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Toxigenic *Vibrio* baselines and optimum storage, transport and shelf-life conditions to inform cold supply chains in the north Australian Tropical Rock Oyster industry

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Toxigenic *Vibrio* baselines and optimum storage, transport and shelf-life conditions to inform cold supply chains in the north Australian Tropical Rock Oyster industry

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Contents

Tables

Figures

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Abbreviations

ASQAAC	Australian Shellfish Quality Assurance Committee
ASQAP	Australian Shellfish Quality Assurance Program
ASVs	Amplicon sequence variants
BRO	Blacklip Rock Oyster
CAP	Canonical analysis of principal coordinates
CDC	Centres for Disease Control
CDU	Charles Darwin University
CFU	Colony forming units
CI	Condition index
DO	Dissolved oxygen
DoH	Department of Health (NT)
DPIRD	Department of Primary Industries and Regional Development
FRDC	Fisheries Research and Development Corporation
FSANZ	Food Standards Australia New Zealand
GAMs	Generalized additive models
Hsp60	Heat shock protein 60
IMAS	Institute for Marine and Antarctic Studies
MLST	Multi-locus sequence typing
MPN	Most-Probable-Number
NSSP	National Shellfish Sanitation Program
PERMANOVA	Permutational multivariate analysis of variance
PIRSA	Department of Primary Industries and Regions
PO	Pacific Oysters
qPCR	Quantitative PCR
SAOGA	South Australian Oyster Growers Association
SARDI	South Australian Research and Development Institute
SD	Standard deviation

SIMPER	Similarity Percentages - species contributions
SRA	Sequence Read Archive
SRO	Sydney Rock Oysters
SVs	Sequence Variants
TDH	Thermostable Direct Haemolysin
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorus
TRH	*trh* gene, linked to TDH related haemolysin
TRO	Tropical rock oysters
TSB	Tryptic soy broth
TVC	Total Viable Counts
UTas	University of Tasmania
UTS	University of Technology Sydney
VBNC	Viable but non-culturable cells
WGS	Whole Genome Sequencing
YAC	Yagbani Aboriginal Corporation

1 Executive Summary

1.1 **Background**

Internationally, tropical rock oysters (TROs) have a poor safety reputation and a pro-active rather than reactive approach to Vibrio food safety is essential for product assurance and branding. Further, risk assessment needs to be informed by real data to ensure appropriate and proportional responses. There are certainly knowledge gaps for north Australia, but from previous work, seawater in Darwin Harbour was shown to contain up to 42 Vibrio spp. including several known toxigenic species in addition to the human pathogens Vibrio parahaemolyticus and V. vulnificus. Studies in the seasonal tropics (Darwin Harbour) showed that V. parahaemolyticus responds to temperature, despite being in the tropics where seawater temperatures are consistently high. V. vulnificus concentrations in seawater are higher in the wet season compared to the dry, and more shellfish are positive for V. parahaemolyticus and V. vulnificus in the wet season compared to the dry season. So if Vibrio diversity and abundance in TRO is seasonal (as shown elsewhere), it is likely that Vibrio spp. infections in humans will also follow a seasonal trend which has implications for risk management. A major bottleneck is that we do not know how vibrio numbers are affected by storage and transport temperatures in TRO. Studies have shown that Pacific and Sydney Rock Oysters have different vibrio growth curves for example, so it is not one size fits all and it is probable TROs will be different again. In addition to identifying Vibrio baselines in TRO and developing tests for toxigenic species, we will identify the best post-harvest storage and transport temperatures and assess TRO shelf life at realistic storage temperatures. This will provide fundamental information to inform cold supply chains that will support farmers, wholesalers and retailers of TROs from northern Australia. We will also use this information to prepare an appropriate and regionally relevant Vibrio risk profile for TRO in northern Australia to assist initial risk management activities. The work described in this report will provide the developing TRO industry with the knowledge needed to make informed decisions about *Vibrio* in particular, and food risk more generally, and help ensure an exemplary reputation with access to premium markets.

1.2 Aims/objectives

The objectives of this study were to:

- Measure *Vibrio* baselines (community diversity and relative abundance) in TROs both spatially and temporally, and optimise/develop tests for *vibrio* species that are toxigenic to oysters and humans.
- Measure *V. parahaemolyticus* growth in TROs to identify optimum storage and transport temperatures.
- Assess TRO shelf life at realistic storage temperatures to maximise product quality and inform cold supply chains.
- Use the knowledge gained in the previous objectives to produce a risk profile for *Vibrio* in northern Australia TRO that will support the industry as it seeks to deliver a safe, premium product.

1.3 Methodology

1.3.1 *Vibrio* spatial baselines in TROs

To understand what *Vibrio* and other bacteria are present in TRO across northern Australia we characterised the bacterial community in healthy wild and farmed TROs covering a 3,500 km

longitudinal and 1,027 km latitudinal expanse across remote northern Australia. In this spatial study, both Blacklip rock oysters (BROs) (*Saccostrea echinata*/lineage J) and Milky oysters (*Saccostrea mordax*/lineage A) were sampled, farmed and wild, across multiple locations. BROs were the primary target species, but Milky oysters were also collected when BROs were not present in the wild, and because small numbers of Milky oysters are being farmed in some regions.

1.3.2 *Vibrio* temporal baselines in BROs

To understand how the *Vibrio* community changes in BROs over time, BROs were collected from a farm on South Goulburn Island on six occasions during 2021/22. We used high throughput amplicon sequencing to identify bacterial taxa and *Vibrio* species in these oysters, including potential human or oyster pathogens, and their relative abundance. We also quantified *V. parahaemolyticus* and *V. vulnificus* in a subset of wild oysters by MPN-qPCR. To gain insights into risk associated with virulence strains, we tested for virulence genes *trh* and *tdh* in samples that were positive for *V. parahaemolyticus.*

1.3.3 Storage temperatures – implications for *V. parahaemolyticus* growth and shelf life

To measure growth of *V. parahaemolyticus* in BROs*,* local strains of the bacteria previously isolated from BROs and Milky oysters were used as the inoculum. BROs obtained from a commercial farm in Bowen (Qld Australia) were injected and growth measured at 4°C, 13°C, 18°C and 25°C. To measure growth, oyster meat and liquor was homogenized and serial dilutions of the homogenate were plated and colony forming units (CFU) per gram oyster homogenate calculated. Growth rates ($\log_{10}$ CFU/h) were calculated from best fit lines and a negative binomial generalized linear model was used to assess whether *V. parahaemolyticus* growth varied significantly at different temperatures. To assess shelf-life, live BROs sent from Bowen at 18°C were held at 4°C, 13°C, 18°C or 25°C. BROs were assessed on multiple days and tested for gaping, condition and spoilage microbes. Sensory quality of the oysters was assessed by six panellists using an oyster quality guide. Panellist were asked to assess odour, body colour and appearance, liquor clarity, texture, and appearance of the mantle, gills and adductor muscle. The panellists were also asked if they considered the oysters fit for sale or consumption.

1.3.4 Risk profile for *Vibrio* spp. in TROs

The risk profile was compiled following CODEX risk assessment guidelines of hazard analysis. Uncertainties were identified and considered during the evaluation of risk and knowledge gaps were identified. Standard web search engines were used to identify information on pathogenic *Vibrio* spp. globally and TRO production in Australia. Results from studies undertaken in this FRDC project 2020-043 were used to inform this risk profile. Records of illness from non-choleragenic vibriosis were requested from the health departments in WA, NT, and Qld, after obtaining the appropriate ethics approvals where necessary.

1.4 Results/key findings

1.4.1 *Vibrio* spatial baselines in TROs

Bacterial composition significantly differed by sites but not by oyster species suggesting a geographical microbial signature. Forty-eight *Vibrio* species were identified in oyster tissue and the most abundant *Vibrio* species (relative abundance) across all oyster samples and sites were *V. parahaemolyticus* (18%), *V. harveyi* (10%), *V. diabolicus* (9%), *V. alginolyticus* (7%), and *V. mediterranei* (6%). The oyster *Vibrio* community was significantly different within and between sites suggesting it is likely influenced by specific features of their surrounding environment. Apart from *V. parahaemolyticus*, other known potential human pathogenic species were *V. vulnificus*, *V. alginolyticus*, and *V. fluvialis.*

V. parahaemolyticus was detected in 77% of oysters with most samples in the range 3-100 MPN/g and 22% with levels >1,110 MPN/g, but virulence genes *trh* or *tdh* were not detected. *V. vulnificus* was detected in 31% of oyster samples, with 10% of samples having levels >100 MPN/g. Eleven potential oyster *Vibrio* pathogens were identified with *V. harveyi* and *V. alginolyticus* the most abundant.

1.4.2 *Vibrio* temporal baselines in BROs

Thirty-five *Vibrio* species were identified in water and oysters, and *Vibrio* abundance and diversity changed over time in both water and oysters. *Vibrio* diversity in water was dissimilar to oysters. In the wet season, the *Vibrio* community in oysters and water was more variable than in the dry season but overall, the *Vibrio* community in water was more variable than that in oysters. While no physicochemical variables were significantly correlated to *Vibrio* abundance in water, turbidity contributed to shaping the wet season *Vibrio* water community, and water temperature and salinity contributed to shaping the dry season *Vibrio* water community. The oyster *Vibrio* community was significantly correlated to turbidity, temperature and total nitrogen. *V. parahaemolyticus* was detected in some wet season oyster samples but rarely in water, whereas *V. alginolyticus* occurred in the dry season, and *V. harveyi* and *V. campbellii* were abundant in oysters and water year-round.

Our results showed that at this location, the water *Vibrio* community diversity is not a surrogate for the oyster *Vibrio* community. This has implications for routine surveillance because detection in water is more sensitive, less technologically demanding and more cost effective than for oysters. This result however does not rule out water as a surrogate for particular species. In future studies, we will build on these results by measuring whether pathogens such as *V. parahaemolyticus* and *V. harveyi* in water are associated with levels in oysters, and whether there are associations between season/rain events and the detection of virulence genes.

1.4.3 Storage temperatures – implications for *V. parahaemolyticus* growth and shelf life

We showed that storage of BROs at 4°C will prevent growth of *V. parahaemolyticus*, unlike at 13°C and higher temperatures. Although there was originally concern that very low temperature storage would have a detrimental effect on shelf life, given they are a tropical species, in fact those stored at 4°C were considered more favorable based on texture and appearance. Although oyster quality attributes declined slightly throughout the storage trial at all storage temperatures, the independent seafood processor's assessment was that BROs held at all storage temperatures were suitable for sale/consumption.

1.4.4 Risk profile for *Vibrio* spp. in TROs

This risk profile highlighted the following:

- Vibriosis is a common risk associated with bivalve shellfish world-wide.
- Potentially pathogenic *Vibrio* spp. have been found in northern Australia in sediments, water, and biota (including seafood).
- Locally acquired illnesses (wound and food) have been reported from WA, NT, and Qld. Where illness rates were able to be calculated they were similar to those found in the USA (temperate oyster species), where vibriosis is a serious consideration for public health officials.
- The above data lead us to conclude that there is a credible risk for aquaculture across northern Australia.

The conclusion from the risk profile was that the TRO industry should undertake active risk management to mitigate the risk of human illness and market incidents. Post-harvest cooling and maintenance of the cool chain during transport and distribution represent the most effective critical control points that if managed, will avoid exposing stock to temperatures that may favour growth of vibrios. This method is the primary risk control implemented in many countries and followed in South Australia and Tasmania, along with growing area closures following reports of illness.

1.5 Implications for relevant stakeholders

TRO shelf life and cold chain requirements were major unknowns when we started this project. *Vibrio parahaemolyticus* did not grow in injected BROs held at 4 °C but did grow at 13°C. Not surprisingly, growth rates were higher at higher temperatures, but what was surprising was that there was no difference between 18°C and 25°C. Using these same temperatures in shelf life trials, BROs were robust and resilient to all storage temperatures, but there was a greater tendency for BROs to gape (open) at 13°C. The independent seafood processor's assessment was that BROs held at all storage temperatures were suitable for sale/consumption. However, those stored at 4°C were considered more favorable based on texture and appearance and as we showed in separate experiments, is also the temperature at which *V. parahaemolyticus* did not grow. This has important implications for the industry because it shows that a tropical oyster species can be held at 4°C and still retain product quality.

Potentially pathogenic *Vibrio* spp. were detected in the spatial and temporal baseline studies, and while this may be of concern to the industry, the knowledge gained from these studies shows that these potential pathogens are more prevalent in the wet season, and thus their seeming predictability could be exploited by not harvesting in the wet season. These patterns provide insights that can be used to manage this potential risk - particularly if further multi-year data also show that the high risk seasons are confined to the wet season when TROs spawn. However this does raise complexity because depending on location of farms and timing with respect to rain, TRO may in fact have a higher gonad index during this period and be in good market condition. The industry in different locations across northern Australia, which does include a range of climates, will ultimately decide on the balance between risk and market condition. However, this complexity does make the case for location specific baseline seasonal *Vibrio* data.

Importantly, virulent strains of *V. parahaemolyticus* were rarely detected, and when they did occur it was associated with the wet season and particularly monsoons. This highlights the need to focus on virulence rather than species presence or abundance to obtain an accurate measure of risk for *V. parahaemolyticus*, particularly for tropical rock oyster species where *V. parahaemolyticus* may prevail in some locations at some times of the year.

Data obtained as part of this project's risk analysis found that locally acquired illnesses (wound and food) have been reported from WA, NT, and Qld. Taken together with the results from this project and similar studies, there is a credible risk for aquaculture across northern Australia which potentially impacts end users including management, industry and consumers in Australia. This has implications for the TRO industry, particularly the recommendation that active risk management is required to mitigate the risk of human illness and market incidents.

1.6 Recommendations

Results from this project have led us to recommend that active risk management is required to mitigate the risk of human illness and market incidents. Specifically, we recommend that post-harvest cooling and maintenance of the cool chain during transport and distribution represent the most

effective critical control points that if managed, will avoid exposing stock to temperatures that may favour growth of vibrios. While this has implications for end users – it also provides guidance and legitimacy to establish a credible and evidence-based post-harvest strategy. This establishes the TRO industry as best practice from the outset - a defendable and appropriate position for an emerging industry that is known to be high-risk in terms of food safety.

The ability to use reliable ecological data to inform food safety considerations is going to be increasingly important in the uncertainty associated with our changing climate. The *Vibrio* baseline temporal study was intended to determine whether *Vibrio* pathogens are associated with particular times of the year, important data needed to inform risk-centric surveillance. Although more multi-year data are needed, *V. parahaemolyticus* in oysters appeared to be associated with the wet season, and this pattern will be examined in further studies, particularly the impacts of first big rains and monsoon events.

1.7 Keywords

Tropical Rock Oysters, northern Australia, Blacklip Rock Oysters (BROs) (*Saccostrea echinata*/lineage J), Milky oysters (*Saccostrea mordax*/lineage A), *Vibrio parahaemolyticus*, *V. vulnificus*, amplicon sequencing, *hsp60,* vibriosis*, Vibrio* community diversity*,* shelf life, cold supply chains, *Vibrio* risk profile, food safety.

2 Measure *Vibrio* baseline in Tropical Rock Oysters

2.1 **Introduction**

Vibrio bacteria occur naturally in warm coastal marine and estuarine waters, as well as in or on the surfaces of marine animals including oysters. Several *Vibrio* species are pathogenic to humans, causing extraintestinal infections via exposure to seawater or gastroenteritis by eating seafood containing these species. Virulent strains within species such as *Vibrio parahaemolyticus*, *V. vulnificus*, *V. cholerae* and *V. alginolyticus* are emerging as a significant problem in temperate oyster growing regions likely due to rising sea temperatures (Baker-Austin et al., 2012; Martinez-Urtaza et al., 2010; Vezzulli et al., 2020). In Australia, *Vibrio* pathogens are now also accepted as a significant risk to human health following foodborne outbreaks traced back to oysters over the last decade (Harlock et al., 2022; Lesseur and Taylor, 2022). There is increasing evidence of metabolic, physiological and immunological stress in bivalves including oysters exposed to elevated temperatures (Ericson et al., 2022; Green et al., 2019; He et al., 2022; Scanes et al., 2021) which would likely increase their susceptibility to opportunistic bacterial pathogens including *Vibrio* spp. Furthermore, increased sea temperatures would favour increased abundance of *Vibrio* spp. in the marine environment (Vezzulli et al., 2011) available for bivalve filtration.

As well as the human health aspect, several *Vibrio* species also cause disease in other animals including oysters. Mass mortality events in Pacific Oysters *Magallana gigas*(=*Crassostrea gigas* (Thunberg 1793)) larvae and adults associated with heat stress, collectively known as 'summer mortality', have been occurring in many countries including Australia (Go et al., 2017). While ostreid herpesvirus (OsHV-1) has been associated with oyster spat and juvenile mortality events (Segarra et al., 2010), *Vibrio* species have been implicated in adult oyster summer mortalities: *V. harveyi*, *V. alginolyticus* (Cavallo and Stabili, 2002; William L King et al., 2019a; Lafisca et al., 2008; Yang et al., 2021), *V. aestuarianus* (Coyle et al., 2023; Labreuche et al., 2006; Saulnier et al., 2010) and *V. splendidus* (Arias et al., 1999; Cowan et al., 2023; Garnier et al., 2007; Gay et al., 2004b; William L King et al., 2019a; Travers et al., 2015). Eleven *Vibrio* species as well as *Pseudoalteromonas piscicida*, *P. shioyasakiensis*, *Shewanella insulae*, and *Photobacterium damselae* were isolated from Pacific Oysters during a summer mortality event in Australia during 2013-2014 (Worden et al., 2022). Other bacteria such as *Arcobacter* (Lasa et al., 2019; Richard et al., 2021) and *Shewanella* (Saulnier et al., 2010) have also been reported in Pacific Oysters during mortality episodes. In a study utilizing mesocosm experimental infections of juvenile Pacific Oysters, core genera (*Amphritea*, *Arcobacter*, *Marinobacterium*, *Marinomonas*, *Pseudoalteromonas*, as well as *Vibrio*) were found to infect oysters during a mortality event (Clerissi et al., 2022). Necrosis and yellow and green lesions in the adductor muscle and other organism was identified in diseased Pacific Oysters in Canada, caused by *Nocardia crassostrea* (Friedman et al., 1998). In Eastern Oysters, *Crassostrea virginica*, the α-proteobacteirum *Roseovarius* was identified as the likely cause of what is now called roseovarius oyster disease (Boettcher et al., 2005). A *Roseovarius* species has also been described as a significant component of microbiota associated with Pacific Oyster families with low OsHV-1 disease resistance (William L. King et al., 2019), although there have been no reports of it causing disease in *M. gigas*.

Blacklip Rock Oysters (BROs) (*Saccostrea echinata*/lineage J) occur naturally in the Indo-Pacific region including Australia (Nowland et al., 2019a) and are grown commercially on a limited scale. Recent investment in BRO spat supply and grow out methods (Nowland et al., 2021, 2019b) coupled with vibriosis outbreaks and sporadic illnesses from Pacific Oysters in temperate areas of Australia (Harlock et al., 2022), has led to increasing interest in expanding BRO production in northern Australia. This represents an unchartered area for commercial tropical rock oyster production and given the preference of *Vibrio* spp. for warmer waters, the increased likelihood of marine heatwaves (Oliver et al., 2019) and increased intensity of tropical storms (Knutson et al., 2015), there is concern about

Vibrio risks in BRO and other tropical rock oyster species, in terms of both food security and food safety. In 2015-2016, north Australia experienced extended marine heat wave conditions for several months (Benthuysen et al., 2018) which coincided with large-scale mangrove dieback (Duke et al., 2017) and poor mud crab fisheries (Grubert et al., 2016). Intertidal molluscs have physiological and immunological adaptations to deal with conditions that can change quickly over a tidal cycle where they tolerate periods (hours) of emersion characterized by extremes in oxygen availability and temperature (Meng et al., 2018; Zhang et al., 2014) but it is unclear what impact the longer lasting climate anomalies (i.e. days compared to hours) will have on tropical rock oyster health and their subsequent ability to interact with microbes.

To support this growing aquaculture interest and ensure the ongoing supply of safe, healthy oysters, there is a need to understand bacterial and *Vibrio* diversity in oysters from tropical northern Australia, to identify *Vibrio* species than can potentially affect oyster health or be transmitted to humans. Over 40 *Vibrio* species were detected in northern Australian estuarine waters, including potentially virulent strains of *V. parahaemolyticus* and *V. vulnificus* (Padovan et al., 2021; Williams et al., 2022). However, relatively little is known about the occurrence and diversity of bacteria including *Vibrio* species in tropical rock oysters (Matté et al., 1994). To date, no large-scale adult BRO mortalities have been reported in Australia in either wild or farmed settings, however, this could change as BRO aquaculture production grows and pressure increases in a changing climate.

2.2 **Objectives**

The objective of this study was to characterise the bacterial community, and in particular the *Vibrio* community, in healthy wild and farmed tropical rock oysters (TRO) covering a 3,500 km longitudinal and 1,027 km latitudinal expanse across remote northern Australia. Additional objectives were to compare farmed and wild oysters, and identify bacterial taxa and *Vibrio* species that may be of concern to both food security and food safety. Our motivation was to provide new information on *Vibrio* and bacterial community diversity on tropical rock oysters, and also report for the first time, levels of *V. parahaemolyticus* and *V. vulnificus* in wild tropical rock oysters that will inform future *Vibrio* shellfish quality assurance considerations and guidelines.

2.3 **Methods**

2.3.1 Oyster collection and handling

BROs were the primary target species, but Milky oysters (*Saccostrea mordax*/lineage A) were also collected when BROs were not present in the wild, and because small numbers of these oysters are being farmed in some regions. The two species of wild TROs were collected from intertidal zones at nine locations in the Northern Territory (NT) between 2019-2021 (Figure 2-1A). In addition, farmed oysters from hatchery stock were also collected from Goulburn Island (NT) and Flying Foam Passage, Cossack and West Lewis in the Pilbara, Western Australia (Figure 2-1B), while farmed oysters from Bowen, Queensland (Figure 2-1C) were from wild caught spat. Oysters were obtained under Permit Nos 2021-2022/S11/279 and 2021-2022/S11/296. Details of sites and collections are given in Supplement 2-1.

Three oysters were collected in triplicate from four sites within a location where possible (e.g., oysters from Maningrida were collected from Crab Creek, Outstation, First Point and Rolling Bay). At many of the remote locations, access to different sites occurred over several days to accommodate tides and travel time, so oysters were individually wrapped in moist Chux® wipes placed into open plastic bags, and stored in a cool, shaded area. Where possible, oysters were delivered to Charles Darwin University (CDU) within 24 h from collection however this was not always possible due to COVID 19 related

reduced flights and lockdowns. Upon receipt, the oysters were scrubbed under running potable water and any gaping or damaged oysters discarded. Oysters were aseptically opened, and the weight of meat recorded. Three individual oysters were pooled to form one sample and there were a total of 147 samples. The oyster meat was homogenized using an Ultra-Turrax® IKA T18 (IKA® Works, Malaysia) and approximately 1 g aliquots stored at -80°C. DNA was extracted from approximately 25 mg oyster tissue homogenates using the DNeasy® Blood and Tissue kit (Qiagen) and nucleic acid quantity and quality determined spectrophotometrically (NanoDrop™).

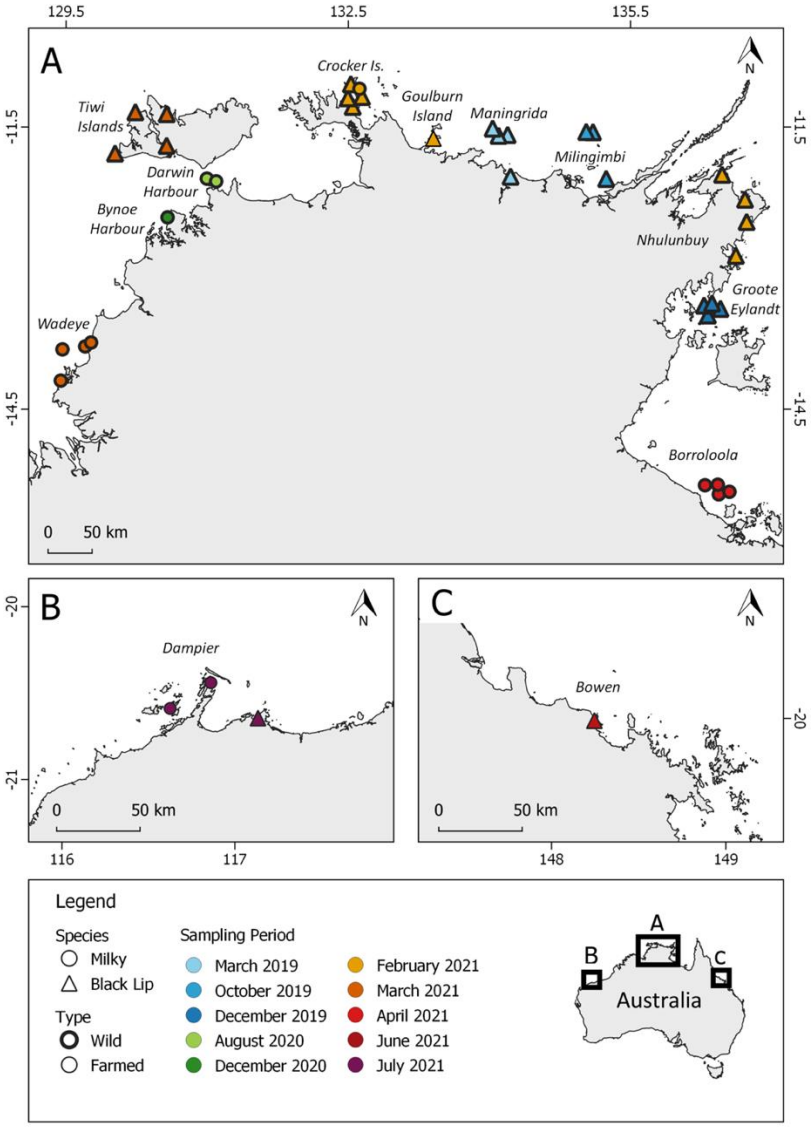


Figure 2-1: Tropical rock oyster sampling locations across northern Australia; A. Northern Territory; B. Western Australia; C. Queensland

2.3.2 Quantification of bacterial and total *Vibrio* abundance

Quantification of the bacterial 16S rRNA gene was performed using a TaqMan assay as previously described (Green et al., 2018). Total *Vibrio* abundance was measured by a SYBR Green quantitative PCR (qPCR) assay to quantify Vibrio-specific 16S rRNA gene copies in each sample as previously described (Thompson et al., 2004; Vezzulli et al., 2011).

2.3.3 Enumeration of *V. parahaemolyticus* and *V. vulnificus* by MPN-qPCR and detection of virulence genes

V. parahaemolyticus and *V. vulnificus* were enumerated in a sub-set of oyster samples (n=78) using the Most-Probable-Number (MPN) qPCR method. Fresh homogenate (10 g) prepared as described above was diluted with alkaline peptone water (APW; 10 g/L peptone, 5 g/L NaCl, pH 7.8) for a 3×3 most probable number (MPN) assay (Kaysner et al., 2004). For some samples where the oysters were small, less than 10 g of homogenate was used. In tubes showing growth, a 1 mL aliquot was boiled for 10 min, centrifuged for 3 minutes and 2 uL of the supernatant tested for *V. parahaemolyticus* and *V. vulnificus* by qPCR targeting the *tlh* (Nordstrom et al., 2007) and *vvhA* genes (Campbell and Wright, 2003), respectively. Concentrations were determined using MPN tables (Blodgett, 2010). To gain insights into risk associated with virulence strains, we tested for virulence genes *trh* and *tdh* in samples that were positive for *V. parahaemolyticus* using published qPCR protocols (Nordstrom et al., 2007).

2.3.4 DNA sequencing to characterise the *Vibrio* community

We used high throughput amplicon sequencing to identify bacterial taxa and *Vibrio* species in these oysters, including potential human or oyster pathogens, and their relative abundance. Amplicon sequencing provides relative abundance of specific taxa, so to more accurately quantify some species and compare these results to relative abundance, we quantified *V. parahaemolyticus* and *V. vulnificus* in a subset of wild oysters by MPN-qPCR. To characterize the composition and diversity of the *Vibrio* community, DNA diluted 1:10 was amplified from the 147 samples using the *Vibrio hsp60* primers Vib-hspF3-23 and Vib-hspR401-422, as previously described (William L King et al., 2019b). For total bacterial community analysis, primers Bakt_341F and Bakt_805R, which amplify the V3-V4 region of the bacterial 16S rRNA gene (Herlemann et al., 2011), were used for PCR.

Amplicons were sequenced using the Illumina NovaSeq SP 500 platform according to the manufacturer's guidelines (Australian Genome Research Facility, Melbourne). Raw data files in FASTQ format will be deposited in the NCBI Sequence Read Archive (SRA).

2.3.5 Processing *hsp60* and 16s rRNA gene sequences

The quality of *hsp60* amplicon sequences was assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and due to the low quality of the reverse sequences, only the forward sequences were processed using the Quantitative Insights into Microbial Ecology (QIIME 2 version 2022.8) pipeline (Bolyen et al., 2018) (https://qiime2.org/). DADA2 within QIIME2 was used to denoise the sequences (left trim 4 bp; length truncation 150 bp; max expected error 2) and create sequence variants (SVs). The taxonomy was assigned to the sequence variants in a two-step process based on the *Vibrio hsp60* database (repset_final_130219) provided by (William L King et al., 2019b). The first step used the Blast taxonomy classifier (https://blast.ncbi.nlm.nih.gov/Blast.cgi) set to 90% identity to filter for *Vibrio* sequences and exclude all non-*Vibrio* sequences, while the second step used the sklearn-based taxonomy classifier to identify *Vibrio* species.

Sequences were further processed using the PhyloSeq package (McMurdie and Holmes, 2013) in R (version 4.1.2; Copyright (C) 2017 The R Foundation for Statistical Computing). SVs which only occurred in one sample were excluded for nMDS, PERMANOVA and richness relative comparisons. All SVs were used for *Vibrio* species analyses. Due to the large variability of the total number of *Vibrio hsp60* reads across samples and because total richness was reached for the majority of samples regardless of sequencing depth (see results below), the reads were not rarefied to the lowest common sequence count, but relative abundance was used. Samples with less than 10 reads were excluded

(three samples) with a final dataset for PERMANOVA and relative richness models of 262 SVs in 113 samples with 33 to 116,100 reads per sample.

