration of enzyme per gram of tissue it was assumed that weight of tissue and volume of PBS correspond in order to standardize the results.

The oysters used for this analysis originated from two different locations (n=5 Cowell and n=5 Smokey Bay). Due to this the effects of geographic location was analysed to assess homogeneity of variance. The Shapiro-Wilk test was initially performed to evaluate the distribution pattern of data. To assess for homogeneity, the Levene's Test was then applied to normally distributed data while the non-parametric Levene's Test was used on data sets failing to show a normal distribution pattern. This protocol was applied to each analyte for each tissue type using IBM SPSS Statistics 25 for Windows.

2.4 Establishment of Reference Range:

Eighty adult Pacific Oysters (*C. gigas*) were used in the establishment of the reference range for haemolymph. Oysters were opened using a hinge approach where the adductor muscle was carefully cut as close to the upper shell as possible using a disposable scalpel blade. The upper shell was then completely removed. Haemolymph was collected directly from the heart or pericardial cavity using a 25g needle and 1mL syringe following the method described by Olafsen (1992). Between 0.3 – 0.5mL was collected from each individual oyster, vortexed and then centrifuged at 10,000g for 5mins to remove debris from the sample. The supernatant was then traNSFerred into analysis sample tubes and refrigerated at 4°C until analysed. All haemolymph samples were analysed within two hours of collection. These oysters were obtained from the two locations previously described. The location affect was investigated for each analyte using the same protocol applied in the tissue enzyme distribution study where the Shapiro-Wilk Test, Levene's Test and the non-parametric Levene's Test were used to assess homogeneity of variance. Once analysed the Reference Value Advisor V2.1 was used to create the haemolymph reference range (Geffré *et al.* 2011). Haemolymph reference ranges of analytes showing statistical variance were also computed using the Reference Value Advisor software for each location (n=40 Cowell and n=40 Smokey Bay).

2.5 Stress Trial:

An additional twenty oysters, ten from each cohort, not used for the establishment of the reference intervals, were manually stressed using two techniques. The first stress trial was completed on ten oysters, five from each cohort, where oysters were left out of water at room temperature (approximately 22-24°C) for 72 hours. The second stress trial was completed on the remaining ten oysters, five from each cohort, where the hepatopancreas was damaged. A small hole was drilled into the shell above the hepatopancreas. The organ was then damaged via fine

needle aspiration for approximately 10 seconds each oyster. These oysters were then recovered and place back into the flow through tank system for 72 hours. Haemolymph samples were collected three days post insult and analysed similar to that for the reference range samples.

2.6 Data Analysis:

Statistical analysis was performed using the Reference Value Advisor V2.1 software package and Microsoft Excel 2018. Nonnumerical results were not included in the data analysis. Analyte results which returned with a negative value were zero'ed and these values were not included in calculations for means or significant data points. Data values that were a zero naturally were included in calculations for means and significant data points.

3. RESULTS

3.1 Raw Data:

The Beckman Coulter AU480 Chemistry Analyser analysed 100 haemolymph samples and 70 tissue samples for 35 analytes. Three analytes (LIP, ICT, HEM) presented as nonnumerical 'N' on the biochemistry panel, subsequently these analytes were not included in the results.
From the 100 haemolymph samples used for establishment of the reference range, 44 Ca:P and 10 D3-Hydroxybutyrate data values were blank after analysis by the Chemistry Analyser, the existing values were still included in the results. From the 70 tissue samples, 14 Ca:P, 13 Na:K and 13 anion gap values were blank after analysis by the Chemistry Analyser, subsequently mean values for these analytes were not included in the results.

3.2 Tissue Enzyme Distribution:

Variance amongst the two locations were analysed for each analyte and revealed no significant statistical variance effect between geographic locations. The hepatopancreas was the most notable organ analysed, found to have the highest activity for Glucose, Creatinine, Albumin, Total Protein, ALP, AST, ALT, Cholesterol, AMY, GGT, Iron, Uric acid, Lactate and Globulin analytes. Exceptionally high levels of AST were also found in the heart and muscle tissue, while GLDH has the highest reading in the heart (Figure 1). The palp and leydig tissues were found to contain the highest levels of bicarbonate, TBA and triglyceride. While the GIT contained the greatest activity of lipase and the mantle had the highest CK reading (Table 1).

Table 1: Mean enzyme activity (U/g) in seven tissue types from Pacific Oysters (*C. gigas*) (n=10). Highlighted areas indicate notable levels of analytes in respective tissue.

	Heart	*Muscle*	*Palps*	*Mantle*	*GIT*	*Leydig*	*Hepatopancreas*
Sodium	1.0010	0.8442	0.8890	0.9457	1.0725	0.9896	0.7788
Potassium	0.0490	0.0716	0.0443	0.0587	0.0647	0.0691	0.0812
Chloride	0.9479	0.8455	0.8881	0.9383	1.0764	0.9939	0.7512
Bicarbonate	0.0023	0.0021	0.0160	0.0108	0.0042	0.0140	0.0019
Glucose	0.0011	0.0024	0.0034	0.0019	0.0011	0.0053	0.0234
Urea	0.0025	0.0022	0.0196	0.0190	0.0169	0.0176	0.0053
Creatinine	0.0000	0.0148	0.0000	0.0000	0.0000	0.0000	0.0213
Calcium	0.0005	0.0027	0.0023	0.0025	0.0015	0.0018	0.0021
Inorganic Phosphate	0.0542	0.0484	0.0378	0.0391	0.0562	0.0352	0.0568
Albumin	0.0008	0.0044	0.0000	0.0000	0.0008	0.0020	0.0103
Total Protein	0.0065	0.0220	0.0093	0.0060	0.0076	0.0178	0.0312
Total Bilirubin C	0.0027	0.0060	0.0202	0.0089	0.0120	0.0160	0.0065
ALP	0.0576	0.0396	0.2414	0.0776	0.2158	0.3237	5.0191
AST	13.6410	13.0763	4.0805	7.6910	4.0704	3.6365	14.8396
ALT	13.0686	9.3210	9.1265	12.9471	8.2723	8.8670	25.8033
Creatinine Kinase	0.2706	0.8258	0.4385	1.3345	0.3252	0.3095	0.6335
Cholesterol	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0004
Amylase	0.0426	0.0586	0.3200	0.2317	0.0216	0.0964	6.5762
Lipase	0.0156	0.0258	0.9560	0.0955	1.5858	0.0955	0.4930
Total Bile Acids	0.0678	0.0018	0.5210	0.0000	0.2461	0.6228	0.0376
Magnesium	0.0160	0.0194	0.0287	0.0363	0.0230	0.0305	0.0192
GGT	0.0164	0.0161	0.3208	0.1668	0.0989	0.2197	0.7453
Iron	0.0555	0.0575	0.0835	0.0609	0.0425	0.0421	0.1133
Triglyceride	0.0007	0.0006	0.0016	0.0000	0.0004	0.0016	0.0007
GLDH	0.6401	0.4020	0.2376	0.4298	0.3041	0.2337	0.5576
D-3 Hydroxybutyrate	0.0001	0.0003	0.0005	0.0005	0.0003	0.0007	0.0004
Uric acid	0.0198	0.0173	0.0835	0.0560	0.0858	0.1125	0.1411
Lactate	0.0001	0.0001	0.0000	0.0000	0.0001	0.0000	0.0002
Globulin	0.0054	0.0158	0.0000	0.0000	0.0024	0.0044	0.0210

LIP, ICT and HEM were not included in table as these analytes were N in biochemistry panel.

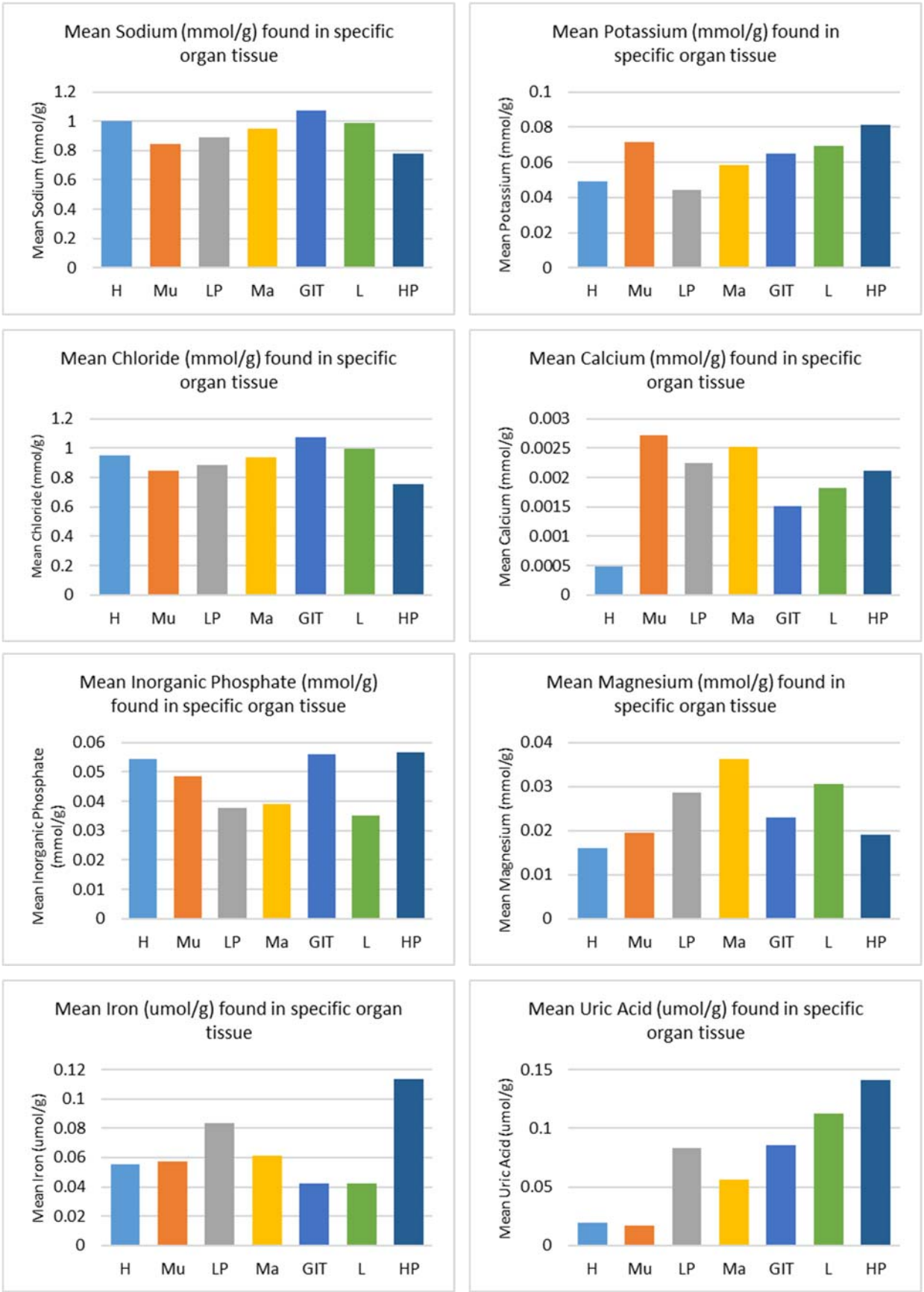

Figure 1: Mean enzyme activity (U/g) found in all tissues at similar and non-subjective concentrations. H indicates heart; Mu, muscle; LP, Labial palps; Ma, mantle; GIT, gastrointestinal system; L, Leydig tissue; and HP, hepatopancreas

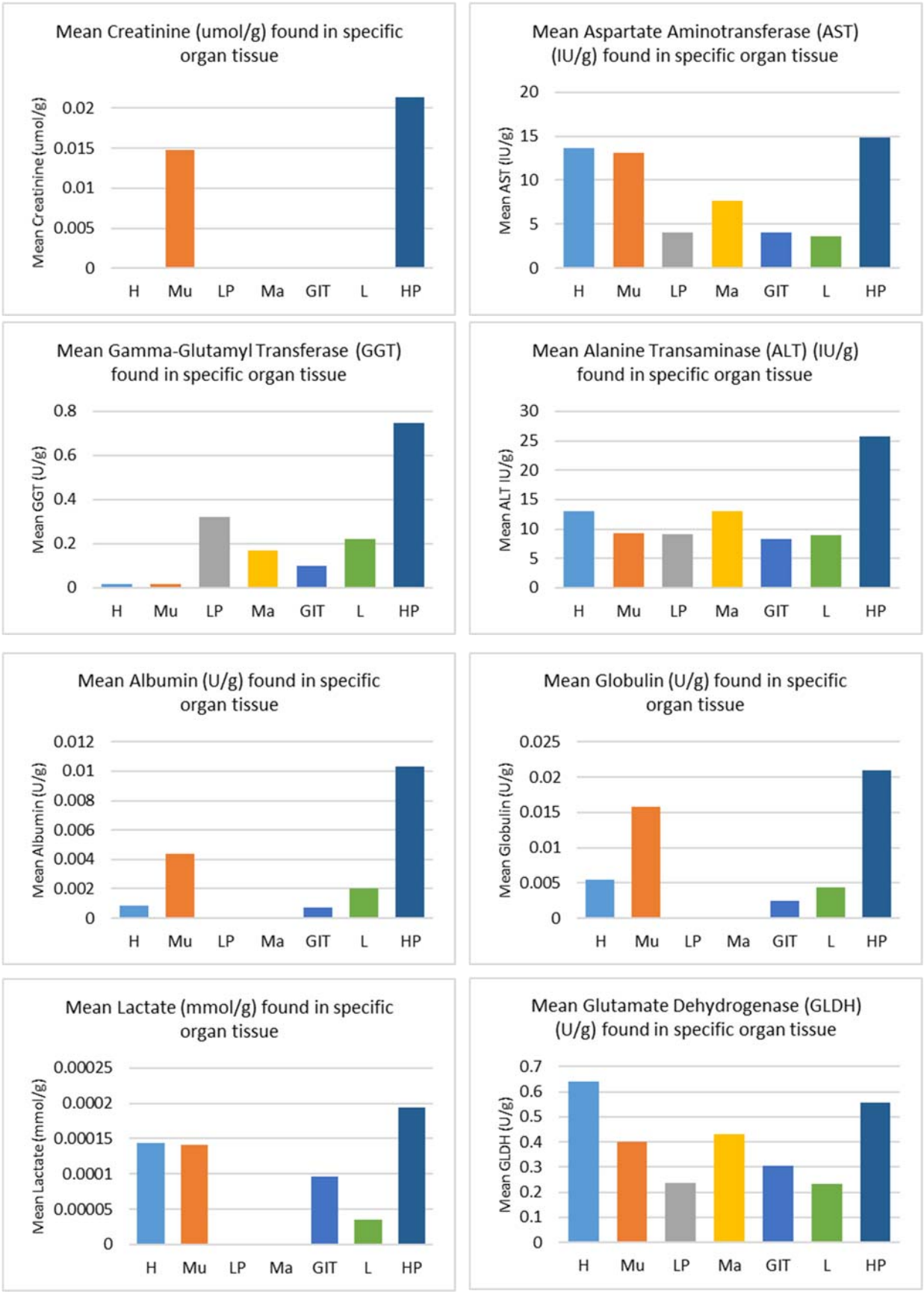

Figure 2: Mean enzyme activity (U/g) similar to that of other domestic animals. H indicates heart; Mu, muscle; LP, Labial palps; Ma, mantle; GIT, gastrointestinal system; L, Leydig tissue; and HP, hepatopancreas.

Figure 3: Notable mean enzyme activity (U/g) for tissues including labial palps, mantle and leydig tissue not commonly found in domestic species. H indicates heart; Mu, muscle; LP, Labial palps; Ma, mantle; GIT, gastrointestinal system; L, Leydig tissue; and HP, hepatopancreas.

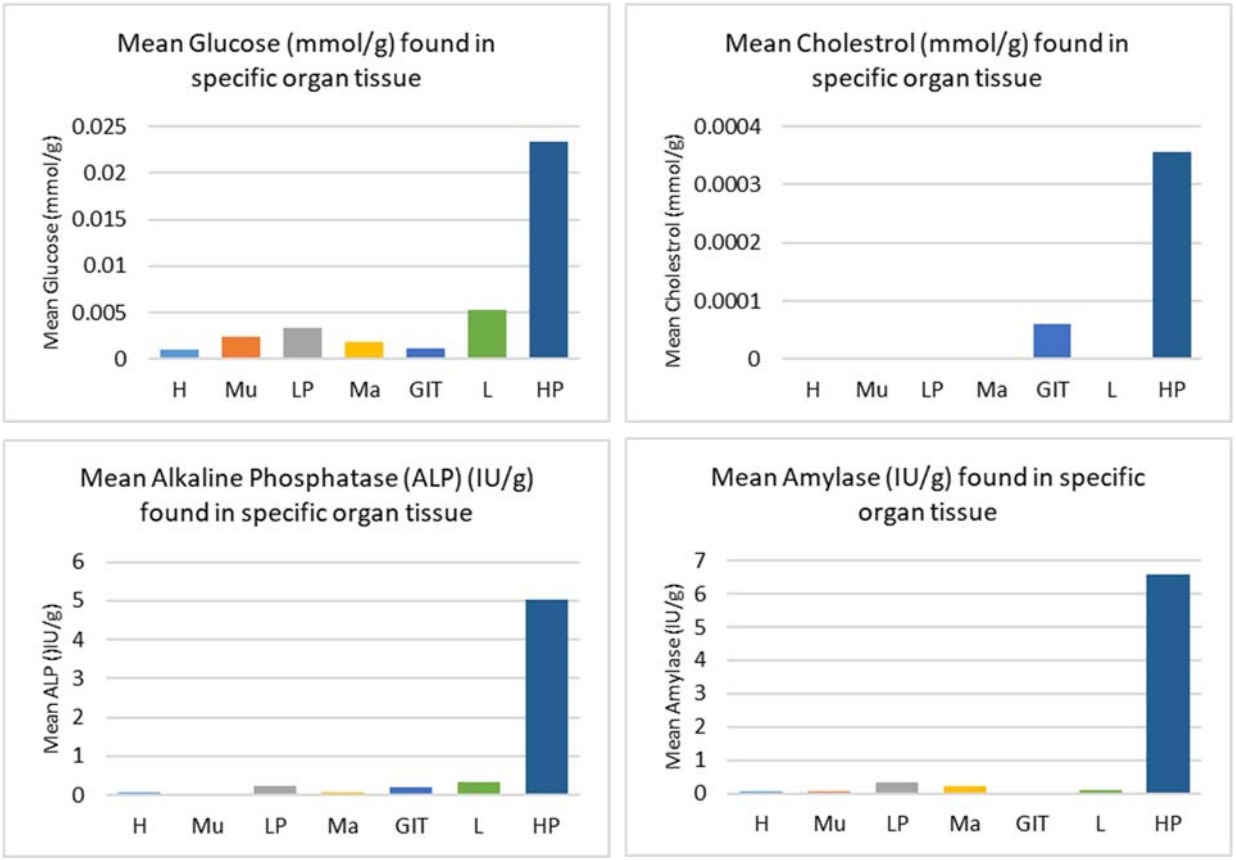

Figure 4: Significant mean enzyme activity (U/g) for tissues seen to be enzyme specific. H indicates heart; Mu, muscle; LP, Labial palps; Ma, mantle; GIT, gastrointestinal system; L, Leydig tissue; and HP, hepatopancreas.

3.3 Establishment of Reference Range and Stress Trial:

3.3.1 Reference Range:

The Reference Value Advisor was utilised to evaluate a set of 80 hemolymph samples obtained from healthy oysters. Statistically significant haematological reference intervals were established for 32 analytes (Table 2). All analytes samples sizes were large enough to compute the nonparametric reference interval, excluding Ca:P ratio (n<40) where the robust method with a Box-Cox traNSFormation was used (Geffré et al. 2011). For all analytes (excluding ALP, cholesterol, AMY, triglyceride and lactate) the 90% CI limit was larger than recommended in International Federation of Clinical Chemistry (IFCC) and the Clinical and Laboratory Standards Institute (CLSI) C28-A3 (Geffré et al. 2011).

Possible outliers were detected according to Turkey or Dixon in a number of analytes (Na, K, Glucose, Urea, Creatinine, CK, Cholesterol, Amylase, Lipase, TBA, GGT, GLDH, Na:K ratio). IFCC and CLSI recommends that unless outliers are known to be aberrant observations, the emphasis should be on retaining rather than deleting them, therefore all data values were retained (Geffré et al. 2011). Suspect data was also detected according to Tukey in a number of analytes (TP, Total Bilirubin C, D-3 Hydroxybutyrate). IFCC and CLSA recommends that unless these data are known to be aberrant observations, the emphasis should be on retaining rather than deleting them, therefore all data values were retained (Geffré et al. 2011). The effect of geographic location showed statistically significant variance for analytes Urea and Inorganic Phosphate (Table 2). The reference intervals for these two analytes showing variance were then tabulated (Table 3).

Analyte	Reference Interval n=80	P-Value Φ
Sodium (mmol/L)	353.23 – 559.44	NV
Potassium (mmol/L)	10.4 – 15.8	NV
Chloride (mmol/L)	394.03 – 654.23	NV
Bicarbonate (mmol/L)	0.7 – 6.7	NV
Glucose (mmol/L)	0 – 0.1	NV
Urea (mmol/L)	0.1 – 0.6	0.002
Creatinine (umol/L)	8.58 – 127.89	NV
Calcium (mmol/L)	7.83 – 11.42	NV
Inorganic Phosphate (mmol/L)	0 – 0.3	0.000
Albumin (g/L)	0	NV
Total Protein (g/L)	0.3 – 3.6	NV
Total Bilirubin C (umol/L)	0 – 0.7	NV
ALP (IU/L)	0 – 47.31	NV
AST (IU/L)	0 – 36	NV
ALT (IU/L)	0.11 – 55.05	NV
Creatinine Kinase (IU/L)	0 – 5	NV
Cholesterol (mmol/L)	0	NV
Amylase (IU/L)	0 – 105.6	NV
Lipase (IU/L)	2 – 196.8	NV
Total Bile Acids (umol/L)	0 – 13.79	NV
Magnesium (mmol/L)	4.79 – 6.55	NV
GGT (U/L)	0 – 2.6	NV
Iron (umol/L)	1.32 – 32.94	NV
Triglyceride (mmol/L)	0	NV
GLDH (U/L)	0 – 1.2	NV
D-3 Hydroxybutyrate (mmol/L)	0 – 0.02	NV
Uric Acid (umol/L)	0 – 9	NV
Lactate (mmol/L)	0	NV
Globulin (g/L)	0 – 4	NV
CA:P ratio*	20.12 – 140.59	NV
Na:K ratio	32.9 – 40.9	NV
Anion Gap (mmol/L)	0	NV

Table 2: Haemolymph biochemical reference range for Pacific Oysters (*C. gigas*)

ϕ TraNSFormed data with homogeneous variances (Parametric & Non-Parametric Levene's Test) for fresh and frozen samples. NV indicates statistical analysis did not have homogeneous variance (value > .005)
Abbreviations: ALT- alanine aminotraNSFerase, AST- aspartate aminotraNSFerase, ALP – alkaline phosphatase, GGT- gamma glutamyl traNSFerase, GLDH – glutamate dehydrogenase Na:K – sodium & potassium ratio, Ca:P – calcium and phosphorus ratio.

Table 3: Haemolymph biochemical reference range of Pacific Oysters (*C. gigas)* from two geographic locations for analytes Urea (mmol/L) and Inorganic Phosphate (mmol/L) Pacific Oysters (*C. gigas*)

Analyte	Reference Interval Cowell n=40	Reference Interval Smokey Bay n=40
Urea (mmol/L)	0.1 – 0.7	0.1 – 0.6
Inorganic Phosphate (mmol/L)	0.1 – 0.3	0.0 – 0.3

Table 4: Reference range for Pacific Oysters (*C. gigas*) haemolymph compared with haemolymph clinical chemistry results for manually stressed animals; left out of water at room temperature and hepatopancreases damage via fine needle aspiration.

Analyte	Reference Interval n=80	Stress 1 (Left Out) n=10		Stress 2 (HP Damage) n=10	
		Mean**	Min-Max	Mean**	Min-Max
Sodium (mmol/L)	353.23 – 559.44	528.15	515 – 552.7	527.04	519.6 – 535.6
Potassium (mmol/L)	10.4 – 15.8	9.35 (8)	7.7 – 11.1	7.17 (10)	6.4 – 8.3
Chloride (mmol/L)	394.03 – 654.23	584.55	563.9 – 615.5	578.88	565.4 – 595.8
Bicarbonate (mmol/L)	0.7 – 6.7	4.87 (2)	3.4 – 6.9	0.95	0.7 – 2
Glucose (mmol/L)	0 – 0.1	0.11 (2)	0 – 0.3	0.08 (1)	0 – 0.2
Urea (mmol/L)	0.1 – 0.6	1.07 (10)	1 – 1.2	1.02 (10)	1 – 1.1
Creatinine (umol/L)	8.58 – 127.89	53.56	17.14 – 96.19	23.82 (3)	0 – 59.7
Calcium (mmol/L)	7.83 – 11.42	9.93	8.91 – 11.25	6.57 (10)	6.33 – 6.71
Inorganic Phosphate (mmol/L)	0 – 0.3	0.15 (1)	0 – 0.6	0.2	0.1 – 0.3
Albumin (g/L)	0 – 0	0	0 – 0	0	0 – 0
Total Protein (g/L)	0.3 – 3.6	1.47	0 – 3	0.3	0 – 0.5
Total Bilirubin C (umol/L)	0 – 0.7	0.27	0.1 – 0.5	0.18	0 – 0.5
ALP (IU/L)	0 – 47.31	58.86 (2)	0.4 – 440.3	53.24 (2)	0.2 – 389.3
AST (IU/L)	0 – 36	67.5 (5)	0 – 276	41.7 (4)	11 – 115
ALT (IU/L)	0.11 – 55.05	98.92 (6)	24.9 – 248.3	48.93 (2)	11.4 – 115.2
Creatinine Kinase (IU/L)	0 – 5	7.2 (5)	0 – 27	3	0 – 9
Cholesterol (mmol/L)	0 – 0	0	0 – 0	0	0 – 0
Amylase (IU/L)	0 – 105.6	155.7 (2)	0 – 1052	141.1 (2)	0 – 770
Lipase (IU/L)	2 – 196.8	3.7 (2)	0 – 11	14.4	1 – 67
Total Bile Acids (umol/L)	0 – 13.79	2.94	0 – 9.4	5.06	0.1 – 9.7
Magnesium (mmol/L)	4.79 – 6.55	5.13	5.03 – 5.22	5.14	5.1 – 5.21
GGT (U/L)	0 – 2.6	2.18 (2)	0 – 7.5	0.98 (1)	0 – 3.1
Iron (umol/L)	1.32 – 32.94	7.82 (2)	1.2 – 20.8	0.88 (6)	0 – 2
Triglyceride (mmol/L)	0 – 0	0	0 – 0	0	0 – 0
GLDH (U/L)	0 – 1.2	1.26 (5)	0 – 2.6	0.93 (3)	0.1 – 2.3
D-3 Hydroxybutyrate (mmol/L)	0 – 0.02	0.01	0 – 0.02	0.01	0 – 0.02
Uric Acid (umol/L)	0 – 9	1.1	1 – 2	1	0 – 3
Lactate (mmol/L)	0 – 0	0	0 – 0	0	0 – 0
Globulin (g/L)	0 – 4	1.5	0 – 3	0.17	0 – 1
CA:P ratio*	20.12 – 140.59	67.43	17.2 – 103.2	37.31	21.1 – 66.8
Na:K ratio	32.9 – 40.9	57.21 (10)	46.5 – 69	73.99 (10)	63.5 – 83.3
Anion Gap (mmol/L)	-104 – -23.5	-51.89	-60.1 – -42.1	-45.62	-54.1 – -37.2

*Box-Cox traNSFormation (all others reference intervals are nonparametric method)

** The number of individual samples outside RI is noted within brackets ()

Highlighted data is lower than RI

Highlighted data is higher than RI

For haemolymph samples obtained from the stress trials, mean and minimum and maximum values for 32 analytes were tabulated and compared against the formulated reference intervals (Table 3).