16S rRNA gene amplicon raw demultiplexed data was processed using QIIME2. Briefly, paired-end sequences were trimmed and denoised using the DADA2 version 1.6, which also removes chimeras (Callahan et al., 2016). Taxonomy was assigned on the rep-set-dada2 output at the single nucleotide level using the sklearn qiime feature classifier against the Silva v132 database (Quast et al., 2013). The dataset was further cleaned by removing SVs with only one read and those identified as non-bacterial, chloroplasts or mitochondria. SVs which only occurred in one sample were excluded (41% of reads) and two samples with less than 8,000 sequences were excluded. Rarefaction analyses indicated that the majority of samples reached their full richness at 8,000 sequences and the samples were rarefied to 8,140 sequences in PhyloSeq with a final dataset of 143 samples and 3,951 SVs.

2.3.6 Data analysis

Permutational ANOVA (PERMANOVA) models were used to assess differences in the vibrio community and total bacterial composition across groups of samples. nMDS ordinations and PERMANOVAs were based on the Bray Curtis dissimilarity matrix of the square root of the relative abundance of *hsp60* or 16s rRNA gene SVs. Fixed factors for the PERMANOVAs where oyster species (two levels) and farm vs wild while sites were included as random effect nested in oyster species and farm vs wild. All permutations of the main test were above 900 permutations.

For correlation analyses, MPN-qPCR values were changed as follows: values <3 MPN/g were changed to 1 MPN/g (n=17; 23%) and values >1,100 MPN/g to 2,000 (n=16; 22%). Various relationships were explored between *V. parahaemolyticus* MPN-qPCR values and *V. parahaemolyticus hsp60* total reads and relative abundance using negative binomial models as well as Fisher's Exact test for frequency comparisons of *V. parahaemolyticus hsp60* presence/absence vs. MPN-qPCR presence/absence. Negative binomial models included oyster species and farm vs wild as fixed factors and sites as random intercept (glmmTMB package) (Brooks et al., 2017). Model residuals were checked for lack of patterns across fitted values and predictors and influential outliers using the DHARMa package (Hartig, 2022) in R.

A multivariable general linear mixed model was used with family Gamma (log link) to assess differences in the *Vibrio* total abundance measured by qPCR across sample groups; and family beta regression (logit link) to assess differences in the *V. parahaemolyticus* relative abundance (based on *hsp60* reads), across oyster species and farmed vs wild (incorporated as an interaction) with sites as random intercept. Pairwise comparisons were adjusted for multiple testing using the Tukey method (lsmeans package) (Lenth, 2016). *Vibrio* richness comparisons were based on a negative binomial model with an interaction between oyster species and farmed vs wild and sites as random effect.

2.4 **Results**

2.4.1 *Hsp60* sequencing summary

Of the 147 oyster DNA samples obtained, 1,464 *vibrio* SVs were obtained from 116 samples and further data cleaning as described above resulted in 262 *Vibrio hsp60* SVs. In samples where sequences were not obtained, there was no evidence of inhibition in the PCR amplification step (results not shown), which suggests that there were low levels of *Vibrio* bacteria in these samples. The *Vibrio hsp60* diversity was captured for most samples (rarefaction curves not shown). The Spearman *rho* correlation between total *Vibrio hsp60* reads and *Vibrio* 16S rRNA gene copies by qPCR was 0.51 ($P<0.001$).

2.4.2 *Vibrio* diversity and relative abundance

Forty-eight *Vibrio* species were identified in oysters (Supplement 2-2), with 9 of these making up 90% of the total *hsp60* sequences. The most abundant *Vibrio* species (and relative abundance) across all samples and sites were *V. parahaemolyticus* (17.7%), *V. harveyi* (9.6%), *V. diabolicus* (8.8%), *V. alginolyticus* (7.1%), *V. mediterranei* (6.4%), *V. kanaloae* (5.8%), *V. toranzoniae* (5.7%) and *V. owensii* (3.3%). Approximately 25% of sequences could not be resolved to species.

2.4.3 *Vibrio* community composition differed by site but not by oyster species

The relative abundance of total *Vibrio* varied across sites as indicated by the *Vibrio* 16S rRNA gene copy values based on qPCR, with the lowest values at Goulburn Island and the highest at Millingimbi (Figure 2-2A). There were significantly more *Vibrio* 16S rDNA gene copies in wild BROs compared to wild Milky oysters ($P=0.003$), but not between farmed versus wild oysters ($P=0.86$) while accounting for variability across sites. Of the *Vibrio* species comprising more than 5% total relative abundance, *V. parahaemolyticus* was often the most abundant *Vibrio* species and occurred in most samples except for oysters from Wadeye and Bowen (Figure 2-2B). *V. harveyi* was also prevalent, occurring in most samples. The *Vibrio* community in oysters from Bowen had a distinctive composition compared to all other samples, with *V. kanaloe*, *V. toranzoniae* and *V. diabolicus* comprising most of the *Vibrio* community (Figure 2-2B).

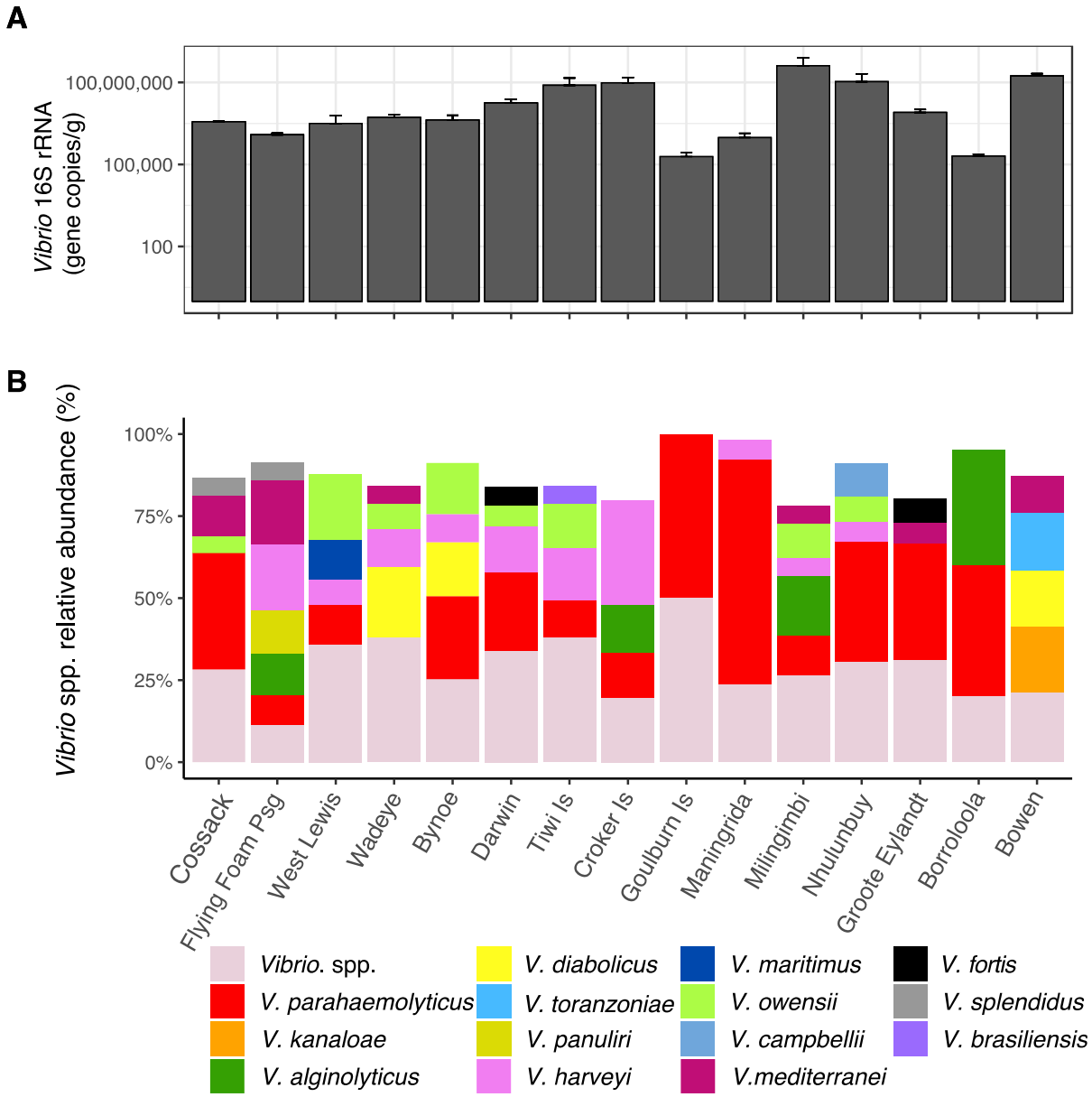


Figure 2-2: Vibrio abundance and community composition in tropical rock oysters from different locations across northern Australia. A. Vibrio 16S rRNA gene copy number per gram. B. Taxa bar plot showing the most abundant Vibrio species identified.

The *Vibrio* community from farmed oysters at Bowen formed a distinct cluster but it was difficult to distinguish other sites as clusters across the dataset (Figure 2-3). There was no clear clustering by oyster species or by farmed versus wild oysters (Figure 2-3).

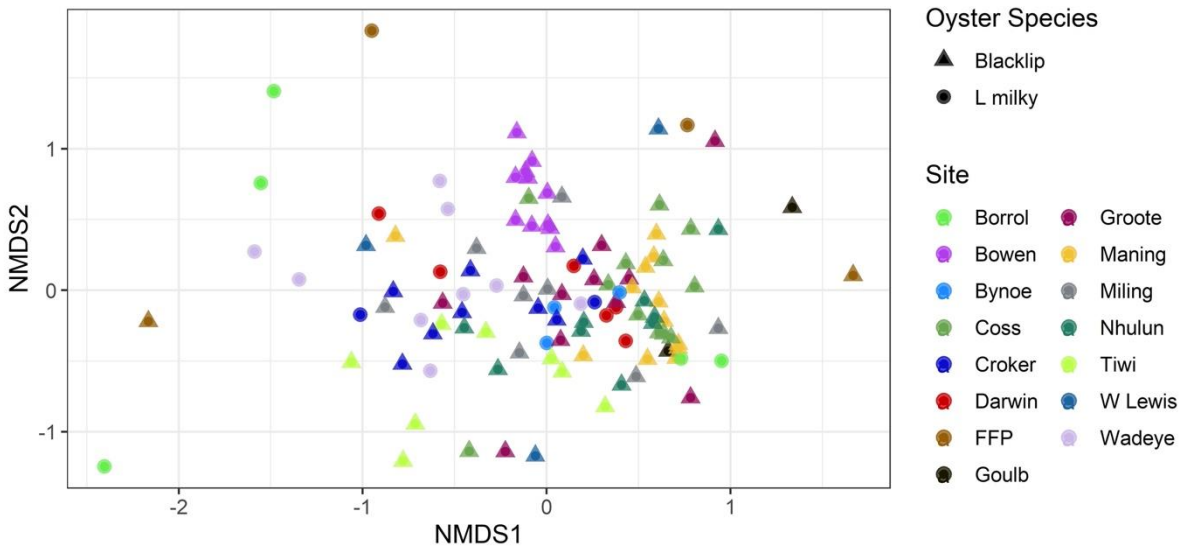


Figure 2-3: Non-metric Multidimensional scaling (nMDS) graph where each symbol represents Vibrio abundance and community composition in tropical rock oysters from different locations. The closer the symbol, the more similar the Vibrio community.

PERMANOVA analysis confirmed that the *Vibrio* community composition significantly differed by site (P=0.001), but not by oyster species (P=0.22) or between farmed and wild oysters (P=0.079) (Table 2-1). Oysters from Flying Foam Passage and West Lewis had the lowest similarity (<7%) to other sites.

Table 2-1: PERMANOVA of the Vibrio community showing the effects of oyster species and sites. All permutations of the main test were above 900 permutations and the residual ECV was 54.7.

Factor	Pseudo.F	df	P.Value	ECV
Farmed vs wild	2.1	1	0.079	20.9
Oyster species	1.3	1	0.220	11.6
Site	2.9	13	0.001	28.6

Overall, the *Vibrio* community composition varied between individual samples (supplementary Figure S1). Within-site similarity values were variable, but displayed very high similarity for Bowen (53%), Bynoe Harbour (40.6%) and Maningrida (35.2%) sites. This decreased to <5% for Goulburn Island, Flying Foam Passage and West Lewis.

The number of *Vibrio* SVs across sites varied from a median of 2 SVs at Borroloola to 50 at Bowen (Figure 2-4) (with SVs only occurring in one sample excluded). There were no significant differences in the *Vibrio* SV richness between farmed versus wild oysters, or between oyster species (P> 0.2 for all).

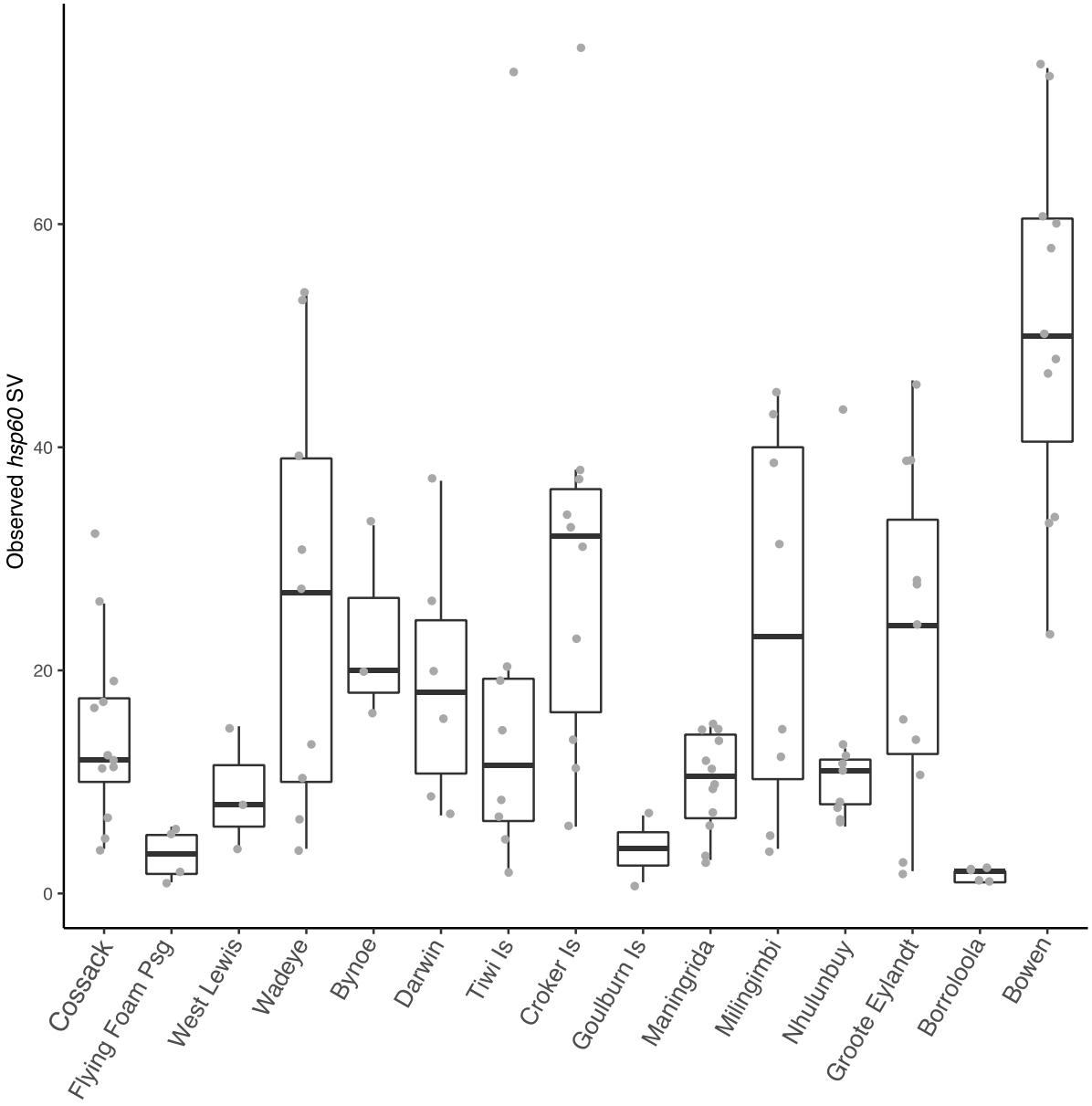


Figure 2-4: Number of Vibrio SVs in oysters from different sites. Horizontal bar in box indicates the median, the box the interquartile range and the whiskers the min/max (excluding outliers).

2.4.4 *Vibrio* human pathogens

Oysters contained sequences from the potential human pathogenic species *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. diabolicus* and *V. antiquarius* (Supplement 2-2). Of these species, *V. parahaemolyticus* was the most prevalent and abundant, present in 97 samples at <2.2%-100% relative abundance. *V. alginolyticus* was the next most prevalent occurring in 52 samples with a relative abundance ranging from 0.1 to 88%. There was a weak trend for higher *V. parahaemolyticus* relative abundance in wild compared to farmed BROs (P=0.045) but not for Milky oysters (P=0.6187). Pairwise testing between all combinations of BROs and Milky oysters accounting for multiple comparisons showed no significant difference in the relative abundance of *V. parahaemolyticus* (P=0.1869). *V. cholerae* or other species isolated from clinical cases in the Northern Territory, *V. metschnikovii* and *V. mimicus* (McAuliffe et al., 2015) were not detected.

2.4.5 *Vibrio* oyster pathogens

There were several potential animal *Vibrio* pathogens, the most prevalent being *V. harveyi* and *V. alginolyticus*. Less common potential pathogens were *V. brasiliensis, V. splendidus, V. tubiashii, V. coralliilyticus, V. neptunius, V. lentus, V. bivalvicida, V. europaeus,* and *V. crassostrea.* (Supplement 2-2). Of the two *Vibrio* species identified in summer mortality of Pacific Oysters (Gay et al. 2004), *V. splendidus* sequences were present in the tropical rock oysters, but *V. aestuarianus* was not identified in the sequence dataset (Supplement 2-2).

2.4.6 Enumeration of *V. parahaemolyticus* and *V. vulnificus* by MPN-qPCR, detection of virulence genes and correlation to *hsp60* sequence data

Of the 78 oyster samples tested, 60 samples (77%) were positive for *V. parahaemolyticus,* and 24 samples (31%) were positive for *V. vulnificus* (Table 2-2). Concentrations ranged from 3.6 to >1,100 MPN/g for both *Vibrio* species. Sixty three percent of the oyster samples tested had *V. parahaemolyticus* values <100 MPN/g. For *V. vulnificus*, most of the oysters were <3.0 MPN/g (Table 2-2).

Table 2-2: Number and percent (%) of oyster samples within the given range of V. parahaemolyticus *and* V. vulnificus *MPN/g values.*

Range (MPN/g)	*V. parahaemolyticus*	*V. vulnificus*
< 3.0	18 (23%)	54 (69%)
3-100	31 (40%)	16 (21%)
101-1100	12 (15%)	7 (9%)
>1100	17 (22%)	1 (1%)
Total	78	78

<3.0 means below the detection limit.

Neither the *trh* nor *tdh* virulence gene was detected in any of the oyster samples (results not shown).

There was no linear relationship between *V. parahaemolyticus* MPN/g values and total *V. parahaemolyticus hsp60* reads (log transformed), *V. parahaemolyticus* relative abundance (P>0.7 for both), or *V. parahaemolyticus hsp60* presence/absence (Fisher's exact test, P=0.13). Of the 18 samples that were below detection by MPN-qPCR for *V. parahaemolyticus*, all but one sample contained *V. parahaemolyticus hsp60* reads.

Only 2 oyster samples contained *hsp60 V. vulnificus* reads. These were both samples from Wadeye in large Milky oysters, and both of these samples also had the highest MPN *V. vulnificus* values of 1,100 and 2,000 MPN/g. A third Wadeye replicate sample had 460 MPN/g *V. vulnificus,* but this sample did not produce any *hsp60* sequences. There were too few data points for any statistical analyses.

2.4.7 Bacterial diversity in oysters

After cleaning the sequencing dataset as described in the methods, 143 oyster samples were retained for analysis, resulting in the identification of 3,951 unique SVs. Rarefaction plots showed that for most samples, the diversity plateau was reached (results not shown). The number of SVs varied between sites with lowest richness in samples from Maningrida and highest richness in samples from Croker

Island (Figure 2-5). There were no differences in the SV richness between farmed vs wild nor Milky vs BROs (P>0.2 for all) while accounting for sites.

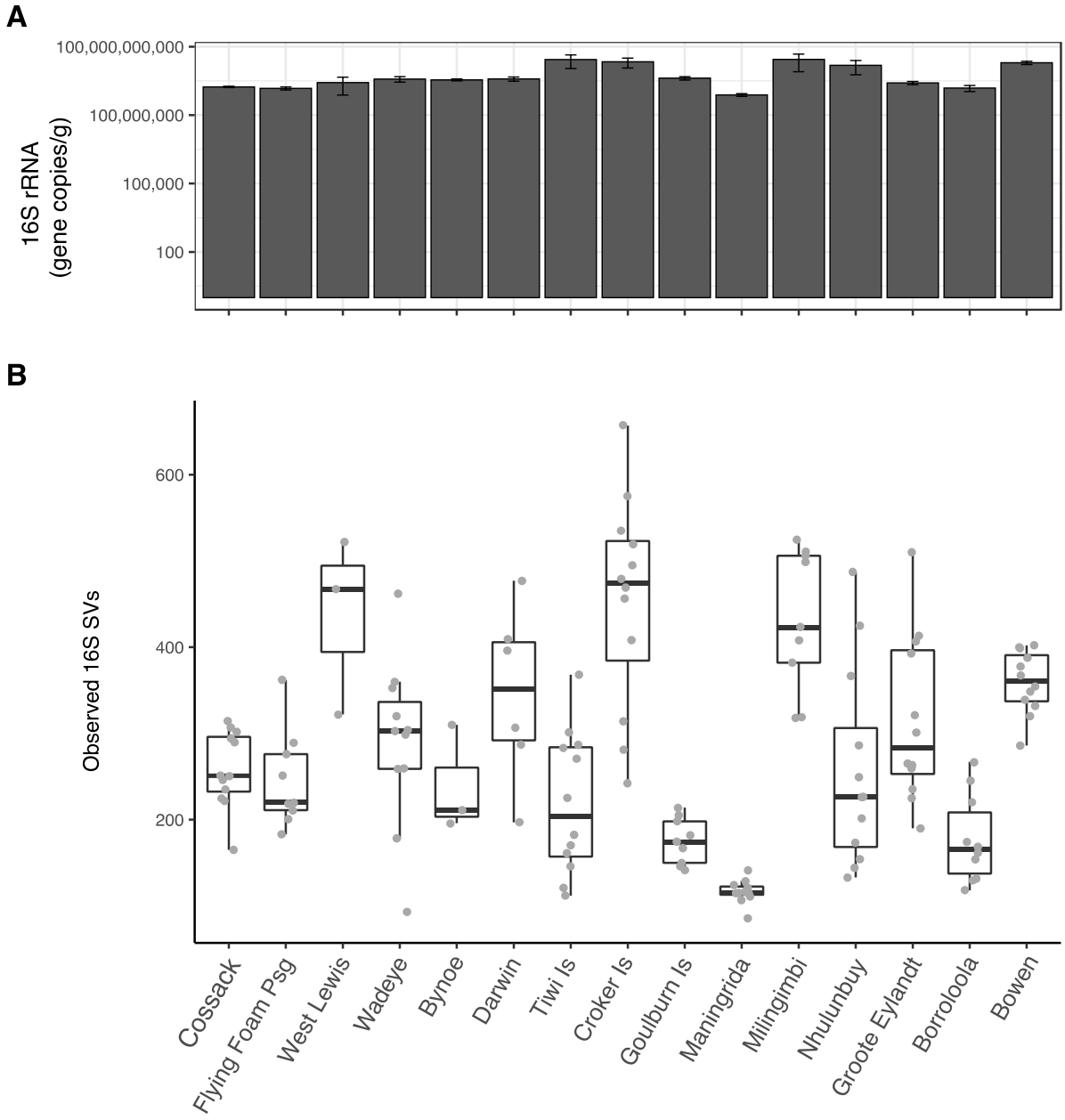


Figure 2-5: Number of bacterial SVs identified in tropical rock oyster tissue across sites.

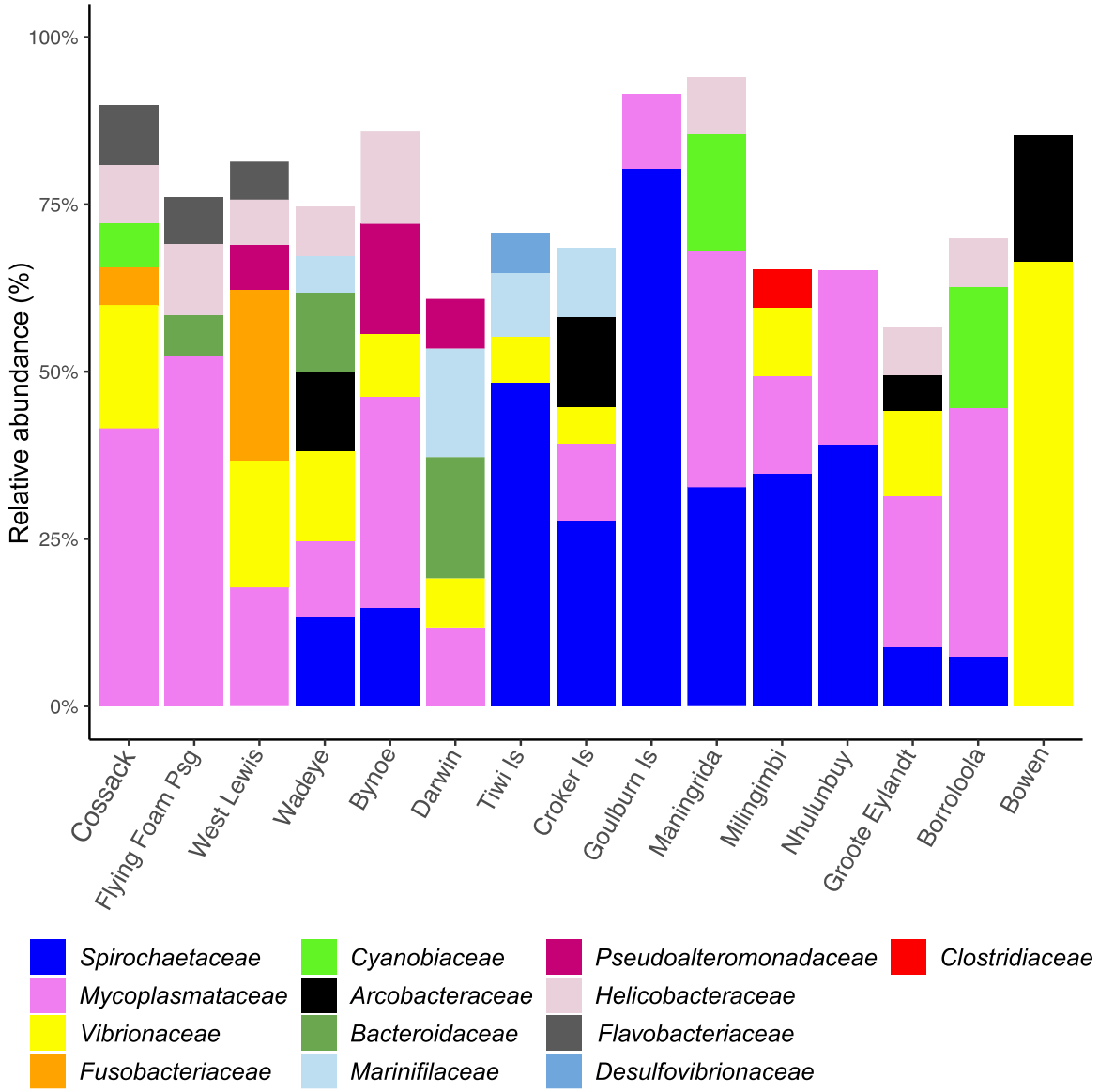


Figure 2-6: Taxa bar plot showing most abundant bacterial families in oysters from different locations across northern Australia. Only abundances >5% are shown.

The dominant bacterial families were *Spirochaetaceae* (21%), *Mycoplasmataceae* (20%) and *Vibrionaceae* (12%) (Figure 2-6). The genus *Mycoplasma* occurred in >75% of all oyster samples, ranging in abundance from 0.01% to 81%. Four *Vibrio* species were identified in the 16S rDNA dataset: *V. aestivus*, *V. kanaloae*, *V. nereis*, *V. ponticus*, and *Vibrio* spp., and of these, *V. aestivus* was not detected in the *hsp60* sequence dataset. The genus *Vibrio* occurred in all 120 oyster samples, comprising 0.01-85% relative abundance.

Bacterial community diversity in each individual sample was compared by site, oyster species and wild versus farmed (Figure 2-7). Unlike the *Vibrio* community where only the oyster samples from Bowen could readily be distinguished from other sites, total bacterial community patterns for Borroloola, Maningrida and Bowen oyster samples were clearly distinguished from the other sites (Figure 2-7).

Farmed oysters from Bowen, Goulburn Island, Cossack, West Lewis and Flying Foam Passage (both oyster species) had high within site similarity and thus formed tighter clusters in the nMDS compared to some of the wild oyster sites such as Groote Eylandt, Milingimbi and Tiwi (Figure 2-7). PERMANOVA

analysis showed that bacterial composition differed by sites (P=0.001) but not by oyster species (P=0.085) or farmed versus wild (P=0.132) (Table 2-3).

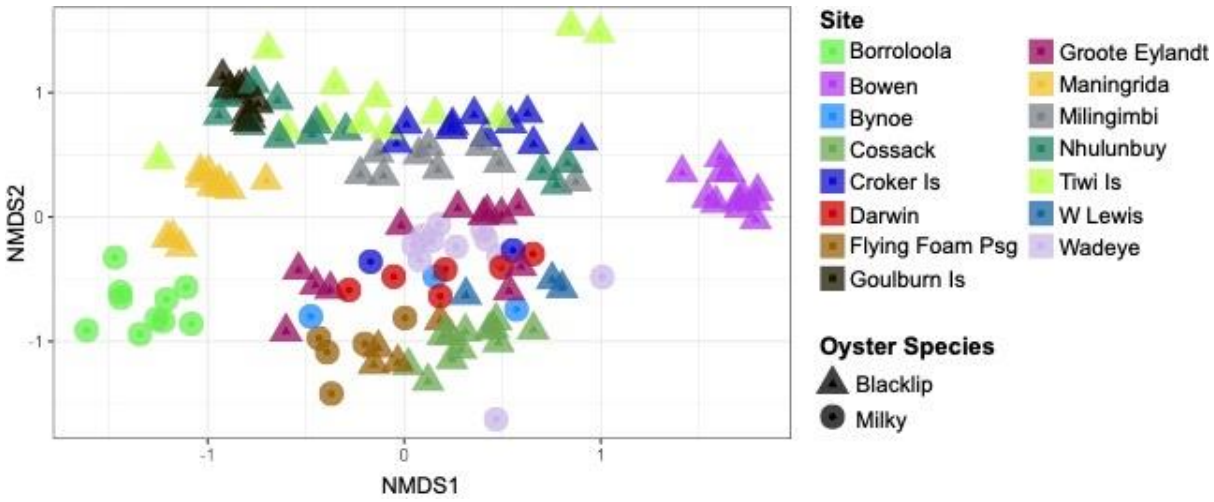


Figure 2-7: nMDS of bacterial community composition relationships in oyster samples from across northern Australia by site and oyster species. Stress value 0.23.

Table 2-3: PERMANOVA of the 16S rRNA gene-based bacterial community. All permutations of the main test were above 900 permutations and the residual ECV was 43.5.

Factor	Pseudo.F	df	P.Value	ECV
Oyster species	1.8	1	0.085	17.0
Farmed vs wild	1.5	1	0.132	14.0
Site	7.6	13	0.001	38.6

The average bacterial community similarity within a site ranged from ≥60% for Bowen and Goulburn Island farmed oysters, to 26% and 28% for Tiwi and Groote Eylandt wild oysters respectively (Supplements 2-3 and 2-4). Overall, the similarity of the oyster bacterial community within sites was greater than for the *Vibrio* community.

2.4.8 Bacterial human and oyster pathogens

Potential human pathogenic taxa identified in the 16S rDNA sequence dataset included *Bacteroides*, *Arcobacteraceae* (including *Halarcobacter* and *Malaciobacter*), *Shewanella*, *Acinetobacter*, and *Escherichia-Shigella*. Sequences from Bacteroides and *Arcobacteraceae* were the most abundant of these, with levels reaching 63% and 59%, respectively, in some oyster samples (Table 2-4). Overall, sequences from *Shewanella*, *Acinetobacter*, and *Escherichia-Shigella* were much less abundant (<3% relative abundance) and less prevalent across samples, except for one oyster sample from Groote Eylandt which contained *Escherichia-Shigella* at a relative abundance of 32% (Table 2-4). No *Aeromonas* sequences were identified in the 16S rDNA dataset.

Table 2-4: List of potential oyster and human pathogenic taxa identified in the oyster 16S rDNA dataset, including relative abundance, and number of samples containing those taxa.

Taxon	Reference	Relative abundance range	No. samples
Associated with oyster mortality			
Amphritea	Clerissi et al. 2023	<1%	2
Arcobacteraceae	Clerissi et al. 2023; Lasa et al. 2019; Richard et al. 2021	0.01-59.40%	86
Marinobacterium	Clerissi et al. 2023	0.02-2.18%	20
Marinomonas	Clerissi et al. 2023	0.01-1.02%	18
Photobacterium	Worden et al. 2022	0.01-31.80%	93
Pseudoalteromonas	Clerissi et al. 2023; Worden et al. 2022	0.03-46.38%	76
Roseovarius	Boettcher et al. 2005	0.12-0.63%	5
Shewanella	Saulnier et al. 2010; Worden et al. 2022	0.01-2.80%	34
Vibrio	Worden et al. 2022	0.01-85.44%	120
Potential human pathogens			
Acinetobacter		0.02-0.66%	11
Bacteroides		0.01-63.35%	83
Escherichia-Shigella		0.01-32.19%	24
Shewanella		0.01-2.80%	34

Of bacterial genera previously isolated from diseased Pacific oysters, including *Amphritea*, *Marinobacterium*, *Marinomonas*, *Photobacterium, Pseudoalteromonas* and *Roseovarius* sequences were detected in the tropical rock oyster dataset but generally at low abundances (Table 2-4) except for *Phtobacterium* and *Pseudoalteromonas* where only a small number of samples had high relative abundances. While the family *Arcobacteraceae* was prevalent in the oyster samples, it only consisted of the genera *Halarcobacter* and *Malaciobacter*, with no *Arcobacter* sequences detected in the dataset. The genus *Vibrio* occurred in all 120 oyster samples, comprising 0.01-85% relative abundance. The genus *Nocardia* was not detected in the dataset.

2.5 **Discussion**

We identified 48 *Vibrio* species in two tropical oyster species, including five known human pathogens and 12 oyster pathogens. The most abundant *Vibrio* species were *V. parahaemolyticus, V. harveyi* and *V. diabolicus*, except for oysters collected from one site where *V. kanaloae*, *V. toranzoniae* and *V. diabolicus* dominated. It is not uncommon for *Vibrio* spp. to be detected in natural healthy oysters (Arias et al., 1999; Buck, 1990; Dahanayake et al., 2018; Matté et al., 1994) including in the haemolymph (Bruto et al., 2017; Gay et al., 2004a; King et al., 2021; Lokmer et al., 2016a; Roux et al., 2004; Wendling et al., 2014). The diversity of *Vibrio* species identified in tropical rock oysters was not unexpected given the diversity present in tropical seawater (Padovan et al., 2021).

We were particularly interested in assessing Vibrio diversity in oysters within and between locations, as well as between farmed and wild oysters and oyster species. We acknowledge that any inferences are based on one sampling event per site and that oysters were collected over two years, so the oysters would have been exposed to different seasonal conditions at the time of sampling.

The *Vibrio* community composition significantly differed by site but this was driven by samples from the Bowen site, which had the lowest similarity to other sites. A potential reason for this was the 5 days taken for them to reach the laboratory, favouring the proliferation of bacteria that were not dominant in samples from other locations. We cannot however, rule out that genetic differences in the Bowen oysters compared to the NT and WA populations, may have impacted differences in the microbiome. The high *Vibrio* diversity and relative abundance also explains why there were no significant differences between oyster species or in wild compared to farmed oysters. The large diversity observed between replicates and sites suggests that *Vibrio* species have little host preference in shellfish where their abundance is likely influenced by their surrounding environment (Bruto et al., 2017; Preheim et al., 2011; Wendling et al., 2014) There are examples of *Vibrio* spp. having stable relationships with their host, for example *V. fischerii* in the Hawaiian bobtail squid (Boettcher and Ruby, 1990) and *V. splendidus* strains have consistently been found in association with cultured *Ostrea edulis* in the Mediterranean Sea over the years (Macián et al., 2001), but this does not appear to be the case with tropical rock oysters.

Of the *Vibrio* species emerging as a significant food safety risk in temperate oyster growing regions (Baker-Austin et al., 2017), *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* sequences were detected in the tropical rock oysters, but *V. cholerae* was not detected. *V. parahaemolyticus* was the most prevalent and abundant of the potential pathogens, and occurred in both farmed and wild oysters. At the beginning of this study, oyster growers were concerned that the wild oysters of indeterminate and possibly older age would have a greater *Vibrio* burden, particularly *V. parahaemolyticus*, and potentially misrepresent levels in farmed oysters. By including ~2 year old farmed oysters from Bowen (Qld), Goulburn Island (NT) and even younger oysters from WA in the sampling program, we were able to alleviate these concerns, with no significant differences in either the relative abundances of *V. parahaemolyticus* or total *Vibrio* levels between farmed and wild oysters. The identification of *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* species does not necessarily indicate a human hazard as the presence of *Vibrio hsp60* sequences do not indicate viability or virulence. However, using these tools to understanding *Vibrio* dynamics is critical to identify responses to global warming, either through changes in the water column or through oyster stress.

In a subset of the wild oysters, *V. parahaemolyticus* and *V. vulnificus* levels were measured using the MPN-qPCR method. Most of the tropical rock oysters tested had *V. vulnificus* levels which were below detection, but detectable levels were measured in the remaining third of the samples including one sample from a Milky oyster with >1,100 MPN/g. This is within the range reported in other oysters and locations (Audemard et al., 2022; DePaola et al., 1994; Givens et al., 2014; Jones et al., 2014; Kirs et al.,

2011; Motes et al., 1998) but it is not yet known whether levels fluctuate in the tropical rock oysters with season as for the temperate species. Nearly a quarter of the tropical rock oysters tested in the present study had no detectable levels of *V. parahaemolyticus*, with 40% of oyster samples having concentrations below 100 MPN/g, but over a third were in the range 100 to >1,100 MPN/g which would be considered unsatisfactory according to the Australian compendium of microbiological criteria for food (FSANZ, 2018). In Australia, *V. parahaemolyticus* levels from 0.4-10^4 CFU/g have been measured in Pacific Oysters (Lewis et al., 2002; Madigan et al., 2007) and Sydney Rock Oysters (Bird et al., 1992; Eyles et al., 1985; Fernandez-Piquer et al., 2010; Lewis et al., 2002). The abundance of *V. parahaemolyticus* in seawater from northern Australia has been previously reported (Padovan et al., 2021; Williams et al., 2022) so it is not unexpected that filter feeding organisms such as oysters reflect this in their microbiome.

Virulence genes *trh* and *tdh* were not identified in any of the oyster samples that were found to contain *V. parahaemolyticus*. This highlights the need to focus on virulence rather than species presence or abundance to obtain an accurate measure of risk for *V. parahaemolyticus*, particularly for tropical rock oyster species where *V. parahaemolyticus* may prevail. Pathogenicity, however, cannot be explained by these two genes alone, as a small percentage of clinical *V. parahaemolyticus* isolates lack *trh* and/or *tdh* (Ottaviani et al. 2012; Saito et al. 2015) indicating that virulence in this species is complex and it is likely that some strains use different tactics to cause disease. Toxin-encoding prophages (Castillo et al. 2018) and recently identified virulence factors such as *hly* (Zha et al. 2023) have recently been shown to contribute to *V. parahaemolyticus* pathogenicity. In the absence of a simple marker for virulence, the presence of *trh* and/or *tdh* remains the best predictor of disease risk.

For *V. parahaemolyticus*, we found no relationship between the number of sequence reads or relative abundance or presence/absence and MPN values. There are several reasons why this may be the case; the MPN culture-based approach may encourage overgrowth of non-target *Vibrio* species, interfering with detection of the target; on the other hand, amplicon sequencing would include DNA from non-viable cells and may overestimate abundance compared to culturing. A rapid and sensitive approach to estimate *V. parahaemolyticus* and other human pathogens in oysters on farms is still lacking. Transport of live oysters is an issue for culture based and molecular enumeration methods, particularly for remote regions where infrequent or expensive freight prohibits the timely delivery of oysters to testing laboratories.

Vibrio diabolicus was the third most abundant species in the tropical rock oyster dataset. *V. diabolicus* was originally identified from a deep sea hydrothermal vent polychaete (Raguénès et al., 1997) and considered an environmental strain. However, genome sequencing and comparisons to published sequences revealed that *V. diabolicus* also occurs in Pacific Oysters and in fact, another sequenced *Vibrio* strain associated with gastroenteritis from spoiled horse mackerel originally identified as *V. harveyi* (Cao et al., 2013) was found to be *V. diabolicus* (Turner et al., 2018). In this same publication, whole genome sequences of two *V. antiquarius* strains and four *V. alginolyticus* strains including the one associated with food poisoning, supported their assignment to the species *V. diabolicus* (Turner et al., 2018). These strains were found to contained genes associated with virulence means that *V. diabolicus* (and *V. antiquarius*) can be considered potential food pathogens.

Vibrio species identified in the tropical rock oyster sequence dataset associated with mortalities in adult oysters include *V. harveyi* (Cowan et al., 2023; Wang et al., 2021a) *V. splendidus*, *V. tubiashi* (Elston et al., 2008; Garnier et al., 2008; Gay et al., 2004b; Vezzulli et al., 2015) and *V. brasiliensis* (Wang et al., 2021b). *V. aestuarianus* isolated from moribund *M. gigas* and associated with deaths in Europe (Garnier et al., 2007) was not identified in the current data set. *V. diabolicus* formed ~9% of the *Vibrio* community in the present study, and while it is not a known human or oyster pathogen, it has been reported in *M. gigas* and genomic studies have identified virulence genes (Turner et al., 2018). Based on the *Vibrio* species identified in this study and oyster pathogens reported in the

literature, *V. harveyi*, *V. splendidus*, *V. alginolyticus*, *V. tubiashii* and *V. brasiliensis* are likely the biggest threat to adult tropical oyster species health. All except *V. splendidus* have been identified in tropical northern Australia coastal waters (Padovan et al., 2021). Laboratories equipped to provide targeted assays for these key *Vibrio* species would be valuable to provide rapid identification.

Vibrio aestivus was one of four *Vibrio* species identified in the tropical rock oyster 16S rDNA sequence dataset, but was not detected in the *hsp60* sequences. *V. aestivus* was first isolated from seawater in Spain (Lucena et al., 2012) and has been reported from seawater in Malaysia (Wong et al., 2019) and in association with the green algae, *Caulerpa cylindrica*, native to Australia but invasive in the Adriatic Sea (Rizzo et al., 2016) and to the best of our knowledge represents the first report of *V. aestivus* in a marine animal.

The dominant bacterial families identified in the tropical rock oysters belonged to the families *Spirochaetaceae*, *Mycoplasmataceae* and *Vibrionaceae* with the genus *Mycoplasma* occurring in at least 75% of all tropical rock oyster samples. These bacterial families have previously been reported in other oyster species from different countries (Akter et al., 2023; Clerissi et al., 2020; Diner et al., 2023; Fernandez-Piquer et al., 2012; King et al., 2012; Lokmer et al., 2016a, 2016b; Pierce and Ward, 2019; Wegner et al., 2013). Considering the samples in our study comprised two different, predominantly wild oyster species of unknown genetics, and were collected at different time points, it strongly suggests that these bacterial families and in particular, *Mycoplasma*, are core, resident taxa, rather than chance encounters, substantiating their role in maintaining tropical rock oyster health, although their function remains unknown. *Mycoplasma* were also found to be a core taxon in *Crassostrea virginica* (King et al., 2012; Pierce and Ward, 2019) and abundant in the digestive gland of Sydney Rock Oysters (Green and Barnes, 2010), and *M. gigas* gills (Wegner et al., 2013) and gut (Lokmer et al., 2016b). Mycoplasmas have a small genome with reduced metabolic function and likely have a symbiotic relationship with their oyster host, however, the genus includes several animal pathogens. Mycoplasmas may also contribute to disease when oysters are under stress, possibly by allowing other opportunistic bacteria to proliferate. Spirochaetes include vertebrate pathogens, but are also well known in invertebrate guts where they breakdown lignocellulose and fix nitrogen.

In our study, the genus *Vibrio* constituted most of the *Vibrionaceae* component of the total bacterial community, with *Photobacterium* and *Allivibrio* constituting only a small fraction. The prevalence of the *Vibrio* genus in the tropical rock oysters suggest they constitute part of the normal oyster flora and may possibly have a role in the breakdown of complex substrates, with many species producing extracellular enzymes that can degrade, for example, algin, lipids, chitin and starch. The large *Vibrio* species diversity between replicates, sites and oyster species measured in this study suggests a tolerance of oysters to multiple *Vibrio* species offering functional diversity which may be advantageous to the oyster host in different conditions. To establish whether they are truly core oyster taxa or transient, oyster microbial communities need to be assessed over different seasons and multiple years.

Bacterial community composition in oysters based on the 16S rRNA gene was useful in discriminating geographical relationships. Bacterial communities were generally more similar in oysters within rather than between sites, with farmed oysters showing higher within site similarity than wild oysters. This may be due to the similar age (1- 2 years) of farmed oysters compared to wild oysters of unknown and possibly varied age or may reflect the distance of replicates within the sites, with shorter distances between replicates in a farm set up compared to wild collections that may have been several kilometres apart. The greater bacterial diversity in wild oysters may also reflect seasonal exposure to higher sediment loads and disturbance, including during their collection.

The bacterial community composition did not significantly differ between oyster species in this study and similar results were found comparing Eastern Oyster (*C. virginica*) to the Blue Mussel (*Mytilus galloprovinicialis*) (Pierce and Ward, 2019). Potential human pathogenic taxa identified in the 16S rDNA sequence dataset included *Bacteroides*, *Arcobacteraceae* (including *Halarcobacter bivalvorium* and *Malaciobacter*), *Shewanella*, *Acinetobacter*, and *Escherichia-Shigella* but were generally at low abundance. Bacteroides, on the other hand, were abundant in some of the oyster samples but further research is needed to determine if they are pathogenic or environmental.

The only bacterial genus detected that is reported to be associated with oyster disease was *Roseovarius* and its presence in wild, healthy oysters in northern Australia has previously been reported (Padovan et al., 2017). *Arcobacter* species have been recovered from marine environments including bivalves (Collado et al., 2009; Levican et al., 2020; Ottaviani et al., 2017) and oysters (Figueras et al., 2011) and are an emerging gastrointestinal pathogen (Ramees et al., 2017) as well as associated with spoilage (Chen et al., 2019) so they are a genus worth tracking in future tropical rock oyster studies, particularly for postharvest management.

This study represents a major step forward in our understanding of the dominant *Vibrio* and bacterial taxa in healthy farmed and wild north Australian tropical rock oysters and has enabled an assessment of potential human and oyster pathogens, before any potential outbreaks. A project is underway to understand the impact of seasonal and weather-based events (e.g., monsoons, heat waves, algal blooms) on *Vibrio* and bacterial community diversity in farmed BROs oysters to identify high and low risk periods for food safety and oyster health.

2.6 **Conclusion**

Vibrio spp. are prevalent in tropical waters from northern Australia. There is increasing interest in tropical rock oyster farming in this region but limited information on the microbial communities existing naturally in wild or farmed oysters. Knowing which bacteria, particularly *Vibrio* spp., occur in oysters can help with managing both oyster disease outbreaks and risks to human health. Two species of wild and farmed tropical rock oysters from northern Australia were analysed for microbial community composition using high throughput sequencing. Blacklip Rock Oysters (BROs) (*Saccostrea echinata*/lineage J) were the intended target species, but Milky oysters (*Saccostrea mordax*/lineage A) were also collected when BROs were not present. Microbial diversity was assessed by sequencing *hsp60* amplicons for *Vibrio* spp. and the bacterial 16S rRNA gene for bacteria. *V. parahaemolyticus* and *V. vulnificus* levels were measured in a subset of oyster samples by MPN-qPCR. Forty-eight *Vibrio* species were identified in oyster tissue. The most abundant *Vibrio* species (relative abundance) across all oyster samples and sites were *V. parahaemolyticus* (18%), *V. harveyi* (10%), *V. diabolicus* (9%), *V. alginolyticus* (7%), and *V. mediterranei* (6%). The oyster *Vibrio* community was significantly different within and between sites suggesting it is likely influenced by their surrounding environment. Apart from *V. parahaemolyticus*, other known potential human pathogenic species identified included *V. vulnificus*, *V. alginolyticus*, and *V. fluvialis. V. parahaemolyticus* was detected in 77% of oysters with most samples in the range 3-100 MPN/g and 22% with levels >1,110 MPN/g, but virulence genes *trh* or *tdh* were not detected. *V. vulnificus* was detected in 31% of oyster samples with 10% of samples having levels >100 MPN/g. Eleven potential oyster *Vibrio* pathogens were identified with *V. harveyi* and *V. alginolyticus* the most abundant. Analysis of the whole bacterial community identified *Spirochaetaceae*, *Mycoplasmataceae* and *Vibrionaceae* as the dominant bacterial families with *Mycoplasma* a core genus, occurring in more than 75% of all oyster samples. Bacterial composition significantly differed by sites but not by oyster species suggesting a geographical microbial signature. In future studies, oyster bacterial species that are negatively correlated with *Vibrio* pathogenic species could be identified to serve as a source of probiotics in production systems.

2.7 Supplementary information

Supplement 2-1: Details of sampling locations and dates, oyster species collected and enumeration of *V. parahaemolyticus* and *V. vulnificus* by MPN-qPCR. Samples positive for *V. parahaemolyticus* were also tested for *trh* and *tdh* virulence genes, with none of these genes detected (results not shown); nt is not tested.

Sample ID	Location	Local Name	Species	Common name	Latitude	Longitude	Collection date	*V. parahaemolyticus* (MPN/g)	*V. vulnificus* (MPN/g)
CRB1	Maningrida	Crab Creek	*S. echinata* lineage J	Blacklip	S12.049586	E134.379639	19/3/19	43	3.6
CRB2	Maningrida	Crab Creek	*S. echinata* lineage J	Blacklip	S12.049586	E134.379639	19/3/19	75	7.2
CRB3	Maningrida	Crab Creek	*S. echinata* lineage J	Blacklip	S12.049586	E134.379639	19/3/19	43	11
OTS1	Maningrida	Outstation	*S. echinata* lineage J	Blacklip	S11.977806	E134.288694	19/3/19	23	<3.0
OTS2	Maningrida	Outstation	*S. echinata* lineage J	Blacklip	S11.977806	E134.288694	19/3/19	1100	3.6
OTS3	Maningrida	Outstation	*S. echinata* lineage J	Blacklip	S11.977806	E134.288694	19/3/19	43	
FRS1	Maningrida	First Point	*S. echinata* lineage J	Blacklip	S11.957861	E134.175722	20/3/19	43	<3.0
FRS2	Maningrida	First Point	*S. echinata* lineage J	Blacklip	S11.957861	E134.175722	20/3/19	43	15
FRS3	Maningrida	First Point	*S. echinata* lineage J	Blacklip	S11.957861	E134.175722	20/3/19	1100	9.2
RLL1	Maningrida	Rolling Bay	*S. echinata* lineage J	Blacklip	S11.888833	E134.014472	20/3/19	3.6	3.6
RLL2	Maningrida	Rolling Bay	*S. echinata* lineage J	Blacklip	S11.888833	E134.014472	20/3/19	23	20
RLL3	Maningrida	Rolling Bay	*S. echinata* lineage J	Blacklip	S11.888833	E134.014472	20/3/19	23	3.6
HNG1	Groote Eylandt	Hanging Rock	*S. echinata* lineage J	Blacklip	S13.681556	E136.6115	8/12/19	<3.0	<3.0
HNG2	Groote Eylandt	Hanging Rock	*S. echinata* lineage J	Blacklip	S13.681556	E136.6115	8/12/19	<3.0	<3.0
HNG3	Groote Eylandt	Hanging Rock	*S. echinata* lineage J	Blacklip	S13.681556	E136.6115	8/12/19	<3.0	<3.0
LTT1	Groote Eylandt	Little Jagged	*S. echinata* lineage J	Blacklip	S13.718583	E136.718944	8/12/19	<3.0	<3.0
LTT2	Groote Eylandt	Little Jagged	*S. echinata* lineage J	Blacklip	S13.718583	E136.718944	8/12/19	<3.0	<3.0
LTT3	Groote Eylandt	Little Jagged	*S. echinata* lineage J	Blacklip	S13.718583	E136.718944	8/12/19	<3.0	<3.0
TMR1	Groote Eylandt	Tamarind Passage	*S. echinata* lineage J	Blacklip	S13.788361	E136.542917	8/12/19	43	<3.0
TMR2	Groote Eylandt	Tamarind Passage	*S. echinata* lineage J	Blacklip	S13.788361	E136.542917	8/12/19	3.6	<3.0
TMR3	Groote Eylandt	Tamarind Passage	*S. echinata* lineage J	Blacklip	S13.788361	E136.542917	8/12/19	23	<3.0
WNC1	Groote Eylandt	Winchelsea Island	*S. echinata* lineage J	Blacklip	S13.701028	E136.509667	8/12/19	<3.0	<3.0
WNC2	Groote Eylandt	Winchelsea Island	*S. echinata* lineage J	Blacklip	S13.701028	E136.509667	8/12/19	<3.0	<3.0
WNC3	Groote Eylandt	Winchelsea Island	*S. echinata* lineage J	Blacklip	S13.701028	E136.509667	8/12/19	9.2	<3.0
MME1	Milingimbi	Murrunga East	*S. echinata* lineage J	Blacklip	S11.925861	E135.122056	2/10/19	<3.0	<3.0
MME2	Milingimbi	Murrunga East	*S. echinata* lineage J	Blacklip	S11.925861	E135.122056	2/10/19	<3.0	<3.0
MME3	Milingimbi	Murrunga East	*S. echinata* lineage J	Blacklip	S11.925861	E135.122056	2/10/19	<3.0	<3.0
MMW1	Milingimbi	Murrunga West	*S. echinata* lineage J	Blacklip	S11.919833	E135.057194	2/10/19	9.2	<3.0
MMW2	Milingimbi	Murrunga West	*S. echinata* lineage J	Blacklip	S11.919833	E135.057194	2/10/19	11	<3.0
MMW3	Milingimbi	Murrunga West	*S. echinata* lineage J	Blacklip	S11.919833	E135.057194	2/10/19	<3.0	<3.0

Sample ID	Location	Local Name	Species	Common name	Latitude	Longitude	Collection date	*V. parahaemolyticus* (MPN/g)	*V. vulnificus* (MPN/g)
LAN1	Milingimbi	Langarra	*S. echinata* lineage J	Blacklip	S12.083417	E135.403417	1/10/19	23	<3.0
LAN2	Milingimbi	Langarra	*S. echinata* lineage J	Blacklip	S12.083417	E135.403417	1/10/19	43	<3.0
LAN3	Milingimbi	Langarra	*S. echinata* lineage J	Blacklip	S12.083417	E135.403417	1/10/19	9.2	<3.0
VIEST1	Darwin Harbour	Vernon Island East	*S. mordax* lineage A	Large milky	S12.07724	E131.09537	20/8/20	>1100	<3.0
VIEST2	Darwin Harbour	Vernon Island East	*S. mordax* lineage A	Large milky	S12.07724	E131.09537	20/8/20	>1100	<3.0
VIEST3	Darwin Harbour	Vernon Island East	*S. mordax* lineage A	Large milky	S12.07724	E131.09537	20/8/20	>1100	<3.0
VIWST1	Darwin Harbour	Vernon Island West	*S. mordax* lineage A	Large milky	S12.04827	E130.99634	20/8/20	>1100	<3.0
VIWST2	Darwin Harbour	Vernon Island West	*S. mordax* lineage A	Large milky	S12.04827	E130.99634	20/8/20	>1100	<3.0
VIWST3	Darwin Harbour	Vernon Island West	*S. mordax* lineage A	Large milky	S12.04827	E130.99634	20/8/20	>1100	<3.0
TPP1	Bynoe Harbour	Tapa Bay	*S. mordax* lineage A	Large milky	S12.46045	E130.57866	16/12/20	3.6	<3.0
TPP2	Bynoe Harbour	Tapa Bay	*S. mordax* lineage A	Large milky	S12.46045	E130.57866	16/12/20	240	<3.0
TPP3	Bynoe Harbour	Tapa Bay	*S. mordax* lineage A	Large milky	S12.46045	E130.57866	16/12/20	43	<3.0
MMN1	Croker Is	MeiMine	*S. echinata* lineage J	Blacklip	S11.07575	E132.59801	3/2/21	23	29
MMN2	Croker Is	MeiMine	*S. mordax* lineage A	Large milky	S11.07575	E132.59801	3/2/21	240	<3.0
MMN3	Croker Is	MeiMine	*S. mordax* lineage A	Large milky	S11.07575	E132.59801	3/2/21	9.2	<3.0
BGC1	Croker Is	Alamirra	*S. echinata* lineage J	Blacklip	S11.04365	E132.52708	3/2/21	23	<3.0
BGC2	Croker Is	Alamirra	*S. echinata* lineage J	Blacklip	S11.04365	E132.52708	3/2/21	93	<3.0
BGC3	Croker Is	Alamirra	*S. echinata* lineage J	Blacklip	S11.04365	E132.52708	3/2/21	43	<3.0
WHT1	Croker Is	Gulgui	*S. echinata* lineage J	Blacklip	S11.23851	E132.52939	2/2/21	240	<3.0
WHT2	Croker Is	Gulgui	*S. echinata* lineage J	Blacklip	S11.23851	E132.52939	2/2/21	38	<3.0
WHT3	Croker Is	Gulgui	*S. echinata* lineage J	Blacklip	S11.23851	E132.52939	2/2/21	1100	<3.0
LNG1	Croker Is	Ardburrdj	*S. echinata* lineage J	Blacklip	S11.20594	E132.50072	2/2/21	<3.0	<3.0
LNG2	Croker Is	Ardburrdj	*S. echinata* lineage J	Blacklip	S11.20594	E132.50072	2/2/21	<3.0	<3.0
LNG3	Croker Is	Ardburrdj	*S. echinata* lineage J	Blacklip	S11.20594	E132.50072	2/2/21	<3.0	<3.0
GKS1	Nhulunbuy	Guku Is	*S. echinata* lineage J	Blacklip	S12.86794	E136.61986	10/2/21	23	<3.0
GKS2	Nhulunbuy	Guku Is	*S. echinata* lineage J	Blacklip	S12.86794	E136.61986	10/2/21	240	<3.0
GKS3	Nhulunbuy	Guku Is	*S. echinata* lineage J	Blacklip	S12.86794	E136.61986	10/2/21	240	21
BRR1	Nhulunbuy	Barrkira	*S. echinata* lineage J	Blacklip	S12.00620	E136.47372	11/2/21	23	9.2
BRR2	Nhulunbuy	Barrkira	*S. echinata* lineage J	Blacklip	S12.00620	E136.47372	11/2/21	93	240
BRR3	Nhulunbuy	Barrkira	*S. echinata* lineage J	Blacklip	S12.00620	E136.47372	11/2/21	23	240
DLP1	Nhulunbuy	Dolphin Rocks	*S. echinata* lineage J	Blacklip	S12.27075	E136.71712	8/2/21	nt	nt
DLP2	Nhulunbuy	Dolphin Rocks	*S. echinata* lineage J	Blacklip	S12.27075	E136.71712	8/2/21	nt	nt
DLP3	Nhulunbuy	Dolphin Rocks	*S. echinata* lineage J	Blacklip	S12.27075	E136.71712	8/2/21	nt	nt
YNW1	Nhulunbuy	Yiniwuy	*S. echinata* lineage J	Blacklip	S12.50778	E136.73427	9/2/21	nt	nt
YNW2	Nhulunbuy	Yiniwuy	*S. echinata* lineage J	Blacklip	S12.50778	E136.73427	9/2/21	nt	nt
YNW3	Nhulunbuy	Yiniwuy	*S. echinata* lineage J	Blacklip	S12.50778	E136.73427	9/2/21	nt	nt
NGM1	Wadeye	Ngumbala	*S. mordax* lineage A	Large milky	S14.19640	E129.44356	1/3/21	>1100	460