3.3.2 Stress Trial 1:

For the majority of individual and mean oyster haemolymph (22 analytes), enzyme activity stayed within the previously established reference intervals. However, mean enzyme activity for ten analytes did not fall into their respective RI (Table 4). Most notably all ten samples for urea and Na:K ratio were above their RIs, elevated enzymes ranged from 1-1.2 and 46.5-69 respectively (Table 4). Only two of the ten samples for ALP and AMY were elevated above the calculated RIs, however they were significantly elevated (137.5, 440.3) and (1052, 502) respectively. Six ALT (88.1-248.3), Five AST (53-276), CK (6-27) and GLDH (1.3-2.6) as well as two glucose (0.2, 0.3) were all above their respective RIs as well. Potassium had eight samples that were below the RI, lowered enzymes ranged between 7.7-10.1 (Table 3).

Individual enzyme activity for Lipase (two – 0, 0), Inorganic Phosphate (one – 0.6), Bicarbonate (two – 6.8, 6.9), GGT (two – 7.5, 3.5) and Iron (two – 1.2, 1.2) were also found outside the RIs but did not impact on mean values (Table 4).

3.3.3 Stress Trial 2:

For the majority of individual and mean oyster haemolymph (24 analytes), enzyme activity stayed within the previously established reference intervals. However, mean enzyme activity for eight analytes did not fall into their respective RI (Table 4). Most notably all ten samples for potassium, urea, calcium and Na:K ratio were outside their RIs, enzyme activity ranged from 6.4-8.3, 1-1.1, 6.33-6.71 and 63.5-83.3 respectively (Table 4). Only two of the ten samples for ALP and AMY were elevated above the calculated RIs, however they were significantly elevated (389.3, 132.6) and (770, 482) respectively. Four AST (39, 49, 97, 115) were above and six iron (0.2-1) were below their respective RIs as well (Table 4).

Individual enzyme activity for Creatinine (three – 0, 6.32, 7.68), Glucose (one – 0.2), GLDH (three – 2.3, 2.2, 1.7), ALT (two – 115.2, 109.9) and GGT (one – 3.1) were also found outside the RIs but did not impact on mean values (Table 4).

4. DISCUSSION

4.1 Tissue Enzyme Distribution:

In order to evaluate haemolymph enzyme activity, an understanding of their distribution in various organ tissue is required. While various tissues have been pooled to for DNA extraction in previous studies (Jenkins *et al.* 2013, Go *et al.* 2017). To our knowledge, no previous reports have been published on clinical chemistry values in which individual values were provided for analytes in tissues for the Pacific Oyster (*C. gigas*). The understanding of enzyme tissue concentrations is important as this and organ mass have a direct correlation with haemolymph enzyme activity. Hence, organs with high concentrations of certain enzymes can increase haemolymph enzyme concentrations when they are damaged or diseased (Hoffmann and Solter, 2008).
Tissue distribution of enzymes has been studied in domestic animals for many decades and while magnitude of activity can vary between species some common patterns are observed (Hoffmann and Solter, 2008). Enzymes known to be found in the liver include ALT, AST, GLDH, lactate dehydrogenase (LDH), ALP, GGT, Bilirubin, Bile acids and coagulation factors including albumin, cholesterol and urea,

while pancreatic enzymes include AMY, lipase and GGT (eClinpath, 2013; Hoffmann and Solter, 2008). Skeletal and cardiac muscle tissues have high levels of CK, ALT, AST and LDH (Hoffmann and Solter, 2008).
Overall, the results of the tissue analysis for pacific oysters suggests that organ enzyme activity is similar to that of terrestrial mammals, with some exceptions. CK activity is usually at greatest concentration in skeletal muscle in domestic animals (Hoffmann and Solter, 2008), however activity of CK was a third higher in mantle tissue than skeletal muscle in the oyster. As the mantle is responsible for the secretion of shell material and respective growth of the oyster (Fougerouse *et al.* 2008), and CK catalyses the production of ATP (eClinpath, 2013), high energy requirements for shell growth could explain the elevated levels of CK within mantle tissue. Cells from liver, bone, kidney and intestinal mucosa have greatest ALP activity per gram, with intestinal mucosa having highest levels (Hoffmann and Solter, 2008). Oyster ALP tissue activity was found chiefly in the hepatopancreas with minimal levels found in GIT tissues.

Leydig and palp tissues are not found in all species and hence are difficult to compare and analyse. The functions of these tissues in molluscs is vague and limited research further impedes evaluation of results. Currently it is theorised that leydig is a component of connective tissue that functions as storage of parenchyma for oysters (Eble and Scro, 1996), while palps are thought to be responsible for restricting food and mucous uptake into the GIT in (Fougerouse *et al.* 2008; Bernard, 1795) and little is known on their enzyme distribution.

Additionally, limitations in sample size, preanalytical and analytical conditions pose further restraints on bias of results. Tissue enzyme distributions were derived from only ten animals, all sourced from South Australian waters. All attempts were made to keep sampling consistent and obtain pure samples of tissue, however collection of individual tissues was difficult due to the size of animals and respective association between certain tissues. Hence, contamination of tissue samples could have occurred leading to the presence of outliers within the data series. Likewise, dilution of tissue supernatant with PBS for five of the ten tissues and the resultant calculation for accounted bias could also have impacted on the results. Therefore, it is uncertain that these results are a true representation of tissue enzymes in the general population of *C. gigas*. However, as current literature on tissue enzyme distribution is limited for the Pacific Oyster, these results still provide a foundation resource for tissue enzyme distribution in the *C. gigas.*

4.1 Establishment of Reference Intervals:

Presently international recommendation for the determination of reference intervals is through the use of the robust method and Box-Cox transformation (Geffré *et al.* 2011). The Reference Value Advisor used on this series of data currently uses gold standard analysis as it calculates reference limits using a nonparametric method when $n \geq 40$ and by parametric and robust methods from native and Box–Cox transformed values, as well as testing for normality of distribution using the Anderson–Darling test and outliers using Tukey and Dixon–Reed tests (Geffré *et al.* 2011). While this software program provides a simple solution regarding the determination of reference intervals, it cannot compensate for flaws in selection, handling and analysis of the sample group. This is an issue when assessing the data's representation to the total population of *C. gigas* and hence the usefulness of the reference range as a tool. Variance from geographic location from total haemolymph analysis was statistically present for analytes urea (mmol/L) and inorganic phosphate (mmol/L), however reference intervals produced when locations were analysed separately showed a 0.1 difference between locations for urea and no difference in intervals for inorganic phosphates. Ideally data obtained during this research would be compared against current figures present in the literature to ascertain accuracy of the results. However, to our knowledge there is currently no known haemolymph reference range for the Pacific Oysters *C. gigas* or any other closely related molluscs. Hence, we are unable to compare our findings accuracy and precision with respect to population values.

Additionally, while all attempts have been made to obtain healthy animals that suffice as true representatives of the *C. gigas* population, the ability to quantify a 'healthy' oyster is exceptionally challenging. With limited literature, resources and techniques at our disposal the likelihood that the constructed reference range represents all *C. gigas* individuals is highly unlikely. Moreover, the limited geographical representation (n=2; Cowell and Smokey Bay, South Australia) and small sample size (n=80) of the study further reduce this likelihood of a population representation. The presence of suspect data and outliers may also skew data findings from population norms. Further investigation into the impact of location, gender, and maturity would also be beneficial. However, while these findings are unlikely to be a true representative of the *C. gigas* population, they still provide a foundation reference range resource for *C. gigas* haemolymph, which may aid in health assessment.

4.3 Stress Trial

The aim of the stress trials was to test the hypothesis that the analysis of haemolymph enzyme activity could also be applied in the Pacific Oyster *C. gigas*. In order to assess the viability of this technique, detection of notable alteration in haemolymph enzyme activity levels due to a 'treatment' was investigated.

4.3.1 Stress Trial 1:

Oysters are exceptionally hardy animals that can maintain water tight integrity and survive for extended periods of time out of the water (Howard *et al.* 2004). While survival is possible, the inability to filter can impact on osmoregulation and homeostasis within the oyster. Oysters like all living animals require oxygen to maintain life and while oysters can survive out of water, they are unable to exchange important molecules such as oxygen (O_2) and carbon dioxide (CO_2). As the cells metabolic activities continues within the oyster, the concentration of O_2 is depleted while CO_2 levels rise. In domestic animals, excess CO_2 allows the generation of bicarbonate that can lead to alkalosis, triggering the release of hydrogen ions into the blood while potassium moves from the extracellular fluid into the intracellular fluid to maintain electroneutrality (eClinpath, 2013).

This was reflected in the trial results as the presence of an electrolyte imbalance was shown with elevated bicarbonate and Na:K ration and decreased potassium (Table 2). In domestic animals' electrolytes are essential to proper functioning of cells and concentrations are usually maintained within narrow limits, even small changes in potassium concentration in the blood can have marked effects on organ function (eClinpath, 2013).

Changes in electrolyte concentrations can also be through excretion or loss including evaporative water loss, possible fluid loss due to a loss of watertight integrity may also have affected the electrolyte imbalance. Other waste products such as urea and inorganic phosphate are also unable to be excreted and there is a resultant increase in these analytes present in circulation which can be seen in the results (Table 2). As the concentration of analytes increases, the strain placed on the major organs involved in circulation and homeostasis impacts on their respective functions. Elevated levels of ALP, ALT, AST, CK, AMY, GLDH and glucose indicate the liver and heart are not functioning correctly and showing signs of systemic stress.

Overall, what this trial does show is that after oysters were environmentally stressed by leaving them out of water at room temperature for three days, there were changes in the haemolymph enzyme activity levels that could be explained due to the treatment they had been subjected to.

4.3.2 Stress Trial 2:

The hepatopancreas is the digestive gland in the oyster and functions as the liver and pancreas through the production of digestive enzymes and absorption of digested food (Fougerouse *et al.* 2008). It was a notable organ as it was found to have the highest activity for a number of analytes.

Hepatocellular damage in domestic animals usually results in elevated levels of ALT, AST and GLDH, this was mirrored in the oyster haemolymph samples (eClinpath, 2013). Elevated levels of urea was also present, as hepatocytes are responsible for detoxification and excretion as well as the synthesis of urea, damage to the liver may have impacted membrane integrity allowing leakage of urea into the haemolymph (eClinpath, 2013; Hoffman and Solter, 2008). Reduced membrane integrity can also explain elevated levels of other enzymes found in high concentrations in the liver, including ALP, glucose and GGT (Table 1). High concentrations of AMY are found in the pancreas of domestic animals and the hepatopancreas of the pacific oyster, further supporting this theory. However, not all enzymes found in the hepatopancreas were elevated, which is something that may require further investigated.

It must also be noted that it is difficult to keep FNA damage consistent throughout the trial and there is the respective risk that other organs may have been damaged by accident. This may have led to elevated levels of enzymes from these organ systems as well as the hepatopancreas, leading it outliers. While this needs to be mentioned, the aim of this trial was to prove the theory that damage to an organ would lead to a respective elevation of some tissue specific enzymes to also be elevated which was found in this trial.

5. CONCLUSION

The study has established a foundation reference range resource for *C. gigas* haemolymph of 32 analytes, while also providing preliminary understanding of tissue enzyme levels in seven tissues. While this information is more likely to be used in research situations, it has still demonstrated itself to be a useful tool in analysis of haemolymph and organ health in *C. gigas.* Continued research to gain further understanding of biochemical composition and physiological pathways is necessary for successful treatment and management of *C. gigas* in the aquaculture industry. However, this information can be used as a starting point to aid in interpretation of clinical findings and monitoring of oyster health.

ACKNOWLEDGEMENTS

We wish to acknowledge Simon Turner (Turner's Oysters, Cowell), Judd Evans (Kiwi's Oysters, Smoky Bay) and Colleen and Jeff Holmes (SA Premium Oysters, Smokey Bay) for organising and donating the Pacific Oysters used in this research. Funding for this project was provided by the University of Adelaide. Additional funding for this project was supplied by Future Oysters CRC Project.

REFERENCES

Adelson, H., 2016, *Crassostrea Gigas Surveillance in South Australia – Histopathology and Health of the Pacific Oyster,* Bachelor of Science (Animal Science) with honours, The University of Adelaide, Australia.

Battison, A 2006, 'Tissue distribution and hemolymph activity of six enzymes in the American lobster (Homarus Americanus): Potential markers of tissue injury', *Journal of Shellfish Research, 25*(2), 553-560.

Bernard, F 1974, 'Particle sorting and labial palp function in the Pacific Oyster crassostrea gigas (thunberg, 1795)', *The Biological Bulletin, 146*(1), pp. 1-10.

Chávez-Villalba, J, Pommier, J, Andriamiseza, Pouvreau,S, Barret, J, Cochard, J & Le Pennec, M 2002, 'Broodstock conditioning of the oyster Crassostrea gigas: Origin and temperature effect', *Aquaculture,* 214(1), pp. 115-130.

Courtney, P, Breen, F, Cameron, B, Crawford, C, Cunningham, M, Goc, J and Poke, J 2017, 'Super Oyster: The oyster that could save Tasmania's industry', *Landline*. RMIT Publishing, Melbourne (Vic.).

Department of Primary Industries and Regions 2016, 'Help protect SA's oyster industry', *Government of South Australia*, viewed 1 October 2018, http://www.pir.sa.gov.au/fishing/publications/fishfacts_e-newsletter/fish_facts_july_2016/help_protect_sas_oyster_industry

Department of Primary Industries and Regions 2018, 'Pacific Oyster Mortality Syndrome', *Government of South Australia*, viewed 1 October, 2018, http://www.pir.sa.gov.au/aquaculture/aquatic_animal_health/pacific_oyster_mortality_syndrome

eClinpath 2013, 'Chemistry', Cornell University, viewed 11 October 2018, http://www.eclinpath.com/chemistry/

Eble, A and Scro, R 1996, 'Chapter 2 – General Anatomy', in V Kennedy, R Newell and A Eble (eds.), *The Estern Oyster: Crassostrea virginica*, 2nd Edn, pp 19-30.

EconSearch, 2018, *The Economic Contribution of Aquaculture in the South Australian State and Regional Economie, 2017/17.* A report to PIRSA Fisheries and Aquaculture.

Fougerouse, A, Rousseau, M, and Lucas, J 2008, 'Chapter 3 - Soft Tissue Anatomy, Shell Structure and Biomineralization', in P Southgate and J Lucas (eds), *The Pearl Oyster*, Elsevier, pp. 77-102.

Geffré, A, Concordet, D, Braun, J and Trumel, C 2011, 'Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel', *Veterinary Clinical Pathology*, 40(1), pp. 107-112.

Go, J, Deutscher, A, Spiers, Z, Dahle, K, Kirkland, P, & Jenkins, C 2017, 'Mass mortalities of unknown aetiology in Pacific Oysters Crassostrea gigas in Port Stephens, New South Wales, Australia', *Diseases of Aquatic Organisms*, 125(3), pp. 227-242.

Gustafson, L, Stoskopf, M, Showers, W, Cope, G, Eads, C, Linnehan, R, and Levine, J 2005, 'Reference ranges for hemolymph chemistries from Elliptio complanata of North Carolina', *Diseases of Aquatic Organisms, 65*(2), 167-76.

Hoffmann, W and Solter, P 2008, 'Chapter 12 - Diagnostic Enzymology of Domestic Animals', in J Jerry Kaneko, J Harvey and M Bruss (ed.), *Clinical Biochemistry of Domestic Animals,* 6th Edn, Elsevier Academic Press, USA, pp. 351-378.

Howard, D, Lewis, E, Keller, B and Smith, C 2004, 'Histological techniques for marine bivalve mollusks and crustaceans', *NOAA Technical Memorandum NOS NCCOS* 5, pp. 218

Jefferson, R 2018 'Aquaculture Production Report 2016-2017'. *NSW Department of Primary Industries*, viewed 18 October 2018, https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0009/750726/Aquaculture-Production-Report-2016-2017.pdf

Li, Y, Qin, J G, Li, X, & Benkendorff, K 2009, 'Monthly variation of condition index, energy reserves and antibacterial activity in Pacific Oysters, Crassostrea gigas, in Stansbury (South Australia)', *Aquaculture*, 286(1), pp. 64-71.

Madin, B., Gordon, R. & Caraguel, C., 2013, *South Australia Oyster Mortality Workshop Report*, South Australian Oyster Research Council, AusVet Animal Health Services.

McGowan, T.,2013, *South Australia Mortality Workshop (FRDC 2013/721),* The Seafood CRC Company LTd, the Fisheries Research and Development Corperation.

Olafsen, J Flectcher, T and Grant, P 1992, 'Agglutinin activity in Pacific Oyster (Crassostrea gigas) hemolymph following in vivo Vibro anguillarum challenge', *Developmental and comparative immunology*, 16, pp. 123-138.

Patrick, S, Faury, N & Goulletquer, P 2006, 'Seasonal changes in carbohydrate metabolism and its relationship with summer mortality of Pacific Oyster Crassostrea gigas (Thunberg) in Marennes–Oléron bay (France)', *Aquaculture*, 252(2), pp. 328-338.

Primary Industries & Regions SA PIRSA, 2017, *ZONING IN: South Australian Aquaculture Report 2015/16.*

3.6.3. Appendix 3 – Histopathology findings from Case 1 investigation

HISTOLOGY FINDINGS:
Cohort 1:
Fish 1:
Mantle:
Shell gland dense epithelium. Multifocal areas of epithelial erosions and associated connective tissue oedema.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
There is a small style within the stomach with no stomach contents.
GIT:
No GIT contents. Mild and moderate transmigration across the git epithelium. Cilia intact.
Leydig Tissue:
Dense with peri-intestinal haemocyte aggregations. Some are vacuolated.
Hepatopancreas:
Focal tubular dilation. Evidence of germinal tubules.
Gonad:
Focal germinal cells present. Mild development.
Heart:
Thin myocardial fibres. Otherwise NSF.
Muscle:
Poorly preserve and preparation.
Kidney:
Two parts present – there glandular area and dilated tubule area. Both NSF.

Fish 2:

Mantle:
Thin shell areas on the mantle. Multifocal epithelial erosions.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
Moderate sized style present. Epithelium intact. No content present.
GIT:
E-gut is good but no content present.
Leydig Tissue:
Reduced dense amount of leydig tissue with some focal vacuolisation. Haemocytes aggregations present in vacuolated areas.
Hepatopancreas:
Zonal areas of tubular dilation throughout.
Gonad:
Immature not developed.
Heart:
NSF
Muscle:
NSF.
Kidney:
NSF.

Fish 3:
Mantle:
Active shell gland area. Focal erosions of the epithelium.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
No gut contents and no style present.
GIT:
Tract appears dilated as well as haemocyte channels. No GIT contents.
Leydig Tissue:
Dense but reduced.
Hepatopancreas:
Regional area has round cells within the lumen (sloughed enterocytes or amoeba?). Generally appears degenerative.
Gonad:
Non-developed
Heart:
Dense. Multifocal aggregations of haemocytes on the myocardial wall
Muscle:
No sample.
Kidney:
NSF.

Cohort 2:
Fish 1:

Mantle:
Epithelium of the mantle is hyperplastic. There is focal oedema of the mantle. There is no erosion of the body of this animal.
Gill:
NSF
Palps:
No sample.
Oesophagus & Stomach:
Mild translocation of haemocytes. No obvious style and no stomach contents.
GIT:
Mild vacuolisation but otherwise NSF.
Leydig Tissue:
Mild amounts present.
Hepatopancreas:
Focal areas of duct degeneration and atrophy. There is dilated lumens in some regions of the hepatopancreas. Some columnar epithelium has become cuboidal.
Gonad:
Immature gonad.
Heart:
No sample.
Muscle:
NSF.
Kidney:
NSF.

Fish 2:
Mantle:
Active shell gland areas. One focal section of mantle appears dilated but is attached to an active gland cell area.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
There is a small thin style and stomach epithelium is thin. No luminal contents in stomach. Moderate transmigrational haemocytes throughout stomach wall.
GIT:
No luminal contents in e-gut.
Leydig Tissue:
Leydig tissue is markedly thin throughout
Hepatopancreas:
Majority of tubules are dilated.
Gonad:
Very immature under developed gonad.
Heart:
No sample.
Muscle:
NSF.
Kidney:

No sample.

Fish 3:
Mantle:
Active mantle associated with leydig tissue.
Gill:
NSF.
Palps:
Marked presence of haemocytes within epithelium.
Oesophagus & Stomach:
No style observed. Moderate amounts of transmigrational haemocytes. One area of epithelial loss. Marked bacteria present.
GIT:
NSF
Leydig Tissue:
Focal denseness and focal vacuolisation. High levels of bacteria throughout that are bacillary or filamentous in nature.
Hepatopancreas:
Autolysed sample.
Gonad:
Not developed.
Heart:
No sample.
Muscle:
NSF.
Kidney:
Post mortem degeneration.

Cohort 3:
Fish 1:
Mantle:
Hyperplasia of the shell glands of the distal mantle. Multifocal haemocyte aggregations on the shell surface of the mantle within the connective tissue. Focal erosion and oedema of the epithelium around the viscera.
Gill:
NSF
Palps:
No sample.
Oesophagus & Stomach:
There is a style present. There is mild transmigration of haemocytes across the epithelium with haemocyte bands in the lumen of the stomach.
GIT:
E-gut appears degenerative. One area with coagulative necrosis associated with a population of bacillary bacteria. There is a granular pink matrix a feat haemocytes and some plate material within the lumen.
Leydig Tissue:
Generally dense in nature with haemocyte aggregations around the periphery of GIT and stomach. Dilated haemocyte channels throughout. One layer that is sub-epithelial which is markedly vacuolated.

Within some of the haemocyte aggregations there are moderate numbers of brown cells and multinucleated cells.

Hepatopancreas:

Well differentiated ducted cells. Moth eaten appearance.

Gonad:

Juvenile gonad.

Heart:

There is atrophy/ a loss of fibres within the heart with marked dense peripheral aggregation of haemocytes around the endocardium. The pericardium appears thickened with vacuolated epithelium.

Muscle: The adductor muscle shows artefactual vacuolisation throughout. Focal areas of degeneration and bacterial invasion throughout the muscle.

Kidney:

NSF.

Fish 2:

Mantle:

Focal epithelium erosions. Secretory epithelium of mantle appears active. Leydig tissue in mantle appears degenerative.

Gill:

NSF.

Oesophagus & Stomach:

Dense style present. Mild transmigration. One focal area of coagulative necrosis with associated bacteria in the stomach epithelium.

GIT:

NSF.

Leydig Tissue:

Moderate numbers of brown cells. Peri-intestinal aggregations on haemocytes within dense leydig tissue.

Hepatopancreas:

NSF.

Gonad:

Immature gonad.

Heart:

NSF.

Muscle:

NSF. There is a large ganglia present next to muscle and kidney.

Kidney:

Interstitium is dilated with oedema but otherwise NSF.

Fish 3:

Mantle:

Multifocal to focally extensive areas of epithelial erosion and ulceration. There is focal oedema of the underlying connective tissue and multifocal to coalescing aggregations of haemocytes on the shell surface of the mantle. There is marked erosion of the epithelium around the body of the oyster. There is intra-lesional bacteria associated with haemocyte aggregations. There are two types present: Filamentous bacillary form and stumpy bacillary form that appear to be sporing. Moderate numbers of brown cells throughout the mantles connective tissue.

Gill:

A focal erosion if the epithelial lining of the gills which is more common on the proximal ends of the gills compared to distal. Variable vacuolisation in the connective tissue of the gills. Focal haemocyte aggregations in the proximal extent of the gills associated with dilated haemolymph channels. Majority of the haemocytes are large and granulated.

Palps:
Not present.
Oesophagus & Stomach:
Small irregular style present. There is moderate transmigration of haemocytes across the stomach epithelium. There is no stomach contents apart from haemocytes and a granular pink matrix. One bacterial plaque is noted. There is a focus of long bacterial invasion into the style with associative necrosis.
GIT:
Two focal areas where the normal intestinal epithelium is lost and there is an aggregation of GIT content material with copious bacterial elements within. There is no significant haemocyte reaction around them. The hepatopancreatic tissue against it is undergoing coagulative necrosis.
Leydig Tissue:
There is copious filamentous to bacillary bacteria in the tissue. There is multifocal areas of haemocyte aggregation some associated with bacteria.
Hepatopancreas:
There is general degeneration (autolysis) of the tubules. Intact proportions appear to be within a normal range.
Gonad:
Immature gonad. NSF.
Heart:
Peripheral haemocyte aggregation around the heart. Otherwise NSF. There is haemocyte aggregations in the linning of the pericardium.
Muscle:
No sample.
Kidney:
No sample.