Sample ID	Location	Local Name	Species	Common name	Latitude	Longitude	Collection date	*V. parahaemolyticus* (MPN/g)	*V. vulnificus* (MPN/g)
NGM2	Wadeye	Ngumbala	*S. mordax* lineage A	Large milky	S14.19640	E129.44356	1/3/21	>1100	<3.0
NGM3	Wadeye	Ngumbala	*S. mordax* lineage A	Large milky	S14.19640	E129.44356	1/3/21	>1100	<3.0
EMU1	Wadeye	Emu Reef	*S. mordax* lineage A	Large milky	S13.86433	E129.46120	1/3/21	<3.0	<3.0
EMU2	Wadeye	Emu Reef	*S. mordax* lineage A	Large milky	S13.86433	E129.46120	1/3/21	<3.0	<3.0
EMU3	Wadeye	Emu Reef	*S. mordax* lineage A	Large milky	S13.86433	E129.46120	1/3/21	<3.0	<3.0
TJN1	Wadeye	Tjinpili	*S. mordax* lineage A	Large milky	S13.83307	E129.70293	2/3/21	460	150
TJN2	Wadeye	Tjinpili	*S. mordax* lineage A	Large milky	S13.83307	E129.70293	2/3/21	460	240
TJN3	Wadeye	Tjinpili	*S. mordax* lineage A	Large milky	S13.83307	E129.70293	2/3/21	>1100	93
CHN1	Wadeye	Chinin	*S. mordax* lineage A	Large milky	S13.80611	E129.74422	2/3/21	>1100	>1100
CHN2	Wadeye	Chinin	*S. mordax* lineage A	Large milky	S13.80611	E129.74422	2/3/21	>1100	240
CHN3	Wadeye	Chinin	*S. mordax* lineage A	Large milky	S13.80611	E129.74422	2/3/21	460	1100
WLM1	Tiwi Is	Walama	*S. echinata* lineage J	Blacklip	S11.34097	E130.23645	17/3/21	>1100	75
WLM2	Tiwi Is	Walama	*S. echinata* lineage J	Blacklip	S11.34097	E130.23645	17/3/21	>1100	<3.0
WLM3	Tiwi Is	Walama	*S. echinata* lineage J	Blacklip	S11.34097	E130.23645	17/3/21	>1100	<3.0
MDL1	Tiwi Is	Mudlow Island	*S. echinata* lineage J	Blacklip	S11.36084	E130.56984	18/3/21	>1100	<3.0
MDL2	Tiwi Is	Mudlow Island	*S. echinata* lineage J	Blacklip	S11.36084	E130.56984	18/3/21	>1100	<3.0
MDL3	Tiwi Is	Mudlow Island	*S. echinata* lineage J	Blacklip	S11.36084	E130.56984	18/3/21	460	3.6
MND1	Tiwi Is	Mindiloo	*S. echinata* lineage J	Blacklip	S11.69793	E130.56871	15/3/21	nt	nt
MND2	Tiwi Is	Mindiloo	*S. echinata* lineage J	Blacklip	S11.69793	E130.56871	15/3/21	nt	nt
MND3	Tiwi Is	Mindiloo	*S. echinata* lineage J	Blacklip	S11.69793	E130.56871	15/3/21	nt	nt
FRC1	Tiwi Is	Cape Fourcroy	*S. echinata* lineage J	Blacklip	S11.78027	E130.02025	16/3/21	nt	nt
FRC2	Tiwi Is	Cape Fourcroy	*S. echinata* lineage J	Blacklip	S11.78027	E130.02025	16/3/21	nt	nt
FRC3	Tiwi Is	Cape Fourcroy	*S. echinata* lineage J	Blacklip	S11.78027	E130.02025	16/3/21	nt	nt
NI1	Borroloola	North Island	*S. mordax* lineage A	Large milky	S15.635217	E136.881833	15/4/21	nt	nt
NI2	Borroloola	North Island	*S. mordax* lineage A	Large milky	S15.635217	E136.881833	15/4/21	nt	nt
NI3	Borroloola	North Island	*S. mordax* lineage A	Large milky	S15.635217	E136.881833	15/4/21	nt	nt
WI1	Borroloola	West Island	*S. mordax* lineage A	Large milky	S15.519517	E136.511767	15/4/21	nt	nt
WI2	Borroloola	West Island	*S. mordax* lineage A	Large milky	S15.519517	E136.511767	15/4/21	nt	nt
WI3	Borroloola	West Island	*S. mordax* lineage A	Large milky	S15.519517	E136.511767	15/4/21	nt	nt
ER1	Borroloola	Eagle Rocks	*S. mordax* lineage A	Large milky	S15.645850	E136.726450	22/4/21	nt	nt
ER2	Borroloola	Eagle Rocks	*S. mordax* lineage A	Large milky	S15.645850	E136.726450	22/4/21	nt	nt
ER3	Borroloola	Eagle Rocks	*S. mordax* lineage A	Large milky	S15.645850	E136.726450	22/4/21	nt	nt
BCI1	Borroloola	Black Craggy Is	*S. mordax* lineage A	Large milky	S15.589650	E136.678267	15/4/21	nt	nt
BCI2	Borroloola	Black Craggy Is	*S. mordax* lineage A	Large milky	S15.35.379	E136.40.696	15/4/21	nt	nt
BCI3	Borroloola	Black Craggy Is	*S. mordax* lineage A	Large milky	S15.35.379	E136.40.696	15/4/21	nt	nt
GI A1	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
GI A2	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt

Sample ID	Location	Local Name	Species	Common name	Latitude	Longitude	Collection date	*V. parahaemolyticus* (MPN/g)	*V. vulnificus* (MPN/g)
GI A3	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
GI B1	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
GI B2	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
GI B3	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
GI C1	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
GI C2	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
GI C3	Goulburn Is	n/a	*S. echinata* lineage J	Blacklip	S11.622071	E133.403951	25/2/21	nt	nt
BFO1	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO2	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO3	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO4	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO5	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO6	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO7	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO8	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO9	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO10	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO11	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
BFO12	Bowen	n/a	*S. echinata* lineage J	Blacklip	S20.012096	E148.246267	27/6/21	nt	nt
COS1	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS2	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS3	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS4	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS5	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS6	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS7	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS8	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS9	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS10	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS11	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
COS12	Cossack	Cossack	*S. echinata* lineage J	Blacklip	S20.644324	E117.134112	7/7/21	nt	nt
FFP1	Flying Foam Passage	Flying Foam Passage	*S. echinata* lineage J	Blacklip	S20.439513	E116.861323	12/7/21	nt	nt
FFP2	Flying Foam Passage	Flying Foam Passage	*S. echinata* lineage J	Blacklip	S20.439513	E116.861323	12/7/21	nt	nt
FFP3.1	Flying Foam Passage	Flying Foam Passage	*S. mordax* lineage A	Large milky	S20.439513	E116.861323	12/7/21	nt	nt
FFP3.2	Flying Foam Passage	Flying Foam Passage	*S. mordax* lineage A	Large milky	S20.439513	E116.861323	12/7/21	nt	nt
FFP4	Flying Foam Passage	Flying Foam Passage	*S. mordax* lineage A	Large milky	S20.439513	E116.861323	12/7/21	nt	nt
FFP5	Flying Foam Passage	Flying Foam Passage	*S. echinata* lineage J	Blacklip	S20.439513	E116.861323	12/7/21	nt	nt

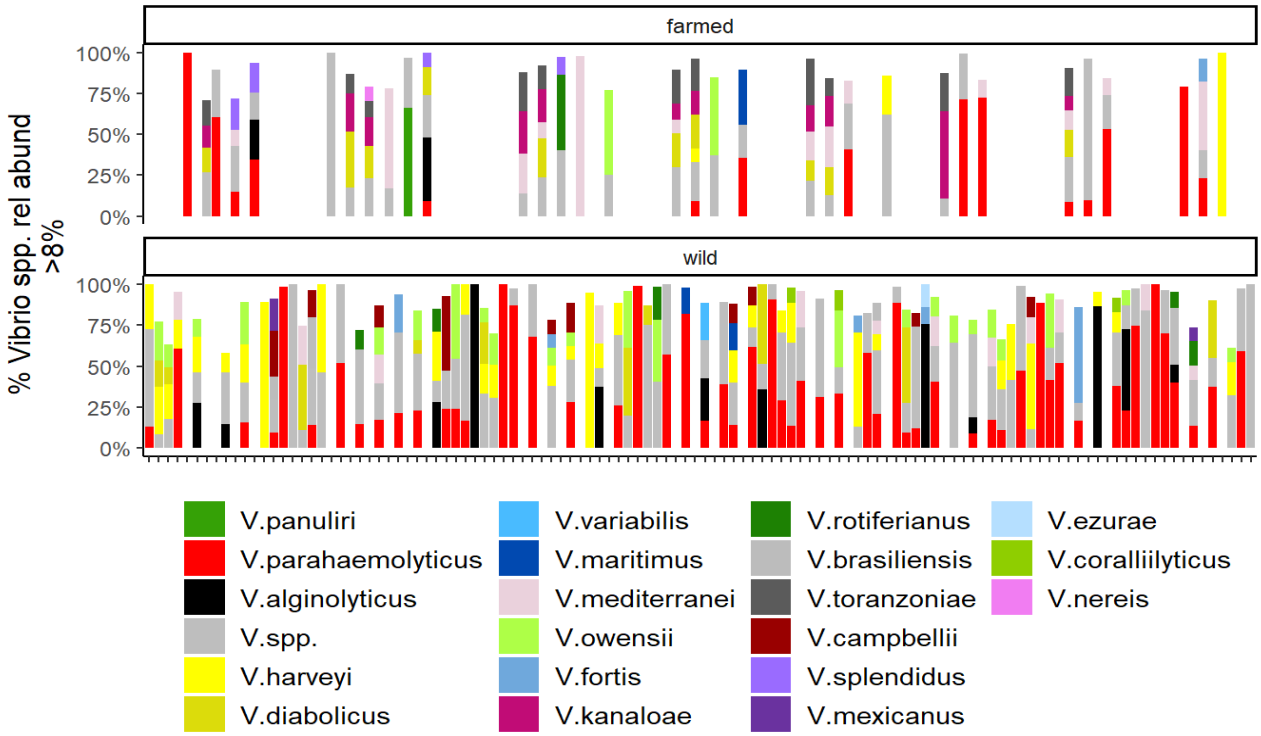
Sample ID	Location	Local Name	Species	Common name	Latitude	Longitude	Collection date	*V. parahaemolyticus* (MPN/g)	*V. vulnificus* (MPN/g)
FFP6	Flying Foam Passage	Flying Foam Passage	*S. echinata* lineage J	Blacklip	S20.439513	E116.861323	12/7/21	nt	nt
FFP7	Flying Foam Passage	Flying Foam Passage	*S. mordax* lineage A	Large milky	S20.439513	E116.861323	12/7/21	nt	nt
FFP8	Flying Foam Passage	Flying Foam Passage	*S. mordax* lineage A	Large milky	S20.439513	E116.861323	12/7/21	nt	nt
WL1	West Lewis	West Lewis	*S. echinata* lineage J	Blacklip	S20.588609	E116.625855	29/7/21	nt	nt
WL2	West Lewis	West Lewis	*S. echinata* lineage J	Blacklip	S20.588609	E116.625855	29/7/21	nt	nt
WL3	West Lewis	West Lewis	*S. echinata* lineage J	Blacklip	S20.588609	E116.625855	29/7/21	nt	nt

Supplement 2-2: Relative abundance of *Vibrio* species sequences identified in tropical rock oyster tissue across all sites. Species highlighted in bold contributed to 90% of all sequences identified. **h** indicates potential human pathogen; **o** indicates potential oyster pathogen.

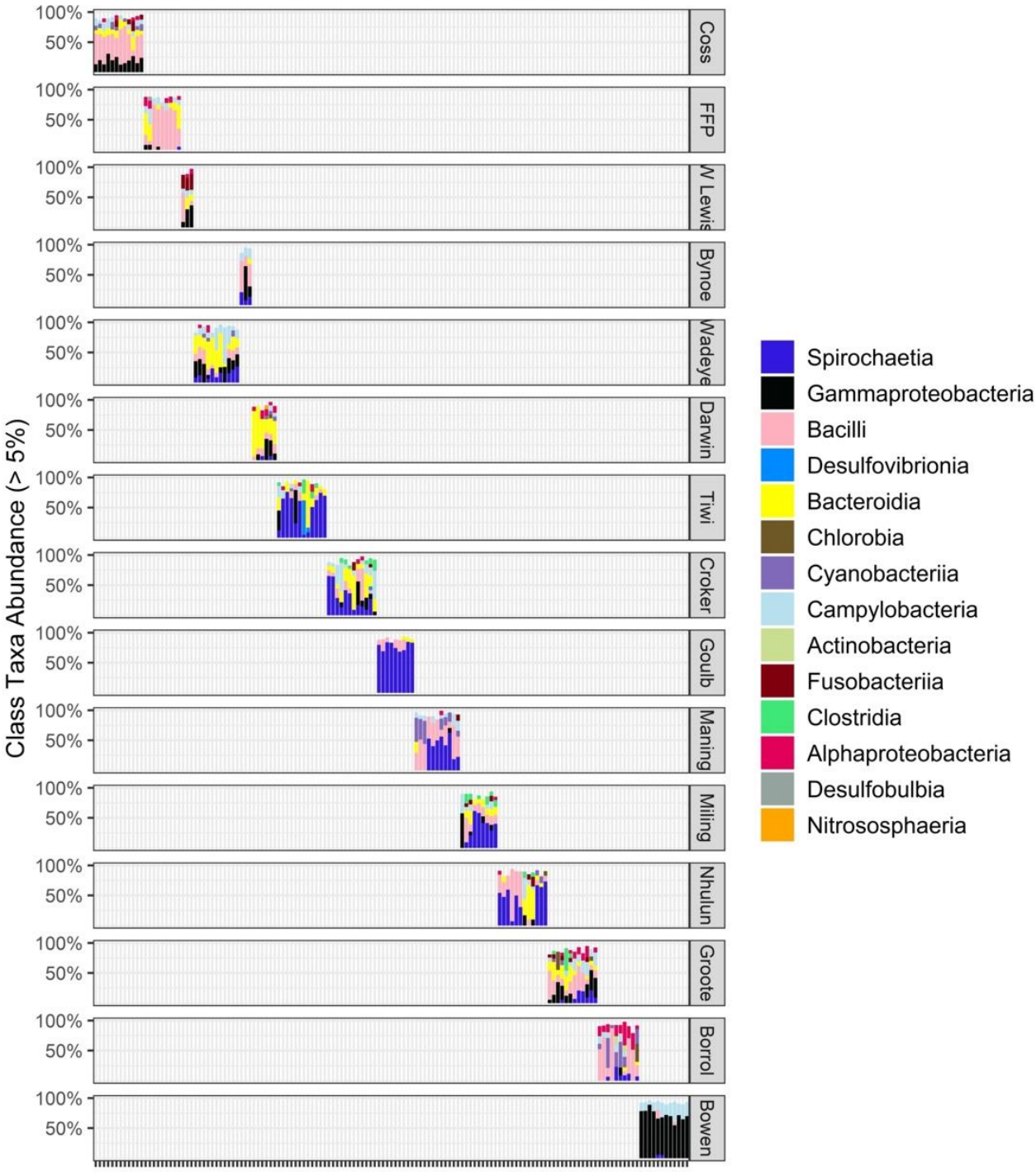
Species	freq samples	% total reads	Reference including species and life stage
Vibrio spp.	107	25.1	
V. parahaemolyticus [h]	97	17.7	
V. harveyi [o]	65	9.6	*M. gigas* spat (Saulnier et al. 2010) *M. gigas* adult (Wang et al. 2021; Cowan et al. 2023)
V. diabolicus	39	8.8	
V. alginolyticus [h,o]	52	7.1	*Ostrea edulis* larvae (Tubiash et al. 1965) *C. virginica* larvae (Tubiash et al. 1970) *M. gigas* larvae (Luna-Gonzalez et al. 2002) *M. gigas* adult (Yang et al. 2021; Wang et al. 2021)
V. mediterranei	50	6.4	
V. kanaloae	11	5.8	
V. toranzoniae	11	5.7	
V. owensii	57	3.3	
V. fortis	47	3.2	
V. campbellii	27	1.2	
V. rotiferianus	26	1.1	
V. nereis	12	0.7	
V. brasiliensis [o]	30	0.7	*M. gigas* adult (Wang et al. 2021)
V. splendidus [o]	28	0.6	*M. gigas* spat (Gay et al. 2004; Saulnier et al. 2010; Waechter et al. 2002) *M. gigas* juvenile (Lacoste et al. 2001) *M. gigas* adult (Saulnier et al. 2010; Garnier et al. 2007) *M. gigas* larvae (Sugumar et al. 2008)
V. natriegens	20	0.5	
V. maritimus	26	0.4	
V. antiquarius (recently classified as *V. diabolicus*)	9	0.3	
V. sinaloensis	22	0.3	
V. tubiashii [o]	29	0.2	*C. virginica* larvae (Tubiash et al. 1965; Richards et al. 2015) *M. gigas* (Takahashi et al. 2000) *M. gigas* larvae (Elston et al. 2008; Travers et al. 2014 Richards et al. 2015)
V. coralliilyticus [o]	18	0.2	*M. gigas* larvae (Richards et al. 2015; Genard et al. 2013; Kesarcodi-Watson et al. 2012) *C. virginica* larvae (Richards et al. 2015) *O. edulis* (Kesarcodi-Watson et al. 2012)
V. mexicanus	23	0.2	
V. cyclitrophicus	10	0.1	
V. panuliri	7	0.1	
V. ezurae	2	0.1	
V. variabilis	13	0.1	

V. neptunius [o]	16	0.1	*Ostrea edulis* larvae (Prado et al. 2005); *M. gigas* (Kesarcodi-Watson et al. 2012)
V. vulnificus [h]	2	0.1	
V. ishigakensis	9	0.1	
V. hepatarius	11	0.1	
V. diazotrophicus	6	<0.05	
V. alfacsensis	6	<0.05	
V. scophthalmi	3	<0.05	
V. furnissii [h]	4	<0.05	
V. lentus [o]	3	<0.05	*M. gigas* spat (Saulnier et al. 2010) *M. gigas* juveniles (Oyanedel et al. 2023)
V. ponticus	7	<0.05	
V. barjaei	3	<0.05	
V. chagasii	2	<0.05	
V. bivalvicida [o]	3	<0.05	*Ostrea edulis* larvae (Dubert et al. 2016)
V. coralliirubri	2	<0.05	
V. xiamenensis	1	<0.05	
V. orientalis	1	<0.05	
V. hangzhouensis	1	<0.05	
V. europaeus [o] (previously described as *V. tubiashii* subspecies)	1	<0.05	*Ostrea edulis* larvae (Prado et al. 2005; 2015) *M. gigas* larvae (Mersni-Achour et al. 2014; 2015; Travers et al. 2014)
V. gigantis	3	<0.05	
V. crassostreae [o]	2	<0.05	*M. gigas* spat (Faury et al. 2004; Saulnier et al. 2010); *M. gigas* adult (Bruto et al. 2017)
V. nigripulchritudo	2	<0.05	
V. fluvialis [h]	1	<0.05	

Supplement 2-3: Taxa bar plots for individual samples comparing farmed versus wild oysters.



Supplement 2-4: Stacked bacterial taxa bar plots of all samples.



3 Develop tests for *Vibrio* species that are toxigenic to oysters and humans

3.1 Introduction

During previous *Vibrio* research we had spent considerable time finding and optimising qPCR methods for species that are either food safety targets, or which are known aquatic animal pathogens. We thought that it would be useful to start a database of published method references and standard operating protocols (SOPs) for species that are not yet published. These could be disseminated via this report and more informally later with interested parties. We focussed on qPCR methods because these are a useful workhorse for ecological studies, and as an early diagnostic test. There are a range of additional *Vibrio* species that could be added over time – for example *V. mediterranei* has been associated with TRO larval mortality and would be useful additional target (Tinning et al unpublished data).

3.2 Published and optimised qPCR methods for four *Vibrio* species

3.2.1 Vibrio parahaemolyticus

A standard operating protocol for qPCR detection of *Vibrio parahaemolyticus tlh* (thermolabile hemolysin) gene and virulence genes *tdh* (thermostable direct hemolysin) and *trh* (thermostable-related hemolysin) was based on the method published by:

Nordstrom, J. L., Vickery, M. C. L., Blackstone, G. M., Murray, S. L. & DePaola, A. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic Vibrio parahaemolyticus bacteria in oysters. Applied and Environmental Microbiology 73, 5840–5847 (2007). DOI: https://doi.org/10.1128/AEM.00460-07

3.2.2 Vibrio vulnificus

A standard operating protocol for qPCR detection of *Vibrio vulnificus vvhA* (cytolysin) gene was based on the method published by:

Campbell, M. S. & Wright, A. C. Real-time PCR analysis of *Vibrio vulnificus* from oysters. *Applied and Environmental Microbiology* 69, 7137–7144 (2003) DOI: https://doi.org/10.1128/AEM.69.12.7137-7144.2003

3.2.3 Vibrio alginolyticus

A SOP for qPCR detection of *Vibrio alginolyticus* was optimised in this project as follows:

Document No.	X.Y
Effective Date	15/12/19
Revision No.	X
Author	JOHN DOE
Reference	Eg. (EN) - DNeasy Blood & Tissue Handbook

Environmental Microbiology & Chemistry Unit (ECMU)
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RIEL
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STANDARD OPERATING PROCEDURE		
***Vibrio alginolyticus* qPCR SOP**		
Final version:	1	17/1/2022

1. PURPOSE

1.1. SOP of SYBR Green assay for *Vibrio alginolyticus* adapted using PCR primers from Zhou et al. 2007:

F-gyrB: 5'-ATT GAG AAC CCG ACA GAA GCG AAG-3'
R-gyrB: 5'-CCT AAT GCG GTG ATC AGT GTT ACT-3'

2. OVERVIEW OF METHOD MODIFICATIONS

2.1. Zhou et al. did not specify cycling conditions or primer concentrations, so we used conditions from Wei et al. 2014 paper.

Cycling Conditions:
Initial - 94 °C for 3 min
30 cycles - 94 °C for 30 s, 60 °C for 30 s and 72 °C for 120 s
Final - 72 °C for 10 min

Primer Concentration: 0.2 µmol/L

Product Size: 337 bp

Minimum concentration of *V. alginolyticus* detected: 10 CFU

3. PROCEDURE

3.1. Mastermix Preparation

Mastermix for 2 µl template	
	1x
2x SYBR Green Mastermix	12.5 µl
For primer (20 ∝M)	0.5 µl
Rev primer (20 ∝M)	0.5 µl
Nuclease-free water	9.5 µl
DNA template	2 µl
TOTAL	25 µl

1 Last updated: 15/12/19

4. QUALITY CONTROL

Optimised qPCR Summary:
Rotor-Gene Q - Vibrio alginolyticus gyrB qPCR4

Threshold	0.03019
R^2 Value	0.99384
Reaction Efficiency	0.92569 (92.6%)

5. REFERENCES

Wei, S., Zhao, H., Xian, Y., Hussain, M. A. & Wu, X. Multiplex PCR assays for the detection of *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae* with an internal amplification control. *Diagnostic Microbiology and Infectious Disease* **79**, 115–118 (2014). doi: 10.1016/j.diagmicrobio.2014.03.012

Zhou, S., Hou, Z., Li, N. & Qin, Q. Development of a SYBR Green I real-time PCR for quantitative detection of *Vibrio alginolyticus* in seawater and seafood. *Journal of Applied Microbiology* **103**, 1897–1906 (2007). doi: 10.1111/j.1365-2672.2007.03420.x

3.2.4 Vibrio harveyi

A SOP for qPCR detection of *Vibrio harveyi* was optimised in this project as follows:

Document No.	1
Effective Date	07/06/22
Revision No.	0
Author	ZARAH TINNING
Reference	Mougin et al. 2021

Environmental Microbiology & Chemistry Unit (ECMU)
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Darwin, 0909, NT, Australia

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RIEL Research Institute for the Environment and Livelihoods

STANDARD OPERATING PROCEDURE		
***Vibrio harveyi* qPCR SOP**		
Final version:	1	07/06/2022

1. PURPOSE

1.1. SOP of probe-based assay for *Vibrio harveyi* adapted using RT-PCR primers from Mougin *et al*. 2021 and probe developed by Nahshon Siboni 2022 (University of Technology Sydney).

mreB11F: TGAAGCTGTGATCAACTACG
mreB9bisR: TGACAGTGGCTCTTGTAA

mreB probe 5'- FAM - AACTACGGCAGCTTGATCGGTGAA - ZEN - IBFQ -3'

2. OVERVIEW OF METHOD

Cycling Conditions:
Initial - 95 °C for 5 min
40 cycles - 95 °C for 10 s, 60 °C for 20 s and 72 °C for 10 s

Primer Concentration: 0.4 µmol/L
Probe Concentration: 0.2 µmol/L

Positive Control: *Vibrio harveyi* ATCC 14126 or mreB gene block

3. PROCEDURE

3.1. Mastermix Preparation

Mastermix for 5 µl template	
	1x
2x PerfeCTa Tough Mix	10 µl
For primer (10 ∝M)	0.8 µl
Rev primer (10 ∝M)	0.8 µl
Probe (10 ∝M)	0.4 µl
Nuclease-free water	3 µl
DNA template	5 µl
TOTAL	20 µl

1 Last updated: 07/09/22

4. QUALITY CONTROL

Optimised qPCR Summary:
Rotor-Gene Q - Vibrio harveyi qPCR1

Threshold	0.04305
R^2 value	0.99577
Reaction Efficiency	1.03 (103%)

5. REFERENCES

Mougin, J., Roquigny, R., Travers, M., Grard, T., Bonnin-Jusserand, M. & Le Bris, C. (2021). Development of a mreB-targeted real-time PCR method for the quantitative detection of *Vibrio harveyi* in seawater and biofilm from aquaculture systems. Aquac.10.1016/j.aquaculture.2020.735337. doi: 10.1016/j.aquaculture.2020.735337

4 Measure *Vibrio* community diversity and relative abundance in farmed Blacklip Rock Oysters (BROs) and surrounding water over the course of one year

4.1 Introduction

The Blacklip Rock Oyster (BRO) industry in northern Australia has significant potential and in the Northern Territory, Indigenous communities are leading this emerging aquaculture enterprise. Globally, the oyster industry is faced with food safety concerns due to the presence of pathogenic bacteria associated with the *Vibrio* genus. This problem is increasing because *Vibrio* bacteria are positively associated with climate change driven increases in seawater temperature. Objective 1 of FRDC Project 2020-043, was to obtain a *Vibrio* baseline in BRO and other Tropical Rock Oysters (TRO) and develop tests for *Vibrio* species identified as potentially toxigenic to oysters and humans. The results of this objective have been detailed in chapters 2 and 3 of this report with *Vibrio* and bacterial taxa, including potential human and oyster pathogens, detected in wild and farmed tropical rock oysters collected across northern Australia. A limitation to this research was that TRO were collected from each location at one point in time, and there remains a knowledge gap about how *Vibrio* community diversity, particularly in farm settings, changes over seasons, in both oysters and surrounding seawater.

Understanding the *Vibrio* community profile and how it changes with time, including potential pathogenic species, will underpin future decision-making about harvest, risk and shellfish quality assurance procedures and compliance. This is particularly important in the tropics where TRO is a relatively new product for the region, so food safety is critical at this early stage of the development of this enterprise. An additional important driver for this time series study was to determine whether the water *Vibrio* community can be considered a surrogate for the oyster *Vibrio* community. Water is much easier to monitor for *Vibrio* than oysters, and a program of water surveillance to provide early warning and follow up oyster analysis would open the door to Indigenous-led biosecurity monitoring generally, and *Vibrio* specifically.

We developed a strong collaboration with the Yagbani Aboriginal Corporation (YAC) during the course of this FRDC project, and the possibility of doing a seasonal study was endorsed by the YAC Board during an on-country workshop for this FRDC project. Due to their enthusiasm and support for sampling, we felt enabled to develop a proposal to track the *Vibrio* community in BRO from South Goulburn Island over the course of one year, to identify shifts in the microbial communities in oysters and seawater and identify possible environmental conditions that favour pathogens such as *Vibrio parahaemolyticus*. This proposal was funded by Charles Darwin University (CDU) based on the existing support of the current FRDC project, and endorsed by the Principal Investigators and Steering Committee. With the endorsement of the FRDC, this study was added as an additional objective to FRDC Project 2020-043.

4.2 **Objectives**

The objective of this study was to measure *Vibrio* community diversity and relative abundance in farmed Blacklip Rock Oysters (BROs) and surrounding water over the course of one year. This objective was not included in the original FRDC application due to budget constraints, however, the need for a *Vibrio* baseline in oysters and water over a one-year time series became apparent to inform risk analysis. This time series baseline was made possible through additional funding by CDU. Following a request to FRDC in 2021, this study was included in the FRDC reporting timetable as it is intrinsically linked to other data generated in this project. This objective was quite broad so the study was broken down into smaller objectives that facilitated communication of the outcomes:

- Measure the number of *Vibrio* species in oysters and surrounding water
- Characterise the changes in *Vibrio* abundance and richness in oysters and water over time
- Identify which *Vibrio* species dominate the community in oysters and water
- Determine how the *Vibrio* community in oysters and water changed over time
- Identify which *Vibrio* species drove the differences between oysters, water and time
- Can water *Vibrio* community diversity be used as a surrogate for oysters?
- Identify which environmental variables drive shifts in the *Vibrio* community
- If present, determine whether *Vibrio* pathogens are associated with particular times of the year

This seasonal study was conducted in collaboration with the Yagbani Aboriginal Corporation (YAC) on South Goulburn Island.

4.3 **Methods**

4.3.1 Sampling site

Blacklip Rock Oysters (BROs), *Saccostrea echinata* (*Saccostrea* lineage J), were collected from a farm on South Goulburn Island on six occasions during 2021/22. Sampling dates were originally designed to cover the Dry season (May-September), the Wet season (October-April), and significant weather events such as monsoons, however, factors such as tides, personnel availability, bad weather, and dangerous wildlife, constrained sampling efforts.

4.3.2 Sample collection and processing

Photographs were taken of shucked BROs collected in 2022 and expert grower knowledge was used to estimate BRO condition.

Seawater and oysters were collected in triplicate from 3 randomly chosen baskets designated A, B, C, within the farm and stored in an esky kept in the shade until delivered to the CDU laboratory on the same day of sampling. Seawater (1 L) was collected in sterile bottles and oysters were placed in plastic ziplock bags.

A Multi-Parameter Testr 35 Series (Eutech Instruments) was used to measure seawater temperature and pH, and a LAQUAtwin EC-33 Compact Conductivity Meter (Horiba Scientific) was used to measure conductivity on site. Back in the laboratory, turbidity was measured in a 500 mL aliquot of seawater using a HYDROLAB® Quanta® water quality instrument.