Cohort 4:
Fish 1:
Mantle:
Mild vacuolisation of the epithelial cells on the oyster side of the mantle. Otherwise NSF.
Gill:
NSF.
Palps:
No sample.
Eosophagus & Stomach:
No digesta present within lumen of eosphagus and stomach. Moderate transmigration of haemocytes.
GIT:
Mild amounts of digesta present.
Leydig Tissue:
Dense leydig tissue. NSF.

Hepatopancreas:
There is epithelial slough in some areas of the hepatopancreas. Hepatopancreatic leydig cells / amoeba? Otherwise NSF, good height on tubules and evidence of tubular development.
Gonad:
Juvenile non-developed.
Heart:
There is a general increase mural attached haemocytes and there are multifocal aggregations of haemocytes within the myocardium that are reactive.
Muscle:
No sample.

Kidney:
Cellular luminal debris of the kidney. Cytoplasmic vacuolisation of the lining cells with mild sloughing of cells.

Fish 2:
Mantle:
Generalised erosion of the epithelium of the mantle and the body of the oyster. The shell gland appears dense and active but there is marked loss of epithelium.
Gill:
Tissue appears autolytic – (suspect dead oyster)
Palps:
Ciliated epithelium appears intact. There is haemocyte clots in the large haemocyte channels (thrombus?). There is some connective tissue oedema.
Eosophagus & Stomach:
Appears degenerative with no style or digesta present.
GIT:
Autolysed tissue
Leydig Tissue:
Dense but lack of structural integrity – autolytic. Leydig tissue oedema present.
Hepatopancreas:
There is marked dilated tubules that are flattened. Autolytic changed present.
Gonad:
Juvenile under developed.
Heart:
No sample.
Muscle:
NSF.
Kidney:
NSF appears autolytic.

Fish 3:
Mantle:
NSF
Gill:
NSF
Palps:
No sample.
Eosophagus & Stomach:
Moderate to high haemocyte migration. No content present but there is a well-developed style.
GIT:
Multifocal aggregations of a feat and reactive haemocytes associated with a remnant degenerative epithelium (e-gut?) One section is also associated with ingesta material.
Leydig Tissue:
Generalised infiltration of different types of haemocytes. With a loss of leydig tissue activity (replacement?)
Hepatopancreas:
NSF.
Gonad:
Juvenile non-developed.
Heart:
Thin myocardium with multifocal aggregations of haemocytes in the myocardial tissue itself.
Muscle:

NSF.
Kidney:
NSF.

Cohort 5:
Fish 1 - 4:
Mantle:
Productive mantle.
Gill:
One fish has autolytic gills otherwise NSF.
Palps:
NSF
Eosophagus & Stomach:
NSF
GIT:
NSF.
Leydig Tissue:
NSF.
Hepatopancreas:
NSF.
Gonad:
NSF.
Heart:
One animal has a thin heart.
Muscle:
NSF.
Kidney:
NSF.

Fish 5-8:
Fish were dead on arrival so autolysis noted.

3.6.4. Appendix 4 – Histopathology findings from Case 2 investigation

Cohort B:

Oyster 1:

Mantle:

Multifocal areas of sub-epithelial oedema.

Gill:

No significant findings (NSF). Generalised hyper-cellularity (haemocytes)

Palps:

Not observed

Oesophagus & Stomach:

No style observed (Angle). Mild ingesta content. Nil transmigration across epithelial wall.

GIT:

Mixed ingesta in the small intestine that is primarily bacteria.

Leydig Tissue:

Scant and dense. Some haemocyte aggregations.

Hepatopancreas:

50% of the gland has thin epithelial walls with dilated lumens.

Gonad:

Not observed

Heart:

Thin atrium.

Muscle:

NSF.

Kidney:

Kidney epithelium is vacuolated.

Nerve:

NSF in central ganglia.

Oyster 2:

Mantle:

Multifocal areas of sub-epithelial oedema.

Gill:

No significant findings (NSF). Generalised hyper-cellularity (haemocytes)

Palps:

NSF. Ciliate intact.

Oesophagus & Stomach:

Mixed ingesta within stomach in moderate to copious amounts. Areas of foci effete haemocytes seen within the lumen of the stomach. NSF in stomach epithelium with rare haemocyte transmigration and copious basophilic mucin cells. There is a style present.

GIT:

Copious mixed ingesta.

Leydig Tissue:

Sparse tissue. Dense with focal oedema. In the tissue close to the pericardial sac there are moderate numbers of ciliated protozoa resembling uronema sp. (circular to ovoid in shape with cilia ranging from 72 to 120 micron in diameter or length with a large central to acentric nucleus containing pale and focally clumped chromatin and a granular basophilic cytoplasm. Nuclear to cytoplasmic ratio is 1 to 2 approximately) they are position in the leydig tissue with mild to moderate associated haemocyte aggregations. In this sample they are only around the pericardial area.

Hepatopancreas:

Generalised vacuolatative (autolytic) change to the hepatopancreas.

Gonad:

Not observed
Heart:
Thin myocardial strands with moderate numbers of mural macrophages and haemocytes.
Muscle:
NSF
Kidney:
Not observed.

Oyster 3:
Mantle:
Outer epithelium is thin with marked sub-epithelial oedema.
Gill:
NSF. Low cellularity.
Palps:
Not observed.
Oesophagus & Stomach:
Style present with minimal to no GIT contents. Thin walled stomach.
GIT:
Mild faecal matter in distal colon.
Leydig Tissue:
Scant. In one area of the leydig next to the kidney and the heart there is dense aggregations of haemocytes that are sub-epithelial to the stomach as well.
Hepatopancreas:
A third of the surface has dilated thin tubules.
Gonad:
Not observed.
Heart:
Reasonal amount of mural macrophages.
Muscle:
Fibres appear thin with interfibre oedema of the adductor muscle.
Kidney:
NSF
Central ganglia:
NSF.

Oyster 4:
Mantle:
Marked amount of sub-epithelial oedema within he eternal epithelium.
Gill:
Appears mildly autolytic.
Palps:
NSF.
Oesophagus & Stomach:
NSF oesophagus. Remnant style in the stomach with multifocal areas of oedema in the stomach wall. Transmigration nil.
GIT:
Minimal to no gut luminal contents.
Leydig Tissue:
Scant.
Hepatopancreas:
NSF – there is a degree of autolytic change.
Gonad:

Not observed.
Heart:
Very thin generally. Low cellularity.
Muscle:
Dense. NSF.

Kidney:
Not observed.
Central ganglia:
NSF.

Oyster 5: (superficial section)
Mantle:
Mild oedema in the sub-epithelial region.
Gill:
Hyper-cellularity of the gills.
Palps:
Not observed.
Oesophagus & Stomach:
NSF
GIT:
NSF
Leydig Tissue:
NSF
Hepatopancreas:
NSF
Gonad:
Not observed
Heart:
Not observed
Muscle:
NSF
Kidney:
Not observed.

Oyster 6:
Mantle:
NSF.
Gill:
Hyper-cellularity of gills.
Palps:
Not observed.
Oesophagus & Stomach:
Pale amorphic contents.
GIT:
NSF.
Leydig Tissue:
Dense. There is an active focus of haemocytes within the tissue with remnant degenerative digestive gland tubules in the middle. Necrosis of inflammatory cells present. Hyper-cellularity throughout the tissue.
Hepatopancreas:
A third dilated tubules with thin epithelium. Several show autolytic change.

Gonad:
Not observed.
Heart:
Hyper-cellular.
Muscle:
NSF.
Kidney:
Hyalanised epithelium that appears eosinophilic.

Oyster 7, 8 & 9:
Mantle:
Vacuolated sub-epithelial areas, multifocal.
Gill:
Hyper-cellular throughout (haemocytes)
Palps:
NSF.
Oesophagus & Stomach:
Amorphous granular eosinophilic material within lumen. Slight style (undeveloped). Minimal transmigration through the stomach wall.
GIT:
NSF.
Leydig Tissue:
Scant tissue.
Hepatopancreas:
Dense tubules. NSF.
Gonad:
Not observed.
Heart:
NSF
Muscle:
NSF.
Kidney:
NSF

Oyster 10: Non –diagnostic

Oyster 11:
Mantle:
There is a focus of haemocytes within the lumen adjacent to the mantle.
Gill:
NSF.
Palps:
Hyper-cellular.
Oesophagus & Stomach:
NSF – no stomach.
GIT:
Not observed.
Leydig Tissue:
Not observed.
Hepatopancreas:
Not observed.

Gonad:
Not observed.
Heart:
Not observed.
Muscle:
Not observed.
Kidney:
Not observed.

Oyster 12:
Mantle:
Marked sub-epithelium oedema.

Gill:
NSF.
Palps:
NSF
Oesophagus & Stomach:
Pale pink amorphous contents in stomach. Style is present. Small pockets of ingesta.
GIT:
Not observed.
Leydig Tissue:
Scant.
Hepatopancreas:
Compact dense
Gonad:
Gonal crypts present but no gonad.
Heart:
Increased number of mural machophages.
Muscle:
NSF.
Kidney:
Central ganglia:
NSF.

Oyster 13:
Mantle:
Marked sub-epithelium oedema.
Gill:
NSF.
Palps:
Not observed.
Oesophagus & Stomach:
Densely cellular oesophagus. Moderate amounts of variably sourced ingesta within stomach with style present.
GIT:
Faecal matter present in caudal intestine
Leydig Tissue:
NSF
Hepatopancreas:
3-% digestive gland tubule dilated with the rest observed to be very dense.
Gonad:

Not observed.
Heart:
NSF
Muscle:
NSF.
Kidney:
NSF.

<u>Cohort C:</u>
Oyster 1: Non - diagnostic
Generalised autolysis of sample.

Oyster 2:
Interstitium of all tissues appear to be full of haemocytes.

Mantle:
Mild focal subepithelial oedema. Separation of the epithelium from the underlying tissue. There is a general hyper-cellularity of the connective tissues.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
Mixed contents. There diatoms present. There is a well formed style with minimal to no transmigrational sites.
GIT:
Full of mixed content. There is some autolytic change to the loops of bowel (suspect fixation)
Leydig Tissue:
NSF.
Hepatopancreas:
There is some autolytic change to all tubules (suspect fixation), otherwise appears reasonably active.
Gonad:
No sample.
Heart:
Appears to have dense myofibres. NSF.
Muscle:
NSF.
Kidney:
NSF.

Oyster 3:
Mantle:
Active shell glands. Mild oedema in the subcutaneous tissue. There is a degree of vacuolisation in the epithelial lining cells.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
Empty of digesta but style present.
GIT:

NSF.
Leydig Tissue:
NSF.
Hepatopancreas:
Focal areas of necrosis in the tissue. Evidence of autolytic degenerative change (suspect fixation)
Gonad:
No sample.
Heart:
Lightly stained cardiac muscle
Muscle:
No sample.
Kidney:
NSF.

Oyster 4:
Similar to fish 3 – degenerative change bordering autolysis with minimal GIT contents.

Oyster 5:
General haemocytosis around the tissues. Autolytic changes evident (suspect fixation). Lack of GIT content in stomach suggests inappetance or lack of nutrients. Stomach lining height is shortened.

Oyster 6, 7 & 8:
Myocardium appears thinned. Autolysis across hepatopancreas tubules. Less hypercellularity in this oyster. Faecaes is not structures.

Oyster 9, 10, 11 & 12:
Thin amount of digesta that is not structured. Heart appears normal.

Cohort D:
Oyster 1, 2 , 3 & 4:
There is a notable hyperplasia of all types of haemocytes. Haemacytosis across all tissues. There is a low amount of digesta in the intestinal tract and in one or two stomachs that have content it is monomorphic. . There is a generalised hepatopancreatic tubular necrosis across all fish (autolysis / toxic algae – because generally speaking the rest of the tissues are well preserved). In three of the four animals there is rudimentary gonadal crypts undifferentiated but in one of the fish there is WELL differentiated testes containing spermatozoa.

Oyster 5, 6, 7 & 8:
Haemacytosis across all tissues. There is generalised tubular necrosis in the hepatopancreas. There is minimal gastrointestinal content. One of the fish (Fish 6) has a well differentiated fully mature with spermatozoa gonad. Varies degrees of preparation artefact. Heart appears thin in two oysters

Oyster 9, 10, 11 & 12:
Haemacytosis across all tissues. Preservation of the ciliated tubules are better preserved compared to glandular parts. One fish as a mature gonad. There is generalised tubular necrosis in the hepatopancreas. There is minimal gastrointestinal content.

3.6.5. Appendix 5 – Histopathology findings from Case 3 investigation

HISTOLOGY FINDINGS:
Slide 1 (2 oysters):
Mantle:
Moderate number of haemocyte aggregates that are sub-epithelial. Mild occurrence of intralesional rod bacteria present. There is generalised oedema present in both oysters. In one oyster there is a section of disruptive mantle within the pericardium but within that tissue there is moderate to marked mixed bacteria population including filamentous bacteria.
Gill:
Marked to moderate brown cells and melanin deposits noted within the gill tissue.
Palps:
No tissue observed.
Oesophagus & Stomach:
No tissue observed.
GIT:
No tissue observed.
Leydig Tissue:
Depleted to absent. Mixed inflammatory cells observed some of which are degenerative.
Hepatopancreas:
Variable atrophy of the tubular epithelial lining that ranges from atrophy to an almost metaplastic appearance. Variable absorptive vacuoles within the tissue. Granular amorphous material in the tubular lumens. Moderate presence of brown cells within the interstitium.
Gonad:
No tissue observed
Heart:
In one animal there is marked haemocyte aggregates present with large number of brown cell suggesting a myocarditis. There is myocardial necrosis present within the ventricle.
Muscle:
Degenerate muscle with regions of intralesional bacteria surrounding the adductor muscle.
Kidney:
No tissue observed
Overall one animal showing marked changed and the other showing only mild changes.

Slide 2 (4 oysters):
Oyster 1.
Mantle:
Generalised mild oedema of the mantle. Thin outer mantle membrane which is mildly eroded in multiple areas. Mild numbers of brown cells throughout. One focal area of sessile protozoa
Gill:
Oedema of the gills. There are mild numbers of trichodinid in the gills. Acidophilic mucins are lower than expected in this animal.
Palps:
No significant findings (NSF)
Stomach:
Digesta present within stomach. There are protein plaques and filamentous algae as well as mixed zooplankton within stomach. No style observed.
GIT:
Small larval oysters present within.
Leydig Tissue:
Oedematous in appearance. There are small haemocyte aggregations throughout (clump).
Hepatopancreas:

There is a foci of 6 degenerate hepatopancreatic tubules filled with epitheliod haemocytes, metaplasia of the lining epithelium and fibrosis (chronic). Tubules appear mildly depleted.

Gonad:
No sample
Heart:
No sample
Muscle:
NSF.

Oyster 2:
Mantle:
Transmigrational haemocytes through the epithelium.
Gill:
Interstium appears spread out and oedematous.
Palps:
NSF.
Stomach:
Empty of digesta.
GIT:
Contents present and similar to first oyster. Focally extensive area of haemocyte aggregations surrounding one piece of bowel that is reactive. A number of oyster spat present.
Leydig Tissue:
Moderate amount of brown cells present. There is mild sub-epithelium oedema. Depleted tissue between tubules.
Hepatopancreas:
Atrophy and lack of height observed in the majority of the hepatopancreatic lumens.
Gonad:
No sample
Heart:
There is a thickening to the pericardial lining. Almost complete atrophy of the myocardium.
Muscle:
No sample.

Oyster 3:
Mantle:
Focally extensive oedema of the mantle.
Gill:
NSF
Palps:
NSF.
Stomach:
Empty of digesta.
GIT:
Contents present and similar to first oyster. Focally extensive area of haemocyte aggregations surrounding one piece of bowel that is reactive. A number of oyster spat present.
Leydig Tissue:
Mild tissue oedema present in palps. Tissue appears depleted. Within a haemocyte channel on leydig system there is a granular plug (faecal?) containing to myxosporidial spores?
Hepatopancreas:
Moderately depleted tubules of the hepatopancreas.
Gonad:

No sample
Heart:
Mild hypercellularity with increased mural macrophage hyperplasia. Otherwise NSF.
Muscle:
NSF.

Oyster 4
Mantle:
Sub- epithelial focal oedema of the mantle. There is dilation of the haemocyte channels.
Gill:
Moderate number of trichodinids in the gill cavity. Otherwise NSF.
Palps:
NSF.
Stomach:
Style present within stomach.
GIT:
Small amount of ingesta within the digestive tract including spat. Intestine is filled with spore life infiltrates (faeces).
Leydig Tissue:
Depleted tissue with mild focal aggregates of haemocytes. Moderate brown cells.
Hepatopancreas:
Generalised Dilated tubules
Gonad:
No sample.
Heart:
Dense with moderate numbers of mural macrophages.
Muscle:
NSF.

Slide 3 (4 fish):
Oyster 1:
Mantle:
Focally extensive areas of oedemas within the mantle. There are mild aggregations of haemocytes in the subcutaneous areas of the mantle.
Gill:
There are areas of inflammatory haemocyte aggregates in parts of the gill filaments with mild amounts of necrosis present.
Palps:
NSF.
Stomach:
NSF
GIT:
High protein digesta present in the git. A mix of bacteria, algae and oyster spat was observed.
Leydig Tissue:
NSF.
Hepatopancreas:
Leydig tissue around the hepatopancreas is depleted. NSF otherwise. Mild loss of height of tubular epithelium.
Gonad:
No sample
Heart:
No sample

Muscle:
No sample
Kidney:
NSF.

Oyster 2:
Mantle:
Oedema is more extensive compared to oyster 1 of this slide. Mantle ganglia appear normal.

Gill:
NSF. Tricodinids present within the gill.
Palps:
Epithelia cells appear vacuolated. Mild to no haemocytes present.
Stomach:
Style present with scant amount of food. Similar in morphology to fish 1 of this slide.
GIT:
NSF.
Leydig Tissue:
Depleted tissue surrounding hepatopancreas tubules.
Hepatopancreas:
Tubules appear dilated and there is 60 to 70% of traNSFormed flat epithelium.
Gonad:
No sample
Heart:
No sample.
Muscle:
NSF.
Kidney:
NSF

Oyster 3:
Mantle:
Focal oedema present alongside thinning of the epithelium.
Gill:
NSF.
Palps:
NSF.
Stomach:
NSF.
GIT:
NSF.
Leydig Tissue:
Depleted.
Hepatopancreas:
Atrophic in appearance similar to oyster 2
Gonad:
No sample
Heart:
No sample
Muscle:
No sample

Oyster 4:
Mantle:
Subcutaneous oedema generalised within the mantle.
Gill:
Tricodinids present. Otherwise NSF.
Palps:
NSF
Stomach:
NSF.
GIT:
No sample.

Leydig Tissue:
NSF.
Hepatopancreas:
NSF.
Gonad:
No sample
Heart:
No sample
Muscle:
No sample.

Slide 4 (3 oysters):
Oyster 1
Mantle:
Focally extensive areas of oedema throughout the mantle.
Gill:
Multifocal areas inflammatory focci within the gills. Triconids are present in large numbers.
Palps:
NSF.
Stomach:
NSF. Minimal content
GIT:
NSF. Minimal content
Leydig Tissue:
Depeted
Hepatopancreas:
NSF.
Gonad:
No sample
Heart:
No sample.
Muscle:
No sample.

Oyster 2:
Mantle:
Focal areas of oedema. Otherwise NSF.
Gill:
Marked number of tricodinids present. Gill structure otherwise NSF.
Palps:

NSF.
Stomach:
Minor amounts of intestinal contents.
GIT:
NSF.
Leydig Tissue:
Focal aggregates of haemocytes present. Otherwise NSF.
Hepatopancreas:
Amoeba present within lumens. Otherwise NSF.
Gonad:
No sample
Heart:
NSF.
Muscle:
NSF.
Kidney:
NSF.

Oyster 3:
Mantle:
Generalised oedema of the mantle. Some areas of brown cells and haemocytes aggregates present.
Gill:
Focal areas of inflammation. Oedema present throughout. Mild numbers of tricodinids present.
Palps:
NSF.
Stomach:
Mild content most of which is proteinacious fluid.
GIT:
Content similar to early samples observed. There is good amount of content.
Leydig Tissue:
NSF.
Hepatopancreas:
Healthy, NSF.
Gonad:
No sample.
Heart:
Myocardium is excessively thin. There is a significant amount of fluid within the pericardial sac.
Muscle:
NSF.

Slide 5 (2 oysters):
Oyster 1:
Mantle:
Focal oedema.
Gill:
Trichodinids through gills which also appear oedematous.
Palps:
No sample.
Stomach:
No sample.
GIT:
Empty of contents

Leydig Tissue:
Depleted
Hepatopancreas:
Dilated tubules. Marked oedema surrounding body mass.
Gonad:
No sample.
Heart:
No sample.
Muscle:
NSF.
Kidney:
Epithelium of kidney appears mildly thickened.
Oyster 2 (sample is poorly fixed – autolysis) :
High autolysis through sample. There are indications of oedema however sample is poor and undiagnostic.

3.6.6. Appendix 6 – Histopathology findings from Case 4 investigation

HISTOLOGY FINDINGS:
Oyster 1 (EOI A):
Mantle:
There is multifocal areas of oedema throughout the mantle. There is focally extensive areas of haemocytic aggregation. Mild focal erosions of the epithelial layers of the mantle.
Gill:
Rare number of trichodinids present.
Palps:
NSF
Oesophagus & Stomach:
Focal erosion ulcer of the stomach wall with epithelial loss. Moderate to high level of transmigrational haemocytes through the stomach wall.
GIT:
NSF.
Leydig Tissue:
Dense and normal.
Hepatopancreas:
Approximately 50% of tubules are dilated. Within the lumens are granular basophilic material.
Gonad:
Rudimentary gonad development present.
Heart:
Epicardial areas of the ventricle shows proliferation and vacuolisation. There is marked proliferation and vacuolisation of the atrium. Vacuoles are clear or contain a granular light blue material some resemble organisms? Multifocal areas of myocardial degeneration.
Muscle:
Focally extensive areas of fibre contraction with the association of oedema with some parts of the foot muscle.
Kidney:
NSF.
Nerve:
Marked peripheral neuronal proliferation of the adductor ganglia. Multifocal areas of mild inflammatory infiltrate. (Possible neuronal hypertrophy)

Oyster 2:
Mantle:
There is focally extensive dilation and oedema. There is focal erosion of the epithelium with underlying oedema. Focal areas of haemocyte aggregations within and around dense storage tissue. Surrounding oedematous areas the underlying epithelium is thinned and metaplastic (squamous). There are also areas that are thickened and appear spongiotic as well as focal areas of migration of a-feat cells through the mantle epithelium.
Gill:
NSF.
Palps:
NSF.
Oesophagus & Stomach:
NSF. Stomach has minimal content with a thin style present. There is focal erosion of the stomach wall with a moderate level of transmigration of inflammatory cells across the mucosa.
GIT:
Minor amount of content. Areas of apoptotic wall lesions.
Leydig Tissue:

Moderately dense.

Hepatopancreas:
Approximately 30% dilated hepatopancreatic tubules. There rest are within normal range.
Gonad:
Germinal gonads present in the crypts (suspect juvenile ovarian tissue)
Heart:
Marked diffuse vacuolisation of the ectocardium. Within the vacuoles are granular material (microcells?)
Muscle:
Vacuolisation surrounding the skeletal muscle fibres in the adductor muscle. There are focal areas of degeneration (myopathy). Minimal infiltration of inflammatory cells.
Kidney:
No sample.
Nerve:
Ganglia is hyper cellular –irregular arrangement of neuronal bodies

Oyster 3:
Mantle:
In one of the mantle haemocyte channels, there is aggregations of haemocytes within the wall around granular leydig cells. Focal areas of oedema present. Moderate number of trichodinids present.
Gill:
NSF. Moderate to high numbers of trichodinids in section.
Palps:
NSF.
Oesophagus & Stomach:
Clear matrix within the stomach. Mild transmigration across the mucosa. Otherwise NSF.
GIT:
There is faecal material in the distal GIT. Limited to no content cranially. Distal content contain bacteria and singe celled organisms.
Leydig Tissue:
NSF
Hepatopancreas:
Approximately a quarter of the tissue appears dilated, the rest appear normal.
Gonad:
Developmental ovary present.
Heart:
Similar vacuolisations observed in ectocardium as seen in oyster 2.
Muscle:
Uniform – NSF.
Kidney:
No sample
Nerve:
Hyper cellular in appearance. Randomly distributed neurons.

Oyster 4:
Mantle:
Epithelial is multifocally eroded with mild oedema associated. Areas of mantle epithelium that appear sponge like. Marked focal aggregations of haemocytes sub-epithelial.
Gill:
NSF. Mild trichodinids present.
Palps:

No sample.
Oesophagus & Stomach:
NSF. Stomach has style but no content Appears normal.
GIT:
Leydig Tissue:
Dense.
Hepatopancreas:
Within normal range of absorption and digestion.
Gonad:
Heart:
Vacuolated atrium and vacuolated ventricle appearance in myocytes.
Muscle:
Focal myopathy (artefact?)
Kidney:
No sample.
Nerve:
Active ganglia. Vacuolated areas in the periphery close to muscle.

Oyster 5:
Mantle:
Focal erosion of the mantle. Thin all around. There are some areas of oedema sub-epithelial. Vacuolisation on the distal nerves of the mantle. Area of haemocyte aggregation in the sub-epithelial area.
Gill:
Multifocal areas of vacuolisation of the gills. Trichodinids present on histology
Palps:
NSF.
Oesophagus & Stomach:
Minimal GIT contents in the stomach.
GIT:
There are small single cellular faecal contents.
Leydig Tissue:
NSF.
Hepatopancreas:
Approximately 25% of tubules appear dilated otherwise normal.
Gonad:
Male gonad.
Heart:
No sample.
Muscle:
Separation of the muscle fibres in the adductor muscle. Muscle section attached to stray piece of mantle appears to show coagulative necrosis of the muscle-associated piece of glandular material shows two distinct cell populations. One area is arranged in epithelial arrays with acini – cells are columnar epithelium differentiated to acid mucin secreting cells and columnar epithelium with basal nuclei. The other population is arranged intrabecularly and palisades cuboidal cells amongst fibroblast matrix . There is also a basophilic dense hyaline matrix associated closely within the muscle fibres embedded within the mass (neoplasia, teratoma ???)
Kidney:
No kidney
Nerve:
Nerve in palps appear mildly vacuolated.