Water (500 mL) was filtered through 0.2 µm mixed cellulose ester filters (Advantec®) which were then stored at -80°C. Fifty mL of seawater was aliquoted and frozen prior to batch nutrient analysis. Total phosphorus (TP) was measured by Kjeldahl digestion (reporting limit 0.003 mg/L) and total nitrogen (TN) was measured by persulphate digestion (reporting limit 0.02 mg/L) (Forensic and Health Services, Qld Govt). Chlorophyll *a* was measured by fluorometric detection adapted from the Trilogy® Laboratory Fluorometer (Turner Designs, San Jose, CA, USA) and standard acetone extraction methods (APHA, 2005), following filtration of 500 mL onto glass fibre filters. Calibrations were performed using stock concentrations of chlorophyll *a* (Sigma). The Trilogy® Laboratory Fluorometer has an extracted chlorophyll *a* minimum detection limit of 0.0225 µg/L and a reporting limit of 0.1 µg/L. Three oysters per replicate per site (27 total) were collected at each sampling event. In the laboratory, oysters were scrubbed with potable water, and dimensions and weight recorded. Oysters were shucked and the meat and fluid from 3 oysters pooled to give one sample. The pH of the intravalvular fluid was recorded. Each oyster sample was homogenized using an Ultra-Turrax® IKA T18 (IKA® Works, Malaysia) and stored at -80°C. DNA was extracted from approximately 25 mg oyster tissue

homogenates using the DNeasy® Blood and Tissue kit (Qiagen) and nucleic acid quantity and quality determined spectrophotometrically (NanoDrop™).

4.3.3 Vibrio-centric hsp60 amplicon sequencing and analysis

PCR was performed on oyster and water DNA using the *Vibrio*-centric *hsp60* primers Vib-hspF3-23 and Vib-hspR401-422, as previously described (King et al. 2019). Amplicons were sequenced using the Illumina NovaSeq SP 500 platform according to the manufacturer's guidelines (Ramaciotti Centre for Genomics, Sydney). Raw data files in FASTQ format will be deposited in NCBI Sequence Read Archive (SRA).

The approach used to measure the *Vibrio* community relies on identification of species from a DNA sequence, in this case part of the *hsp60* gene. This approach defines each *Vibrio* as a sequence variant or SV, and comparisons to a *Vibrio* species database provides a taxonomic classification. The quality of *hsp60* amplicon sequences was assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and due to low quality of the reverse sequences, only the forward sequences were processed using QIIME2 (2022.8) (https://qiime2.org/). DADA2 within QIIME2 was used to denoise the sequences (left trim 4 bp; length truncation 150 bp; max expected error 2) and create sequence variants (SVs). The taxonomy was assigned to the sequence variants (SVs) in a two-step process based on the *Vibrio hsp60* database (repset_final_130219) provided by (King et al. 2019). The first step used the Blast taxonomy classifier (https://blast.ncbi.nlm.nih.gov/Blast.cgi) set to 90% identity to filter for *Vibrio* sequences and exclude all non-*Vibrio* sequences, while the second step used the sklearn-based taxonomy classifier to identify *Vibrio* species. Phyloseq in R (v4.1.2) was used to exclude *hsp60* SVs which only occurred in one sample or were <0.1% relative abundance.

4.3.4 Vibrio community quantitative PCR (qPCR)

To provide a measure of *Vibrio* abundance, a quantitative PCR (qPCR) assay based on SYBR Green was used to quantify *Vibrio*-specific 16S rRNA gene copies in each sample as previously described (Thompson et al. 2004; Vezzulli et al. 2011).

4.3.5 Data analysis

Hsp60 gene sequences analyses were conducted in R using the phyloseq package (McMurdie and Holmes 2013) (version 4.1.2; Copyright (C) 2017 The R Foundation for Statistical Computing), and Primer v7 (Quest Research Limited, Plymouth UK).

To assess differences in the *Vibrio* community over time and between water and oysters, the relative abundance of SVs was calculated and the Bray Curtis dissimilarity matrix generated. The matrix was visualized with ordinations nMDS and PCoA (packages phyloseq in R and in Primer-E). A PERMANOVA in PrimerE was conducted with fixed factors sampling period (n=6 levels), sample type (n=2) and sites (n=3). All main and pairwise comparisons had 990+ unique permutations. A distance-based test for homogeneity of multivariate dispersions **(**PermDISP**)** was performed to check for equal dispersion of the *Vibrio* community amongst groups of samples.

To assess which *Vibrio* species were mainly contributing to changes in *Vibrio* communities over time and sample type, a "Similarity Percentages - species contributions" (SIMPER) analysis was conducted in Primer (if the PERMANOVA showed a significant effect). A canonical analysis of principal coordinates (CAP) was conducted in Primer to assess the predictive power of the *Vibrio* communities for the

sampling period and sample type i.e. is it possible to predict to what group a sample belonged to based on its *Vibrio* composition and if yes, which *Vibrio* SVs contributed most to clustering.

To explore associations between the water *Vibrio* community, water abiotic factors (water pH, conductivity, temperature, turbidity, chlorophyll *a*, total nitrogen, total phosphorus and sampling period, a DIABLO analysis (Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (Singh et al. 2019) was performed using MixOmics. Dissolved oxygen was excluded due to missing data for September 2021. The SVs were further filtered excluding all SVs which did not occur in a minimum of three water samples and a minimum relative abundance of 0.05% across all samples. A value of 0.5 was used for the design matrix with equal weight to maximize the correlation between the *Vibrio* community and abiotic water profile and a model able to differentiate the sampling periods based on community and water profile.

To assess correlations between the water and oyster *Vibrio* species community and measured environmental variables, redundancy and constrained correspondence analyses (RDA and CCA) were done using the package vegan in R. An RDA analysis was used to explain linear relationships between the *Vibrio* communities and is useful for exploring heterogeneous communities and generally narrower environmental gradients. CCA was used to explore unimodal relationships, often with broader environmental gradients and larger species turnover. CCA also puts more weight on less abundant species. These analyses were done at *Vibrio* species level. Rare species which occurred in less than three samples were excluded. The RDA analyses was done on the Hellinger transformed (square root of relative abundance) *Vibrio* species data (i.e. abundant counts are down-weighted). The data was not transformed for the CCA analyses. The abiotic water variables were assessed for collinearity and skewness using scatter plot matrices and also the variance inflation factor for the former.

Euler diagrams were used to visualize the number of core SVs shared between sample groups (MicEco package in R). A core SV was defined as one that occurred in at least 80% of all oyster or water samples.

Generalized linear models (Gamma family with a log link) were fitted in R to predict the total *Vibrio* abundance (based on *Vibrio* 16S qPCR copies/g or mL) with explanatory variables sampling site, period and type. Sampling round and type were fitted as a multiplicative interaction to account for sampling round having a different effect upon *Vibrio* abundance in water vs oysters. Predictive performance of different models was compared using the Akaika Information Criterion corrected for small sample size (AICc) (bbmle package). For the water *Vibrio* subset of samples, abiotic water variables were also added as predictors. Model residuals were checked for lack of patterns across fitted values and predictors using the DHARMa package. To assess richness, negative binomial models were used with SV richness as outcome and sampling round and site as predictors. P values were adjusted for multiple comparisons using the Tukey method. Model residuals were checked for lack of patterns across fitted values and predictors.

All tests were considered significant at a two-sided alpha threshold of 0.05. The Pearson correlations coefficient was calculated to measure linear relationships between continuous variables (GraphPad Prism Version 9.5.1, GraphPad Software, LLC).

4.4 Results

4.4.1 Environmental conditions during the year of sampling on South Goulburn Island

The BRO lease on South Goulburn Island is located near Fletchers Point with a 3 m tide maximum. There were six collection dates from September 2021 to September 2022 (Table 4-1). During this period seawater temperature ranged from 27-32°C, with the coolest period in August and the hottest in December (Table 4-1). Water temperature during the January 2022 sampling event was notable for being unseasonably cool, which was likely due to rainfall on the sampling day (Supplement 4-1, Bureau of Meteorology rainfall data). pH varied little during the sampling periods with 0.2 pH unit difference between the highest and lowest values. Conductivity was more than double most other values in September 2021 (Table 4-1) which received no rain, nor in the preceding four months. Turbidity ranged between 2.7 and 9.5 NTU, with the highest in December 2021 (Table 4-1). Chlorophyll *a* remained relatively constant for all sampling periods except for low levels in August 2022. TP values ranged from 0.006 mg/L to 0.022 mg/L, the highest measured in December 2021, and the highest TN value was also measured in December 2021 (Table 4-1).

Table 4-1: Average ± standard deviation (SD) physicochemical variables measured in seawater during six sampling events at Goulburn Island during the course of this study.

Date	Season	Temperature (C°)	pH	Salinity (ppt)	Turbidity (NTU)	Chlorophyll *a* (µg/L)	TP (mg/L)	TN (mg/L)
13/9/21	Dry	30.0±0.0	8.3±0.0	34.60±0.00	4.1±0.1	1.27±0.10	0.008±0.002	0.15±0.02
2/12/21	Wet	31.8±0.2	8.1±0.0	11.87±0.06	9.5±0.0	1.14±0.86	0.022±0.005	0.16±0.03
27/1/22	Wet	29.7±0.1	8.1±0.0	15.73±0.32	5.8±0.0	1.26±0.39	0.012±0.002	0.13±0.01
27/4/22	Wet	30.7±0.1	8.1±0.0	10.83±0.45	5.5±0.5	0.98±0.14	0.014±0.002	0.14±0.02
30/8/22	Dry	27.5±0.1	8.2±0.0	16.27±0.38	4.6±0.2	0.37±0.01	0.008±0.001	0.11±0.01
27/9/22	Dry	30.8±0.2	8.3±0.0	17.93±0.64	2.7±1.1	1.35±0.14	0.006±0.001	0.09±0.03

Comparisons between physicochemical variables showed that turbidity had a strong positive correlation with TP (r=0.874, P<0.0001) and a strong negative correlation with pH (r=-0.75, P<0.0001) (Fig 4-1).

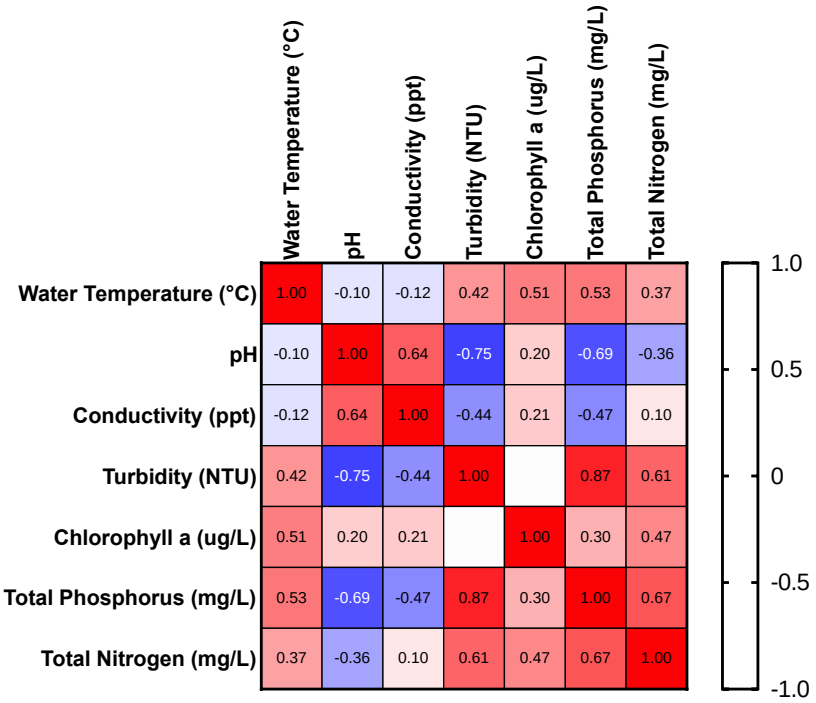


Figure 4-1: Correlation matrix of physicochemical variables. Numbers refer to Pearson's r correlation coefficient.

4.4.2 BRO condition during the year of sampling on South Goulburn Island

Oyster condition was determined by visual assessment by an experienced BRO grower on South Goulburn Island. He used a percentage valuation where increasing % meant increasingly good condition based on oyster reproductive condition, mantle condition and shell fullness. The wet season month January was rated as 10% (Fig 4-2A), April 30%, August 90-100%, September 90-100% (Fig 4-2B) and December 60%.

Figure 4-2: Typical oyster condition: A. January (not ideal) and B. September (excellent) – qualitative assessment by grower.

4.4.3 Measure the number of *Vibrio* species in oysters and surrounding water

In this approach *Vibrio* sequence variants (SVs) were classified to species where possible. Of the 108 samples, 4 oyster samples did not amplify in the *Vibrio hsp60* PCR assay, 2 other oyster samples contained no *Vibrio* sequences, and one oyster sample was excluded as it contained <0.1% SVs. Two water samples contained <100 reads and were also excluded leaving 99 samples. Some *V. owensii* and *V. campbellii* SVs shared almost identical sequences showing identical similarity metrics in NCBI Blast alignments to Genbank sequences of these species.

Across both water and oyster samples, 763 SVs were detected, spanning 35 *Vibrio* species. This does not include 87% of the SVs detected, which were excluded because they only occurred in one sample. The data were not rarefied as the number of reads per sample varied greatly, ranging from 110 to 350,407. The rarefaction curves indicated that the SV richness was reached for all samples with a distinct flattening of curves including those samples with an abundance of sequences (Supplement 4-2) indicating that relative abundance is a good measure of community composition regardless of sequencing depth for this dataset. The total read counts also showed a strong correlation with *Vibrio* 16S qPCR data (Supplement 4-3), which meant that read counts were unlikely to be impacted by technical issues. Some sequences could not be resolved and in those cases the SVs were designated *Vibrio* spp.

4.4.4 Changes in *Vibrio* abundance and richness in oysters and water over time

In water, there was significantly more total *Vibrio* in water in September 2021, April 2022 and August 2022 compared to the other months ($P<0.001$ for all) (Fig 4-3A). In oysters, there was significantly more *Vibrio* in the dry season months September 2021, 2022 and August 2022 compared to the wet season months December 2021, January 2022 and April 2022 ($P<0.050$ for all) (Fig 4-3A).

Richness refers to the number of *Vibrio* sequence variants in a sample. In water, the most striking pattern was the significantly higher *Vibrio* SV richness in September 2021 compared to a reduction in December 2021 ($P<0.001$) and January 2022 ($P=0.050$) coinciding with first rains (Fig 4-3B). In oysters, *Vibrio* SV richness in the dry season months was significantly higher than in the wet season months of December 2021, January 2022 and April 2022 ($P<0.001$ for all) (Fig 4-3B).

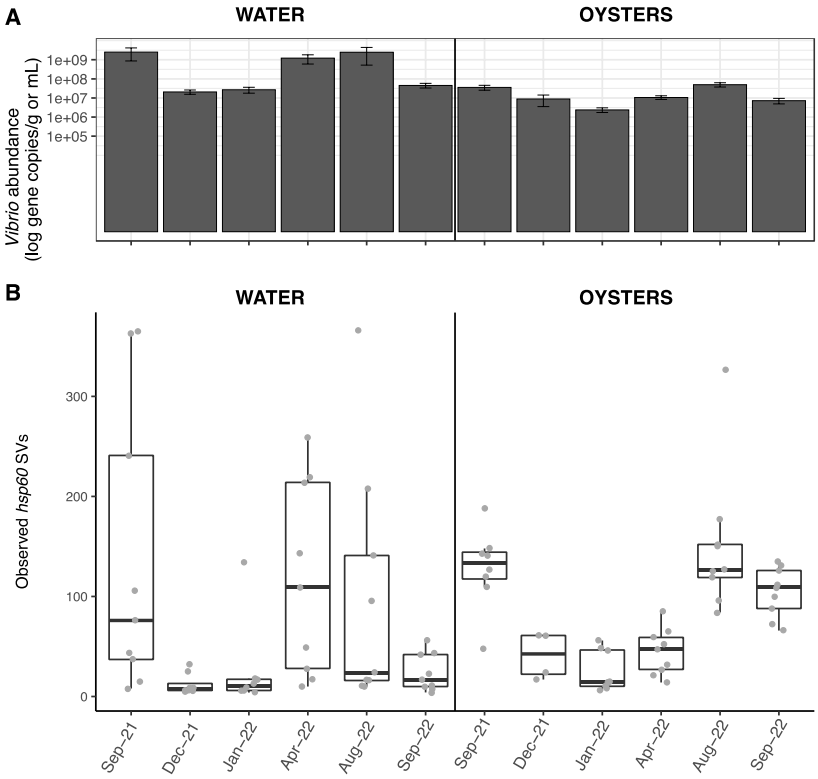


Figure 4-3. Vibrio abundance by 16S qPCR (A) and observed number of Vibrio SVs (B), in oyster (Oy) and seawater (W) across sampling events.

There was a significant positive correlation between the total average *Vibrio* abundance in oysters and water per time point and site (Spearman's correlation *rho* 0.61, P=0.009). There was no significant correlation between the mean SV richness in oysters and water (Spearman correlation rho 0.30, P=0.2).

4.4.5 Identify which *Vibrio* species dominate the community in oysters and water

Of the 35 *Vibrio* species identified in the South Goulburn Island oyster and water communities (see species list (Supplement 4-4), the dominant (>5% relative abundance) identifiable species were *V. owensii*, *V. harveyi*, *V. brasiliensis*, *V. coralliilyticus* and *V. campbellii*. The relative abundance of these species changed with time and sample type (Fig 4-4). *V. owensii* was abundant in both seawater and oysters, particularly in the wet season samples December 2021 and January 2022. *V. harveyi* was common in both water and oysters, but more relatively abundant in oysters, particularly in the late dry season sample September 2021 and the wet season sample April 2022. *V. brasiliensis*, *V. coralliilyticus* and *V. campbellii* mainly occurred in water, with *V. brasiliensis* dominating water in the wet season sample April 2022 and the dry season sample August 2022. While not abundant, *V. alginolyticus* occurred in September 2021 and September 2022 in both water and oysters, and also occurred in oysters in April 2022 and August 2022. *V. fortis* was detected in oysters in January 2022, but not (<2%) at other sample times, and not in water. *V. rotiferianus* occurred in some sites in oysters but not others for the same timepoint.

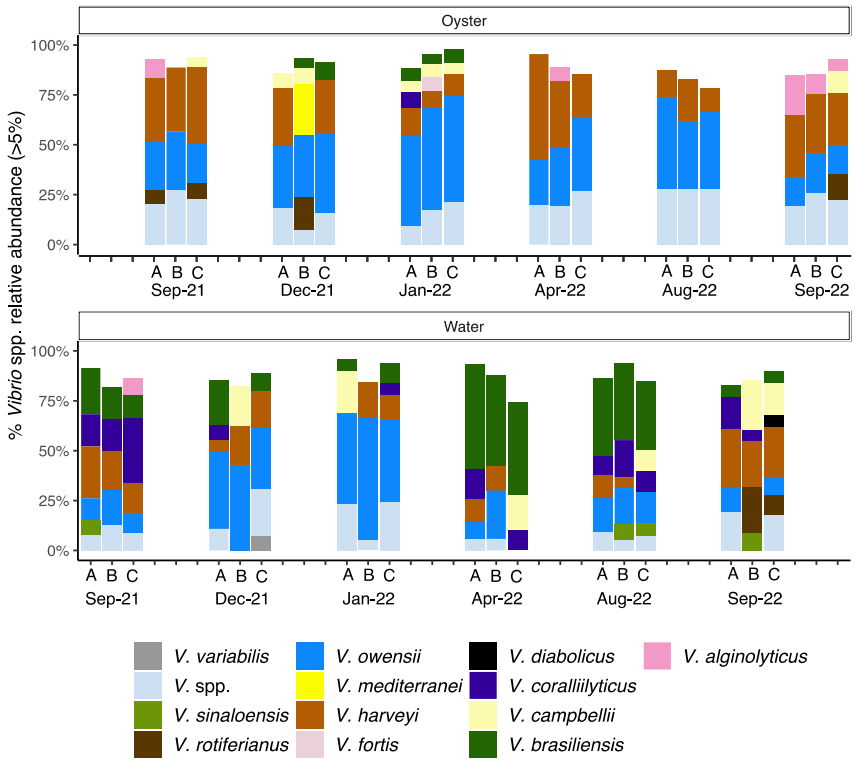


Figure 4-4: Taxa plots showing dominant (>5%) Vibrio species in oyster and water samples at each site A, B, C.

4.4.6 Changes in the *Vibrio* community in oysters and water over time

Over the one year time-series, the *Vibrio* community in water varied more than it did in oysters (Fig. 4-5). When comparing the *Vibrio* community between oysters, there was very little variation in the dry season, illustrated by the tight cluster of stars for the dry season months (Fig 4-5). Following on from that, the *Vibrio* community changed between the dry and wet seasons, and varied more in the wet season, for both oysters and water (Fig 4-5).

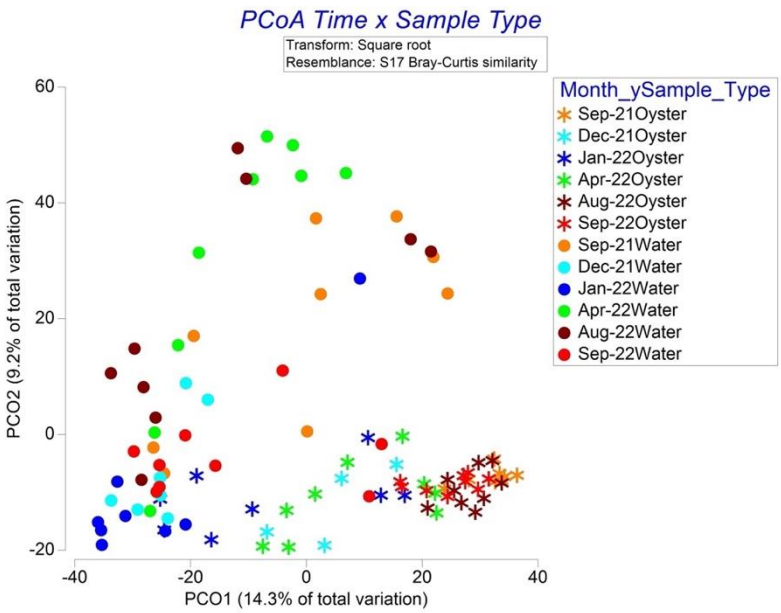


Figure 4-5: PCoA plots of the Vibrio community in oysters and water. Each point on the plot represents a Vibrio community; the closer the symbols, the more similar the Vibrio community.

PERMANOVA results showed a significant difference in the *Vibrio* community by sample type (oyster versus water) and over time, and also the changes over time differed by sample type (Table 4-2). In contrast, there was no significant difference by site or time * site (P=0.173).

There was also a large difference in the variability of samples between oyster and seawater, and to a lesser degree also across sampling rounds (time) but not across sites (PermDisp P values, Table 4-2).

Table 4-2: Permutational multivariate analysis of variance (PERMANOVA) table for Vibrio communities in oysters and seawater collected at 6 sample times. ECV is the estimated components of variation expressed as a percentage of the total variation.

Factor	Pseudo.F	df	*P* value	ECV	PermDisp *P* value
Sample Type (Oyster vs Water)	10.6	1	0.001	24.8	0.001
Sampling Round	2.7	5	0.001	17.8	0.014
IA Type x Round	1.9	5	0.001	18.5	0.001
Site	1.2	2	0.115	4.3	0.116

Pairwise PERMANOVA testing showed that the oyster *Vibrio* communities differed between all months (P<0.010), except not between wet season months when the variability of the oyster *Vibrio* communities was also larger. The water *Vibrio* communities differed between all months except not between some combinations of September, April and August 2022. The variability between replicate water samples was highest in December 2021 and September 2022. *Vibrio* communities differed between oyster and water for all months except not in December 2021 which was likely due to the high dissimilarity in the water samples.

4.4.7 *Vibrio* species driving the differences between oysters, water and time

There was no single *Vibrio* species responsible for the dissimilarity between water and oyster samples. The top species contributing to a cumulative 10% dissimilarity were *V. owensii*, *V. campbelli* and *V. harveyi*, all of which were more abundant in oysters.

Differences between months were mainly driven by *V. owensii, V. harveyi, V. campbelli* or unidentified *Vibrio* spp., which were also the most abundant *Vibrio* species. *V. owensii* SVs contributed to the top 10% cumulative dissimilarity between dry season September 2021 and wet season December 2021/January 2022, and were more abundant in the wet season. *V. campbellii* and *V. harveyi* SVs were both more abundant in September 2021.

CAP analysis was used to better understand the relationship between *Vibrio* diversity for sample type (oyster versus water) and time. Figure 4-6 shows a clear clustering of the water samples in the wet season, mainly due to the high relative abundance of *V. owensii*, and a clear distinction also for the April water samples which were highly correlated with *V. brasiliensis* relative abundance (Fig 4-6). As with the nMDS and PCoA plots of the *Vibrio* community in oysters, there was again a tight clustering showing the relatively conserved nature of *Vibrio* species diversity in the oyster samples. The oyster dry season samples were clearly distinguished from the wet season samples but unfortunately the *Vibrio* SVs responsible for the difference were all unidentified *Vibrio* species.

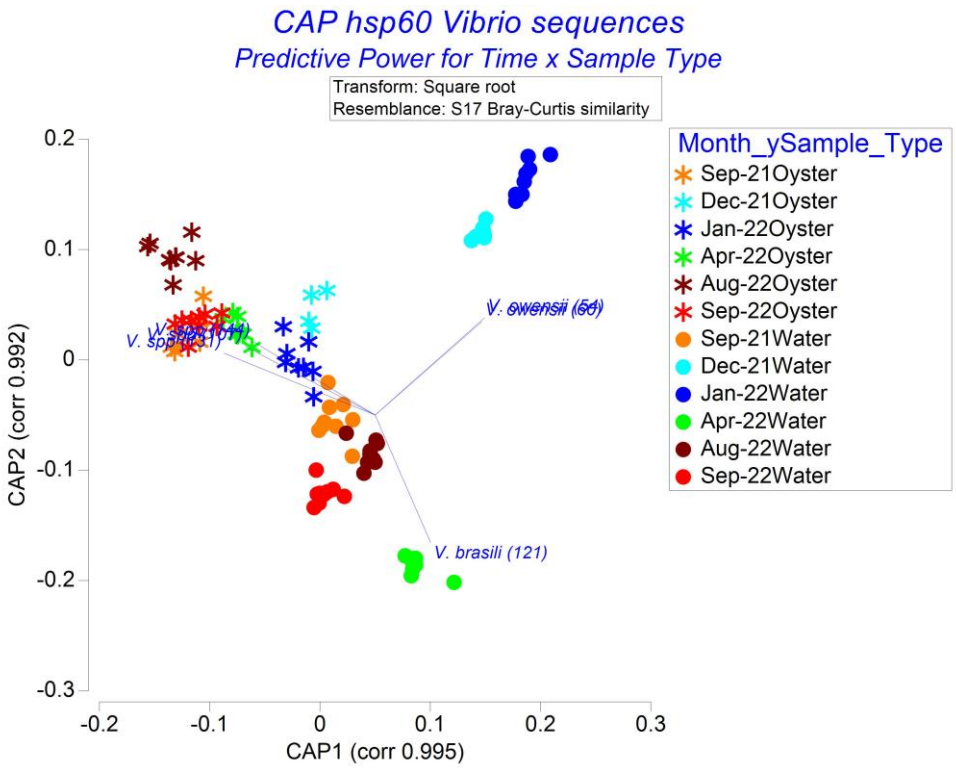


Figure 4-6: Canonical analysis of principal coordinates (CAP) plot ordination showing axes that best discriminate the Vibrio community in oysters and water at different sampling events and correlations with specific Vibrio species.

4.4.8 Can water *Vibrio* community diversity be used as a surrogate for oysters?

The above taxa and ordination plots showed that water is not a good surrogate for oysters for measuring the *Vibrio* community. Another way to assess this is to measure SVs shared between oysters and water. Across the entire dataset, 58% of *Vibrio* SVs were shared between oysters and water, 12% of *Vibrio* SVs were unique to oysters, and 30% of *Vibrio* SVs were unique to water (Fig 4-7). Across the sampling periods there were fluctuations of shared and unique SVs, with 58% of SVs being unique to oysters in September 2022, while only 13% were unique to oysters in September 2021 (results not shown).

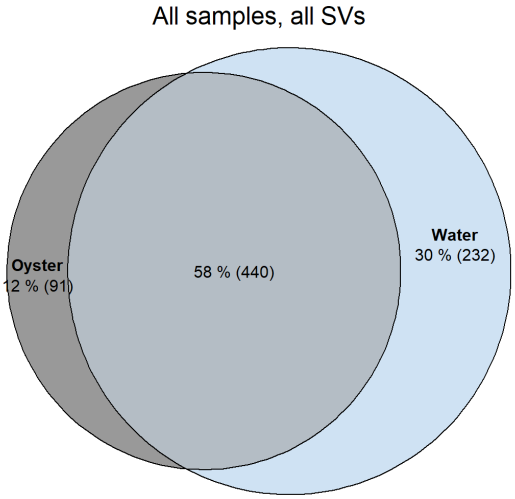


Figure 4-7: Euler diagram showing percent (number) of common and unique Vibrio SVs in oysters compared to seawater.

Unlike 'shared' SVs as discussed above, a core SV was defined as one that occurred in at least 80% of all oyster and water samples. In the present data, there were no SVs that were core across the entire data set. The closest was a single *V. owensii* SV that occurred in 63 (64%) of all samples. For oysters alone, 3 *V. owensii* SVs were core, and they were in fact present in all oyster samples.

4.4.9 Identify which environmental variables drive shifts in the *Vibrio* community

None of the measured physicochemical water variables (temperature, pH, conductivity, turbidity, chlorophyll *a*, TP and TN) showed a significant association with total *Vibrio* abundance in water measured by qPCR if accounting for sampling month and site. There was a weak positive correlation between *Vibrio* abundance and total N but this was not significant (P=0.099). Water temperature showed a weak negative association with *Vibrio* abundance but again, this was not significant (P=0.082).

For SV data analysed by both RDA and CCA, turbidity explained some of the water *Vibrio* community associated with wet season samples (Fig 4-8). Water temperature and conductivity (salinity) explained some of the *Vibrio* community associated with dry season samples (Fig 4-8). Chl*a* and TN explained some linear relationships to particular *Vibrio* species (Fig 4-8A). TP explained unimodal relationships of less abundant *Vibrio* species i.e. likely broader environmental gradients (Fig 4-8B).

V. harveyi was more abundant in the dry season and was associated with conductivity (salinity), and *V. owensii* was more abundant in the wet season and associated with turbidity and TN (Fig 4-8A). Less abundant *Vibrio* are shown on the CCA plot (Fig 4-8B) and *V. maritimus* was associated with a slightly higher turbidity while *V. ishigakensis* and *V. diabolicus* were associated with higher water temperature, salinity, and pH in the dry season (Fig 4-8B).