Oyster 1,2,3 (EOI B):
Mantle:
Mild focal mantle erosion with moderate to marked sub-mantle oedema.
Gill:
There is focal oedema and ballooning of the epithelium.
Palps:
NSF
Oesophagus & Stomach:
Mixed moderate to copious amounts of stomach contents.

GIT:
NSF
Leydig Tissue:
Diluted and loss of architecture to tissue. Appears degenerate.
Hepatopancreas:
Greater than 60% tubules appear dilated in all three oysters. With some squamous metaplasia present in some.
Gonad:
Juvenile gonad in all three samples. Sex indeterminate.
Heart:
No sample in all three oysters.
Muscle:
Separation of muscle fibres across all samples.
Kidney:
Present in two oysters. There is 3 ganglia present in the kidney of 1 fish showing marked vacuolisation.
Nerve:
NSF.

Oyster 4 & 5:
Mantle:
Erosion and thinning of the mantle (across it all). Well differentiated shell gland, sub-epithelial oedema. Occasional focal haemocyte aggregations.
Gill:
Focal epithelial vacuolisation.
Palps:
Focal vacuolisation of the epithelium of the palps.
Oesophagus & Stomach:
Mixed content present. Moderate amounts of granular mixed cells.
GIT:
NSF.
Leydig Tissue:
Depleted and disrupted.
Hepatopancreas:
60 – 70% dilated tubules. Low cuboidal epithelium. Some seen with amoeba within the lumen.
Gonad:
Rudimentary gonad.
Heart:
Moderate vacuolisation of the atrium ectocardium.
Muscle:
Segmentation of the muscle bundles in both fish (oedema?)
Kidney:
Not present.

Nerve:
Hyper cellular, lots of nuclear bodies within a fish. Peripheral nerve appear normal.

Oyster 1,2,3 (EOI C):
Mantle:
Mild focal erosion and loss. Sub-epithelial oedema.
Gill:
Marked trichodinids present within the gill cavity.
Palps:
NSF.
Oesophagus & Stomach:
There is moderate to mild amounts of granular cellular contents of the stomach.

GIT:
NSF.
Leydig Tissue:
Dense and compact across all three samples.
Hepatopancreas:
Occasional dilated tubules, otherwise NSF.
Gonad:
Rudimentary juvenile gonads.
Heart:
No samples present.
Muscle:
Areas of fibre separation (Artefact?)
Kidney:
No samples.
Nerve:
Hypercellular and irregularly distributed.

Oyster 4:
Mantle:
Focally extensive erosion and loss. Sub-epithelial oedema.
Gill:
Focal oedema.
Palps:
NSF
Oesophagus & Stomach:
Stomach is full of granular content.
GIT:
NSF.
Leydig Tissue:
Dense.
Hepatopancreas:
10 – 20% dilated tubules, otherwise, NSF.
Gonad:
Rudimentary male gonad.
Heart:
No sample.
Muscle:
Segmented.
Kidney:

NSF
Nerve:
Hypercellularity present.

Oyster 5:
Mantle:
Focal erosion and sub-epithelial oedema present.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
Stomach empty with style present.
GIT:
NSF.

Leydig Tissue:
Dense
Hepatopancreas:
50% dilated tubules, otherwise NSF.
Gonad:
Rudimentary gonad present.
Heart:
Mild vacuolisation of the ectocardium similar to previously described.
Muscle:
Segmented
Kidney:
No sample.
Nerve:
NSF.

Oyster 1 (EOI D): (Autolysis?)
Mantle:
There is focal loss and erosion of the epithelium in the mantle (Gill side is eroded). There is evidence of cellular necrosis underlying the mantle. One vessel within the sub-epithelial layer showing endothelial necrosis and cell sloughing. Epithelial oedema present in the mantle. Vacuolisation of the epithelial cells and random loss is present. There is shrinking of the muscle fibres within the mantle and the cross-sectioned fibres appear hyaline with mild amounts of myocyte nuclei appearing vacuolated.
Gill:
Trichodinid present throughout. Mild vacuolisation of cells present. No other significant findings.
Palps:
No sample.
Oesophagus & Stomach:
Insufficient sample.
GIT:
Insufficient sample.
Leydig Tissue:
Hypercellularity surrounding leydig tissue and evidence of cell necrosis.
Hepatopancreas:
There is generalised atrophy of the epithelium. Mild to moderate number of amoeba present within the lumens.

Gonad:
There is a rudimentary male gonad.
Heart:
No sample.
Muscle:
There is contraction of fibres with increased spaces surrounding them.
Kidney:
Degenerative nerves associated with kidney that appear necrotic. Otherwise NSF.
Nerve:
Appear hyper-cellular.

Oyster 2:
Mantle:
Vacuolisation of epithelial cells in a random focal arrangement. There is mild erosion with marked numbers of trichodinids present. There is extensive focal oedema present. There is shrinking of the muscle fibres within the mantle and the cross-sectioned fibres appear hyaline with mild amounts of myocyte nuclei appearing vacuolated.
Gill:
Generalised thinning of the gill epithelium with no associated inflammation observed.

Palps:
NSF.
Oesophagus & Stomach:
No structure to contents of stomach and GIT. NSF.
GIT:
NSF
Leydig Tissue:
Thin with loss of cell structure.
Hepatopancreas:
Approximately 80% of the tubules appear atrophied.
Gonad:
Rudimentary gonad present.
Heart:
No sample.
Muscle:
There is a focal shrinkage of fibres, retraction and hyalinised change.
Kidney:
NSF.
Nerve:
There is degeneration of the main ganglia with vacuolisation of the nuclei.

Oyster 3:
Mantle:
Multifocal extensive sub-epithelial oedema present. There is focal haemocyte aggregations within the connective tissue of the mantle.
Gill:
NSF.
Palps:
NSF.
Oesophagus & Stomach:
No content present in the stomach.
GIT:

Minimal content present within the intestines. There is sloughed epithelial cells and basophilic fibrous plant material present within the GIT lumen. The intestinal wall appears autolytic.

Leydig Tissue:

Thin and depleted showing a lack of structure.

Hepatopancreas:

The majority of tubules are atrophic with thinning. Some tubules at the distal part of the organ are almost squamous metaplastic.

Gonad:

Rudimentary gonad present.

Heart:

Significant oedema of the myocardium observed. There is oedema of the pericardial sac wall.

Muscle:

Focal degenerative change observed similar to that seen in the previous fish.

Kidney:

NSF.

Nerve:

NSF.

Oyster 4:

Mantle:

There is focal hypertrophy of the secreting cells within the mantle. There is focally extensive sub-epithelial oedema.

Gill:

Multifocal sub-epithelial oedema in the gill arches but otherwise NSF.

Palps:

NSF.

Oesophagus & Stomach:

There is minimal content within the lumen of the stomach. Scant amount of fibrous plant content present.

GIT:

Within the intestine, a more diverse make up of content is present but basophilic plant fibrous content is still present throughout.

Leydig Tissue:

Focally degenerative. Otherwise NSF.

Hepatopancreas:

30% of tubules show atrophic change. Otherwise NSF.

Gonad:

Gonad is rudimentary

Heart:

Vacuolated change present in atrial sections as noted in previous samples. Vacuolisation appear clear with no debris present within.

Muscle:

Muscle degenerative change as described in previous samples. Degeneration, contraction and hyalinisation of fibres.

Kidney:

No sample.

Nerve:

Appears hyper-cellular however, cutting artefact is present.

Oyster 1 (AOOA):

Mantle:

Focal extensive oedema and associated erosion of the epithelium. There is extensive sub-epithelial oedema across the body of the meat.

Gill:

Generalise sub-epithelial oedema of the gills. There is focal haemocyte aggregations within the connective tissue.

Palps:

There is reasonable vacuolisation of the distal palps.

Oesophagus & Stomach:

There is reasonable vacuolisation of the oesophagus. Moderate amount of stomach content present that has mixed morphology. Haemocytes plaques present within the stomach lumen and a style is present.

GIT:

E-gut appears the same as the stomach. Vacuolisation present of the wall of the E-gut and moderate transmigration of cells.

Leydig Tissue:

Sparse with haemocyte aggregations within.

Hepatopancreas:

60% of atrophic epithelium observed associated with tubules.

Gonad:

Rudimentary female gonad.

Heart:

Extensive myocyte vacuolisation with brown cells and intracellular inclusions on both ventricle and atrium.

Muscle:

There is focal muscle degeneration observed

Kidney:

NSF

Nerve:

Vacuolated nerve as well as peri-nuclear vacuolisation.

Oyster 2:

Mantle:

Extensive sub-epithelial oedema with bulla formation. There are active shell glands on the mantle with mild thinning of the epithelium.

Gill:

Generalise vacuolisation across the gills.

Palps:

Vacuolisation of the epithelial wall to the level of the oesophagus. There is a focal necrotic inflammatory cell in the wall of the proximal palps.

Oesophagus & Stomach:

Vacuolisation present as described in palps. Moderate amount of intestinal contents with mixed morphology. Protein plaques observed within content. Moderate transmigration across stomach wall. Style present.

GIT:

Changes as described as stomach.

Leydig Tissue:

Degenerative tissue with loss of tissue structure. Haemocyte aggregations present within the tissue.

Hepatopancreas:

Atrophy of the epithelium in 70 – 80% of the tubules.

Gonad:

Rudimentary gonad.

Heart:

Vacuolisation of atrium.
Muscle:
Appears degenerative.
Kidney:
NSF
Nerve:
Peri-nuclear vacuolisation present.

Oyster 3:
Mantle:
Focal sub-epithelial oedema with presence of haemocyte aggregations.
Gill:
Extensive severe vesicular oedema.
Palps:
NSF.
Oesophagus & Stomach:
Insufficient sample.
GIT:
Insufficient sample.
Leydig Tissue:
Degenerated with loss of structure.
Hepatopancreas:
NSF.
Gonad:
Rudimentary gonad
Heart:
Atrium vacuolisation as well as vacuolisation on the pericardial lining.
Muscle:
As described in previous sample.
Kidney:
NSF

Nerve:
No sample.

Oyster 1,2,3 & 4 (AOOB):
Mantle:
Extensive sub-epithelial oedema. All have significant focal active shell gland areas.
Gill:
Generalised sub-epithelial oedema.
Palps:
NSF
Oesophagus & Stomach:
Low stomach contents.
GIT:
Low content with mixed morphology with dense plant content.
Leydig Tissue:
Focally depleted to normal.
Hepatopancreas:
40 to 50% dilated tubules with epithelial hypoplasia.
Gonad:
Rudimentary gonads

Heart:
NSF.
Muscle:
As described in previous samples.
Kidney:
No sample.
Nerve:
NSF.

Oyster 1 (AOOC):
Mantle:
Generalised sub-epithelial oedema.
Gill:
NSF.
Palps:
No sample.
Oesophagus & Stomach:
No sample.
GIT:
Moderate amounts of basophilic plant material within.
Leydig Tissue:
Atrophic with loss of integrity
Hepatopancreas:
NSF.
Gonad:
Developed testis
Heart:
Generalised vacuolisation of the ectocardium focally around the atrium
Muscle:
As described as previous samples.
Kidney:
NSF.
Nerve:
NSF.

Oyster 2:
Mantle:
Oedema present in the body of the distal mantle otherwise NSF.
Gill:
NSF.
Palps:
NSF.
Oesophagus & Stomach:
No content present.
GIT:
No content present.
Leydig Tissue:
Depleted with loss of structure.
Hepatopancreas:
NSF.
Gonad:
Rudimentary gonad.

Heart:
NSF. Trichodinids present. Focal area of the internal lining of pericardium that appears to be extensively vacuolated.
Muscle:
As previously described.
Kidney:
No sample.
Nerve:
Hyper-cellular with vacuolisation.

Oyster 3:
Mantle:
Generalised oedema across all external epithelium.
Gill:
Marked number of ciliates present.
Palps:
No sample
Oesophagus & Stomach:
Mild amounts of intestinal contents. Mixed morphology with predominantly plant material.
GIT:
Similar as described to stomach.
Leydig Tissue:
Deplete with minor loss of structure.
Hepatopancreas:
70% of tubules are dilated.
Gonad:
Rudimentary male
Heart:
Atrial vacuolisation and sub-epithelial vacuolisation in the epithelium.
Muscle:
Artefact appears there is myopathy.
Kidney:
No sample.
Nerve:
Peri-nuclear vacuolisation.

Oyster 4:
Generalised vacuolisation and oedema to ALL structures apart from stomach wall. Marked number of trichodinids present.
Gill:
Grossly oedematous.
Palps:
NSF.
Oesophagus & Stomach:
No contents.
GIT:
No contents.
Leydig Tissue:
Dense
Hepatopancreas:

95% tubules are dilated with advanced squamous metaplasia of the epithelial linning.
Gonad:
Rudimentary gonad
Heart:
No sample.
Muscle:
Degenerate.
Kidney:
Thin walled and oedematous.
Nerve:
No sample.

Oyster 1 (BOA):
Mantle:
Hyperplasia of the shell gland.
Gill:
NSF
Palps:
NSF with mild focal oedema.
Oesophagus & Stomach:
No content present.
GIT:
No content present. (Small sample)
Leydig Tissue:
Deplete and unstructured.
Hepatopancreas:
90% tubules are dilated with occasional squamous metaplasia.
Gonad:
Rudimentary male.
Heart:
Vacuolated atrium that are clear.
Muscle:
Hyalinised muscle with a degree of autolytic change.
Kidney:
Dense.
Nerve:
NSF.

Oyster 2 (AUTOLYTIC OYSTER):

Oyster 3:
Mantle:
Multifocal sub-epithelial oedema with occasional bullae observed.
Gill:
NSF
Palps:
No sample.
Oesophagus & Stomach:
Style present in stomach. Scant content.
GIT:

Full of content with mixed morphology dense plant and algae material. Mild haemocyte transmigration through the wall of the E-gut.
Leydig Tissue:
Depleted.
Hepatopancreas:
NSF.
Gonad:
Developing female
Heart:
NSF
Muscle:
As previously described.
Kidney:
NSF
Nerve:
No sample.

Oyster 4:
Mantle:
Focal oedema and hypertrophy of shell gland.
Gill:
NSF
Palps:
NSF
Oesophagus & Stomach:
Empty – no content.
GIT:
No content.
Leydig Tissue:
Depleted.
Hepatopancreas:
30% dilated otherwise NSF.
Gonad:
Rudimentary male.
Heart:
Thin myocardium.
Muscle:
As previously described.
Kidney:
Thin epithelium of the kidney.
Nerve:
NSF.

Oyster 1 (BOB):
Mantle:
Focal oedema of the sub-epithelial layer occasional bullae present. The tissue appears thin.
Gill:
NSF.
Palps:
NSF.

Oesophagus & Stomach:
Pink amorphous material present within stomach lumen.
GIT:
As described as stomach.
Leydig Tissue:
Depleted.
Hepatopancreas:
NSF.
Gonad:
Male rudimentary
Heart:
Loss of structure to the heart (whispy). Gross thinning of the trabeculae. Vacuolisation of the atrium.
Muscle:
Fibre separation.
Kidney:
NSF.
Nerve:
Hyper-cellularity to the ganglia particularly the nerve bodies.

Oyster 2:
Mantle:
Hyperplasia of the gland cells.
Gill:
NSF
Palps:
No sample
Oesophagus & Stomach:
Empty no content but style present.
GIT:
No fill.
Leydig Tissue:
Depleted and loss of structure
Hepatopancreas:
Atrophy of the glands.
Gonad:
Rudimentary male
Heart:
Appears dilated with loss of structure.
Muscle:
Fibre separation and autolytic change present.
Kidney:
No sample
Nerve:
No sample.

Oyster 3:
Mantle:
Marked extensive sub-epithelial oedema throughout the meat. Marked bullae formation and dilatation of haemolymph vessels that appear cell poor. There is shell gland hyperplasia.

Gill:
Dilatation of haemolymph vessels.
Palps:
NSF.
Oesophagus & Stomach:
NSF in the oesophagus. Stomach has scant to moderate contents within.
GIT:
Intestines have scant to moderate polymorphic content. Content is comprised mostly of digestive organisms with mild amounts of filamentous bacteria present.
Leydig Tissue:
Depleted and disrupted in some peripheral areas but dense and compact within the peri-renal area.
Hepatopancreas:
NSF.
Gonad:
Developing male.
Heart:
Small sample present that appears to show clear vacuoles.
Muscle:
NSF
Kidney:
NSF
Nerve:
Peripheral nerves appear normal. Main ganglia absent.

Oyster 4:
Mantle:
Generalised oedema in the mantle. Hypercellularity of the gland cells.
Gill:
NSF
Palps:
Mild vacuolisation in the palp epithelium.
Oesophagus & Stomach:
NSF oesophagus. Stomach is empty with no style present. There is generalised vacuolisation of the stomach wall.
GIT:
Nil GIT content. There is generalised vacuolisation of the intestinal wall.
Leydig Tissue:
Marked depletion.
Hepatopancreas:
Generalised dilation across all tubules with the presence of amoeba noted.
Gonad:
Rudimentary gonad.
Heart:
Clear vacuolisation of atrial wall with thinning of both ventricular and atrial walls.
Muscle:
There is marked shrinkage of the myotomes.
Kidney:
NSF.
Nerve:
NSF.

Oyster 5:

Mantle:
Generalised oedema in the mantle. Hypercellularity of the gland cells.
Gill:
NSF
Palps:
Mild vacuolisation in the palp epithelium.
Oesophagus & Stomach:
NSF oesophagus. Stomach is empty with no style present. There is generalised vacuolisation of the stomach wall.
GIT:
Nil GIT content. There is generalised vacuolisation of the intestinal wall.
Leydig Tissue:
Marked depletion.
Hepatopancreas:
Generalised dilation across all tubules with the presence of amoeba noted.
Gonad:
Rudimentary gonad.
Heart:
Clear vacuolisation of atrial wall with thinning of both ventricular and atrial walls.
Muscle:
There is evidence of anti-mortem myopathy and mild inflammation.
Kidney:
NSF
Nerve:
NSF

Oyster 1 (BOC):
Mantle:
Focal sub-epithelial oedema of the mantle with epithelial erosion.
Gill:
Generalised dilation of the haemolymph canals.
Palps:
No sample
Oesophagus & Stomach:
Insufficient sample.
GIT:
Insufficient sample
Leydig Tissue:
Depleted and degenerated. Appears disrupted
Hepatopancreas:
Generalised dilation of the hepatopancreatic tubules.
Gonad:
Absent
Heart:
Atrial vacuolisation observed. There is oedema of the pericardial wall.
Muscle:
Contracted and separated muscle fibres. Cachectic myopathy.
Kidney:
NSF.
Nerve:
Hyper-cellular with a degree of nuclear vacuolisation.

Oyster 2:
Mantle:
Focal sub-epithelial oedema of the mantle with epithelial erosion.
Gill:
Generalised dilation of the haemolymph canals.
Palps:
NSF.
Oesophagus & Stomach:
Insufficient sample
GIT:
Insufficient sample.
Leydig Tissue:
Depleted and degenerated. Appears disrupted
Hepatopancreas:
Generalised dilation of the hepatopancreatic tubules.
Gonad:
Rudimentary gonad
Heart:
No sample.
Muscle:
Contracted and separated muscle fibres. Cachectic myopathy.
Kidney:
No sample
Nerve:
Marked vacuolisation of the nerve (autolysis?)

Oyster 3: This oyster is in very poor condition.
Mantle:
Marked generalised oedema.
Gill:
NSF
Palps:
NSF
Oesophagus & Stomach:
No content – oedematous
GIT:
No content – oedematous
Leydig Tissue:
Loss of leydig tissue
Hepatopancreas:
Generalised dilation of tubules and vessels. Significant atrophy/
Gonad:
No sample
Heart:
No sample
Muscle:
Shrinkage and separation of muscle fibres.
Kidney:
No sample
Nerve:

No sample

Oyster 4:
Mantle:
Generalised sub-epithelial oedema with bullae present. Scant focal erosion of the epithelium.
Gill:
NSF.
Palps:
NSF
Oesophagus & Stomach:
Small amount of content in stomach with style present. Contents appear polymorphic.
GIT:
Small amount of content present.
Leydig Tissue:
Focally dense surrounding HP but otherwise depleted.
Hepatopancreas:
NSF
Gonad:
Developing male
Heart:
Generalised vacuolisation within the myocardium and vacuolisation of myocytes. Note vessel walls generally appear empty with no haemocytes present.
Muscle:
NSF. Note trichodinids found all through fish.
Kidney:
NSF.
Nerve:
Hypercellularity within the ganglia. Appears to show ganglioneuritis with vacuolisation.

Oyster 1 (BOD):
Mantle:
Focal proliferation of shell glands. There is multifocal areas of sub-epithelial oedema surrounding the body of the oyster with presence of occasional bullae. There is a focal underlying a hyperplasic shell gland there is an aggregation of active haemocytes and fibrosis. Corresponding haemocyte vessel appears thickened with haemocytes present within and shows a hyalinising change.
Gill:
All haemolymph channels appear dilated with scant number of haemocytes present.
Palps:
No sample.
Oesophagus & Stomach:
No content within stomach – insufficient sample.
GIT:
No content - insufficient sample.
Leydig Tissue:
Multifocal areas of haemocytes aggregations surrounding the vasculature. Tissue is depleted and regionally depleted.
Hepatopancreas:
80% dilated tubules and presence of amoeba noted.
Gonad:
Rudimentary gonad.
Heart:
No sample.

Muscle:
Contracted fibres.
Kidney:
NSF
Nerve:
Not present.

Oyster 2:
Mantle:
Dilation and bullae present. There is a section showing erosion with an inflammatory response with increased inflammatory cell presence and vascular cupping.
Gill:
NSF. Scant sample.
Palps:
Not present
Oesophagus & Stomach:
No sample.
GIT:
Insufficient sample.
Leydig Tissue:
Dense in certain areas with presence of haemocytes. There are areas with atrophy
Hepatopancreas:
70% dilated tubules with depleted leydig tissue.
Gonad:
Scant developing gonad.
Heart:
Dilated and appears to show a myopathy.
Muscle:
Atrophic fibres.
Kidney:
NSF
Nerve:
NSF.

Oyster 3:
Mantle:
Marked sub-epithelial oedema with presence of bullae and vascular dilation. Focal inflammation reaction is noted (abscess like) – organising inflammatory focci. There is a focal supprative inflammation lesion noted in an area of the mantle. There is a focus of increased acid and basic mucin cells arrange in papilla form epithelium (Significance?)
Gill:
There is vascular dilation but scant sample.
Palps:
No sample
Oesophagus & Stomach:
Insufficient sample – no contents.
GIT:
Insufficient sample – no contents.
Leydig Tissue:

Depleted with focal aggregation of haemocytes within the leydig tissue. There is haemocyte necrosis and cell debris present with disorientation of the muscle fibres. Significant vacuolisation in the associated nerves (neuritis).

Hepatopancreas:

Moderate degree of generalised atrophy.

Gonad:

Rudimentary

Heart:

Scant sample that is vacuolated.

Muscle:

Contracted fibres – mild atrophy /myopathy.

Kidney:

Appears proliferative. Hyperplasia (Section orientation of actual?)

Nerve:

No sample.

Oyster 4:

Mantle:

There is extensive sub-epithelial oedema with presence of bullae.

Gill:

Heavy trichodinid burden observed. Haemolymph channels appear dilated with scant number of haemocytes present

Palps:

NSF

Oesophagus & Stomach:

Insufficient sample

GIT:

No content present.

Leydig Tissue:

Depleted appearance with loss of structure.

Hepatopancreas:

30% of hepatopancreatic lumens appear dilated. An enlarged vessel is noted in the organ.

Gonad:

Rudimentary gonad

Heart:

No sample

Muscle:

Contracted fibres – mild atrophy /myopathy.

Kidney:

No sample

Nerve:

Nerve tissue appears vacuolated and it's structure appears abnormal (whispy)

Oyster 1 (BOE):

Mantle:

There is marked sub-epithelial oedema with bullae formation noted. There is a focal area of haemocyte aggregations present. There is a focal vessel in the mantle that appears markedly dilated with no haemocyte content within the lumen but large numbers of haemocytes aggregated surrounding the vessel.

Gill:

Moderate number of trichodinids observed. There is mild oedema present in the haemolymph channels.

Palps:
NSF
Oesophagus & Stomach:
Insufficient sample
GIT:
Mild mixed content noted
Leydig Tissue:
Areas of reactive haemocyte aggregations noted within the tissue.
Hepatopancreas:
Approximately 70% of hepatopancreatic tubules appear dilated. Scant numbers of amoeba observed within lumens.
Gonad:
Rudimentary gonad present
Heart:
Epicardium is thickened and vacuolated. Thinning of the myocardial fibres noted. Marked clear vacuolisation of the atrium wall. Significant number of melanin pigmented large brown cells observed within the ectocardium.

Muscle:
Contracted fibres – mild atrophy /myopathy.
Kidney:
NSF
Nerve:
NSF

Oyster 2:
Mantle:
There is sub-epithelial oedema with bullae formation noted.
Gill:
Moderate number of trichodinids observed. There is mild oedema present in the haemolymph channels.
Palps:
NSF
Oesophagus & Stomach:
No sample
GIT:
No content observed in intestinal tracts. Uncertain if one was cut at different angle with no view of lumen – marked mucin cells observed.
Leydig Tissue:
Depleted
Hepatopancreas:
Approximately 80% of hepatopancreatic tubules appear dilated. lumens.
Gonad:
No sample
Heart:
No sample
Muscle:
Contracted fibres – mild atrophy /myopathy.
Kidney:
NSF.
Nerve:
NSF

Oyster 3:
Mantle:
Small sample on slide that appears oedematous.
Gill:
NSF – mild number of trichodinids observed.
Palps:
No sample
Oesophagus & Stomach:
Amorphous basophilic content present throughout.
GIT:
Moderate mixed content present. Haemocyte aggregations at the surosal surface of most gut.Fibrous plant like material noted. (Bacterial presence?)
Leydig Tissue:
Depleted with loss of structure.
Hepatopancreas:
Approximately 80% of hepatopancreatic tubules appear dilated. lumens.
Gonad:
No sample
Heart:
Vacuolated ectocardium.