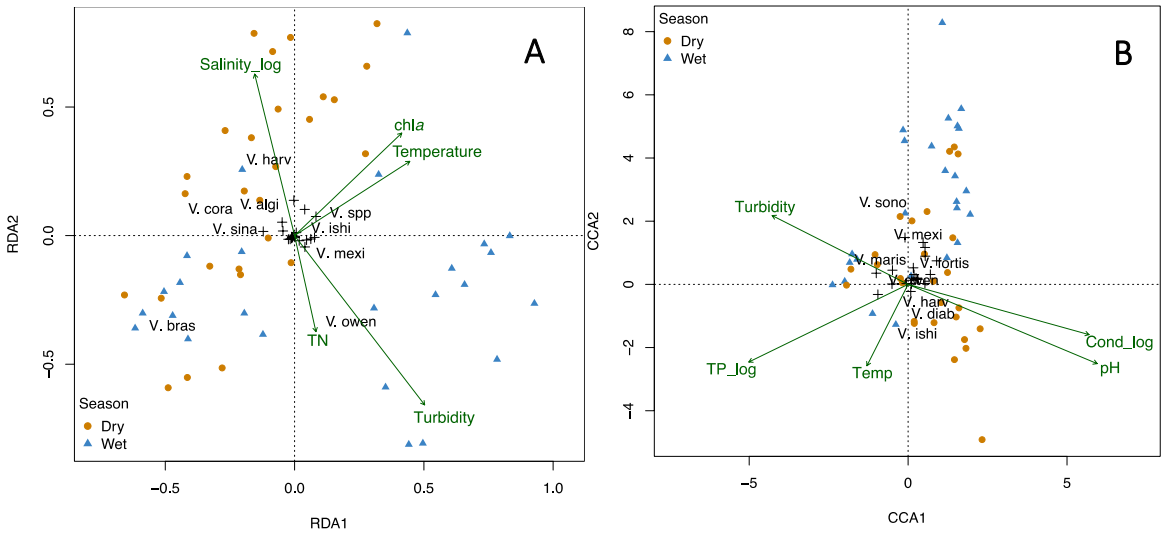


Figure 4-8: RDA correlation biplot (A) and CCA (B) of the Vibrio community in water and environmental variables. Type II scaling was used with angles between vectors including species reflecting their linear correlation. The RDA explained 24.1% of the variability in the Vibrio community with the 1st axis explaining 11.6% (P=0.001) and the 2nd axis 8.4% (P=0.001). The CCA explained 37% of the variance in the Vibrio community with the first axis explaining 22% (P=0.001) and the 2nd 6.2% (P=0.006). Species abbreviations are V. harveyi, V. coralliilyticus, V. brasiliensis, V. owensii, V. mexicanus, V. alginolyticus, V. ishigakensis, V. sinaloensis, V. diabolicus, V. sonorensis, V. marisflavi.

The RDA model with temperature, turbidity, chl*a*, TN and pH (all P<0.050) showed a clear separation of *Vibrio* by season with a more alkaline pH in the dry season, and higher turbidity, TN and chl*a* in the wet season (Fig 4-9A). There was no strong association with a particular *Vibrio* species, however, *V. brasiliensis* was associated with wet season samples, which is supported by the taxa plots (Fig 3-4) showing this species in the December and January samples, while *V. alginolyticus* and *V. harveyi* were associated with dry season samples. The CCA showed no clear separation of the *Vibrio* community in the oyster samples by environmental variable but temperature (P=0.001), turbidity (P=0.005), TN (P=0.001) and salinity (P=0.025) contributed significantly to the model. While there was no strong association with any species, *V. parahaemolyticus* was associated with slightly higher TN and *V. rotiferianus* was associated with higher water temp and more turbid water (Fig 4-9B).

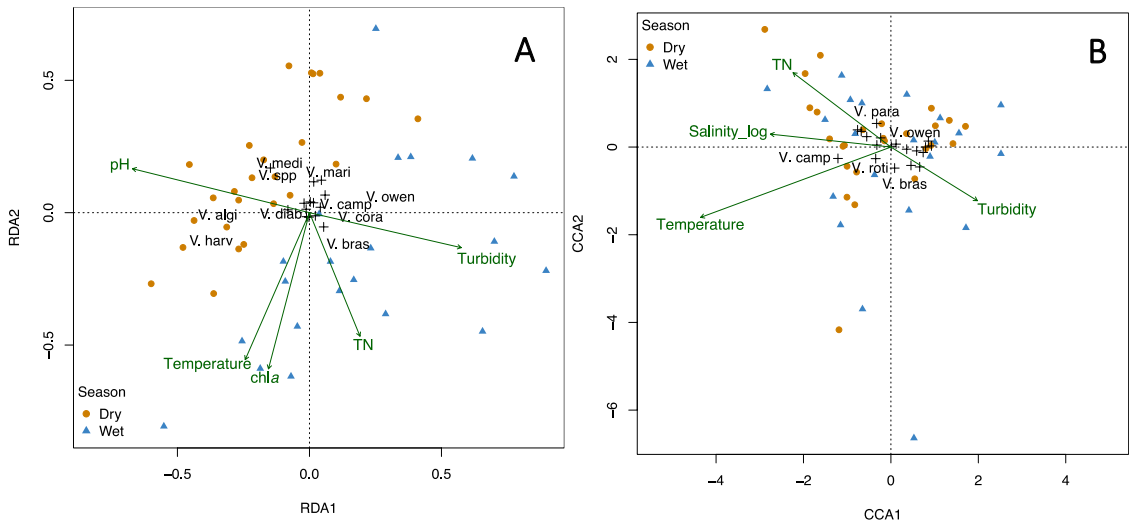


Figure 4-9: RDA correlation biplot (A) and CCA (B) of the Vibrio community in oysters and environmental variables. Type II scaling was used with angles between vectors including species reflecting their linear correlation. The RDA explained 27.5% of the variability in the Vibrio community with the 1st axis explaining 15.7% (P=0.001) and the 2nd axis 5.7% (P=0.071). The CCA explained 29.5% of the variance in the Vibrio community with the first axis explaining 16.8% (P=0.001) and the 2nd 7.2% (P=0.002). Species abbreviations are V. harveyi, V. coralliilyticus, V. brasiliensis, V. owensii, V. parahaemolyticus, V. alginolyticus, V. campbelli, V. rotiferianus, V. diabolicus, V. mediterranei, V. marisflavi.

4.4.10 Seasonality of *Vibrio* pathogens

V. parahaemolyticus (0.18%) was detected in oysters at one of the three sites each in January and April 2022, but not in water (Fig 4-10A). Notably, *V. parahaemolyticus* was not detected in water in January 2022 and at <0.01% relative abundance at one site in April (Fig 4-10A). The animal pathogen *V. harveyi* (>5%) was abundant in water and oysters across all sampling periods (Fig 4-10B). In contrast to *V. parahaemolyticus*, the potential human pathogen *V. alginolyticus* (1.24%) was mostly absent in December 2021 and January 2022 (except in water at site A) and at higher levels in the dry season samples for both oysters and water (Fig 4-10C). The animal pathogen *V. campbellii* (>5%) was abundant in water and oysters across all sample periods. Neither *V. cholerae* nor *V. vulnificus* were detected in the sequence dataset (Fig 4-10D).

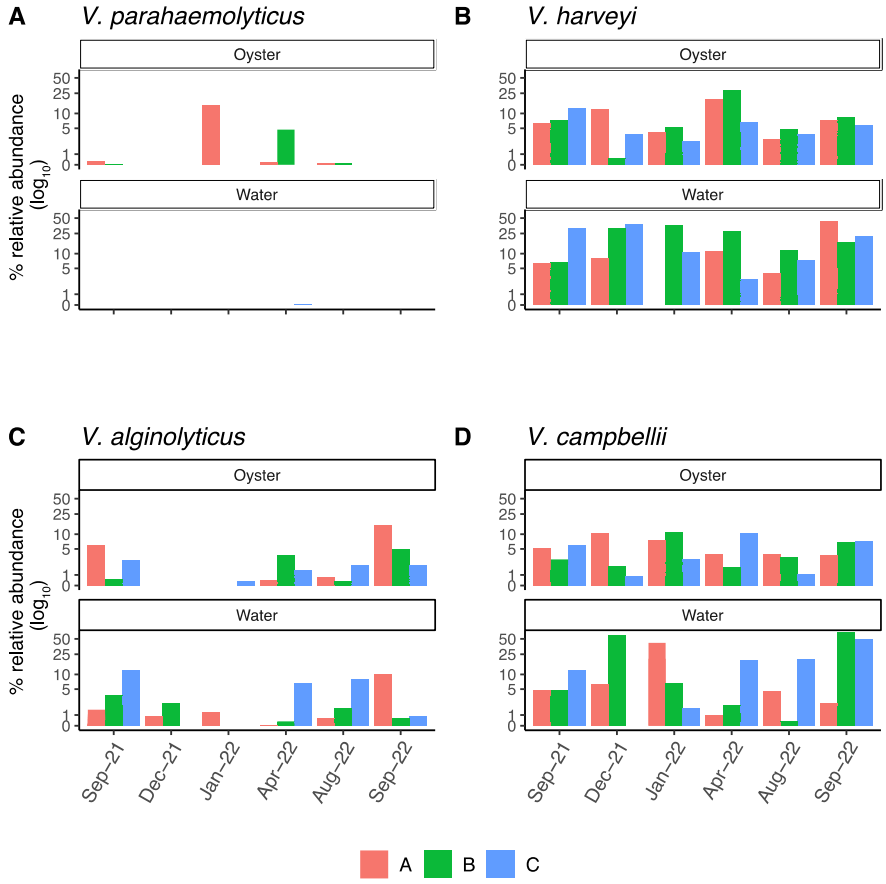


Figure 4-10: Bar plots of the relative abundance of known human and oyster pathogens detected in oysters and water at different sample times at three sites (A, B, C).

V. parahaemolyticus detection in oysters coincided with high rainfall in the preceding days before sampling (Fig 4-11).

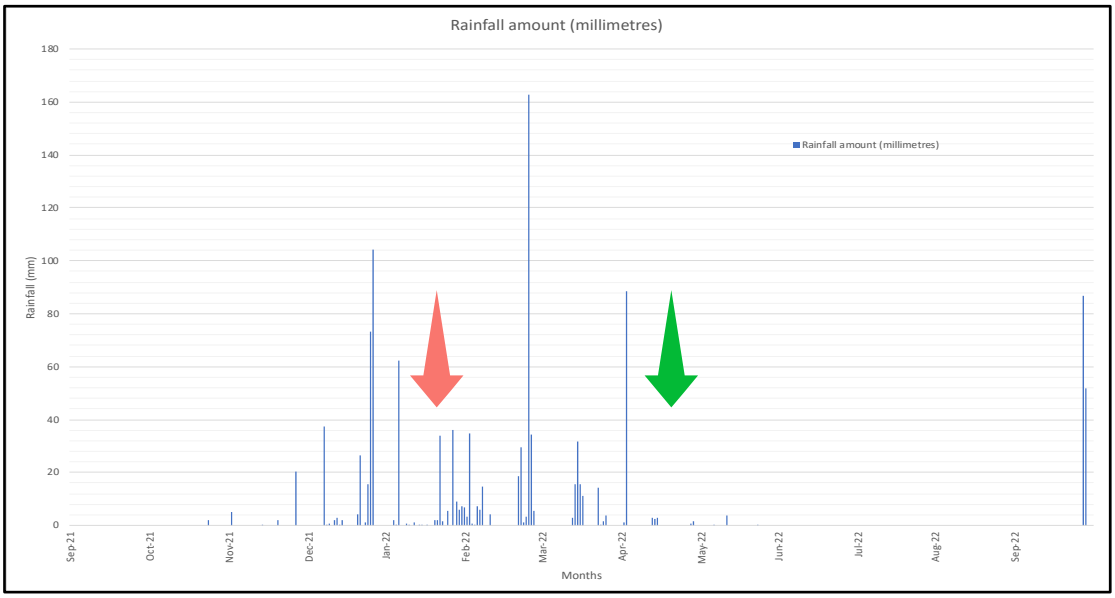


Figure 4-11: South Goulburn Island rainfall during the sampling period showing the V. parahaemolyticus positive oyster samples in January (orange arrow) and April (green arrow).

4.5 **Discussion**

In this study, *Vibrio* community diversity and relative abundance were measured in farmed Blacklip Rock Oysters (BROs) and surrounding water over the course of one year. We measured the number of *Vibrio* species in oysters and surrounding water and characterised the changes in *Vibrio* abundance and richness in oysters and water over time. We identified the dominant *Vibrio* species in oysters and water and identified which *Vibrio* species drove the differences between oysters, water and time. An important question we hope to answer was whether the water *Vibrio* community diversity can be used as a surrogate for the oyster *Vibrio* community. This has implications for routine surveillance because detection in water is more sensitive, less technologically demanding and more cost effective than for oysters. It therefore lends itself to on-country testing. It was also important during this study, to identify which environmental variables drive shifts in the *Vibrio* community. If routine water quality monitoring can be linked to high *Vibrio* risk periods in terms of human and animal health, then not only does this save on the intensity of sampling, but means that routine water quality measures are validated indicators of the need for further testing, again a saving on time and cost. Lastly, this study was intended to determine whether *Vibrio* pathogens are associated with particular times of the year which again adds to the data needed to inform risk-centric surveillance.

During this project we also took account of oyster condition as determined by visual assessment. The consensus on 'best-eating' times that will inform commercial harvest is still being developed and so in this study we used assessment by an experienced BRO grower on South Goulburn Island. He used oyster reproductive condition, mantle condition and shell fullness to make a decision on condition. Poorest condition was in the wet season and 'best-eating' was the dry season months August and September. However this is not the case in all potential commercial farms in northern Australia. For example in WA the TROs are in good condition in the lead up to wet (Oct-Nov) and best condition over the wet (Dec- Mar) with poor condition in the dry (May -July) (Steven Gill pers. comm.)

Thirty-five *Vibrio* species were identified in water and oysters and the number of SVs varied greatly between samples. *Vibrio* abundance and richness in oysters and water changed significantly with time. Total *Vibrio* levels were lower in the wet season (December, January) in both oysters and water, indicating a positive correlation between these two sample types. However, the lower *Vibrio* levels in the wet season is counterintuitive because globally, *Vibrio* are positively associated with warmer temperatures (Pfeffer et al. 2003) and lower salinity (Zimmerman et al. 2007), even in the tropics (Padovan et al. 2021) where water temperature ranges are narrower than in temperate regions. A possible ecological explanation is that levels were reduced by predators (Worden et al. 2006) or phages (Baross et al. 1978). A temporal analysis of plankton and *Vibrio* phage communities could provide insights into this speculation.

Of the 35 *Vibrio* species identified in the South Goulburn Island oyster and water communities, the dominant species were *V. owensii*, *V. harveyi*, *V. brasiliensis*, *V. coralliilyticus* and *V. campbellii*. The relative abundance of these species changed with time and sample type. *V. owensii* was abundant in both seawater and oysters, particularly in the wet season and *V. harveyi* was also common in both water and oysters. *V. brasiliensis*, *V. coralliilyticus* and *V. campbellii* mainly occurred in water. While not abundant, *V. alginolyticus* occurred in both water and oysters. *V. fortis* was detected in oysters in the wet season. *V. rotiferianus* occurred in some sites in oysters but not others for the same timepoint. *Both V. fortis and V. harveyi* were detected in pearl oysters (*Pinctada maxima)* from northern Australia (King et al. 2021) and both increased dramatically in simulated heat wave experiments associated with Pacific Oyster mortality, which implicates them as pathogens, cooperatively or independently (Green et al. 2019). Pearl oysters had an average relative abundance (16%) of *V. owensii* (King et al. 2021) while in a previous study in BROs across northern Australia (chapter 3), the average relative abundance of *V. owensii* ranged from 0-0.15%. *V. owensii* was originally isolated from diseased crustaceans in Australia (Cano-Gómez et al. 2010) and has been

isolated from the haemolymph of moribund Pacific Oysters along with other *Vibrio* species, although challenge experiments showed that *V. owensii* had low pathogenicity (Wang et al. 2021).

The *Vibrio* community in water was more variable than in oysters, and this was particularly obvious in the dry season, when the *Vibrio* community in oysters was very similar to each other, forming a tight cluster compared to both the wet season, and to water. The high variability of the *Vibrio* community in water was further supported by comparisons of *Vibrio* sequence variants (SVs). In this study, an SV was defined 'core' if it was present in at least 80% of samples. There were in fact no core SVs in water comparing all months, but within months there were only 2 core SVs (both *V. brasiliensis*) in April and one core SV (*V. owensii*) in January. In support of the abovementioned dry season cluster in oysters, this period also had the highest number of oyster core SVs. For example, in the 2021 dry season there were 45 core SVs in oysters (but not water), a mix of *V. harveyi, V. owensii, V. rotiferianus* and *Vibrio* spp. In the dry season samples the following year, there were 24 core SVs in August, a mix of *V. harveyi, V. ishigakensis, V. maritimus* and *Vibrio* spp., and in September there were 29 core SVs comprising *V. alginolyticus, V. harveyi, V. owensii and Vibrio* spp.. In the dry season (September) for both years, there was only 1 core SV for oysters and water, and this was *V. harveyi*.

Along with other measured differences in the Vibrio community between water and oysters, the lack of core SVs exclusively shared between oyster and water samples over all sample times provides further evidence that the water *Vibrio* community is not a surrogate for oysters. This analysis of SVs between water and oysters, and over time, provides a more detailed sub-species view of the *Vibrio* community. It provides insights that may be obscured by just looking at species. The lack of core SVs shared between oysters and water, and the dynamic nature of the water *Vibrio* community shines a light on the dynamic nature of *Vibrio* ecology in the tropics. Further research on water / oyster surrogacy for particular species may be helpful for future monitoring. For example, there may be potential pathogens such as *V. harveyi* that, as we have shown, are shared between water and oysters during some months, in which case water motoring would be of value.

While the nutrient levels were at the lower end compared to those for Darwin Harbour (Wilson et al. 2004), the other physicochemical values were typical for northern Australia (Padovan 2003; Duggan 2006; McKinnon et al. 2006; Burford et al. 2008; Nowland et al. 2019b). The physicochemical variables measured at South Goulburn Island did not show the same level of variability for turbidity, salinity and nutrients measured in three tidal creeks in macrotidal Darwin Harbour (Padovan et al. 2021). In that study, the input of freshwater and run-off during the wet season and treated effluent at one site extended the range of physicochemical parameters and explained much more of the variability in the *Vibrio* community. The elevated salinity was likely associated with preceding periods of no rain. Spikes in turbidity and cool water temperatures were likely associated with rainfall. The relationship between turbidity and TP has previously been reported for Darwin Harbour (Wilson et al. 2004) and given the relative ease of measuring turbidity, it's capacity to serve as a surrogate for TP should be considered when developing on-country water quality surveillance.

The BRO farm in Fletcher Bay on South Goulburn Island received run-off after rains but is not macrotidal, so it is possible that the physicochemical measurements are not as seasonally extreme as those measured in the Darwin Harbour study. However, the wet season, especially opening rains, would lead to nutrient-bearing runoff in the oyster growing area. This fact, along with changing currents and winds, might be expected to show a strong relationship between physicochemical factors and particular *Vibrio* species. The fact that this was not reflected in the current data might be more to do with the times sampled rather than any real difference between locations (eg compared to the Darwin Harbour results). Greater frequency or event-based sampling of oysters and water and continuous monitoring of physicochemical measurements over time would help better understand the drivers of the *Vibrio* community.

Of the pathogens detected, *V. parahaemolyticus* is most notable as an increasing threat to shellfish food safety worldwide including Australia (Harlock et al. 2022), compounded by the impacts of climate

change and warming seawater temperatures (Froelich and Daines 2020). In the present study *V. parahaemolyticus* was detected in oysters at one of the three sites each in the January and April 2022 wet season sample times, at a time when *Vibrio* diversity in oysters was low. Both of these events coincided with high rainfall in the preceding days before sampling and although infrequent, the levels detected were high, suggestive of the 'Hot oyster' phenomenon (Klein and Lovell 2016). Notably, *V. parahaemolyticus* was not detected in the water in January 2022 and at <0.01% relative abundance at one site in April (Fig 11). This finding for oysters supports a previous study in Darwin Harbour (Padovan et al. 2020), where *V. parahaemolyticus* was detected in more molluscs (mostly gastropods) in the wet season. However, in a *Vibrio* ecology study in water in Darwin Harbour, Padovan et al (2021) reported detectable levels of *V. parahaemolyticus* in both wet and dry seasons and higher levels in the wet season, associated with temperature, whereas we found barely detectable levels in water, and on only one occasion. Neither *V. cholerae* nor *V. vulnificus* were detected in the sequence dataset.

In contrast to *V. parahaemolyticus*, *V. alginolyticus* was mostly absent in the wet season, and at higher levels in the dry season samples for both oysters and water. *V. harveyi* and *V. campbellii* were abundant in water and oysters across all sampling periods. In contrast, *V. harveyi* was more abundant in the dry season in the Darwin Harbour *Vibrio* ecology study (Padovan et al. 2021). In a spatial study of *Vibrio* communities in 2020, samples of oysters from South Goulburn Island were collected in the wet season on one occasion, and levels of *V. parahaemolyticus* were significantly higher than those detected in the present study. These results suggest that each location will be unique and for shellfish production, this supports the Australian Shellfish Quality Assurance Program's recommendation of individual harvest area risk management (ASQAAC 2022). While this guidance document does not currently include *Vibrio*, when it does in the future, these data would support such a recommendation. Other reports also suggest that locations within regions can have very different detection profiles with Bockemuhl and Triemer (1974) reporting that "whereas *V. parahaemolyticus* was rarely found in water from the Atlantic ocean, the lagoons along the coast proved to be important reservoirs."

4.6 **Conclusion**

The aim of this study was to measure *Vibrio* community diversity and relative abundance in farmed Blacklip Rock Oysters (BROs) and surrounding water over the course of one year. Thirty-five *Vibrio* species were identified in water and oysters. There were five dominant species, and of these, *V. owensii*, *V. campbelli* and *V. harveyi* drove the differences between both sample type (more abundant in oysters) and sample time (months), with *V. owensii* more abundant in the wet season, and *V. cambellii and V. harveyi* more abundant in the dry season.

The number of sequence variants (SVs) varied greatly between samples, and *Vibrio* abundance and diversity (richness) changed significantly over time in both water and oysters. In the wet season, *Vibrio* abundance and richness (diversity) in oysters were lower compared to the dry season. In the wet season, the *Vibrio* community in oysters and water was more variable than in the dry season. The oyster dry season samples were very similar to each other and clearly distinguished from the wet season samples. Overall, the *Vibrio* community in water was more variable than that in oysters.

We conclude from these data that at this location that the water *Vibrio* community diversity is not a surrogate for the oyster *Vibrio* community. This difference between oysters and water was supported by the lack of core SVs (occurring in at least 80% of sample) exclusively shared between oysters and water samples. This has implications for routine surveillance because detection in water is more sensitive, less technologically demanding and more cost effective than for oysters. This result however does not rule out water as a surrogate for particular species.

While no physicochemical variables were significantly correlated to *Vibrio* abundance in water, turbidity helped shape the wet season *Vibrio* water community, and water temperature and salinity

helped shape the dry season *Vibrio* water community. The oyster *Vibrio* community was significantly correlated to turbidity, temperature and total nitrogen.

We also found that the potential human pathogen *V. parahaemolyticus* was detected in some wet season oyster samples but rarely in water, whereas *V. alginolyticus* occurred in the dry season, and *V. harveyi* and *V. campbellii* were abundant in oysters and water year-round. Future studies could could target pathogens such as *V. parahaemolyticus* and *V. harveyi* and determine whether levels in water are associated with levels in oysters, and whether there are associations between season/rain events and the detection of virulence genes.

Lastly, this study was intended to determine whether *Vibrio* pathogens are associated with particular times of the year which again adds to the data needed to inform risk-centric surveillance. Although more data are needed, *V. parahaemolyticus* in oysters was associated with the wet season, and this will also be pursued in further studies, particularly associations with first big rains and monsoon events. The ability to use reliable ecological data to inform food safety considerations is discussed further in the final chapter 'Risk profile for *Vibrio* spp. in Tropical Rock Oysters'.

4.7 Supplementary information

Supplement 4-1: Monthly rainfall for Warruwi Airport, South Goulburn Island, 2021-2022 (Bureau of Meteorology, accessed 18 July 2023). Cells highlighted in green are the sampling dates. Empty cells indicate where a valid observation is not available. This is frequently associated with the observer being unavailable (where observations are undertaken manually), a failure in the observing equipment, or when an event has produced suspect data.

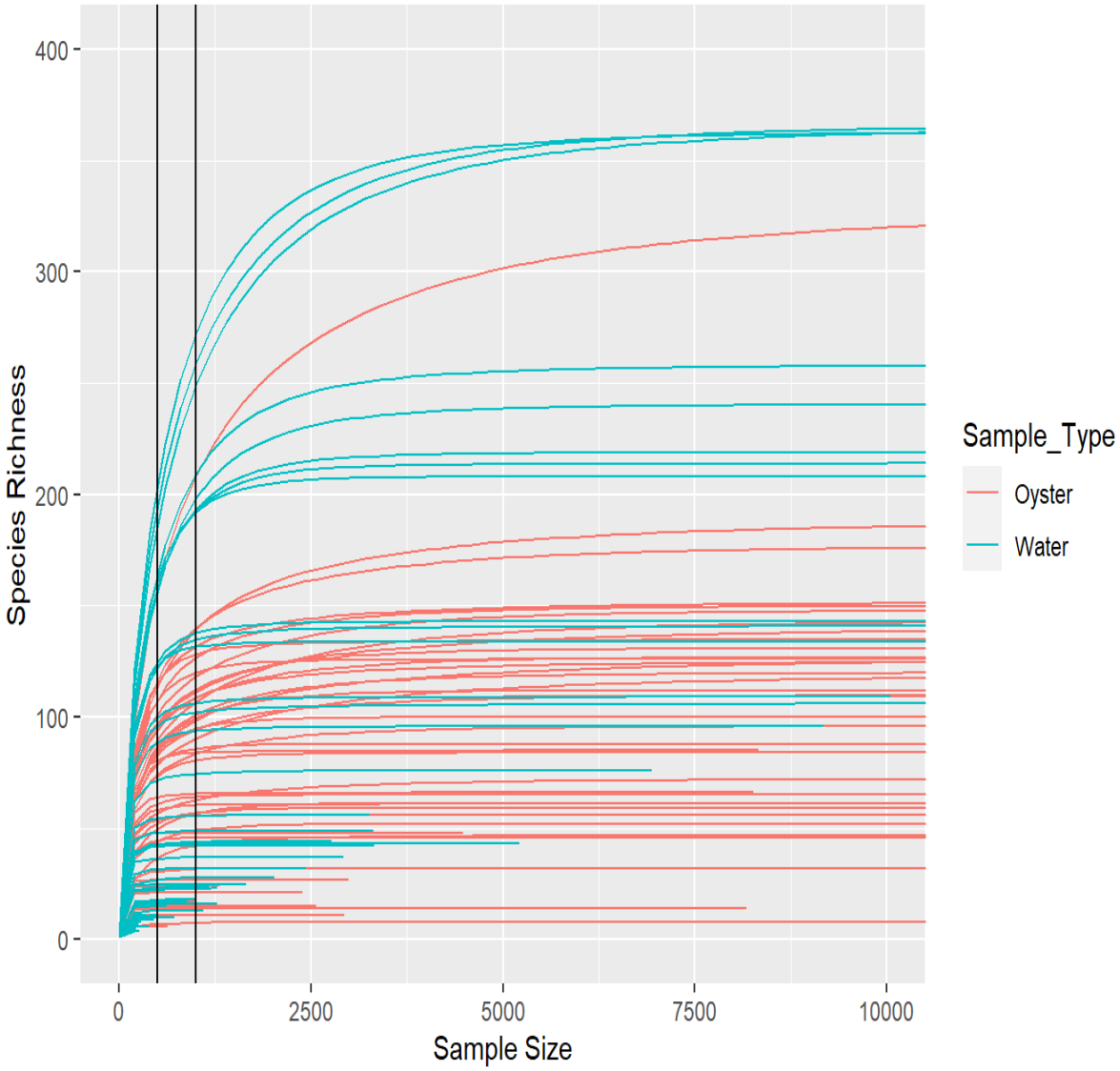
2021

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1st		2.4	0	0	0	0	0	0	0		0	0
2nd		1	0.2	2	0	0	0	0	0		5	0
3rd		2	0	3.2	0	0	0	0	0		0	
4th		22	0	6.4	0	0	0	0	0		0	0
5th		1.2	24.4	15	0	0	0	0	0		0	0
6th		0.2	0	0	0	0	0	0	0	0	0	0
7th	0.4	32.4	0	0	0	0	0	0	0	0	0	37.2
8th	0	9.2	13.2	0	0	0	0	0	0	0	0	0.2
9th	0	1.4	0.2	3.6	0	0	0	0	0	0	0	0.8
10th	0	1	0.4	0	0	0	0	0	0	0		0
11th	0	0	7	0	0	0	0	0	0	0		2.2
12th	5.8	0	24.2	8.6		0	0	0	0	0		2.8
13th	0.2	3.8	1	0	0	0	0	0	0	0	0	0.2
14th	0.2	17	0.2	0	0	0	0	0	0	0	0.4	1.8
15th	0.6	0.2	0	0	0	0	0	0	0	0	0	0
16th	0.6	0	0	0	0	0	0	0	0	0	0	0
17th	11.2	2.6	0.2	0		0	0	0	0	0	0	0
18th	1.6	13.4	2.4	0		0	0	0	0	0		0
19th	54.6	0	0	0	0	0	0	0	0	0	0	0
20th	10.2	0	0	0	0	0	0	0	0	0	2	4.4
21st	0.2		0	0	0	0	0	0		0	0	26.4
22nd	2.2		0.2	0	0	0	0	0	0	0	0	0
23rd	12.6		1.4	0	0	0	0	0	0	0	0	1.2
24th	2.4		5	0	0	0	0	0	0	1.8	0	15.4
25th	1.4		13	0	0	0	0	0	0	0	0	73
26th	6.8	0.2	0.2	0	0	0	0	0		0	0	104.4
27th	4.8	20.8	0.6	0	0	0	0	0		0	20.2	
28th	22.2	0	2.2	0	0	0	0	0		0	0	
29th	1		33.8	3.8	0	0	0	0		0	0	
30th	6.6		10.8	0	0	0	0	0		0	0	0
31st	0.2		1.4		0		0	0		0		0