Muscle:
Contracted fibres – mild atrophy /myopathy.
Kidney:
No sample
Nerve:
NSF

Oyster 4:
Mantle:
Sub-epithelial oedema noted with presence of bullae formation.
Gill:
Haemolymph channels are oedematous with reduced haemocyte numbers.
Palps:
NSF
Oesophagus & Stomach:
Moderate fill with pink tinged amorphous content present.
GIT:
One section of GIT appears to have aggregation of haemocytes within the lumen.
Leydig Tissue:
Areas of depleted tissue with loss of structures and areas (surrounding hepatopancreas) that appear dense. Mineralised concretion within a membrane spotted in focal area of the leydig tissue (stray mantle or angle of cut?)
Hepatopancreas:
Extensive dilation and atrophy of lumens with loss of tubular architecture. Extensive uniform vacuolisation in the absorptive cells and secretory cells of the hp. There are aggregations of haemocytes in the intertubular spaces there is also random patchy degeneration of ciliated tubules (apoptotic)
Gonad:
No sample
Heart:

No sample
Muscle:
Multifocal areas of hyalination of fibres. There is increased spread between contracted muscle fibres.
Kidney:
No sample
Nerve:
No sample

Chapter 4 Winter Mortality

Cheryl Jenkins

Summary

The Sydney rock oyster (SRO) industry is the largest aquaculture industry in New South Wales (NSW) with a value of $40 million per annum. Oyster growers frequently cite winter mortality as a major health issue affecting SRO production and product quality with 10-20% mortalities often observed in market-sized oysters during outbreaks of the disease. Despite its impact to industry, winter mortality is a poorly understood disease with many discrepancies in the literature regarding the causative agent. The lack of a clear case definition for winter mortality has also confounded investigations into this disease with oysters from all mortality events occurring during winter submitted for diagnosis, many of which do not align with descriptions of the disease in the literature.

This study aimed to improve clarity of the definition of winter mortality disease and to investigate causative agents implicated in the literature including *Bonamia* spp. and *Mikrocytos* spp. parasites, and bacterial agents which may have been overlooked in investigations.

SROs from mortality events south of Port Stephens, NSW, during winter and spring (July-November) were received at the Elizabeth Macarthur Agricultural Institute for testing. Oysters were examined for gross pathology and by histopathology for microscopic lesions. Oysters with lesions consistent with the initial disease investigation (Roughley 1926) were examined further using a mix of bacterial culture and molecular tests specific for *Bonamia* spp., *Mikrocytos* spp., *Perkinsus* spp. and *Vibrio splendidus*. Archival material collected from a longitudinal winter mortality investigation in 2010 was also examined for shifts in the microbiome of oysters before and during a winter mortality outbreak to determine whether dysbiosis was a feature of winter mortality.

Seven submissions of oyster samples were received for winter mortality exclusion over two winter/spring seasons with only 2 of the 7 submissions meeting the case definition for the disease. The case definition for winter mortality was based on a review of the relevant literature and observations made in this study regarding the correlation of gross lesions with histological observations which were consistent with that literature. Winter mortality is defined as:

Mortalities in 18+ month old Saccostrea glomerata *between July and November, from Port Stephens to the southerly reaches of the range of* S. glomerata*, in water with higher than average salinity. Affected cohorts display gross lesions consisting of brown or yellow pustules on the external surfaces of the gonad, mantle, palps or gills or internally in the adductor muscle or gut epithelium. The lesions are focal and consist of accumulations of haemocytes.*

Microcell-like structures were only rarely observed in affected oysters, but molecular testing did not reveal the presence of any of the microcell parasites that were associated with winter mortality including *Mikrocytos* spp. and *Bonamia* spp. All samples also tested negative for *Perkinsus* spp. Characterisation of the microcell-like parasite observed in this and earlier studies of winter mortality should be pursued to enable examination of its epidemiology and relationship (if any) to disease.

Bacterial culture on material aspirated from gross lesions provided profuse pure growth of *Vibrio splendidus*. Quantitative PCR demonstrated a statistically higher load of *V. splendidus* in oysters meeting the case definition for winter mortality compared to those that did not. Genome sequencing of two of the winter mortality-associated *V. splendidus* isolates demonstrated the presence of a number of mobile genetic elements including a plasmid with high sequence homology to the pGV1512 plasmid that confers virulence on *V. crassostreae*. Strains of *V. splendidus* isolated from abscesses of the yesso scallop (*Patinopecten yessoensis*), furthermore, display cold-induced virulence in experimental challenge studies. Analysis of the microbiomes of oysters before and during a winter mortality event demonstrated a shift in the microbiome during disease which was associated with the histopathologicaly severity of the lesions.

These data combine to suggest that there is a primary or secondary role for bacterial agents in winter mortality.

A case definition for winter mortality will assist farmers and diagnosticians to recognise this disease and help to direct research efforts investigating its cause and management.

Further research into the epidemiology and role of *Vibrio splendidus* in winter mortality as a primary or secondary agent contributing to winter mortality should be considered.

4.1. Introduction

Sydney rock oysters (SROs; *Saccostrea glomerata*) are one of three key edible oyster species farmed in Australia along with the Pacific Oyster (*Crassostrea gigas*) and the Native Oyster (*Ostrea angasi*). SROs are a warm water species, and are cultivated in NSW, southern Qld and the south west of WA. *Saccostrea glomerata* is the major oyster species cultivated in NSW and constitutes the largest aquaculture industry in the state, comprising more than 90% of all commercial oyster production with a value of approximately $40 million per annum.

Disease imposes a major economic burden on oyster production globally and the SRO industry in Australia is no exception. QX disease and winter mortality are considered the two key diseases affecting SRO production. In the early 1990s and 2000s, SRO production collapsed in the major production areas of the Georges and Hawkesbury Rivers with up to 90% mortalities observed due to QX disease. Tools for QX disease diagnosis have enabled the identification of high risk estuaries and enabled strategies for disease management. Winter mortality episodes are less predictable geographically due to gaps in knowledge about the disease and a lack of suitable diagnostic tools, however outbreaks occur sporadically in winter and spring across the southern half of the SRO cultivation range (south of Port Stephens), typically causing mortalities of 10-20% and up to 80% in severe cases. Oysters near market size (2-3 years of age) are more susceptible than juvenile oysters.

Winter mortality was described in the winter and spring of 1924 and 1925 in the Georges River system by T. C. Roughley. Despite a number of studies since, his disease investigation remains the most thorough. Roughley attempted to replicate the disease by exposing SROs to cold temperatures and concluded that temperature was an indirect rather than a direct cause of the mortalities. He also highlighted the importance of growing height in disease expression, suggesting that lower growing heights were associated with increased disease severity and surmising that prolonged exposure of the SROs to an agent in the water was a factor in disease (Roughley 1926). Wolf (1967) emphasised the link between winter mortality episodes and salinity, with higher mortalities generally observed near the mouth of an affected estuary and the most severe outbreaks occurring in years of low autumn rainfall. Wolf attributed the increased mortalities in oysters at low growing height to exposure to high salinity water for prolonged periods (Wolf 1967).

Winter mortality can be identified by the presence of 1-6 mm surface lesions in the form of yellow or brown spots on the palps, gills, mantle, or gonad. Lesions can also be located internally, such as within the digestive gland or adductor muscle (Roughley 1926). Roughley described these lesions at the microscopic level as being analogous to abscesses due to the abundance of inflammatory cells (haemocytes) contained therein. There has been substantial debate in the literature about the cause(s) of winter mortality, but Roughley did not report associated parasites, and both he and Wolf suggested that the disease was of bacterial origin (Roughley 1926; Wolf 1967).

In 1988, Austin Farley undertook histological examination of material collected by Peter Wolf from the Georges River in the 1960s (Farley *et al.* 1988). Farley conducted a comparative assessment of the winter mortality specimens (n=7) in relation to that from Pacific Oysters (*Crassostrea gigas*) suffering Denman Island disease. Denman Island disease is caused by a microcell parasite, *Mikrocytos mackini*, which infects cells of vesicular connective tissue, and presents with similar gross lesions consisting of 1-5mm green or brown spots and in the later stages of disease, yellow pustules. Denman Island disease occurs when water temperatures are below 10°C for extended periods, affects oysters over 2 years of age and causes approximately 30% mortalities (OIE 2019). The predisposing conditions and the presentation of Denman Island disease is therefore similar to those observed for winter mortality. Histologically, Farley noted the focal abscesses described by Roughley, and also observed small structures (1-2µm with a 1µm eccentric nucleus) within haemocytes, which he concluded were microcell parasites distinct from *M. mackini,* but which he considered to be sufficiently similar to be assigned to the same genus. The microcells observed were named *Mikrocytos roughleyi.* At the time of his

description of *M. roughleyi*, Farley also undertook histological studies on the haplosporidian microcell parasite *Bonamia*, and specifically excluded *M. roughleyi* as belonging to this genus on the basis that it caused focal lesions rather than systemic disease, and that it infected crassostreid rather than ostreid oysters (Farley *et al.* 1988).

While Farley *et al.* (1988) and later Hine *et al* (2001), described the ultrastructure of *M. mackini*, noting a lack of mitochondria, he did not undertake comparative ultrastructural studies on *M. roughleyi*. These studies were undertaken later by Cochennec-Laureau using fixed material collected by the NSW Department of Fisheries. An organism resembling *Bonamia* was seen in in electron micrographs of this material and molecular testing for *Bonamia* spp. revealed the presence of an organism related to *Bonamia ostreae* and *B. exitiosa*. It was concluded that *M. roughleyi* should be reassigned to the species *Bonamia roughleyi* (Cochennec-Laureau *et al.* 2003); however no gross or histological descriptions were made of the examined material and therefore there was no way to link the presence of *Bonamia* to winter mortality. Later studies of winter mortality, including a longitudinal study of the disease in the Georges River, failed to link *Bonamia* to gross or histological signs of the disease using molecular techniques and in situ hybridisation (Carnegie *et al.* 2014; Spiers *et al.* 2014). As a result, Carnegie *et al.* (2014) suggested that the name "*Bonamia roughleyi*" should be considered nomen dubium.

Adlard and Lester (1995) were apparently able to reproduce gross signs similar to the winter mortality ("light brown diffuse lesions" on the mantle and palps) by experimental transmission using homogenised and filtered material from winter mortality-affected oysters confirmed to be harbouring microcells. Nonetheless, infection with *M. roughleyi* was only observed in 3/20 oysters and at low intensity. Despite developing a protistan PCR for detection of the parasite, no sequence data was obtained from that study and therefore, the identity of the parasite was never confirmed.

Molecular methods have been applied in other studies in an attempt to identify parasites that could be associated with winter mortality, including for haplosporidian parasites in the genera *Haplosporidium*, *Minchinia* and *Bonamia*; *Marteilia sydneyi (*ie: non-sporulating forms), *Mikrocytos mackini* and *Perkinsus* spp. (Carnegie *et al.* 2014; Spiers *et al.* 2014). None of these methods have yielded an answer as to the identity of the microcell parasite described as "*Mikrocytos roughleyi*" which has hindered efforts to confirm them as a causative agent of winter mortality.

A major impediment to the study of winter mortality is the difficulty in obtaining suitable material for further research. Oysters suffering mortality in the colder months are often submitted for winter mortality investigation and yet do not meet key criteria for the disease. This study aimed to establish a clear case definition for winter mortality, investigate potential causes, and was conducted over three seasons to maximise opportunities for obtaining suitable material.

4.1.1. Objectives

This project aimed to refine the understanding of causation of Winter Mortality, either by identifying if Winter Mortality is caused by a single agent, or to improve the understanding of its causes. It aimed to refine the case definition, improve management strategies for Sydney Rock Oysters in Winter Mortality prone areas and validate Winter Mortality resistance assessments for Sydney Rock Oysters

4.2. Methods

4.2.1. Oysters and oyster processing

Oysters from NSW leases south of Port Stephens with reports of mortalities between the months of July 1st and November 30th in 2017, 2018 and 2019 were submitted to the Elizabeth Macarthur Agricultural Institute for winter mortality investigation. Oyster submissions received and the procedures performed on each submission are listed in Table 4.1.

Table 4.1 Oyster samples tested in this study

Submission number	Date collected	Location	n	Analyses performed
1	21st September 2017	Crookhaven River	12	Gross examination, histopathology
2	21 September 2017	Georges River	20	Gross examination
3	26th September 2017 (follow up from submission 1)	Crookhaven River	36	Gross examination, histopathology
4	11th October 2017	Nelsons Lagoon	20	Gross examination, histopathology
5	27th August 2018	Clyde River	38	Gross examination, histopathology, PCR, bacteriological culture
6	19th September 2018	Berrys Bay, Shoalhaven River	27	Gross examination, histopathology, PCR
7	10th October 2018	Shoalhaven River	24	Gross pathology, histopathology, PCR
Archived material	February-November 2010	Georges River	24	Microbiome analysis (this study), histopathology and PCR performed previously (Spiers *et al*. 2013).

All oysters were cleaned by scrubbing and rinsing the external surfaces and opened with shucking knives which had been rinsed in bleach followed by MilliQ water. The adductor muscle of each oyster was severed and the oyster meat placed in a sterile petri dish to enable examination for gross lesions typical of winter mortality. Any gross lesions were noted and then a sterile scalpel blade was used to collect a cross section of each oyster for histopathological examination (Figure 4.1). If possible, lesions were included in the section collected for histopathology with some of the affected tissue also reserved in a 1.5 mL microfuge tube for molecular testing and tissue imprints. Where lesions were suppurative, purulent material was aspirated aseptically for bacteriological culture. For molecular testing, the surfaces of each organ (gonad, gill, mantle, digestive gland and adductor muscle) were swabbed and the swabbed material resuspended in sterile phosphate buffered saline (PBS) for later DNA extraction. The remaining oyster tissue was stored at -80°C.

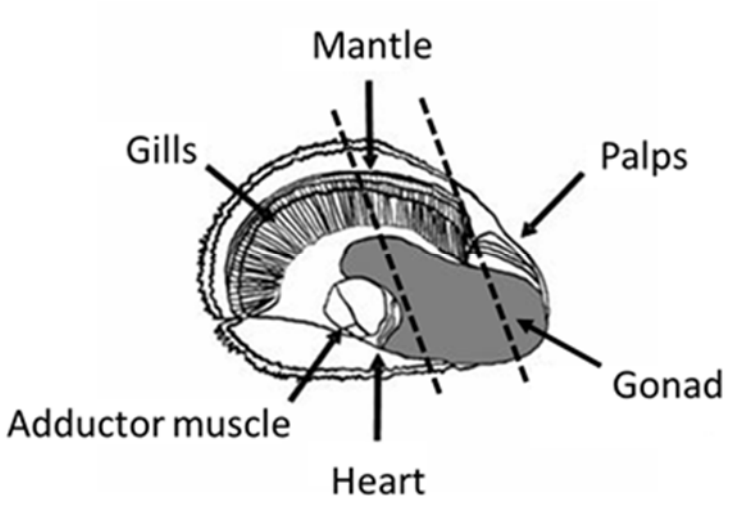

Figure 4.1 Diagram of oyster anatomy showing the major organs and the cross section sampled for histopathological examination (dotted lines), including mantle, palps, gills, gonad and underlying digestive gland. Where gross lesions were observed, an attempt was made to include these in the histological section.

4.2.2. Histopathology

Oyster sections were placed into a histology cassette and fixed in neutral-buffered formalin for 24 h prior to paraffin embedding in a tissue processor. Sections (5 µm thick) were stained with haematoxylin and eosin (H&E) and examined by a pathologist with experience in diagnosis of molluscan diseases.

4.2.3. Tissue imprints

Tissue imprints were made from affected tissue (tissue displaying lesions on surface examination) by dabbing the tissue onto a glass slide and allowing to air dry. The slides were fixed with methanol and stained with Diff-Quik stain (Australian Biostain).

4.2.4. Bacteriological culture

Purulent material from suppurative lesions was cultured on marine salt blood agar (Edwards Group) and thiosulfate citrate bile salts agar (TCBS; ThermoFisher Scientific). Plates were incubated aerobically at room temperature (20°C) for 48 hr.

4.2.5. DNA extraction

For PCR testing DNA was extracted from oyster swabs or oyster lesions using the MagMAX^{TM} CORE nucleic acid purification kit (ThermoFisher Scientific). Material from swabs and lesions was prepared for extraction by adding 500µL of PBS, pH 7.4 to 1.5mL screw capped tubes, vortexing and transferring 200 µL of the suspension to a new tube. Proteinase K solution (100 µL per sample) was added and the samples were vortexed and incubated at 55°C for 30 min. The samples were then centrifuged at 13 000 rpm for 2 min and 200 µL of the digested sample was then mixed with 20 µL of magnetic bead suspension in a deep-well plate. The DNA extraction was performed on a KingFisher Flex robotic magnetic particle processor (ThermoFisher Scientific) according to Workflow D of the manufacturer's instructions.

For microbiome analysis, DNA was extracted from archival tissues using the DNeasy Power Soil kit (Qiagen), which has been shown to yield greater alpha diversity in microbiome studies than other methods (Mattei *et al.* 2019). DNA was extracted according to the manufacturer's instructions. Archived material consisted of oysters collected during a prior longitudinal study of winter mortality (Spiers *et al.*

2014) which had been stored at -80°C. In that study, focal lesions were largely internal occurring adjacent to the gut; therefore cubes of oyster midgut were sampled for microbiome analysis.

For preparation of DNA from pure bacterial cultures for whole genome sequencing (WGS), DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in MilliQ water for downstream WGS analysis.

4.2.6. PCR testing

DNA extracts from oyster swabs and lesions were subjected to PCR testing for a variety of parasitological and bacteriological agents. Conventional PCRs were performed using BioTaq DNA polymerase (Bioline) and were run in a total reaction volume of 25 µL on a Master Cycler (Eppendorf). Real-time PCRs were run using the TaqMan Environmental Master Mix 2.0 in a total volume of 20 µL. Primers probes and PCR conditions used in this study are summarised in Table 4.2.

Statistical analysis of PCR results was performed in GraphPad Prism 8 using the Kruskal Wallis test.

Table 4.2 Primers, probes and PCR conditions used in this study.

PCR target	Primer/probe sequences 5'-3'	Primer/probe concentrations	PCR conditions	Amplicon size (bp)	Reference
Bonamia genus	Bo1: CCTAGGGTGACCCCTCTG Bo4: GGGTCAAACTCGTTGAACG	Primers: 0.4 µM	94°C, 3 min (x1) 94°C, 30 s 58°C, 30 s (x40) 72°C, 45 s 72°C, 5 min (x1)	237	Marty *et al*. 2006
Mikrocytos genus	Msp443F: ATACCTAAGCGTTACTGCGTTT Msp746R: TCGCCACTGGTAGTCCTGTTT	Primers: 0.5 µM	94°C, 3 min (x1) 94°C, 40 s 60°C, 40 s (x35) 72°C, 60 s 72°C, 5 min (x1)	281-305	Adapted from Gagne *et al*. 2008; Garcia *et al*. 2018
Perkinsus genus	PerkF: TCCGTGAACCAGTAGAAATCTCAAC PerkR: GGAAGAAGAGCGACACTGATATGTA Probe: FAM-CCCTTTGTGCAGTATGC-MGB	Primers: 0.9 µM Probe: 0.25 µM	50°C, 2 min (x1) 95°C, 10 min (x1) 95°C, 15 s 60°C, 60 s (x 45)	87	Gauthier *et al*. 2006
Vibrio splendidus cluster	16S SpF2: ATCATGGCTCAGATTGAACG 16S SpR2: CAATGGTTATCCCCCACATC 16S SpProbe: FAM-CCCATTAACGCACCCGAAGGATTG-BHQ1	Primers: 0.3 µM Probe: 0.2 µM	95°C, 10 min (x1) 95°C, 315 s 60°C, 90 s (x40)	154	Saulnier *et al*. 2017

4.2.7. Whole genome sequencing (WGS)

WGS was carried out on the Ion Torrent S5 with Ion Chef reagents (ThermoFisher Scientific). Prior to library preparation, DNA samples were quantified using the Qubit dsDNA High Sensitivity (HS) kit (ThermoFisher Scientific). HS reagent was prepared by mixing 199 µL of HS buffer and 1 µL HS reagent per sample analysed. The prepared buffer was then added to standard tubes (190 µL per standard) and sample tubes (198 µL per sample) with either 10 µL of standard or 2 µL of sample. All tubes were vortexed and incubated for 2 min at room temperature. Each sample and standard was then read on a Qubit Fluorometer 2.0 (ThermoFisher Scientific). The volume of sample required to yield 50-100 ng of DNA was then calculated for genome sequencing.

The DNA was fragmented with Ion Shear Plus enzyme in a total volume of 50 µL containing the DNA sample and 1 × Ion Shear Plus reaction buffer at 37°C for 7 min to generate 350-450bp fragments. Tubes were placed on ice and 5 µL of Ion Shear Stop buffer added to terminate the reaction.

The fragmented DNA was purified by mixing with 99 µL of AMPure beads and incubating for 5 min at room temperature. Tubes were placed on a magnetic rack for 3 min or until the solution had clarified. The supernatant was removed and the beads mixed with 500 µL of 70% ethanol, incubated for 30 s and turned on the magnetic rack to wash the beads. This step was repeated, residual ethanol removed and the tubes were then allowed to air dry for 5 min. The tubes were removed from the magnetic rack and resuspended in 25 µL of low Tris-EDTA (TE) buffer. Samples were returned to the magnetic rack for 1 min and the eluted DNA removed to a new tube.

Ligation of adapters to the DNA fragments and nick repair was performed in a total volume of 100 µL containing the purified DNA fragments, 1 × ligase buffer, 2 µL of dNTP mix, 2 µL of DNA ligase, 2 µL of Ion P1 adapter, 2 µL of Ion Xpress barcode and 8 µL nick repair enzyme. Samples were incubated at 25°C for 15 min followed by 72°C for 5 min and were then held at 4°C. The ligated DNA was purified again using 100 µL AMPure beads as described above with elution of the samples in 22.5 µL of low TE buffer.

To select the correct fragment sizes for sequencing, the samples were loaded onto an E-gel. 2.5 µL of loading buffer was added to each sample and each sample loaded onto the gel with 25µL of DNA ladder. The 2% SizeSelect protocol was run for 17 min and migration of the reference band monitored. When the reference band reached the reference line, all liquid was removed from the recovery wells and was replaced with 50 µL of nuclease free water. The run was resumed until the reference band was inside the collection well and the samples were recovered from the collection wells and placed in fresh tubes.

The DNA library was amplified using approximately 25 µL of sample, 5 µL of Library Amplification Primer mix and 100 µL of Platinum PCR SuperMix High Fidelity. The samples were then split into two tubes of 65 µL and the following PCR protocol was run: 95°C, 5 min (×1 cycle); 95°C 15s, 58°C 15s, 70°C 60s (×6 cycles); 4°C (hold). The amplified library was then combined into a single tube and purified using 130µL of AMPure beads and eluted in 20 µL low TE buffer.

A 1:100 dilution of the sample was quantified against the supplied *E. coli* standards at 1:10, 1:100 and 1:1000 (6.8 pM, 0.68 pM and 0.068 pM) dilutions using quantitative PCR. DNA samples or standards (4.5 µL) were mixed in a volume of 10 µL with 5 µL 2× TaqMan master mix and 20× Ion TaqMan Assay. The qPCR was run on a QuantStudio5 (ThermoFisher Scientific) for 40 cycles of 95°C for 1 s and 60°C for 20 s. The library concentration was determined by multiplying by the dilution factor (100) and was diluted to 100 pM.

Sample information was then input into the Torrent Suite™ software which set the parameters and instructions for both the Ion Chef™ and subsequent Ion Torrent runs and post processing. The automated preparation, normalisation, and placement of samples onto a 530™ chip was performed by the Ion Chef™. The 520TM chip was then placed in the Ion Torrent and whole genome sequencing (WGS) of two unknown bacterial isolates was successfully performed. The resulting data was then demultiplexed and saved on the Ion Torrent server in UBAM and FASTQ formats. Both FASTQ files were uploaded

to the University of Technology's high performance computing (HPC) cluster and each assembled into a FASTA scaffold file using the "spades" de-novo assembler. Flags used by "spades" were: "--iontorrent --careful -s".

4.2.8. Phylogenetic analysis

All reference bacterial genomes were downloaded from NCBI in FASTA format. Reference genomes and the previously generated isolate (sample) scaffolds were all placed into a single directory. Initial phylogenetic analysis was performed on this collection. An alignment FASTA file was constructed with Phylosift (Darling *et al.* 2014) followed by the construction of a tree file by the FastTree program. The resulting phylogenetic tree was visualised in either Geneious Prime or the web-based "Interactive Tree of Life" (iTOL - https://itol.embl.de/). These initial results showed that the unknown isolates clustered within the *Vibrio* family, so all *Vibrio* genomes and scaffolds were subsequently downloaded from NCBI and the phylogenetic analysis repeated as above. If alignment files generated by Phylosift needed to be combined, a custom script (refineListForPhylo.R) concatenated the FASTA alignment files and removed duplicates. The final phylogenetic tree was constructed manually: A list of identifiers was selected, saved as a tab-delimited file, and the file input into a custom script (refineListForPhylo.sh) that generated the final alignment file and phylogenetic tree.

Calculation of Average Nucleotide Identity (ANI)

Average nucleotide identity (ANI) of genomes was examined using the PYANI program (https://github.com/widdowquinn/pyani). The ANIm algorithm was used to calculate ANI (uses MUMmer (NUCmer) to align the input sequences). Custom heatmaps were also generated via a custom R-script "pyani_heatmap.R".

Identification of mobile genetic elements

Fasta scaffolds from Isolate 1 and Isolate 25A were aligned against the *V. splendidus* reference genome with Mauve using the progressive mauve algorithm (Darling *et al.* 2010). Genome regions representing insertions in the winter mortality isolates that were larger than 20 Mbps were identified and used as input for a nucleotide search of GenBank using BLASTn (discontiguous megablast).

Plasmid discovery was also performed using PlasFlow (Krawczyk *et al.* 2018). A custom script "auto_plasflow" iterated through each genome (FASTA file) and deliver results into individual sub-directories. For each FASTA input the plasflow program would output two FASTA files, listing putative plasmid sequences and unclassified sequences. All plasflow output was then used as input into BLASTN and the XML results examined in Geneious Prime. Any sequences under 5,000bp were disregarded.

4.2.9. Microbiome analysis

DNA samples for microbiome analysis were sent to the Ramiaciotti Centre for Genomics, University of New South Wales where the V1-V3 regions of the 16S rRNA gene were amplified and sequenced using the Illumina MiSeq.

Sequence analysis was performed with QIIME2 (Bolyen *et al.* 2019). The QIIME2 workflow in general followed that described in the QIIME2 tutorial (https://docs.qiime2.org/2019.7/tutorials/moving-pictures/), with a number of changes listed below. The demultiplexed FASTQ files were loaded into qiime2 using the import tool (type was set to 'SampleData[PairedEndSequencesWithQuality]' and input-format set to CasavaOneEightSingleLanePerSampleDirFmt). Sequence quality control was performed by dada2 which trimmed and denoised the sequences (Callahan *et al.* 2016). After a number of test analyses the start and end sequence trimming was chosen to be 10bp and 240bp respectively. The sequence alignment was via MAFFT, the alignments filtered by "mask", and phylogenetic tree was generated by FastTree (Price *et al.* 2009) with the tree rooted using the "midpoint rooting" algorithm. Even sampling or rarefaction depth was set to equal the level of the sample with the lowest depth. Alpha diversity was examined qualitatively using Faith's Phylogenetic Diversity, quantitatively using

Shannon's diversity index, and in terms of evenness by Pielou's Evenness. Beta diversity was examined qualitatively with Jaccard distance, quantitatively using the Bray Curtis distance, and qualitatively and quantitatively incorporating phylogenetic relationships with both weighted and unweighted UniFrac distance. Beta diversity results were visually summarised with Principal coordinate analysis (PCoA) using the "Emperor" program (https://github.com/biocore/emperor). For taxonomic analysis either a pre-trained or custom trained Naive Bayes classifier was used that was based on the SILVA database (Quast *et al.* 2012). The pre-trained file was downloaded from https://data.qiime2.org/2019.7/common/silva-132-99-nb-classifier.qza, or the downloaded files for custom training were the reference sequence and the corresponding taxonomic classifications. For this experiment the chosen reference sequence downloaded from the SILVA website was "silva_132_99_16S.fna" and the file for taxonomic classification "silva_taxonomy_7_levels.txt". The quantitative, taxonomic bar graphs were produced by the default application within qiime2. The Krona graphs were produced by a custom R-script (qiime2ToKrona_GUI.R) that utilised "Krona Tools" and the script "OTUsamples2krona.sh". Customised tables output from qiime2ToKrona_GUI.R were then used in the custom script heatmapForQiime2_V2.R to draw heatmaps that represented the percentage of the species, or other taxon levels, within all samples. For heatmapForQiime2_V2.R the taxon level and sample grouping to be examined was input by the user.

4.3. Results

4.3.1. Winter mortality case definition

One of the major impediments to the study of winter mortality has been the lack of a clear case definition for this disease brought about by the inconsistencies in the literature regarding its cause. In reviewing the relevant literature, we propose that cases of winter mortality be defined as:

Mortalities in adult (at least 18 months) Saccostrea glomerata*, occurring between July and November, from Port Stephens to the southerly reaches of the species range, in high salinity water. Affected cohorts display gross lesions consisting of brown or yellow pustules on the external surfaces of the gonad, mantle, palps or gills or internally in the adductor muscle or gut epithelium. The lesions are focal and consist of accumulations of haemocytes.*

4.3.2. Field, gross and histopathological characteristics

All oysters in this study were received within the July-November period (Table 4.1) in either the 2017 or 2018 season. Up until the time of writing this report (July 31st 2019), no submissions had yet been received in the 2019 season. The submissions received highlight the importance of a clear case definition, with 5/7 submissions lacking gross lesions consistent with the literature and 3/7 submissions of SROs in the wrong age class for winter mortality (Table 4.3).

Of the 7 submissions, only 2 from the Clyde and Shoalhaven Rivers (Submissions 5 & 6) met the case definition for winter mortality. These submissions were received in August and September of 2018 and were from leases experiencing 15-20% mortalities. Adult oysters were affected in both cases, with juvenile oysters on the same lease unaffected (Submission 5) and low rainfall, frosts and higher than usual salinity reported (Submission 6). Some oysters from both Submission 5 & 6 displayed characteristic yellow or brown lesions typical of winter mortality. Lesions were observed on a range of organs, predominantly the gonad (Figure 4.2A-E), but also the mantle (Figure 4.2 E) and internally within the adductor muscle (Figure 4.2D) and the border of the gonad and digestive gland (Figure 4.2F).

Table 4.3. Field, gross and histopathological characteristics of oyster submissions 2017-2018

Submission number	% mortality	Age	Submitter Comments	Gross lesions present?	Key histological findings	Meets case definition?
1 Crookhaven River	80%	<1.5 yr	Prolonged cool and dry conditions reported in area	No	Diffuse moderate-marked haemocyte infiltrates. No evidence of focal lesions.	No. Wrong age class. No gross or focal microscopic lesions observed.
2 Georges River	10-15%	2 yr	Routine submission of oysters deployed for WM investigation	No	Not examined	No. No gross lesions observed.
3 Crookhaven River	80%	<1.5 yr	Prolonged cool and dry conditions reported in area	No	Not examined	No. Wrong age class. No gross lesions observed.
4 Nelsons Lagoon	50%	(Spat) 0.5 yr		No	Alimentary duct hyperplasia. Non-specific haemocyte infiltrates.	No. Wrong age class. No gross or focal microscopic lesions observed
5 Clyde River	15-20%	2 yr	No deaths in juvenile oysters on same lease	Yes	Focal abscessation associated with gross pathology. Haemocytes effacing and replacing normal architecture. Erosion, ulceration and proteinaceous surface crusting. 2-3 µm structures observed in haemocytes (1 oyster)	Yes.
6 Berrys Bay, Shoalhaven River	20%	1.5 yr	Recent frosts and high salinities (28ppt) reported	Yes	Extensive inflammatory gut lesions. Haemocytes effacing and replacing normal architecture. 1-2 µm basophilic bodies observed in multiple haemocytes (1 oyster)	Yes
7 Shoalhaven River	5%	1-2 yr	Routine submission of oysters deployed for WM investigation	No	Minimal pathology evident in these oysters. Where present, multifocal infiltrates and necrosis more consistent with exposure to toxins or bacteria.	No. No gross or focal microscopic lesions observed.

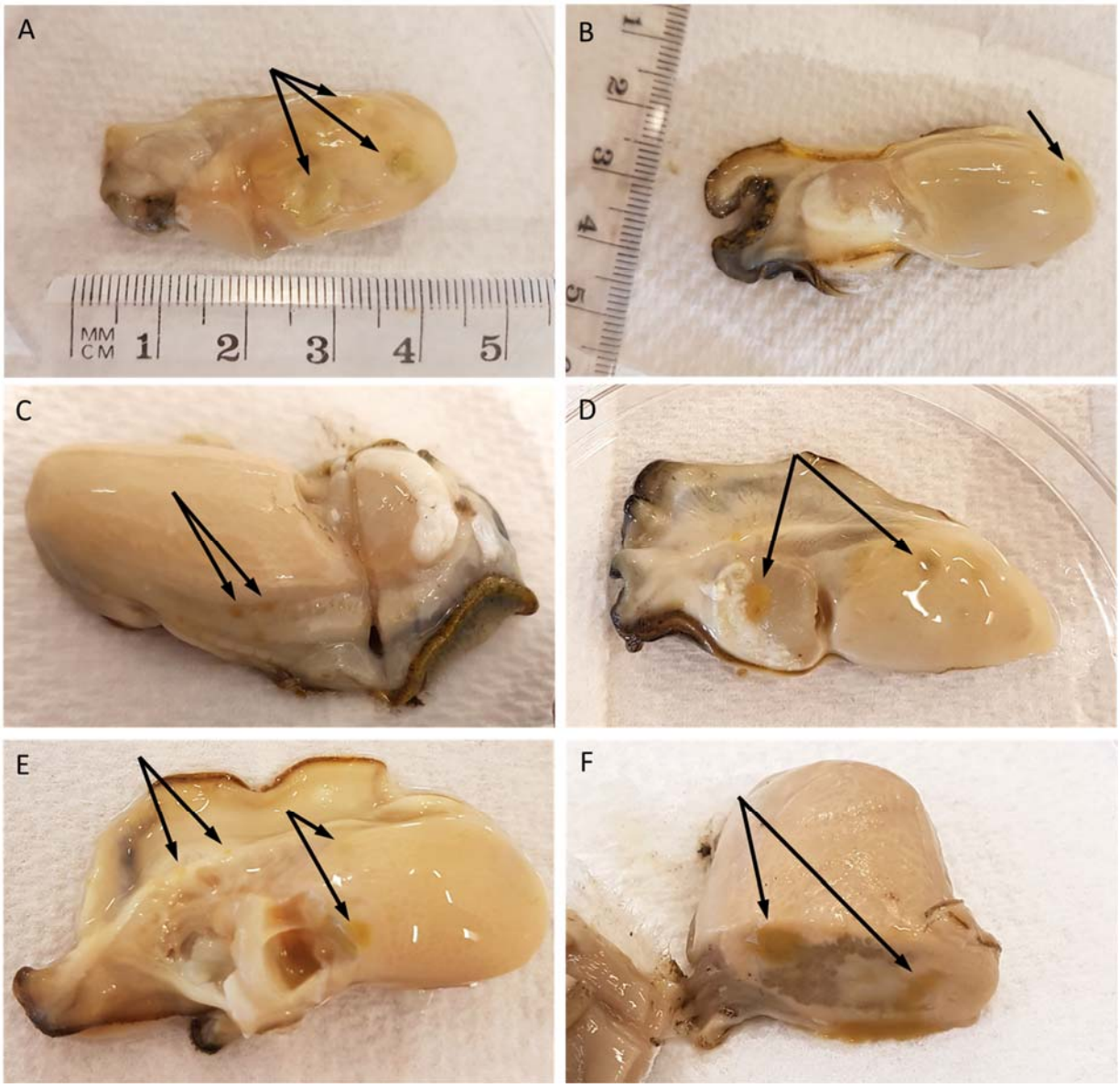

Figure 4.2 Gross pathology of oysters from a submission from the Clyde River meeting the case definition of winter mortality. Yellow or brown lesions (arrows) were observed on the gonad (A-E), mantle (E) and were also located internally, within the adductor muscle (D) or within the gonad bordering the digestive gland (F), and were only evident upon cross section.

Histologically, lesions were consistent with the original descriptions of abscessation (and sometimes ulceration) by Roughley (1926), and were filled with abundant haemocytes, in severe cases completely replacing normal cellular architecture (Figure 4.3). In contrast, submissions containing oysters without gross lesions typical of winter mortality also lacked histological evidence for the disease with haemocyte infiltrations being diffuse and non-specific in nature rather than focal (Table 4.3).

Microcells were observed in oysters from both submissions 5 and 6 which met the case definition for winter mortality, and were absent from submissions that did not. However, the prevalence of microcell-like structures was low, with only a single oyster from each submission identified as containing these structures. The structures were approximately 2µm in diameter with a large eccentric nucleus (Figure 4.4A & B) and were comparable to those seen in the original description of *Mikrocytos roughleyi* made in 1988 by Farley (Figure 4.4C).

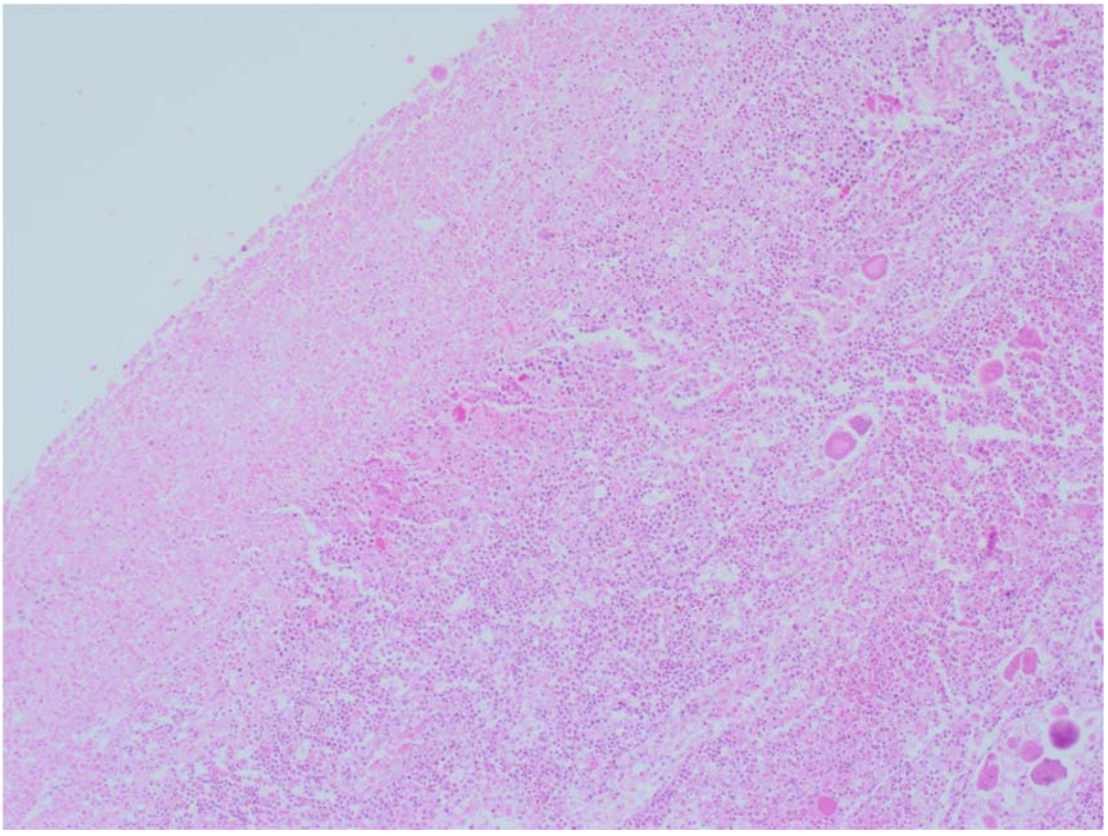

Figure 4.3 Surface lesions in the gonad correlate to focal abscesses in histopathology. Abundant haemocytes have replaced normal architecture and underlie a layer of necrotic tissue.

4.3.3. Bacteriological culture

Given that a bacterial cause for winter mortality was originally suspected (Roughley 1926; Wolf 1967), aerobic culture on a general growth medium (marine salt blood agar) was undertaken from two oysters from Submission 5 that displayed with suppurative lesions including one internal and one external abscess. Profuse growth and near pure cultures of beta haemolytic organisms were obtained from both lesions. Subculture onto TCBS agar suggested that these organisms may be members of the *Vibrionaceae*. Sequencing of the 16S rRNA genes of 4 isolates from the two samples including predominant and non-predominant colony types were all confirmed as *Vibrio* spp. most closely related to *V. splendidus*, suggesting that the different colony types on each plate represented phenotypic variants.

4.3.4. Phylogenomics

Whole genome sequences from two *Vibrio* spp. isolates from external (Isolate 1) and internal (Isolate 25A) winter mortality lesions were generated on the Ion Torrent S5, assembled and the core marker genes analysed using Phylosift. Phylogenetic analysis using a large number of reference genomes from members of the *Vibrionaceae* confirmed the 16S rRNA results which placed the isolates within the *V. splendidus* cluster. A sub-tree generated using genomes of members of the *V. splendidus* cluster (shown in Figure 4.5) demonstrated that these isolates are most closely related to *V. splendidus* within the cluster. The two isolates from Submission 5 were most closely related to each other.

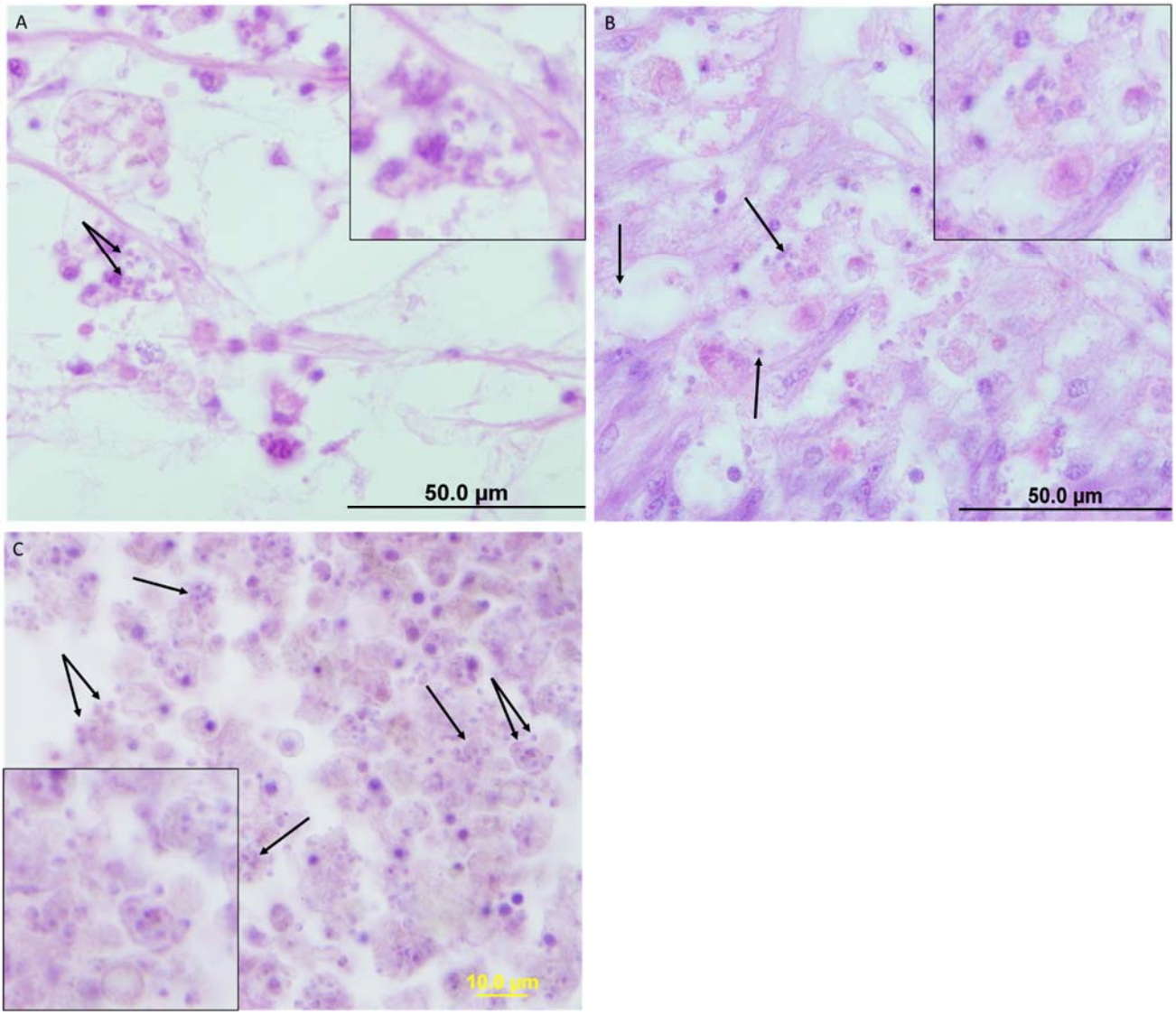

Figure 4.4 Microcell-like structures (arrows and inset) observed in an oyster from Submission 6 (A) and Submission 5 (B). The structures are approximately 2 µm in diameter with an eccentric nucleus. The structures are comparable to those seen in the type material submitted by Farley (1988) but are less abundant. The image used in (C) was generously supplied by R. Carnegie.

4.3.5. Identification of mobile genetic elements (MGEs)

Virulence determinants in bacteria are often located on mobile genetic elements including prophages, integrative and conjugative elements and plasmids. Comparison of the genomes of the *V. splendidus* isolates from this study with the *V. splendidus* reference genome identified a number of insertions present in the winter mortality isolates that were absent in the reference genome (Figure 4.7). BLASTn analysis of these scaffolds revealed that a number of these insertions represented mobile genetic elements including a phage, an extrachromosomal element, an integrative and conjugative element and a plasmid (Table 4.4).

Most notably, a 110 Mbp scaffold in isolate 1 showed high levels of identity (99.9%) with the pGV1512 plasmid identified in *V. crassostreae* as being involved in virulence in *C. gigas* (Bruto *et al.* 2017). This match had a query coverage of 41%, which may be a result of incomplete sequence data from isolate 1, given that the scaffold analysed was 110 Mbps in length while the pGV1512 plasmid is nearly 180 Mbps. The pGV1512 plasmid is known to encode both Type IV and a Type VI secretory systems and novel virulence factors which, when deleted, resulted in virulence attenuation in *V. crassostreae* (Bruto *et al.* 2017).

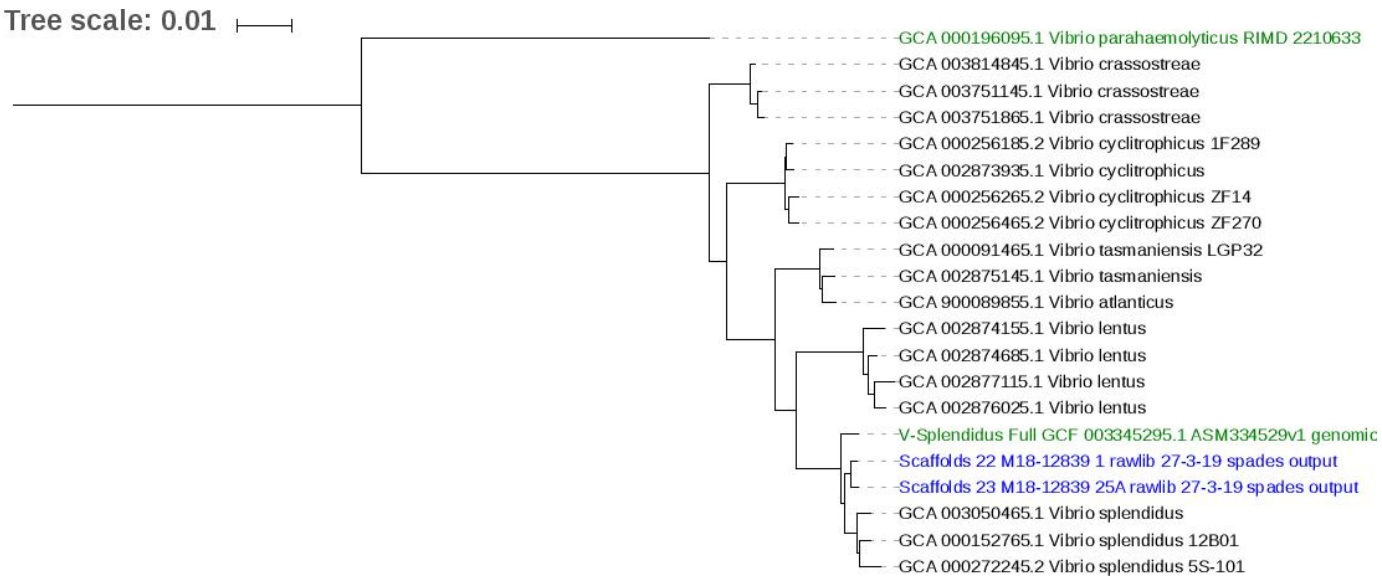

Figure 4.5 Phylogenetic tree generated with Phylosift from various reference genomes from members of the V. *splendidus* cluster and genomes of two isolates obtained in near pure culture from winter mortality lesions. Isolates obtained from this study are shown in blue. A complete reference genome for *V. splendidus* is shown in green. *V. parahaemolyticus*, also shown is green, was used as an outgroup. The tree demonstrates that both isolates obtained in this study are strains of *V. splendidus*. Average nucleotide identity calculations generated using ANIm indicated that the two isolates were 98% identical to each other and 97% identical to the next closest strain of *V. splendidus* (Figure 4.6).

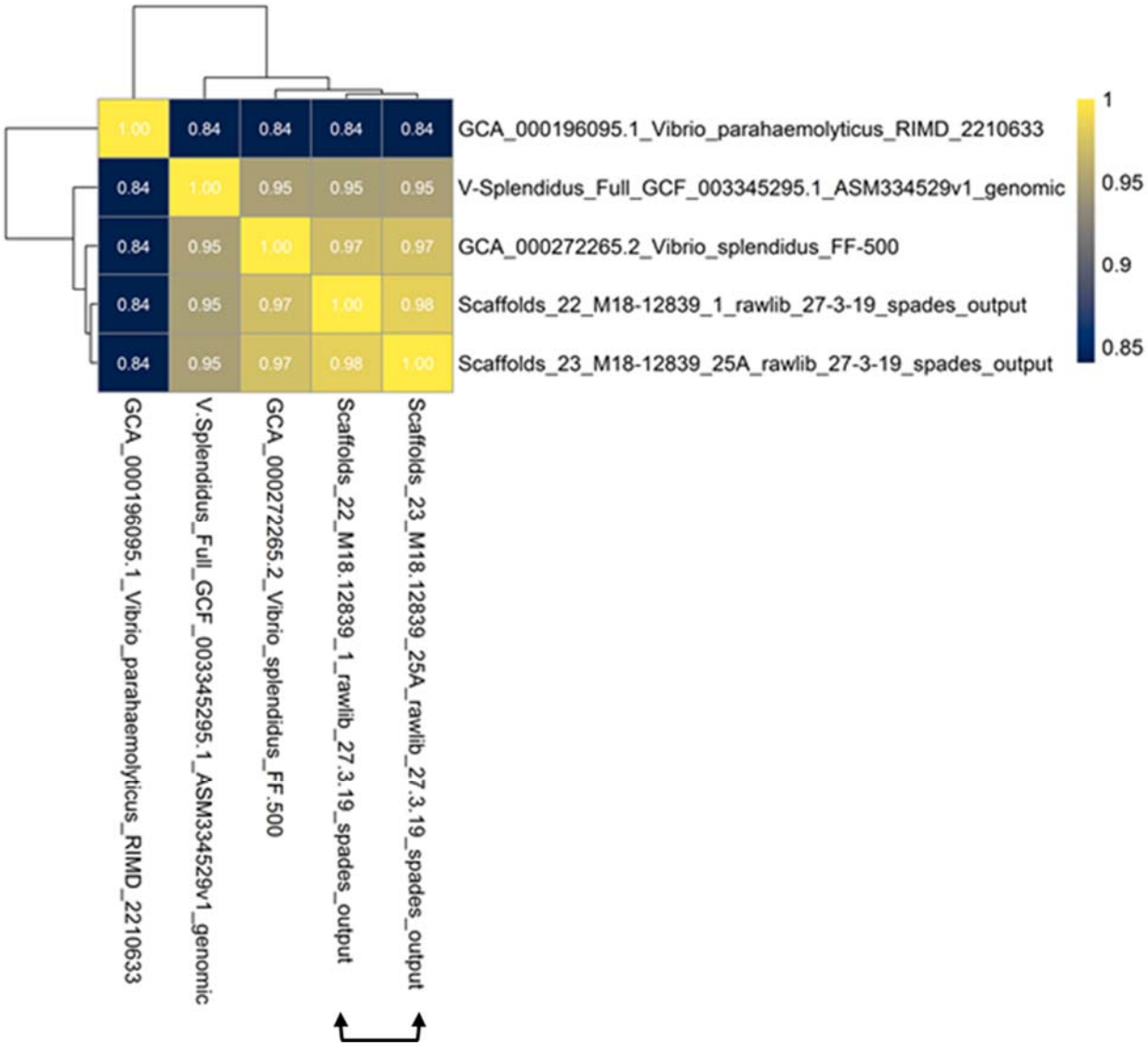

Figure 4.6 Heat map showing the average nucleotide identity scores for the isolates obtained from winter mortality lesions (arrows) and those of other *V. splendidus* (and *V. parahaemolyticus*) strains. The two isolates from this study are most closely related to each other (98% identity).