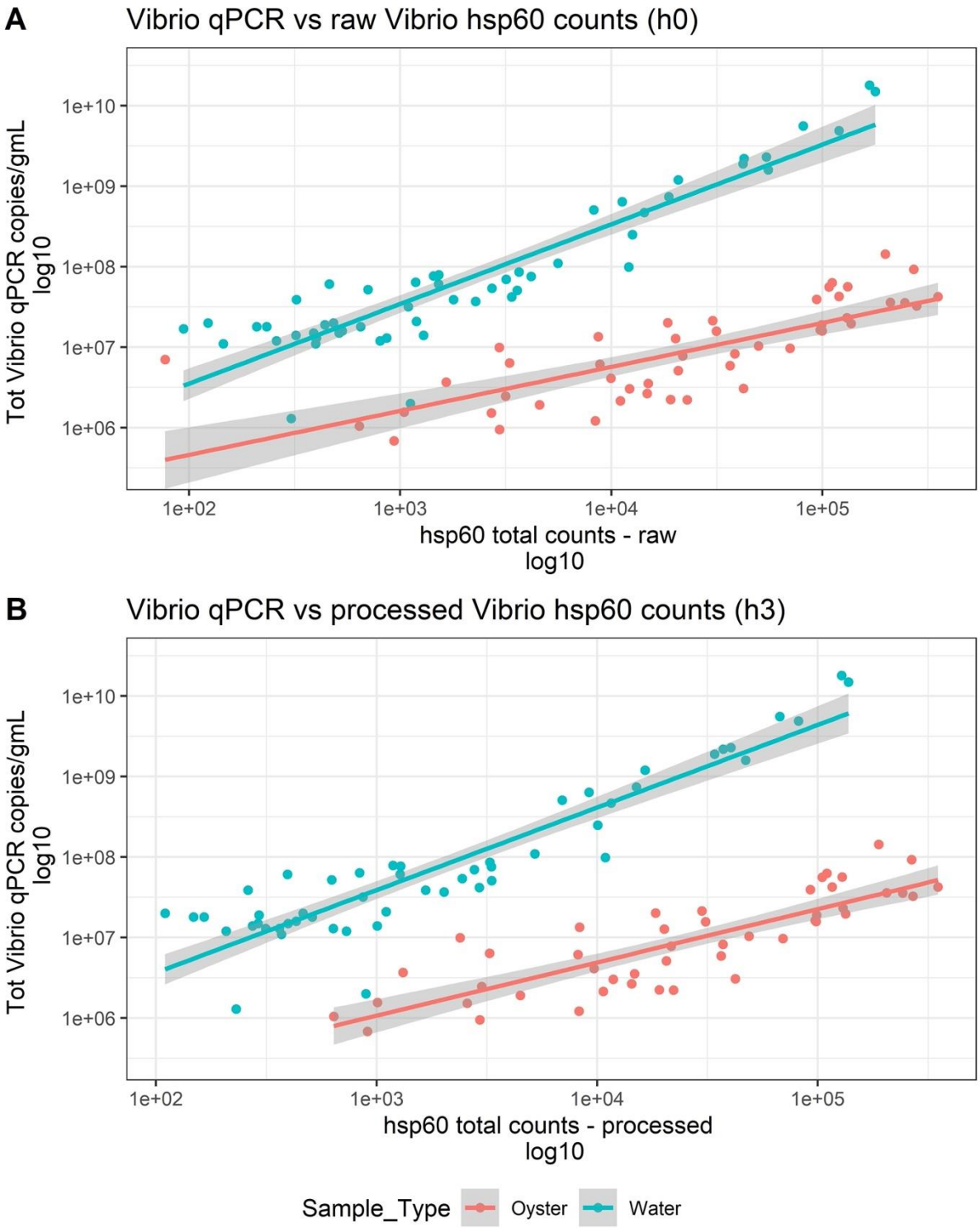
2022

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1st	0	6.6	0	0	0	0	0	0	0	0	0	0.2
2nd	0	3.2	0	1	0	0	0	0	0	2	0	0
3rd	0	34.8	0	88.4	0	0	0	0	0	0.8	0.4	0
4th	2.2	0.6	0	0	0	0	0	0	0	0	7.6	0
5th	0.2	0.4	0	0	0	0	0	0	0	0	0	29.4
6th	62.2	7.4	0	0	0.4	0	0	0	0	0	0	0.2
7th	0	6	0	0	0	0	0	0	0	0	0.4	0.6
8th	0	14.6	0		0	0	0	0	0	0		0
9th	0.6	0	0	0	0	0	0	0	0	0.2	0	0
10th	0.2	0	0	0	0	0	0	0	0	0	0	0
11th	0	4.4	0	0	3.8	0	0	0	0	0	0	0
12th	1	0	0	0	0	0	0	0	0	0		0
13th	0	0	3	2.8	0	0	0	0	0	0	0.4	0.4
14th	0.2	0	15.4	2.6	0	0	0	0	0	0	0.2	0
15th	0.2	0	31.6	3	0	0	0	0	0	0.4	0	37
16th	0	0	15.6	0	0	0	0	0	0	0	0	0.2
17th	0.4	0	11	0	0	0	0	0	0	9	0	2.4
18th	0	0	0	0	0	0	0	0	0	0.4	0	0
19th	0	0	0	0	0	0	0	0	0	0	0	62.4
20th	1.8	0	0	0	0	0	0	0	0	0	0.2	13
21st	1.8	0	0	0	0	0	0	0	0	0	3.8	21.2
22nd	33.8	18.6	0	0	0	0	0	0	0	10		55.4
23rd	1.6	29.4	14.2	0	0.4		0	0	0	0		70.6
24th	0	1	0.2	0	0	0	0	0	0	0	0	25.2
25th	5.4	3.2	1.4	0	0	0	0	0	0	0	0	0
26th		162.8	3.8	0	0	0	0	0	0	0	26.4	0
27th	36	34.2	0	0	0	0	0	0	0	0		1.4
28th	0	5.4	0	0.6	0	0	0	0	86.8	0		61.6
29th	9.2		0	1.6	0	0	0	0	51.8	0	23.4	28.6
30th	6		0	0	0	0	0	0	0	0	38.8	26.8
31st	7.4		0		0		0	0		0		20.6

Supplement 4-2: Rarefaction curves for *hsp60* sequences.



Supplement 4-3: Relationship between number of raw *hsp60* reads (A) or processed (*hsp60* reads B) and *Vibrio* 16s qPCR abundance.



Supplement 4-4: *Vibrio* species identified in BROs and seawater, and their relative abundance.

Species	Relative abundance
V. owensii	26.27
V. harveyi	19.15
V. spp	15.84
V. brasiliensis	12.10
V. campbellii	5.83
V. coralliilyticus	5.54
V. rotiferianus	3.28
V. alginolyticus	2.95
V. sinaloensis	2.06
V. mediterranei	1.01
V. fortis	0.82
V. tubiashii	0.66
V. diabolicus	0.42
V. mexicanus	0.42
V. panuliri	0.34
V. maritimus	0.30
V. ponticus	0.28
V. variabilis	0.25
V. ishigakensis	0.24
V. parahaemolyticus	0.21
V. neptunius	0.18
V. marisflavi	0.14
V. thalassae	0.14
V. aerogenes	0.11
V. nereis	0.11
V. hepatarius	0.07
V. natriegens	0.06
V. alfacsensis	0.05
V. sonorensis	0.04
V. nigripulchritudo	0.03
V. xuii	0.03
V. fluvialis	0.02
V. cidicii	0.01
V. splendidus	0.01
V. orientalis	0.01

5 Direct *Vibrio parahaemolyticus* detection in Blacklip Rock Oysters

5.1 Introduction

A bottleneck for *Vibrio* ecological studies that require quantification is the lack of rapid, specific, sensitive and quantitative tests, particularly of pathogens including *Vibrio parahaemolyticus*, in complex matrices such as shellfish tissues. In particular, we lack protocols that are not overly complicated and do not require prior culturing steps to enable sensitive detection, identification and rapid turn-around-time. Removing the need for culturing would allow for freezing and batch processing at a later date. This would be particularly suitable for remote regions and would place less pressure and costs on communities having to send fresh oysters to laboratories with 24 hours of sampling. If a sufficiently sensitive test could be developed that was not overly technical, it might be possible to develop on-farm test kits for growers and used prior to harvest to check for pathogenic *V. parahaemolyticus* strains. Quantification is not always necessary in which case sensitive presence/absence tests are already available which use an enrichment step that increases sensitivity. This is suitable for presence/absence of toxin genes when any detection is of concern, but where quantitation is required, we still have a gap.

5.2 Objective

The objective of this work was to determine whether *Vibrio parahaemolyticus* could be detected by qPCR directly in oyster tissue, without the need for overnight culturing, and if so, to what sensitivity.

5.3 Methods

V. parahaemolyticus (ATCC® 17802™) was grown overnight at 35°C in marine broth (BD Difco 2216). The following day, decimal dilutions were prepared in 1x PBS. To quantify the pathogen, 100 µL of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread onto marine agar plates in duplicate, incubated at 35°C, and on the following day, the plates showing the best density of cells were counted.

Homogenized Blacklip Rock Oyster tissue (1 g) was spiked with 100 µL of the serial dilutions from the overnight culture of *V. parahaemolyticus* (ATCC® 17802™) in triplicate. A control was included where the oyster tissue was spiked with 100 µL of 1x PBS. DNA was extracted from 25 mg using the DNeasy® Blood and Tissue kit (Qiagen) and nucleic acid quantity and quality determined spectrophotometrically (NanoDrop™).

Quantitative (q) PCR using TaqMan probes for the detection of total *V. parahaemolyticus tlh* gene and *V. parahaemolyticus* virulence gene (*trh*) from extracted DNA was performed as previously described (Nordstrom et al. 2007) Two µL of DNA were used in a 20 µL reaction for each qPCR assay. Each assay was performed in triplicate and real time PCR was performed using a Rotor-Gene Q (Qiagen, Australia).

The spiking experiment was repeated a second time using a greater range of *V. parahaemolyticus* dilutions - 10^{-1} to 10^{-8}.

5.4 Results

The standard curve for the *V. parahaemolyticus tlh* gene enabled detection to 8 cfu/assay, with a reaction efficiency of 0.99 (Figure 5-1).

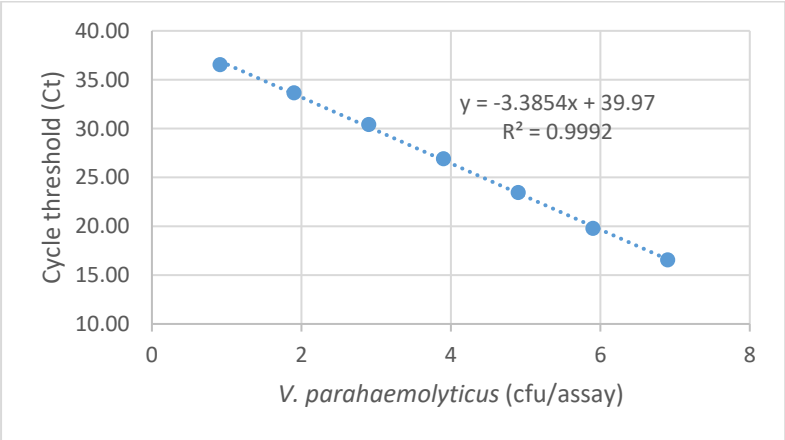


Figure 5-1: Standard curve for V. parahaemolyticus tlh qPCR from purified genomic DNA.

The first spiking experiment resulted in oyster homogenate with *V. parahaemolyticus* concentrations ranging from 4 x 10^2 cfu/g to 4 x 10^7 cfu/g. The resulting standard curves showed variability between the 3 replicates which can be attributed to uneven distribution of the spiked *V. parahaemolyticus* culture in the oyster homogenate, and different extraction efficiencies in each sample (Figure 5-2). At the lowest *V. parahaemolyticus* concentration, the Ct values were more variable than at higher concentrations, or there was no amplification at all (4 out of 9 samples gave Ct values), indicating that the concentration was near the detection limit (Figure 5-2). Reaction efficiencies were within the acceptable range (90-99%) with an estimated detection limit of 4 × 10^2 cfu/g.

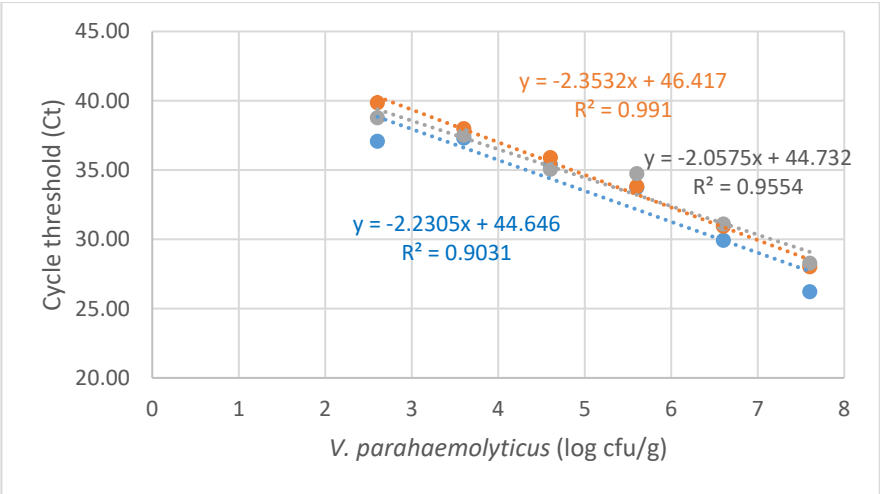


Figure 5-2: Standard curve for V. parahaemolyticus spiked in oyster homogenate. Different colours represent data from 3 replicates. Dashed lines are lines of best fit (Excel).

In the second spiking experiment, *V. parahaemolyticus* concentrations ranged from 2.45 to 2.45 x 10^7 cfu/g, and sensitivity was to 10^4 cfu/g (results not shown). The *trh* qPCR assay was not sensitive enough to detect the *trh* gene in any spiked oyster tissue and had a poor reaction efficiency (56%) when diluted in PBS. Further work is needed to optimize the *trh* qPCR assay.

5.5 **Discussion**

In the present study, the 'direct' *V. parahaemolyticus* qPCR assay using DNA extracted from whole oyster homogenate reliably detected *V. parahaemolyticus* to ~10^3 – 10^4 cfu/g and is therefore only suitable to detect high contamination levels, and is not sensitive enough for food guidelines (FSANZ 2022). The *trh* qPCR assay was not sensitive enough to detect the *trh* gene in any spiked oyster tissue and had a poor reaction efficiency (56%) when diluted in PBS. Further work is needed to optimize the *trh* qPCR assay.

Campbell and Wright (2003) initially found reduced sensitivity in *Vibrio vulnificus* detection in *Crassostrea virginica* by qPCR, but after modifying their methods by reducing the amount of oyster homogenate extracted, increasing the qPCR assay volume, *Vibrio vulnificus* could be detected in oysters down to 10^2 cfu/g compared to 10^3 cfu/g without the modifications. These authors also found no evidence of inhibition of the qPCR assay by oyster tissue. In a separate study, DNA was extracted from digestive glands (not whole oyster homogenate) and nucleic acid purified using magnetic beads resulting in the detection of down to 50 cfu/g of digestive gland (Baker-Austin et al. 2009). When oyster homogenate was spiked with a dilution series of pure *V. vulnificus*, it was possible to achieve detect down to 10^2 to 10^3 cells per assay depending on primers used, while naturally harvested oysters required overnight enrichment to detect *V. vulnificus* (Gordon et al. 2008). Finally, detection limits of 2.5 x 10^4 cfu/g were obtained for *V. vulnificus* in oysters, with a 6 hr enrichment improving the detection limit 100-fold (Han et al. 2011).

Detection of *Vibrio cholerae* in raw oysters by qPCR was found to be very sensitive, in the range of 6-8 cfu/g, however, this was determined by spiking 25 g oyster homogenate diluted in 225 mL buffer with a single, known amount of *V. cholerae*, then serially diluting the whole mixture 10-fold in buffer, prior to DNA extraction (Lyon 2001). This would also dilute out oyster flesh, oyster and other bacterial DNA, and other substances that may be potentially inhibitory, potentially explaining the low detection level. The qPCR detection limit for *Vibrio aestuarianus* in Pacific Oysters was determined in a similar way to the work presented in our FRDC study; oyster tissue was seeded with *V. aestuarianus* cells and serial dilutions prepared using non-spiked oyster homogenate as diluent (rather than buffer as done in Lyon 2001) and 200 µL used for DNA extraction using a commercial kit (Saulnier et al. 2009). The detection limit for *V. aestuarianus* was estimated to be 1.6 x 10^2 cfu/mg of oyster which equated to 1.6 *V. aestuarianus* cells per PCR reaction (Saulnier et al. 2009).

Few publications could be found on direct detection of *V. parahaemolyticus* in oyster tissue by PCR. In one report, mantle fluid was identified as a better matrix (less inhibitory) for estimating *V. parahaemolyticus* concentrations than oyster homogenate. These authors stated that there was a good linear correlation between qPCR Ct values and log concentration of *V. parahaemolyticus* cells spiked into the PCR assay tube, especially at higher concentrations (>10^4 cfu/g), and detection was to 2 log cfu/mL (Kaufman et al. 2004). The advantage of using mantle fluid is that little oyster manipulation is required compared to tissue dissection and a simple boil method was used to lyse cells compared to more time consuming and costly DNA extraction kits. Both mantle fluid and gut were found to have higher *V. parahaemolyticus* densities compared to gills and meat of Pacific Oysters, although 3 individual oysters had extremely high levels of *V. parahaemolyticus* (> 10^4 cfu/g) in the gills (Klein and Lovell 2016). In another study tracking *V. vulnificus* and *V. parahaemolyticus* levels in fish, oyster, sediment and water over winter and spring, qPCR detection limits of ~10^3 copies/g were reported (Givens et al. 2014). Multiplex qPCR assays for enumeration of *Vibrio* spp. and *V. parahaemolyticus*, *V. vulnificus* and *V. anguillarum* were developed for seawater and fish (Kim and Lee 2014). Different DNA extraction methods were trialled and a combination of a specific buffer, chelex-100 and precipitation to concentrate the DNA resulted in a detection limit of 10 CFU/g for fish (Kim and Lee 2014).

For future work, approaches that might increase the sensitivity of *V. parahaemolyticus* qPCR in oyster tissue include (1) targeting specific tissue which is not inhibitory and/or which is likely to contain higher concentrations of the bacteria in question; (2) improving extraction efficiencies; and (3) including an incubation step to multiply levels of the pathogen. This third option means that oysters should not be frozen and quantification is not possible, however, in the case of *V. parahaemolyticus*, detection of strains containing virulence genes may be more beneficial for human risk assessments than being able to quantify the species.

5.6 **Conclusion**

The 'direct' *V. parahaemolyticus* qPCR assay using DNA extracted from whole oyster homogenate reliably detected *V. parahaemolyticus* to ~10^3 – 10^4 cfu/g and is therefore only suitable to detect high contamination levels, and is not sensitive enough for food guidelines (FSANZ 2022).

6 Growth of *V. parahaemolyticus* in Tropical Blacklip Rock Oysters

6.1 **Introduction**

Sydney Rock Oysters (SROs) (*Saccostrea glomerata*) and Pacific Oysters (PO) (*Magallana gigas* (Thunberg 1793)) account for 99% of Australia's oyster production (AUD 114M (ABARES 2021), with farms located in cooler temperate regions of New South Wales, South Australia and Tasmania (Oysters 2020). In contrast, Blacklip Rock Oysters (BROs) (*Saccostrea* lineage J), occur naturally in the Indo-Pacific region including across northern Australia (Nowland et al. 2019a) and are grown commercially on a limited scale. There is increasing interest in expanding production of BROs in this region, particularly in remote Aboriginal communities, with current research focused on securing consistent spat supply and optimizing production methods (Nowland et al. 2019b, 2021).

BROs grow in warm waters, which also support many species of indigenous aquatic microbes, including *Vibrio* spp. that are potentially pathogenic to humans (Padovan et al. 2021). Among these, *V. parahaemolyticus* accounts for most seafood borne gastroenteritis (Ralston et al. 2011) and is amongst the top emerging risks for food safety world-wide (EFSA et al. 2020). There is considerable global concern about the increasing incidences of seafood poisoning due to *Vibrio* blooms and warming sea temperatures in temperate regions (Martinez-Urtaza et al. 2010; Vezzulli et al. 2011; Baker-Austin et al. 2012; Roux et al. 2015; Froelich and Daines 2020). In Australia, *Vibrio* spp. are recognised as an emerging food safety risk (Elvira et al. 2020). This threat to food safety and the emergence of a tropical oyster market for Australia means there is an urgent need to learn as much as possible about *Vibrio* – BRO dynamics, including growth rates of potentially pathogenic *Vibrio* spp. at temperatures likely to be encountered post-harvest.

Post-harvest storage conditions are informed by recommendations to keep oysters as cool as possible to limit pathogen growth, while keeping the animals alive since dead seafood may lead to rapid spoilage and adversely affect microbiological safety (FSANZ 2005). The Australian Shellfish Quality Assurance Program (ASQAAC 2022) provides guidelines for postharvest practices to manage shellfish microbiological quality. The guidelines recommend shell stock intended for raw consumption to be cooled to 10°C or less, within 24 hr of harvest, unless there is evidence that higher temperatures will not support unacceptable growth of human pathogens. POs and other shellfish are generally stored at these temperatures, but SROs are stored at 25°C or less within 24 hr of harvest and then at 21°C or less within 72 hr of harvest (NSW-Food-Authority 2018). These guidelines are based on different responses of SROs and POs to spoilage at different temperatures, measured using aerobic plate counts and sulphide-producing bacteria (Madigan 2008) as well as different *V. parahaemolyticus* growth rates in these oyster species (Eyles et al. 1985; Bird et al. 1992; Madigan 2008; Fernandez-Piquer et al. 2011). To calculate *V. parahaemolyticus* growth rates, researchers have used either oysters naturally infected with *V. parahaemolyticus* at the time of collection (Parveen et al. 2013; Mudoh et al. 2014; Gooch et al. 2016) or inoculated with a culture of *V. parahaemolyticus* (Fernandez-Piquer et al. 2011; Ellett et al. 2022), and measured *V. parahaemolyticus* inactivation or growth at different temperatures over time. The use of naturally infected oysters is more realistic but the large variability of *V. parahaemolyticus* levels that may be present in individual oysters can make the interpretation of results difficult.

While recommended storage temperatures exist for POs and SROs, they may not be relevant for tropical oyster species that host a *Vibrio* community that is adapted to a tropical climate. The objective of this study was to determine the effect of storage temperature on the growth rate of tropical *V. parahaemolyticus* strains in artificially inoculated BROs and in doing so provide the necessary foundation for postharvest temperature control plans for BROs.

6.2 Objectives

The objective of this study was to identify optimum storage and transport temperatures to inform post-harvest cold supply chains

6.3 Methods

6.3.1 Isolation of V. parahaemolyticus strains from oysters and preparation of inoculum

V. parahaemolyticus strains were isolated from BROs collected from the Tiwi Islands (S11.34097 E130.23645) and from Milky oysters (*Saccostrea mordax*/lineage A) collected from Buffalo Creek (S12.33779 E130.908103), in the Northern Territory of northern Australia. During this isolation process, oysters were scrubbed under running potable water and shucked. The meat and liquor from 3 oysters were pooled, homogenized using an Ultra-Turrax® IKA T18 (IKA® Works, Malaysia), diluted 1:1 (w/v) with 1× PBS, 100 µL spread onto CHROMagar™ Vibrio (Dutec Diagnostics, NSW) and the plates incubated overnight at 35°C. Mauve colonies typical of *V. parahaemolyticus* were picked and re-streaked onto fresh CHROMagar™ Vibrio twice more to obtain individual colonies.

Colonies were screened for *V. parahaemolyticus* by qPCR targeting the *tlh* [21]or *toxR* [22] genes using a pick and boil method to extract DNA from plated colonies. Briefly, colonies were dispersed into 50 µl of sterile distilled water, boiled for 3 min, centrifuged at 13,500 x g/10 min and 1 µL template used in a qPCR assay. Isolates positive for the *tlh* or *toxR* gene were grown in liquid broth (tryptic soy broth (TSB) containing 2% (w/v) NaCl) at 30°C. Glycerol stocks of each isolate were prepared and stored at -80°C. DNA from overnight cultures was extracted using the DNeasy® UltraClean® Microbial Kit (Qiagen). A PCR assay targeting the *hsp60* gene [23] was performed on the extracted DNA, the amplicons purified using the ISOLATE II PCR and Gel Kit (Meridian Bioscience) and sequenced in both directions at the Australian Genome Research Facility. The forward and reverse sequences were assembled using MacVector v17.5.6 (MacVector Inc 2020) and identities confirmed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Four isolates were selected, M51 and M56 from BROs from the Tiwi Islands, and M116 and M117 from small Milky oysters from Buffalo Creek. These isolates were also screened for virulence genes *trh* and *tdh* [21], *vscC2*, *vopC* and *vopP* [24] but all assays were negative (results not shown).

One day before the inoculation experiments started, bacterial isolates were streaked onto TSA with 2% NaCl and grown overnight at 30°C. Four mL of sterile TSB/2% NaCl broth was inoculated with 2-3 individual colonies from each isolate separately and incubated with shaking at 30°C for approximately 4-5 hr until visibly turbid. The cultures were centrifuged at 10,000 rpm for 2 min and resuspended in filtered sterile seawater to give a final absorbance at 600 nm of between 0.15-0.25 units. Two mL from each of the 4 cultures were pooled to give the final inoculum. Serial dilutions were prepared using 1× PBS and 100 µl plated onto TSA/2% NaCl to calculate cell numbers which were expressed as colony forming units (CFU) per mL.

6.3.2 Oyster inoculation, incubation and processing

BROs (*Saccostrea* lineage J) were obtained from a commercial farm in Bowen (Queensland Australia) in two shipments of approximately 250 oysters each sent two weeks apart. Average seawater temperatures ranged from 23°C to 25°C at the time of sampling (https://data.aims.gov.au/aimsrtds/datatool.xhtml accessed 10/11/2022). Oyster shell length ranged from 49-74 mm with an average (± standard deviation) of 60 mm (± 6 mm). The first shipment of oysters were used for experiments at 4°C and 13°C and the second shipment was used for experiments at 18°C and 25°C. Oysters were placed in an open plastic bag in a polystyrene box and

kept at 18°C overnight. The following morning, the plastic bag was closed, ice bricks were added over a thick layer of newspaper, and the box sealed. The oysters were airfreighted to the Charles Darwin University laboratory in Darwin and experiments commenced the next morning. On receipt, the temperature of the BROs was 15°C and 18°C in shipments 1 and 2 respectively.

Oysters were scrubbed and washed under running potable water. A 2-5 mm hole was made into the oyster lid approximately halfway along the length of the shell and 100 μL of either filtered sterile seawater (control) or *V. parahaemolyticus* suspension were injected into the adductor muscle using a sterile 1 mL syringe fitted with a 22-gauge needle. The initial inoculum concentration was 2.0×10^7 CFU/mL for the 4°C and 13°C experiment and 4.2×10^5 CFU/mL for the 18°C and 25°C experiment. A higher concentration was used for the cooler temperatures to enable detection as levels were expected to decrease with storage.

Oysters were placed into open plastic bags in trays for storage in incubators set to 4°C, 13°C, 18°C and 25°C. Temperature loggers were used to record temperature. Five replicates were used for oysters injected with *V. parahaemolyticus*, with three oysters pooled per replicate. For the 4°C and 13°C experiments, sampling times were 0, 24 hr, 72 hr, 120 hr, 192 hr and 264 hr. For the 18°C and 25°C experiments, sampling times were 0, 12 hr, 24 hr, 72 hr, 120 hr and 168 hr. The shell width of each oyster was measured, and the total meat and liquor weight of the pooled oysters recorded at time zero and at each time interval when the oysters were harvested. Twenty extra control and *V. parahaemolyticus* injected oysters were prepared and stored at each temperature to allow for losses during the experiment.

Controls were oysters injected with filtered sterile seawater, in duplicate with 5 oysters per replicate. Controls were sampled at the beginning, middle and end of the experiment. The number of controls per replicate were to account for the expected variability in background levels of indigenous *V. parahaemolyticus*. The controls primarily accounted for injuries sustained in the injection process and to track *V. parahaemolyticus* levels during the experiment. Gaping, non-responsive oyster were assumed dead and excluded from sampling.

At each time point oysters were shucked, the meat and liquor pooled, and weight recorded. An equal volume of sterile 1× alkaline peptone water (APW, pH 8.4 (CM1028 Oxoid)) was added and the sample homogenized using an Ultra-Turrax®. The dispersion element was washed between replicates in the following sequence of solutions: potable water, 1% (w/v) Virkon™ disinfectant, potable water, 80% (v/v) ethanol, and sterile high pure water. On each sampling day, control oysters were processed before *V. parahaemolyticus* injected oysters, and blanks were included (APW) to check for adequate tool disinfection. Serial dilutions of the homogenate were made in 1× phosphate buffered saline (PBS; 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and 100 μL of each dilution plated in triplicate onto CHROMagar™ Vibrio. Plates were incubated at 30°C overnight and mauve colonies (*V. parahaemolyticus*) counted and colony forming units (CFU) per gram oyster homogenate calculated. To confirm identity as *V. parahaemolyticus*, 50-60 mauve colonies were randomly picked and assayed by qPCR targeting the *tlh* gene as outlined above.

6.3.3 Analyses

Data were imported into Prism 9 for MacOS (GraphPad Software, LLC 1994-2021). Counts were transformed to $\log_{10}$ values, and lines or curves fitted to the data. Growth rates ($\log_{10}$ CFU/h) were calculated from best fit lines at 4°C and 13°C. To calculate specific growth rates (mu) and maximum population densities ($\log_{10}$ CFU/g) at 18°C and 25°C, data were imported into https://foodmicrowur.shinyapps.io/biogrowth/ and fitted using a modified Gompertz model. Generalized additive models (GAMs) were fitted in R (version 3.6.0 2017-06-30; Copyright (C) 2017

The R Foundation for Statistical Computing) (library mgcv) to assess non-linear changes of *V. parahaemolyticus* counts over time. Models were fitted with a separate smooth term for time (h) for each temperature group, temperature as additional categorical predictor and using a negative binomial distribution (log link). To assess whether *V. parahaemolyticus* growth varied significantly at different temperatures, a negative binomial generalized linear model was performed with outcome *V. parahaemolyticus* counts and the interaction of time and temperature groups as predictors. To account for the nonlinear growth of *V. parahaemolyticus* at 18°C and 25°C, a 2nd degree polynomial function was fitted for time. All tests were 2-tailed and considered significant if P values were < 0.05.