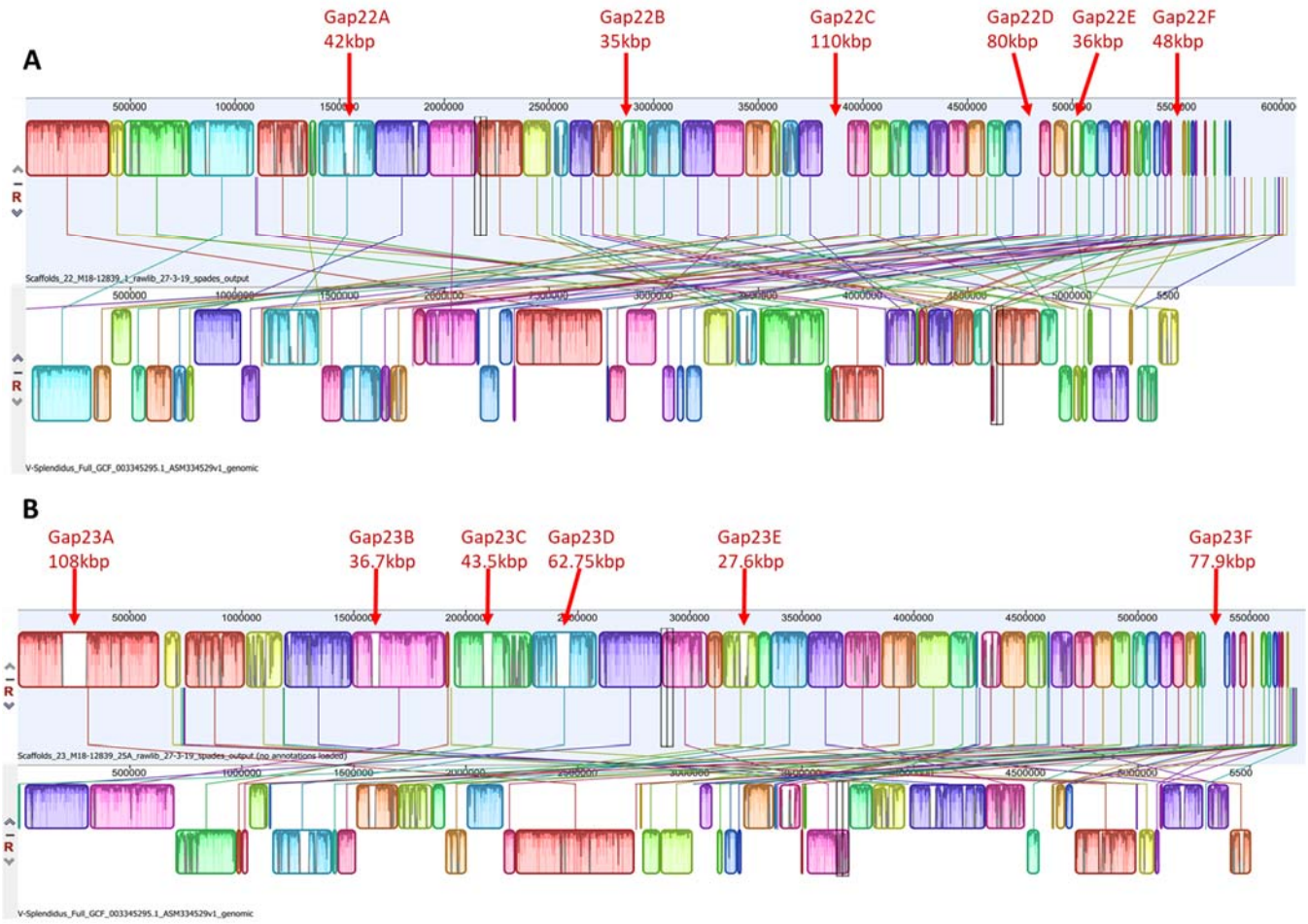

Figure 4.7 Genome alignments of isolate 1 (A) and isolate 25A (B) with the *Vibrio splendidus* reference sequence. Sequences identified in the isolate genomes that were absent from the reference sequence are indicated by the arrows. The size of these insertions/deletions are given in base pairs.

Table 4.4 Insertions in the winter mortality *V. splendidus* isolates with matches to known mobile genetic elements

Insertion/ deletion	Closest match (BLASTn)	% identity	Query Length (Mbp)	Hit Length (Mbp)	Query coverage (%)
Gap 22B	*Vibrio* phage martha 12B12 genomic sequence	89	35	33	32
Gap 22C	*Vibrio crassostreae* strain J5-20 plasmid pGV1512, partial sequence	99.9	110	178	41
Gap 22D	*Vibrio crassostreae* 9CS106 ECE2 extrachromosomal element	84	80	39	8
Gap 23A	*Actinobacillus pleuropneumoniae* ICEApICh1 mobile element, complete sequence (also present in *V. cholerae*)	96.8	108	101	25.17

4.3.6. PCR testing

Although microcells were not observed in abundance histologically in this study, they have frequently been cited as the cause of winter mortality in SROs. "*Mikrocytos roughleyi*" and "Bonamia roughleyi" are the most frequently cited agents. One study, (Carnegie *et al.* 2014) also noted the similarity between the microcell-like structures observed by Farley and *Perkinsus* spp., due to the presence of a large vacuole displacing the nucleus. In this study, we undertook PCR testing, using generic assays designed for each of these oyster pathogens. In addition, to determine the prevalence and intensity of infection of members of the *V. splendidus* cluster in oysters suffering winter mortality, we also undertook quantitative PCR analysis of oyster DNA extracts. For PCR, we analysed 3 submissions of oysters (submissions 5-7), two of which met the case definition of winter mortality and one which did not (Tables 4.1 & 4.3). We also PCR tested material specifically collected from gross lesions to maximise chances of pathogen detection.

PCR for *Bonamia* spp. and *Perkinsus* spp. was negative for all oysters tested. The former result is consistent with two prior studies which suggested that *Bonamia* is not the cause of winter mortality (Carnegie *et al.* 2014; Spiers *et al.* 2014), despite the fact that an organism similar to *Bonamia exitiosa* has been reported occasionally in SROs (Cochennec-Laureau *et al.* 2003; Carnegie *et al.* 2014; Spiers *et al.* 2014). A generic PCR for *Mikrocytos* spp. which was successful in identifying new *Mikrocytos* spp., *M. veneroides* and *M. donaxi* in the wedge clam, *Donax truculus,* also failed to yield any specific amplicons for this genus in any of the oysters tested.

In contrast, PCR revealed 100% prevalence of members of the *V. splendidus* cluster in oysters from all 3 submissions. Vibrios are common inhabitants of the marine environment so this high prevalence was not surprising, however; statistically higher loads of bacteria from this cluster (P<0.0001) were observed in oysters from the two submissions displaying lesions consistent with winter mortality, with the highest loads detected in Submission 5 which had the largest number of oysters with gross and histological signs of disease (Figure 4.8).

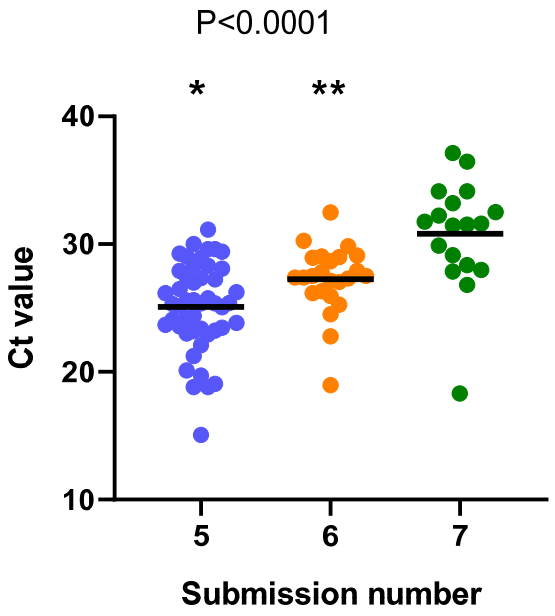

Figure 4.8 Statistically lower mean Ct values corresponding to a higher load of members of the *V. splendidus* cluster were detected in oysters from submissions meeting the winter mortality case definition (submissions 5 & 6). Asterisks indicate that the means statistically different at the level of P<0.0001.

4.3.7. Microbiome analysis of archived specimens

In addition to analysis of oyster samples submitted for winter mortality investigation during this project, we also undertook microbiome analysis on oysters collected during a longitudinal study of winter mortality in 2010 (Spiers *et al.* 2014). Oyster material archived at -80°C was subjected to Illumina sequencing using primers for the V1-V3 regions and phylogenetic diversity assessed using QIIME with SILVA as a reference database for assigning operational taxonomic units (OTUs).

While there was considerable variability in individual oyster microbiomes, a general pattern of loss of microbial species diversity in affected relative to unaffected oysters was observed (Figure 4.9).

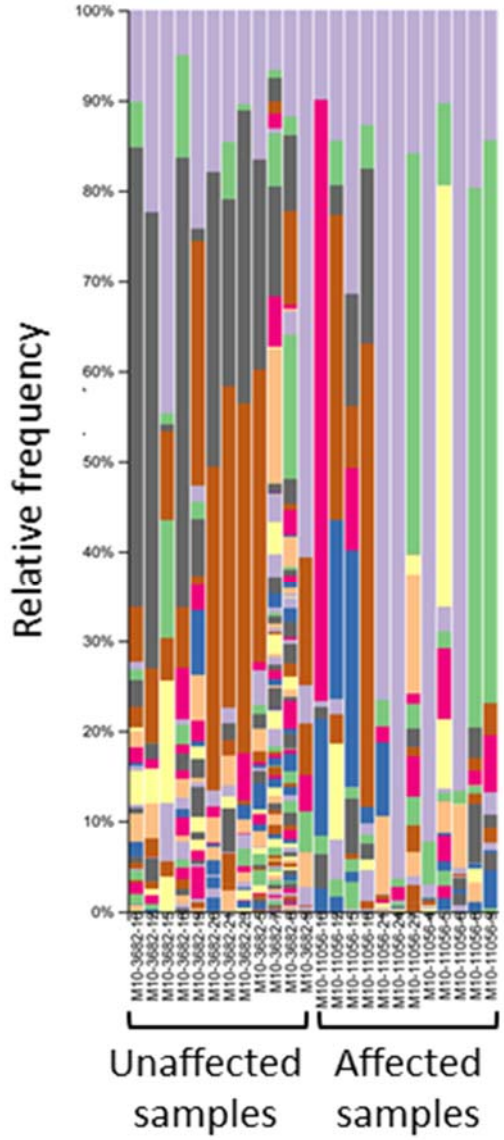

Figure 4.9 Differences in bacterial diversity (coloured bars) and relative frequency of OTUs (Y axis) detected in oysters from the longitudinal winter mortality study. Unaffected samples were collected before the onset of winter mortality season, while affected samples were collected during the peak period for winter mortality with disease confirmed in this cohort via histopathology (Spiers *et al.* 2014).

Microbiome disruption (dysbiosis) is common in human and animals diseases. Loss of species diversity is one of the major hallmarks of dysbiosis, along with loss of key taxa and blooms of pathogenic species (Hooks and O'Malley 2017). In this study, oysters from the same cohort which had been deployed at the same lease and collected before and during a winter mortality event, were examined with respect to their

gut microbiome. There was a significant temporal factor in this experiment in that unaffected oysters were collected within 1 month of the commencement of the longitudinal study (March) and affected oysters were collected during the peak period for winter mortality (September). Thus, differences in microbial diversity could potentially be explained by lower water temperatures resulting in reduced microbial diversity in the water column during spring compared to early autumn. To address this issue, histopathological data collected during the longitudinal study, which showed the presence or absence of focal lesions typical of winter mortality, was used to examine how the microbiome of individual oysters compared within time points with respect to the severity of pathology.

During the 2010 study, the severity of pathological lesions was scored as - (no lesions), + (mild pathology) or ++ (severe pathology). When this data was analysed alongside the OTUs identified in the individual oyster microbiomes using principal component analysis, oysters with lesions clustered away from oysters without lesions, regardless of the time point during which they were collected (Figure 4.10). This suggests that disease was a factor in the observed microbiome shift.

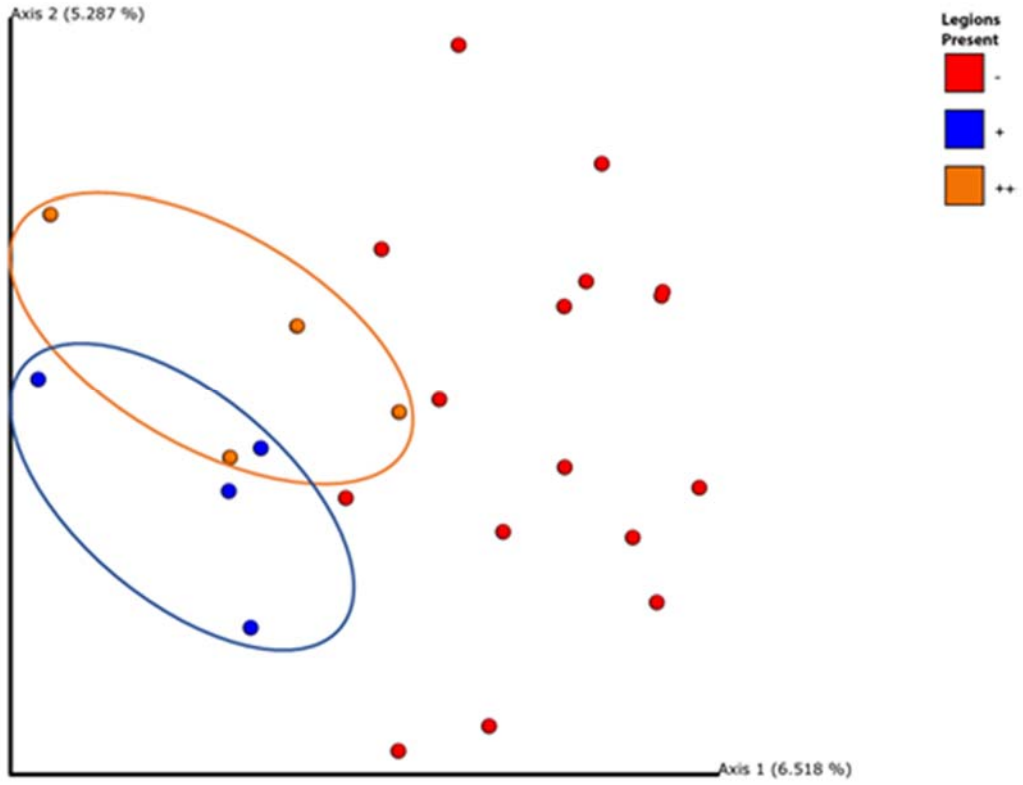

Figure 4.10 Plot from principal component analysis showing clustering of oysters with mild (blue +) or severe (orange ++) pathology away from those with no lesions based on histological examination.

Some clear differences in the prevalence and relative abundance of specific OTUs were also identified in the oysters from the longitudinal study and this is shown in the heatmap in Figure 4.11. Organisms belonging to the *Bradyrhizobium*, *Mesorhizobium* and *Arcobacter* genera increased during the winter mortality event. In contrast members of the *Helicobacter*, *Synechoccocus*, *Mycoplasma*, *Propionibacterium*, *Ilumatobacter*, *Vibrio* genera as well as some unidentified uncultured bacteria all decreased in abundance during the winter mortality event.

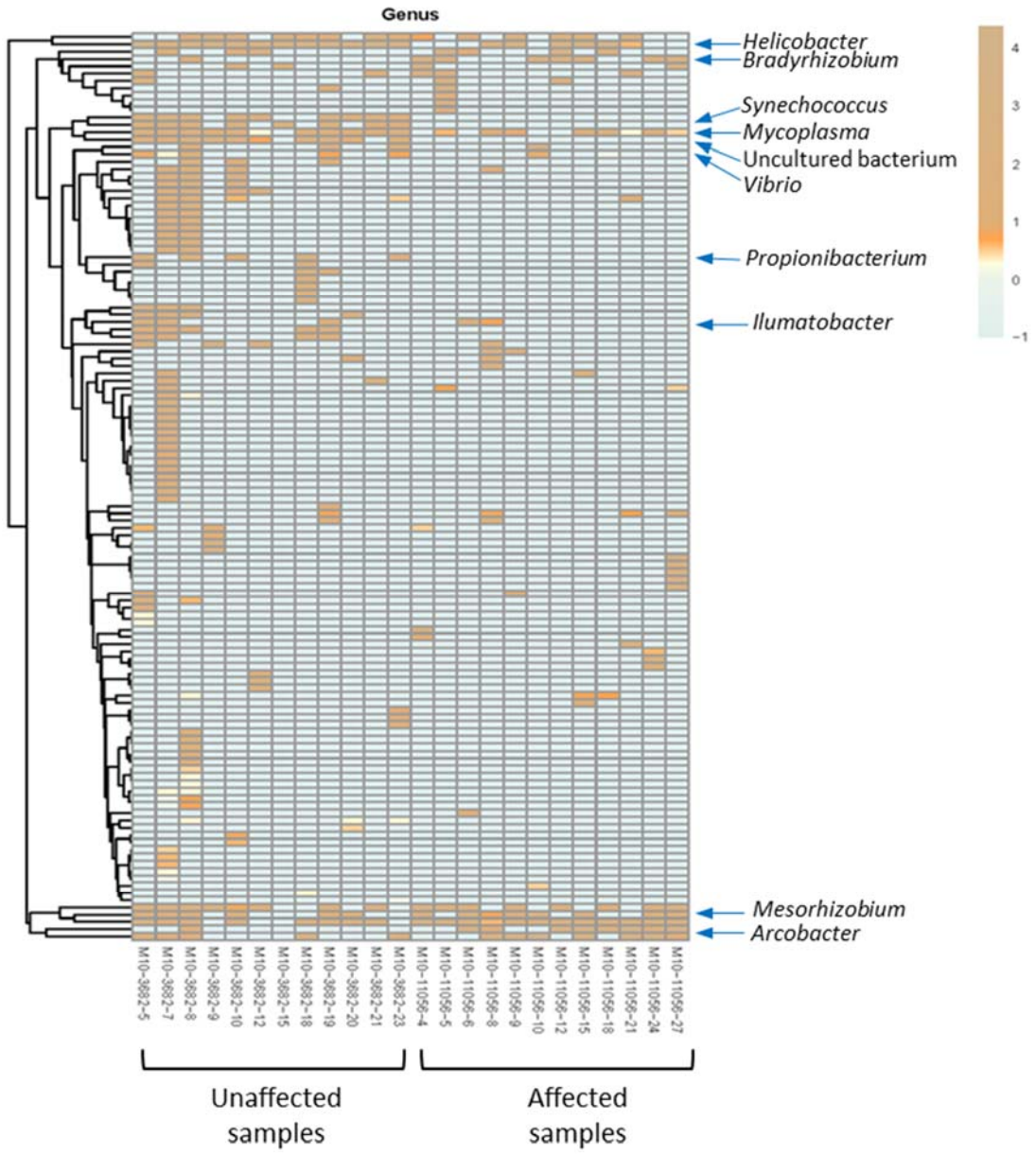

Figure 4.11 Heatmap showing the major OTUs identified in cohorts of oysters affected or unaffected by winter mortality.

4.4. Discussion

The aetiological agent of winter mortality has eluded identification since the first description of the disease in 1926, with both bacterial and parasitic causes posited. In this study we examined the two major parasites which have been linked to the disease ("Bonamia roughleyi" and *Mikrocytos roughleyi* as well as potential bacterial causes for winter mortality.

Several features of winter mortality are very similar to Denman Island disease of *C. gigas* caused by *Mikrocytos mackini*. Expression of disease by *M. mackini* requires extended exposure of oysters to cold temperatures and adult oysters in good condition are more prone to the disease. Similarly, the gross pathology associated with *M. mackini* infection, and indeed infection with other mikrocytids such as *M. mimicus* in *C. gigas* and *Paramikrocytos canceri* in the edible crab, *Cancer pagurus* (Hartikainen *et al.* 2014), is remarkably similar to that of winter mortality. The major gross sign of infection with these

parasites is the presence of yellow, green or brown spots or pustules, although these are not considered pathognomonic.

In infections with *Mikrocytos* spp., gross lesions correspond histologically to focal lesions with intense haemocyte infiltration and with microcells often within or at the periphery of those lesions (Farley *et al.* 1988; Hartikainen *et al.* 2014). In this study, microscopic lesions similar to those seen in *M. mackini* infection (Bower 2015) were observed. Microcells were not observed in oysters from submissions that did not meet the case definition for winter mortality, but were also relatively rare in the 2 submissions that did meet the case definition. Microcells were not abundant and were only observed in one oyster each from Submission 5 and Submission 6. While they were observed in association with focal haemocyte infiltrates, microcells were not found in oysters with gross lesions and were not apparent in tissue imprints made from affected tissue despite the fact that imprints made from pustules are a recommended method for detecting *Mikrocytos mackini* (OIE 2019).

Low prevalence of *Mikrocytos* is generally associated with a lack of gross pathology, such as with *M. boweri* (Abbott *et al*. 2014), raising the question of whether the microcells sometimes observed in winter mortality cases are the cause of the gross pathology and subsequent mortalities or whether they are merely an incidental finding and that there may be an entirely different cause. Indeed, mikrocytids favour cold conditions and their appearance during the Australian winter and spring months may simply be coincidental. Interestingly, neither T. C. Roughley (1926) nor Peter Wolf (1967) identified microcells in their examinations of winter mortality cases and instead suggested that the cause of the disease was likely bacterial. While microcell-like structures were clearly observed in Peter Wolf's material by Farley (1988), the material relied upon by Farley consisted of only 7 oysters in total and it is unclear whether the abundant microcell-like structures observed in his study were representative across affected specimens.

Owing to their small size, microcell infections can be difficult to detect and difficult to identify. The prior assertion that the aetiological agent of winter morality is actually a *Bonamia* species, "Bonamia roughleyi", was based on electron microscopic observations of a *Bonamia* parasite in SROs suffering winter mortality (Cochennec-Laureau *et al.* 2003). While the authors undoubtedly identified a *Bonamia* parasite within SROs, and in fact were able to use molecular methods to confirm the presence of *Bonamia* in the samples tested, there was no histological data linking the EM and molecular observations with winter mortality disease. More recent studies have suggested that *Bonamia* is unlikely to cause winter mortality based on PCR and in situ hybridisation data (Carnegie *et al.* 2014; Spiers *et al.* 2014); however this theory was worth revisiting in this study due to recent reports of *Bonamia ostreae* in the New Zealand oyster, *Ostrea chiliensis* (Lane *et al.* 2016). Prior to this study *Bonamia ostreae* was believed to be confined to the Northern Hemisphere. Like the microcell parasite that has been associated with winter mortality and which was observed in this study, *Bonamia ostreae* is very small in size (1-2µm) with an eccentric nucleus and resides within haemocytes. Nonetheless, molecular testing for members of the *Bonamia* genus undertaken in this study failed to identify this parasite in any samples tested including the 2 submissions that met the case definition for winter mortality.

Characterisation of the microcell-like parasite observed in this and prior studies of winter mortality should continue to be pursued to enable thorough examination of its epidemiology and relationship (if any) to disease. The microcell-like structures observed in 2 oysters from this study were consistent with those observed by Farley (1988), Spiers *et al* (2014) and Carnegie *et al*. (2014) in terms of their size and morphology (approx. 2µm in diameter with a large eccentric nucleus). Most mikrocytids are around 3µm in size with a large central nucleus, although *M. mimicus* has been described as having an eccentric nucleus (Hartikainen *et al.* 2014). Even if the microcell parasite observed in this study were a mikrocytid, the extreme sequence divergence observed amongst known members of this group highlights the need for less targeted and more general diagnostic approaches. While the PCR method used in this study has identified new *Mikrocytos* species in the past (Garcia *et al.* 2018), the parasite in SROs could perhaps represent a more divergent mikrocytid. A deep sequencing approach on material with a high intensity microcell infection is recommended to determine whether this parasite is a new species of mikrocytid or other uncharacterised microcell parasite.

The potential for bacterial involvement in winter mortality was considered in this study using both a microbiome approach, to better understand how the SRO bacterial flora might contribute to disease, and to re-examine the case for bacteria as the causative agent as postulated by both Roughley (1926) and Wolf (Wolf 1967). Two *Vibrio* strains from the *Vibrio splendidus* clade were isolated from purulent material aspirated from suppurative internal and external lesions from 2 separate oysters. The strains were isolated in apparent pure cultures from the lesions, with a non-dominant colony type from each oyster representing phenotypic variants of the same strain according to 16S rRNA sequencing. Further characterisation of the two strains was undertaken using a phylogenomics approach which indicated that the isolates were strains of the species *V. splendidus* within the *V. splendidus* clade. PCR testing of oysters for members of the *V. splendidus* cluster revealed a 100% prevalence in oysters collected between August and October from 2 different estuaries. While members of this clade were present in oysters that did not meet the case definition for winter mortality as well as submissions of oysters that did, loads of vibrio from the *V. splendidus* clade were significantly greater in affected oysters.

Members of the *V. splendidus* clade including the species *V. splendidus* are known pathogens of aquatic animals including bivalves (Travers *et al.* 2015). *Vibrio splendidus* is common in marine and estuarine waters and environmental factors such as temperature, salinity and nutrient levels are considered important drivers of pathogenicity and survival responses. *V. splendidus* is one of several *Vibrio* species known to enter a viable but nonculturable (VBNC) state under stress conditions, in which the cells remain viable but do not divide. In one study, 2 strains of *V. splendidus* were shown to enter a VBNC state in freshwater or brackish conditions but not under conditions of high salinity. A VBNC state could be induced at high salinity only when nutrient concentrations were also high (Armada *et al.* 2003). Thus, ideal conditions for *V. splendidus* survival appear to be those under which winter mortality disease appears (ie: high salinities and low rainfall).

Vibrio splendidus has been linked to outbreaks of summer mortality in *C. gigas* in Europe (Garnier *et al.* 2007), with high temperatures associated with *V. splendidus* infection in those cases. However *V. splendidus* strain JZ6 which is associated with festers disease of the Yesso scallop (*Patinopecten yessoensis*), shows highest virulence at 10°C, with pathogenicity significantly reducing with increasing temperature and being abolished completely at 28°C (Liu *et al.* 2013). Extracellular product of *V. splendidus* JZ6, containing a metalloprotease, has also been shown to be cytotoxic to oyster haemocytes (Liu *et al.* 2016). Transcriptomic analysis of *V. splendidus* JZ6 at 10°C and 28°C revealed that low temperatures enhanced adhesion, activated quorum sensing systems and stimulated virulence factor synthesis and secretion (Liu *et al.* 2016).

One interesting finding from this study is that the *V. splendidus* isolates from the winter mortality lesions contain a number of MGEs which could be linked to virulence. In particular, a scaffold from Isolate 1 displaying high sequence identity to plasmid pGV1512 was identified. This plasmid was described in *V. crassostreae*, another member of the *V. splendidus* clade which has been associated with pathogenesis in oysters. Notably, the pGV1512 plasmid was shown to be responsible for conferring virulence in *V. crassostreae* but was not essential for survival. The specific genes carried by the plasmid that are responsible for virulence appear to be novel with no homology to known virulence factor genes (Bruto *et al.* 2017). Whether the *V. splendidus* strains identified within winter mortality lesions in this study are primary or merely opportunistic pathogens remains uncertain.

Microbiome analysis of archived material from a longitudinal study of winter morality conducted in 2010 (Spiers *et al.* 2014) did not reveal an increase in members of the *Vibrionaceae* in winter mortality affected oysters, although the V1-V3 region of the 16S rRNA gene region examined in this study was not able to discriminate *Vibrio* to the species level due to the close phylogenetic relationships of members of this genus. It is well established that both virulent and non-virulent strains of *Vibrio* are present in oysters with virulent strains replacing non-virulent strains during disease outbreaks (Lemire *et al.* 2015), therefore sequencing of additional V regions may be required to provide further clarity on which strains were present. Another limitation of the microbiome study was that sections of the midgut were sampled for analysis on the basis that lesions in those oysters were found to be most prominent in the gut (Spiers *et al.* 2014); however specific sampling of lesions was not possible due to the use of

archived material where the gross appearance of the lesions was no longer apparent. Nonetheless the microbiome analysis revealed the presence of key OTUs which have been identified in other oyster microbiome studies. Notably, *Arcobacter* spp., which were found to be significantly associated with moribund *C. gigas* oysters in a previous study (Lokmer and Wegner 2015) were more abundant in winter mortality affected SROs in this study. Both *Ilumatobacter* and members of the *Bradyrhizobiaceae* are considered core members of the microbiomes of disease resistant *C. gigas* (King *et al.* 2019); however while *Ilumatobacter* spp. decreased during the winter mortality event, members of the *Bradyrhizobiaceace* increased. While there appear to be commonalities in the composition of key members of the *C. gigas* and *S. glomerata* microbiomes, it is currently unclear whether similar shifts in the microbiome occur during disease events.

4.5. References

Adlard RD, Lester RJG (1995) Development of a diagnostic test for *Mikrocytos roughleyi*, the aetiological agent of Australian winter mortality of the commercial rock oyster, *Saccostrea commercialis* (Iredale & Roughley). *Journal of Fish Diseases* 18, 609-614.

Armada SP, Farto R, Perez MJ, Nieto TP (2003) Effect of temperature, salinity and nutrient content on the survival responses of *Vibrio splendidus* biotype I. *Microbiology-Sgm* 149, 369-375.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37, 852-857.

Bower SM (2015) Synopsis of infectious diseases and parasites of commercially exploited shellfish: *Mikrocytos* spp. of oysters Accessed: 27 August 2019. URL: http://www.dfo-mpo.gc.ca/science/aah-saa/diseases-maladies/mikmacoy-eng.html

Bruto M, James A, Petton B, Labreuche Y, Chenivesse S, Alunno-Bruscia M, Polz MF, Le Roux F (2017) *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J* 11, 1043-1052.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13, 581-3.

Carnegie RB, Hill KM, Stokes NA, Burreson EM (2014) The haplosporidian *Bonamia exitiosa* is present in Australia, but the identity of the parasite described as *Bonamia* (formerly *Mikrocytos*) *roughleyi* is uncertain. *J Invertebr Pathol* 115, 33-40.

Cochennec-Laureau N, Reece KS, Berthe F, Hine PM (2003) *Mikrocytos roughleyi* taxonomic affiliation leads to the genus *Bonamia* (Haplosporidia). *Dis Aquat Organ* 54, 209-217.

Darling AE, Jospin G, Lowe E, Matsen FAt, Bik HM, Eisen JA (2014) PhyloSift: phylogenetic analysis of genomes and metagenomes. PeerJ 2, e243.

Darling AE, Mau B, Perna NT (2010) progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5, e11147.

Farley CA, Wolf PH, Elston RA (1988) A long-term study of "microcell" disease in oysters with a description of a new genus, *Mikrocytos* (g. n.), and two new species, *Mikrocytos mackini* (sp. n.) and *Mikrocytos roughleyi* (sp. n.). *Fishery Bull* 86, 581-593.

Gagne N, Cochennec N, Stephenson M, McGladdery S, Meyer GR, Bower SM (2008) First report of a *Mikrocytos*-like parasite in European oysters *Ostrea edulis* from Canada after transport and quarantine in France. *Dis Aquat Organ* 80, 27-35.

Garcia C, Haond C, Chollet B, Nerac M, Omnes E, Joly JP, Dubreuil C, Serpin D, Langlade A, Le Gal D, Terre-Terrillon A, Courtois O, Guichard B, Arzul I (2018) Descriptions of *Mikrocytos veneroides* n. sp and *Mikrocytos donaxi* n. sp (Ascetosporea: Mikrocytida: Mikrocytiidae), detected during important mortality events of the wedge clam *Donax trunculus* Linnaeus (Veneroida: Donacidae), in France between 2008 and 2011. *Parasites & Vectors* 11.

Garnier M, Labreuche Y, Garcia C, Robert A, Nicolas JL (2007) Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific Oyster Crassostrea gigas. *Microbial Ecology* 53, 187-196.

Gauthier JD, Miller CR, Wilbur AE (2006) Taqman (R) MGB real-time PCR approach to quantification of *Perkinsus marinus* and *Perkinsus* spp. in oysters. *Journal of Shellfish Research* 25, 619-624.

Hartikainen H, Stentiford Grant D, Bateman Kelly S, Berney C, Feist Stephen W, Longshaw M, Okamura B, Stone D, Ward G, Wood C, Bass D (2014) Mikrocytids Are a Broadly Distributed and Divergent Radiation of Parasites in Aquatic Invertebrates. *Current Biology* 24, 807-812.

Hine PM, Bower SM, Meyer GR, Cochennec-Laureau N, Berthe F (2001) The ultrastructure of *Mikrocytos mackini*, the cause of Denman Island disease in oysters *Crassostrea* spp. and *Ostrea* spp. in British Columbia, Canada. *Dis Aquat Organ* 45, 215-227.

Hooks KB, O'Malley MA (2017) Dysbiosis and Its Discontents. *mBio* 8, e01492-17.

King WL, Siboni N, Williams NLR, Kahlke T, Nguyen KV, Jenkins C, Dove M, O'Connor W, Seymour JR, Labbate M (2019) Variability in the Composition of Pacific Oyster Microbiomes Across Oyster Families Exhibiting Different Levels of Susceptibility to OsHV-1 mu var Disease. *Frontiers in Microbiology* 10.

Krawczyk PS, Lipinski L, Dziembowski A (2018) PlasFlow: predicting plasmid sequences in metagenomic data using genome signatures. *Nucleic Acids Res* 46, e35.

Lane HS, Webb SC, Duncan J (2016) *Bonamia ostreae* in the New Zealand oyster *Ostrea chiliensis*: a new host and geographic record for this haplosporidian parasite. *Dis Aquat Organ* 118, 55-63.

Lemire A, Goudenege D, Versigny T, Petton B, Calteau A, Labreuche Y, Le Roux F (2015) Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *Isme Journal* 9, 1523-1531.

Liu R, Chen H, Zhang R, Zhou Z, Hou ZH, Gao DH, Zhang H, Wang LL, Song LS (2016) Comparative Transcriptome Analysis of *Vibrio splendidus* JZ6 Reveals the Mechanism of Its Pathogenicity at Low Temperatures. *Applied and Environmental Microbiology* 82, 2050-2061.

Liu R, Qiu LM, Yu ZA, Zi J, Yue F, Wang LL, Zhang H, Teng WM, Liu XF, Song LS (2013) Identification and characterisation of pathogenic *Vibrio splendidus* from Yesso scallop (*Patinopecten yessoensis*) cultured in a low temperature environment. *Journal of Invertebrate Pathology* 114, 144-150.

Lokmer A, Wegner KM (2015) Hemolymph microbiome of Pacific Oysters in response to temperature, temperature stress and infection. *Isme Journal* 9, 670-682.

Marty GD, Bower SM, Clarke KR, Meyer G, Lowe G, Osborn AL, Chow EP, Hannah H, Byrne S, Sojonky K, Robinson JH (2006) Histopathology and a real-time PCR assay for detection of *Bonamia ostreae* in *Ostrea edulis* cultured in western Canada. *Aquaculture* 261, 33-42.

Mattei V, Murugesan S, Al Hashmi M, Mathew R, James N, Singh P, Kumar M, Lakshmanan AP, Terranegra A, Al Khodor S, Tomei S (2019) Evaluation of Methods for the Extraction of Microbial DNA From Vaginal Swabs Used for Microbiome Studies. *Front Cell Infect Microbiol* 9, 197.

OIE (2019) Infection with *Mikrocytos mackini*. Manual of Diagnostic Tests for Aquatic Animals.

Price MN, Dehal PS, Arkin AP (2009) FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol* 26, 1641-50.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41, D590-D596.

Roughley TC (1926) An investigation of the cause of an oyster mortality on the Georges River, New South Wales, 1924-5. *Proc Linn Soc NSW* 51, 446-491.

Saulnier D, De Decker S, Tourbiez D, Travers MA (2017) Development of a duplex Taqman real-time PCR assay for rapid identification of *Vibrio splendidus*-related and *V. aestuarianus* strains from bacterial cultures. *J Microbiol Methods* 140, 67-69.

Spiers ZB, Gabor M, Fell SA, Carnegie RB, Dove M, O'Connor W, Frances J, Go J, Marsh IB, Jenkins C (2014) Longitudinal study of winter mortality disease in Sydney rock oysters *Saccostrea glomerata*. *Diseases of Aquatic Organisms* 110, 151-164.

Travers MA, Miller KB, Roque A, Friedman CS (2015) Bacterial diseases in marine bivalves. *Journal of Invertebrate Pathology* 131, 11-31.

Wolf PH (1967) Winter mortality hits state's oyster output. *Fisherman* 2, 20-22.

Chapter 5 Conclusions

Testing for OsHV-1 in eDNA using the plankton sampling and qPCR testing approach outlined in Chapter 1 is effective. Compared to testing infected tissues from Pacific Oysters, testing eDNA from plankton with qPCR has lower diagnostic sensitivity, but specificity is high and the eDNA method is an appropriate approach where sentinels cannot be deployed or where suitable oysters to sample cannot be accessed. Despite the lower diagnostic sensitivity (likelihood of detection in a sample) of testing plankton in comparison to tissue, high (0.95) survey sensitivity (likelihood of detection in at least one sample) for detection of a prevalence of <0.1, can be achieved by collection of only a moderate number of samples (~50), making the method feasible to apply , The method facilitates assessment of viral load in water and the risk posed by ballast water uptake and discharge, and is a non-destructive method that could be applied in farming areas to complement other surveillance strategies.

Flow cytometry is a rapid, inexpensive and quantitative test for the presence of OsHV-1. The flow cytometer used for this project, the Merck Muse Cell Analyser, is simple to use, portable and inexpensive. Initial testing of the capacity of the Muse cytometer to detect small particles such as viruses and heterotrophic bacteria was successful, producing abundance values comparable to values recorded on expensive benchtop flow cytometers. The development of custom made OsHV-1 specific primer probes that fluoresce only when bound to OsHV-1 nucleic acid was achieved and when analysed on the Muse cytometer exhibited a clear signal for the presence of OsHV-1 using standardised protocols for detection and enumeration. Guidelines for the interpretation of results have been established. At this point the interpretation guidelines are based on field samples brought into the laboratory. When the Muse cytometer is taken into the field those interpretations may need modification. During this process we have worked with industry and been approached by industry members to develop the OsHV-1-Muse system for use in commercial settings. The developed test for OsHV-1 detection and enumeration will be invaluable to Pacific Oyster farms and hatcheries and enable them to monitor and measure the virus to make informed decisions on the management of their produce. Overall, the development has been successful and the objectives of the project have been met.

Results obtained from the industry survey have revealed a number of key issues that are present in the South Australian oyster industry with regards to SAMS. Results indicated a varied understanding by producers of the definition of SAMS. Only a minority of producers mentioned key aspects of the 2013 case definition (Madin, *et al.* 2013). Unexplained mortalities appear to be a significant issue for oyster producers in South Australia, with 50% of the participants admitting to experiencing ‘SAMS’ on their leases. Investigators electronically communicated with participants who stated a willingness to provide samples to this project and stressed the importance of sample submissions for diagnostic testing to enable patterns of disease to be observed. Results from sample submissions can be found in the diagnostic chapter of this study. The third key issue revealed by the survey, was the marked dissociation between producers accepted mortality rates and actual mortality rates on farm. The majority of participants were experiencing unacceptable rates of mortality. As recommended previously, further effort in surveillance is mandatory to obtain a definitive picture of SAMS. With larger sample numbers comprehensive dataset can be created and utilised to determine relevant patterns of change that can then be associated to current diseases as well as for new syndromes that may occur. This approach will assist the industry to monitor overall oyster health as well as provide improved and efficient identification of triggers associated with disease occurrences such as SAMS.

Chapter 4 provides an updated disease definition for winter mortality disease of Sydney rock oysters which should assist oyster growers in recognising the disease and be used to guide future diagnostic efforts. Parasitic organisms that have historically been associated with winter mortality such as *Bonamia* and *Mikrocytos* spp. were not detected by molecular methods and microcells were observed rarely in diseased cohorts of oysters. There were significant differences in microbiome composition and an increased load of *Vibrio splendidus* in winter mortality affected oysters when compared to those that did not meet the winter mortality case definition. *Vibrio splendidus* is a known oyster

pathogen and a strain isolated in pure culture from winter mortality lesions in this study contains a plasmid which confers virulence to oysters in *V. crassostreae*. Other features of *V. splendidus* may be consistent with winter mortality disease, including cold-induced virulence in some strains; therefore a role for this organism as a primary or secondary agent in winter mortality disease warrants further investigation.

5.1. Implications

Surveillance for OsHV-1 using qPCR testing of oyster tissue or plankton is better understood and can be planned with better epidemiological certainty. In particular, the DSe and DSp data support proof-of-freedom programs, translocation health assessment and analysis of surveillance data.

A flow cytometry method for detecting POMS in seawater and oyster tissue has been developed. The method facilitates detection of OsHV-1 to 5 OsHV-1 particles per ml. The portability and ruggedness of the Muse flow cytometer used in this project means that operation and OsHV-1 detection and quantification can occur in sheds or on leases. The low cost of approximately $2 per sample combined with the 1-hour preparation, with sequential samples being approximately 3 minutes apart, means that changes in OsHV-1 concentration can be tracked over the course of a day. This will permit improved management in endemic areas, and improved research outcomes because detection is precise, in real time and quantitative. The overall implication is that the project has developed the ability for scientists, government agencies and growers to quantitatively measure OsHV-1. This has laid the groundwork for the rapid development of detection of other oyster and aquaculture pathogens. This novel flow cytometry test has the potential to be more sensitive that current PCR techniques. Some PCR methods have a lower detection limit of ~500 OsHV-1 particles per ml, while our flow cytometry method provides a lower detection limit of 5 OsHV-1 particles per ml. This indicates that the flow cytometry method may have analytical sensitivity up to 2 orders of magnitude more sensitive that standard PCR methods. This flow cytometric method combined with current PCR methods will allow future studies to measure how long OsHV-1 survives in the environment, by using PCR to measure OsHV-1 DNA and flow cytometry to measure the number of viral particles and assess what proportion of them are complete, it will be possible to more accurately estimate OsHV-1 survival in the ocean.

The case definition for SAMS remains unclear and should be either redefined or abandoned as an entity and replaced by an approach for the investigation of all un-explained mortalities of Pacific Oysters in commercial production. The impact of mortalities is having a significant impact on grow out and requires an industry approach for understanding any possible causes. Improved diagnostic tools (i.e Oyster haemolymph analysis) have been developed but there is need for a protocol approach for the identification of *Vibrio* spp associated with Pacific Oysters. All cases of un-explained mortalities in Pacific Oysters should be thoroughly investigated which will require funding. This funding can be on a farm by farm basis instigated by lease holders requesting diagnostic services or it can be a holistic industry funded program. There is a need for a comprehensive coordinated approach to farm data collection, diagnostic investigations and case management. There is a need to link pathologies and other diagnostic findings to other factors for improved response capabilities by farmers i.e. trigger point management. There were unique pathologies observed in the investigated cases in this project, which should be characterised more fully.

For winter mortality, the case definition outlined in this report will help to inform oyster growers and diagnosticians with respect to the relevant features of this disease including environmental factors (high salinity), host factors (age ≥1.5 years), gross signs (visible surface pustules in a proportion of affected oysters) and histological features (abscessation characterised by intense foci of haemocytic infiltrates). Histology remains the method of choice for disease diagnosis given that the presence of previously suspected parasitic agents “Bonamia roughleyi” and *Mikrocytos roughleyi* could not be demonstrated with molecular methods in association with gross or histological features of the disease. Diseased oysters displayed a significant increase in the load of the known oyster pathogen, *Vibrio*

splendidus. This organism may represent a primary or secondary agent of the disease given the presence of a virulence plasmid in an isolate from winter mortality lesions and the propensity of cold temperatures to induce virulence in some strains of this organism. However, more work is required to confirm the significance of this organism in disease before detection of this organism could be considered a marker for disease.

5.2. Recommendations

The data developed about tests for OsHV-1 from both oyster and plankton samples should be incorporated into surveillance programs.

For the flow cytometry test: a series of workshops to demonstrate the method for relevant industry members should be developed; a trial program should be implemented where an industry member can have the machine in their facility for up to a week as a test; acquisition, training, set up and calibration for those that wish to adopt the test should be arranged; incorporating the technique into an accessible service should be examined. This technique should be considered for other aquaculture pathogens that can be detected as eDNA in seawater or in tissue.

For SAMS, the following recommendations are given in no order of priority:

1. An assessment of the extent and economic impact of un-explained mortalities should be undertaken for industry to guide the investment for further investigating Pacific Oyster health issues.
2. A holistic approach for the investigation of un-explained mortalities should be developed for the South Australian oyster industry which includes cases which currently fit into the case definition of SAMS. This means an approach to reporting (at least maintenance of existing protocols and further development), submission of diagnostic samples including sample selection and transport and correlation of data and reporting.
3. On farm education and extension for improved health surveillance and sample collection.
4. Assess industries engagement and requirement for investigative services and support for oyster health (i.e. Government, research and service providers)
5. Priority setting for funded research for investigations related to un-explained mortalities.

Surveillance for winter mortality in SRO should be ongoing, with particular attention to the presence of pathogenic *V. splendidus* in winter mortality cases.

5.2.1. Further development

Environmental surveillance would be improved by developing enhanced confirmatory tools that can provide the sensitivity of qPCR but also produce sequence data. An understanding of the behaviour of assays for pathogens in the complex matrix presented by eDNA should be developed using spiking experiments and assessment of PCR amplification efficiency for that matrix. For oyster surveillance analysing autocorrelation between test results would provide an understanding of approaches to maximise OsHV-1 surveillance sensitivity. Developing such high sensitivity approaches would benefit disease management in situations where risk is created by subclinically affected animals, including assessment of applications to translocate stock from hatcheries in infected areas where broodstock may have been exposed to, or infected with, OsHV-1. The feasibility of applying molecular tests for environmental detection of pathogens requires an understanding of capture efficiency and occurrence of the target in plankton (‘prevalence’ in the environment).

For the flow cytometry test refinements could include: optimizing the processing time, reducing the cost (included in a current Honours thesis inspired by this CRC-P project), developing the technique for other oyster and aquaculture pathogens, benchmark this flow cytometry method against other

OsHV-1 detection and measurement methods, processing a large number of samples to validate the flow cytometry method against a completed PCR data set of the same samples.

For winter mortality, further investigation is required to determine the role of *V. splendidus* in winter mortality including passive surveillance, and experimental infection assays. It is recommended that the microcell parasite sometimes seen in association with winter mortality cases be identified to definitively rule in or rule out involvement of this organism in disease. This could be achieved using a deep sequencing approach on material containing a high intensity infection which would help to facilitate the development of suitable diagnostic tools for this organism.

5.3. Extension and Adoption

The project was communicated to industry as it progressed in the following fora:

Oysters Australia presentations

Deveney, M.R. 2017. Advanced surveillance for OsHV-1. Oysters Australia Meeting. Adelaide, Australia 12 April 2017.

Deveney, M.R. 2018. Advanced surveillance for OsHV-1. Oysters Australia Research and Development Meeting. Sydney, Australia 9 April 2018.

Paterson, J.S., Sapula, S., Dann, L.M., Mitchell, J.G., 2018. Rapid POMS development, detection and enumeration using flow cytometry. Oysters Australia Research and Development Meeting. Sydney, Australia.

State based industry presentations

Deveney, M.R. 2016. Advanced surveillance for OsHV-1. South Australian Oyster Industry Seminars:

- Friday 8th July Smoky Bay
- Wed 13th July Coffin bay
- Friday 15th July Cowell
- Friday 22nd July Stansbury

Buss, J.J. 2017. Update on advanced surveillance for OsHV-1. South Australian Oyster Industry Seminar. Coffin Bay, South Australia, 4 August.

Deveney, M.R. 2018. Advanced surveillance for OsHV-1. South Australian Oyster Industry Seminar. Smoky Bay, South Australia, 24 August.

Deveney, M.R. 2019. Advanced surveillance for OsHV-1. South Australian Oyster Industry Seminar. Streaky Bay, South Australia, 23 August.

Paterson, J.S., Sapula, S., Dann, L.M., McIvor, C., Mitchell, J.G., 2019. Rapid POMS development, detection and enumeration using flow cytometry. South Australian Oyster Industry Seminar. Streaky Bay, South Australia.

Data on winter mortality was communicated at the NSW Oyster Industry Biennial Conference (Forster, August 2019). Outcomes will also be submitted for peer review publication for dissemination amongst the scientific community.

Findings of the SAMS project were presented industry members at the SAOGA/SAORC meeting at Streaky Bay in August 2019.

In consultation with industry members from Smoky Bay the format of a farmers oyster health and sampling workshop is under development for delivery later in 2019

Scientific conferences

Deveney, M.R., Perera, R., Jones J.B. January 2016. Aquatic disease biosecurity: moving away from an approach based on pathogens. International Conference on marine Bioinvasions, Sydney, Australia.

Deveney, M.R., Wiltshire, K.H., Ellard K.A. July 2017. Biofouling as a long-distance vector for pathogens. Australasian Aquatic Animal Health and Biosecurity Conference, Cairns.

Deveney, M.R., Wiltshire, K.H., Buss, J.J., Lieu, Y.N., Harris, J.O., Speck P., Yeadon, P.J., Ellard, K.A., Mohr P.G., Moody, N.J.G., Bansemer M.S., Roberts S.D. July 2019. OsHV-1 microvariant in the Port River, South Australia: an unnatural history. Australasian Aquatic Animal Health and Biosecurity Conference, Cairns.

Deveney, M.R., Wiltshire, K.H., Giblot-Ducray, D., Roberts S.D., Moody, N., Crane, M., Ellard, K.A., Livore, J.P. October 2018. Disease translocation in a connected world: the anatomy of a mollusc disease outbreak. International Conference on marine Bioinvasions, Puerto Madryn, Argentina.

Publications

None submitted.

Other presentations

- James Paterson and Jim Mitchell presented findings to Mr Mori, President of the World Oyster Industry, in October 2018 who showed strong interest in the test.

5.3.1. Project coverage

- James Paterson and Lisa Dann gave an interview to ABC Eyre Peninsula at the SAOGA Annual Seminar in August 2018 regarding the flow cytometry test and the potential use in the future.

- Short video on the Oysters Australia website published on 27th February 2019: https://www.oystersaustralia.org/post/video-rapid-poms-development-detection-and-enumeration-using-flow-cytometry