6.4 **Results**

6.4.1 *V. parahaemolyticus* growth rates in injected oysters

At the beginning of the experiment, after initial inoculation of oysters with the *V. parahaemolyticus* cocktail, concentrations and standard deviation of *V. parahaemolyticus* in the control BROs were 5.001 ± 0.282 $\log_{10}$ CFU/g at 4°C and 13°C, and 3.567 ± 0.164 $\log_{10}$ CFU/g at 18°C and 25°C.

Changes in *V. parahaemolyticus* concentrations at 4°C and 13°C were best explained by a linear relationship (GAM model effective degrees of freedom (edf) 1.9, P<0.001 for the latter), while at 18°C and 25°C, a curve best explained the data (Figure 6-1) (GAM model edf >2; P<0.001). No lag phase was observed in the growth curves. At 4°C there was no significant change in *V. parahaemolyticus* levels over time (linear regression on log *V. parahaemolyticus* levels and GAM model P>0.050) although the trend was a gradual decrease (Figure 6-1). At 13°C, 18°C and 25°C, there was a significant increase in *V. parahaemolyticus* levels over time (P<0.001 for all models).

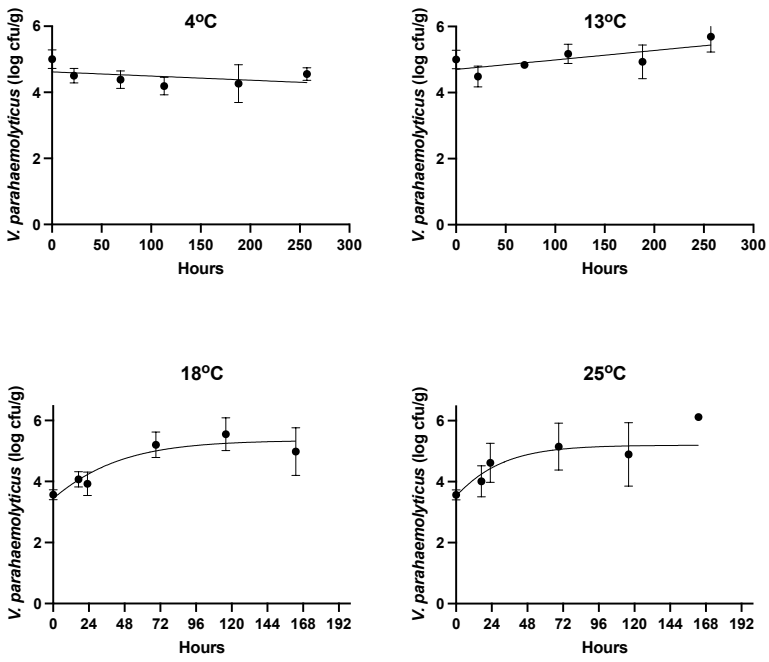


Figure 6-1: Growth profiles of Vibrio parahaemolyticus in Blacklip Rock Oysters stored at 4 ℃, 13 ℃, 18 ℃ and 25 ℃. Points indicate averages of five replicates, bars are standard deviation and the lines indicate fitted curves. The last sample at 25 ℃ consisted of one sample only.

Estimated growth rates of *V. parahaemolyticus* in BROs were -0.001, 0.003, 0.032 and 0.047 $\log_{10}$ CFU/hr at 4°C, 13°C, 18°C and 25°C respectively (Table 6-1). The highest maximum population density of 5.31 $\log_{10}$ CFU/g was achieved at 18°C after 116 hr.

Table 6-1: Kinetic parameters for Vibrio parahaemolyticus growth. Growth rates at 4 ℃ and 13 ℃ were calculated from best fit lines. At 18 ℃ and 25 ℃, the maximum specific growth rate (μ) and maximum population density were estimated from modified Gompertz curves. ND is not determined. RMSE is the root mean squared error.

Storage temperature (°C)	**Growth rate (log CFU/hr ± SE)**	**Maximum population density ($\log_{10}$ CFU/g ±SE)**	**Goodness of fit (RMSE)**
4	-0.0013 ± 0.0007	ND	0.390
13	0.0029 ± 0.0009	ND	0.408
18	0.032 ± 0.011	5.31 ± 0.245	0.463
25	0.047 ± 0.021	5.14 ± 0.394	0.652

V. parahaemolyticus concentration varied significantly between all temperature groups (polynomial GLM model, P<0.05) except between 18°C and 25°C. At 48 hr there was a significant difference in *V. parahaemolyticus* concentrations between 4°C and 25°C only, but at 72 hr, *V. parahaemolyticus* concentrations significantly differed between all temperature groups with the exception of no difference between 18°C and 25°C (Figure 6-2).

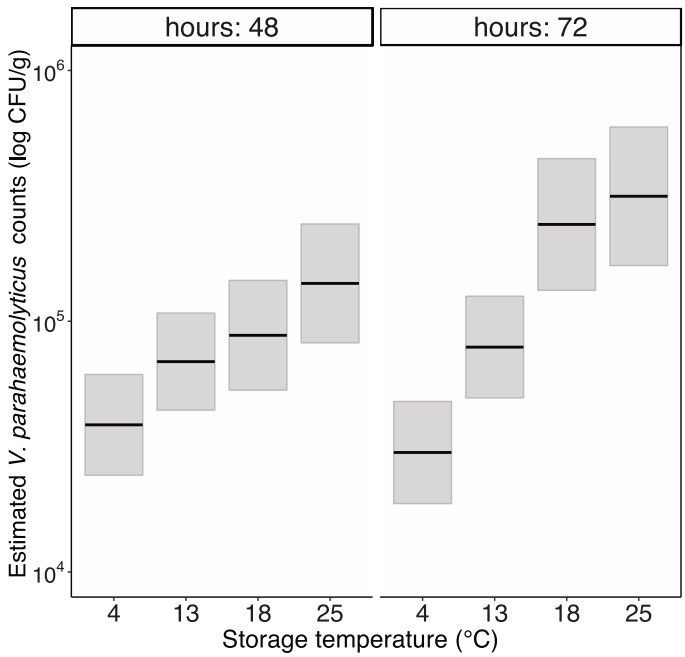


Figure 6-2: Estimated Vibrio parahaemolyticus growth in Blacklip Rock Oysters at each temperature. Black line is estimated average counts and grey area is the 95% confidence interval based on a polynomial generalized linear model. Starting concentrations of V. parahaemolyticus injected into the oysters were 4.2 x 10^5 CFU/mL for the 4 ℃ and 13 ℃ experiment and 2.0 x 10^7 CFU/mL for the 18 ℃ and 25 ℃ experiment.

6.4.2 Control (seawater-injected) oysters

At the beginning of the experiment, concentrations (and standard deviation) of *V. parahaemolyticus* in the control BROs were 3.554 ± 0.136 $\log_{10}$ CFU/g at 4°C and 13°C from the first shipment of oysters, and 3.285 ± 0.167 $\log_{10}$ CFU/g at 18°C and 25°C from the second shipment (Figure 6-3).

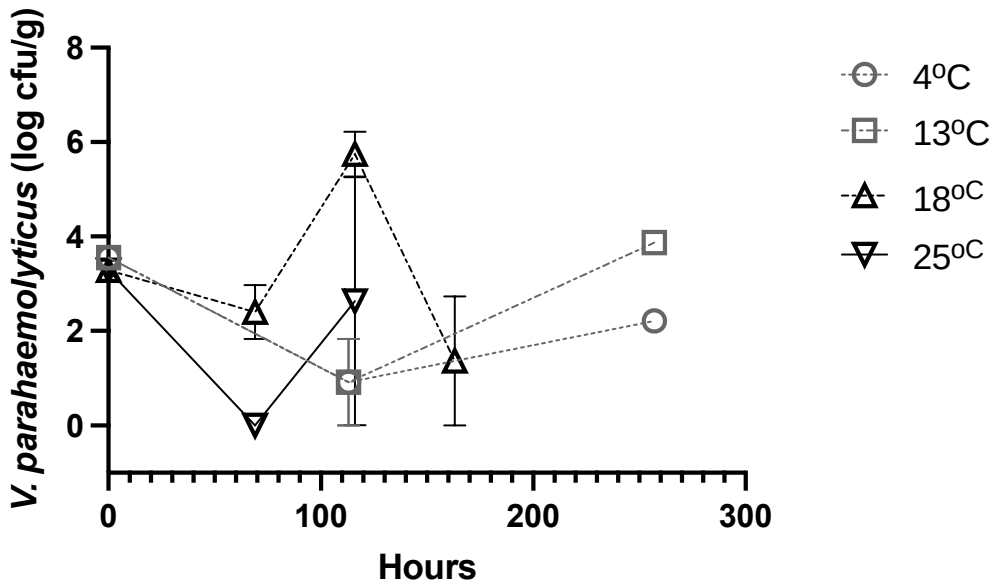


Figure 6-3: Vibrio parahaemolyticus concentrations in Blacklip Rock Oyster injected with filtered sterile seawater and stored at 4 ℃, 13 ℃, 18 ℃ and 25 ℃.

Concentrations of *V. parahaemolyticus* initially decreased at 4°C and 13°C, but then increased again at the end of the storage period, after 11 days (257 hr). Similarly at 18°C and 25°C, the *V. parahaemolyticus* concentration decreased after 3 days (69 hr), and then increased at day 5 (116 hr). A final measurement was taken on day 7 (163 hr) at 18°C where *V. parahaemolyticus* levels again decreased. The variability in concentrations was greatest at the warmer incubation temperatures.

6.5 **Discussion**

Vibrio parahaemolyticus seafood risk management is supported by implementing cold chain temperatures that minimize pathogen growth. Here we present the first *Vibrio* risk data for BROs, which are the focus of a developing aquaculture industry in northern Australia. Following injection into BROs, *V. parahaemolyticus* did not grow at 4°C, but grew at temperatures ≥ 13°C. The tipping point for *V. parahaemolyticus* growth in oyster species is in the temperature range 10-15°C (Wang et al. 2010; Fernandez-Piquer et al. 2011; Parveen et al. 2013; Cook and Ruple 2016; Ellett et al. 2022). The low growth rate for *V. parahaemolyticus* in BROs reported in this study at 13°C fits within this range and is notable given that *Vibrio* strains used here were isolated from warm tropical waters. *V. parahaemolyticus* growth in BROs was minimal at 13°C and significantly lower than growth at the warmer temperatures.

Our results show that storage of BROs at 4°C will prevent growth of *V. parahaemolyticus*, but since this storage temperature may also kill or impair these tropical oysters, shelf life and quality at this temperature needs to be assessed in case death accelerates spoilage by psychrotolerant microorganisms. At 13°C, very low *V. parahaemolyticus* growth rates were measured in BROs which may be a better temperature for BRO survival, however, this is not a standard commercial

refrigeration temperature. Our study showed no significant difference in *V. parahaemolyticus* growth or maximum population densities in BROs at 18°C or 25°C, possibly because the oysters and their microbiome adapted to these warmer temperatures are able to 'manage' introduced *V. parahaemolyticus* levels.

Compared with other oyster species, *V. parahaemolyticus* growth rates at 25°C in Eastern Oysters (*Crassostrea virginica*) and artificially inoculated Pacific Oysters (PO) were higher than those measured in BROs (current study), which had similar rates to Asian oysters (*C. ariakensis*) (Figure 5-4). In addition, *V. parahaemolyticus* growth rates in POs, Eastern Oysters and Asian oysters increased with higher temperatures (25°C compared to ~20°C) (Figure 6-4), but this was not the case for BROs where there was no significant difference between growth at 18°C and 25°C. In contrast, *V. parahaemolyticus* did not grow in SROs stored at temperatures up to 28°C (Eyles et al. 1985; Bird et al. 1992; Fernandez-Piquer et al. 2011), with growth only observed over 30°C (Eyles et al. 1985; Tamplin et al. 2007).

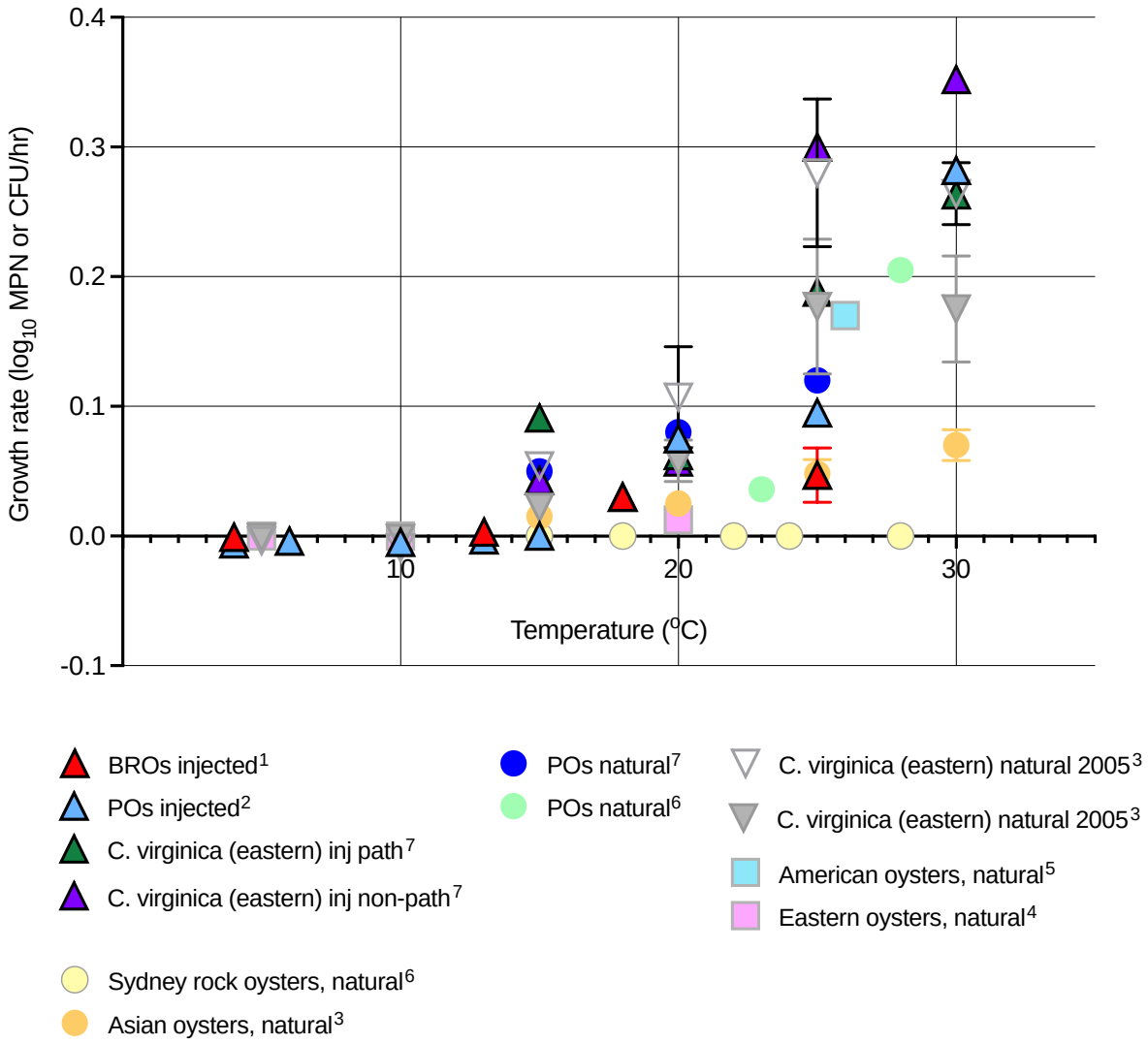


Figure 6-4: Growth rates of Vibrio parahaemolyticus in different oyster species at different temperatures. [1]This study; [2]Fernandez-Piquer et al. 2011; [3]Parveen et al. 2013; [4]Mudoh et al. 2014; [5]Gooch et al. 2016; [6]Fernandez-Piquer et al. 2010; [7]Ellett et al. 2022.

Variations in *V. parahaemolyticus* growth are often attributed to oyster immunology and their responses to substantial changes in their surrounds, the interaction of the introduced pathogen to resident oyster microbes or the use of different experimental bacterial strains. Sydney Rock Oysters are considered a hardy species (FSANZ 2005) and it has been suggested that lower microbial counts measured in SROs stored at 15°C compared to 8°C could be due to a more active immune system at the warmer temperature (Madigan 2008). Intertidal molluscs have physiological and immunological adaptations to deal with conditions that can change quickly over a tidal cycle where they tolerate periods of emersion characterized by extremes in oxygen availability and temperature (Zhang et al. 2014; Meng et al. 2018). The type and extent of these responses (Dunphy et al. 2006; Dudognon et al. 2013) may influence their ability to cope with these stressors and subsequently impact their interaction with microbes (Allen and Burnett 2008; Macey et al. 2008). Wild BROs are intertidal and are also considered a hardy species and may be better able to cope with substantial changes in their surrounds.

A recent study showed that virulent *V. parahaemolyticus* strains injected into *M. gigas* grew faster at 15°C than non-virulent strains (Ellett et al. 2022). In contrast, other studies using broth and *M. gigas* oyster slurry, reported more rapid growth of *V. parahaemolyticus* strains lacking the virulent *trh* gene compared to strains without *trh* (Yoon et al. 2008). Such comparisons between studies can be complicated by the use of different matrices as well as the use of different strains. In our study, a mix of four strains isolated from tropical rock oysters were injected in BROs to account for potential differences in growth between strains. These four strains lacked both the *trh* and *tdh* genes, however, since vibriosis has been reported from trh^{-}/tdh^{-} strains (Saito et al. 2015), these markers are no guarantee of the capacity to cause disease. What constitutes a pathogenic strain is still the subject of much debate and whole genome sequencing is revealing new virulence factors (Park et al. 2004; Hubbard et al. 2016) that contribute to infection. It is also possible that pathogenic strains respond differently in tropical BROs and the investigation of those strains in BROs will further our understanding of the behaviour of *V. parahaemolyticus* in stored tropical oysters.

Maximum *V. parahaemolyticus* population densities in oysters can vary by several orders of magnitude when stored at warmer temperatures. For example, maximum *V. parahaemolyticus* densities were higher in Eastern Oysters stored at 20-25°C (Parveen et al. 2013) and PO injected with *V. parahaemolyticus*, but lower in natural POs and SROs (Fernandez-Piquer et al. 2011) compared to BROs (current study). Maximum population densities for *V. parahaemolyticus* or any pathogen may depend on the type and density of other resident microbiota (Hood et al. 1983; Elvira et al. 2020) including non-pathogenic environmental *Vibrio* species that may inhibit pathogenic *Vibrio* species (Froelich and Oliver 2013; Burks et al. 2017). Work is currently underway to measure the whole microbial community (total bacteria and *Vibrio* species) in stored BROs from this study to assess the impact of inoculated *V. parahaemolyticus* on the resident oyster microbiome compared to the seawater inoculated controls.

Due to the large natural variability in *V. parahaemolyticus* levels in oysters, as evidenced by the seawater injected BRO controls and other reports (Kaufman et al. 2016), the approach used in this study was to inject a known number of cells into the oysters to avoid highly variable measurements between replicates and enable an accurate growth rate to be calculate. This also allowed measurements of *V. parahaemolyticus* levels at cooler temperatures following inactivation. Inoculation of oysters by filtration would better represent ingestion under natural conditions and be less invasive, however, because this can lead to variable uptake (Ellett et al. 2022), injection of bacteria was considered the most suitable inoculation method for this study.

Concentrations around 3.29-3.55 $\log_{10}$ CFU/g were measured before inoculating the BROs, which is at the higher end of the range reported in (Padovan and Gibb 2022) the temperate species POs and SROs

(Eyles et al. 1985; Bird et al. 1992; Lewis et al. 2002; Madigan 2008). *V. parahaemolyticus* is present almost year-round in northern Australia coastal seawater (Padovan et al. 2021) with higher levels than more southern Australian locations (Williams et al. 2022). There are periods of higher density in seawater, often related to season (Robles et al. 2013; Machado and Bordalo 2016; Rivas-Montaño et al. 2018; Padovan et al. 2021) or locations influenced by freshwater run-off (Chávez-Villalba et al. 2010) so it is therefore not unexpected that filter feeding organisms in the tropics may contain higher natural *V. parahaemolyticus* levels than their temperate counterparts. Storage at 18°C and temperatures during transit may also have increased the natural levels of existing *V. parahaemolyticus* in the BROs in this study.

Oyster condition varies with season and is impacted by environmental factors such as algal blooms and oyster reproduction cycles in northern Australia (Nowland et al. 2019c). These major physiological changes in oysters and their microbiome throughout their life cycle and seasons may alter their response to bacterial challenges, so the behaviour of *V. parahaemolyticus* in oyster tissue at various storage temperatures may vary depending on oyster age and condition and this needs to be further explored.

6.6 **Conclusions**

The opportunistic pathogen *Vibrio parahaemolyticus* poses a significant food safety risk worldwide, and understanding its growth in commercially cultivated oysters, especially at temperatures likely to be encountered post-harvest, provides essential information to provide the safe supply of oysters. The Blacklip Rock Oyster (BRO) is an emerging commercial species in tropical northern Australia and as a warm water species, it is potentially exposed to *Vibrio* spp. In order to determine the growth characteristics of *Vibrio parahaemolyticus* in BRO post-harvest, four *V. parahaemolyticus* strains isolated from oysters were injected into BROs and the level of *V. parahaemolyticus* was measured at different time points in oysters stored at four temperatures.

Estimated growth rates were −0.001, 0.003, 0.032, and 0.047 $\log_{10}$ CFU/h at 4 °C, 13 °C, 18 °C, and 25 °C, respectively. The highest maximum population density of 5.31 $\log_{10}$ CFU/g was achieved at 18 °C after 116 h. There was no growth of *V. parahaemolyticus* at 4 °C, slow growth at 13 °C, but notably, growth occurred at 18 °C and 25 °C. *Vibrio parahaemolyticus* growth at 18 °C and 25 °C was not significantly different from each other but were significantly higher than at 13 °C (polynomial GLM model, interaction terms between time and temperature groups $p < 0.05$).

Our results show that storage of BROs at 4°C will prevent growth of *V. parahaemolyticus*, but since this storage temperature may also kill or impair these tropical oysters, shelf life and quality at this temperature needs to be assessed in case death accelerates spoilage by psychrotolerant microorganisms. At 13°C, very low *V. parahaemolyticus* growth rates were measured in BROs which may be a better temperature for BRO survival, however, this is not a standard commercial refrigeration temperature. Our study showed no significant difference in *V. parahaemolyticus* growth or maximum population densities in BROs at 18°C or 25°C, possibly because the oysters and their microbiome adapted to these warmer temperatures are able to 'manage' introduced *V. parahaemolyticus* levels.

These results support BROs storage at both 4°C and 13°C for minimizing *V. parahaemolyticus* growth and sets the foundation for regulators and the Australian oyster industry to develop storage and transport guidelines appropriate for tropical rock oysters to maximize product quality and food safety. Further post-harvest storage trials using pathogenic strains are required to determine if they respond differently to the non-pathogenic strains used in this study to further our understanding of the behaviour of *V. parahaemolyticus* in stored tropical oysters.

7 Assess TRO shelf life at realistic storage temperatures to maximise product quality and inform cold supply chains

7.1 Introduction

Global production of edible oysters is approximately 6.3 million tonnes, with over 98% produced from aquaculture (FAO 2022). Of these, the Pacific Oyster (*Magallana gigas*) and the Eastern or American Oyster (*Crassostrea virginica*) are the predominate cultured species. Tropical species make up only around 1% of total oyster production; with commercial-scale production, of more than 100 t per annum, occurring in a small number of countries while, experimental and subsistence farming occurs more broadly and is reported across 21 tropical countries (Nowland et al. 2020). In Australia, all shellstock intended for consumption as a raw product must be placed under ambient refrigeration at 10°C or less within 24 h of being harvested (Australian Shellfish Quality Assurance Advisory Committee 2022). Although a higher temperature will be considered acceptable only if demonstrated, by scientifically-robust evidence, that such a higher temperature will not support unacceptable growth of human pathogens (Australian Shellfish Quality Assurance Advisory Committee 2022). As an example, the NSW Food Authority, stipulates that Sydney Rock Oysters must be stored at 25°C or less within 24 h of harvest and at 21°C or less within 72 h of harvest (NSW Food Authority 2020). The Sydney Fish Market's 'Seafood Handling Guidelines' recommends that live oysters should be stored and transported in temperatures similar to their natural environment and kept under high humidity conditions (Sydney Fish Market 2015), whereas on their species information sheets they recommend storing Sydney Rock Oysters, Pacific Oysters and Native Oysters at 5°C (Sydney Fish Market).

Whilst Blacklip Rock Oysters (BROs), *Saccostrea echinata/* lineage J, have been harvested from rocky shores for decades in Australia (Boyer 2019), the market for BROs is currently quite local on a spatial scale (Schrobback and Rolfe 2020). Internationally, very few studies have been published on the optimal storage conditions for tropical oysters. It is important that the developing BRO industry understands how the product quality and safety is affected by post-harvest conditions. BROs are considered a very hardy species which can remain alive out of water for at least a week in ambient conditions in the shade (J Collison, S Westley, S Gill; pers. comm). Tropical oysters may have different cold chain requirements compared to temperate species (Schrobback and Rolfe 2020) and handling protocols for one species may not apply to another (Chinnadurai et al. 2013). Objective 3 evaluated the effect of storage temperatures on the product quality and shelf-life of live (closed/whole-shell) BROs.

7.2 Objectives

The objective of this study was to assess Blacklip Rock Oyster (BRO) shelf life at realistic storage temperatures to maximise product quality and inform cold supply chains. Objective 3 activity was completed in December 2022. Blacklip Rock Oysters (BROs) were sourced from a commercial farm (Bowen Fresh Oysters, Bowen, Qld) on two occasions (mid-season and peak-season) and airfreighted to SARDI Food Sciences (Urrbrae, SA). BROs were randomly allocated to four storage temperatures and held at 4°C, 13°C, 18°C and 25°C. Product attributes were assessed over six time points (days 2, 4, 6, 8, 9 and 10 post-harvest). Assessors included a small consumer panel (mid- and peak season trials) and an independent seafood processor (peak season).

7.3 **Methods**

7.3.1 Oyster sampling and storage

Live BROs (*Saccostrea echinata/* lineage J) of market size were sourced from Bowen Fresh Oysters (Bowen, Queensland) on two occasions (August 2022 – mid-season harvest; and November 2022 – peak-season harvest). The salinity and sea-surface temperatures at the time of harvest were approximately 30.2 PSU and 21.0°C for the mid-season and 35.9 PSU and 26.7°C for peak-season. Upon harvest, the oysters were rinsed with potable water, allowed to air-dry overnight in an 18°C airconditioned environment, before being packed into plastic lined polystyrene boxes and airfreighted to SARDI Food Sciences (Urrbrae, South Australia) under ambient conditions. Each shipment consisted of approximately 105 dozen oysters. During transportation the temperature of the shipments were monitored and the average temperature during transportation was 18.0°C (range: 14.1-21.7°C) and 22.7°C (range: 17.2-26.7°C) for shipments 1 and 2 respectively. Upon arrival the oysters were initially held at 18°C before being randomly split into four treatments and held at 4°C, 13°C, 18°C or 25°C. The BROs were assessed on days 2, 4, 6, 8, 9 and 10 (post-harvest). Temperature loggers (Mon-T2, TempRecord International Ltd) were used to record temperature.

7.3.2 Biometry, mortality and intravalvular fluid

Oysters were randomly sampled (n=12) from each treatment per sampling day. For each individual, the shell length (including hinge) and total weight were recorded. The oysters were then placed on a bench for 2 hours and assessed for gaping. If gaping, the oysters were subsequently assessed for an adequate response to percussion (i.e., the ability for the oyster to close by themselves when tapped). Any oysters that did not respond to the percussion test were considered dead. Oysters were then hand shucked, and the meat and shell weight recorded after draining on absorbent paper. The meats and shells were subsequently dried at 105°C overnight to determine the dry weights. The weight of intravalvular fluid was determined by difference. The condition index (CI) was calculated using Equation 1 from Rainer and Mann (1992). The technique was selected as it is independent on the quantity of intravalvular fluid present in the cavity of the oysters. The meat to shell ratio was calculated using Equation 2 as this is used when grading Sydney Rock and Pacific Oysters (Ryan 2008).

$$\text{CI} = \frac{\text{Meat dry weight (g)}}{\text{Shell dry weight (g)}} \text{ x } 100 \qquad (1)$$

$$\text{Meat to shell ratio} = \frac{\text{Drained meat weight (g)}}{\text{Shell length (mm)}} \text{ x } 100 \qquad (2)$$

7.3.3 Microbiological analysis

Live oysters were randomly sampled (n=18) from each treatment per sampling day and delivered to the SA Analytical Laboratory Services (Athol Park, South Australia) in insulated containers for microbiological analysis. Six oysters (meat and intravalvular fluid) were pooled to form each sample. Pooled samples were homogenised in Peptone Saline Solution (PSS) using a stomacher for 30 seconds to form a 1 in 2 dilution. Homogenate (20 mL) was then diluted in PSS (80 mL) to form a 1 in 10 dilution. Total viable counts (TVC) were determined in triplicate on Plate Count Agar (Oxoid, Australia, Thebarton, South Australia) (shipment 1 and shipment 2) and Marine Agar (Difco, Detroit, Michigan, USA) (shipment 2). TVC on Plate Count Agar was measured in accordance with AS 5013.5-2016, whereas the TVC on Marine Agar used an “in-house” method. All agar plates were incubated at 30°C for up to 72 h.

7.3.4 Consumer-panel sensory evaluation

The sensory quality of the oysters (mid- and peak-season trials) were assessed by six panellists using an oyster quality guide (Table 7-1) BROs were manually shucked and turned, before being placed on oyster trays without rinsing. The half-shell oysters were loosely covered with plastic wrap and stored at 4°C for at least 30 min prior to assessment. Panellist were provided two samples from each treatment in a coded-format and asked to assess without consuming for odour, body colour and appearance, liquor clarity, texture, and appearance of the mantle, gills and adductor muscle. The panellists were also asked if they considered the oysters fit for sale or consumption.

Table 7-1: Oyster quality guide. Adapted from He and Morrissey (1999). The scores of 2, 4 and 6 allowed the panellists to provide a more accurate assessment score by having the ability to score between any two attribute descriptors.

Parameter	Attribute score						
	1	2	3	4	5	6	7
Odour	Hay/crisp	-	Strong sea-weedy	-	Spoiled with slight sour smell	-	Sour and putrid smell
Body colour and appearance	Cream white	-	White, a few striations	-	Tan/beige, some striations	-	Yellow/light brown, many striations
Liquor clarity	Clear	-	Clear with small amount of debris	-	Clear with large amount of debris	-	Cloudy
Texture	Firm and elastic	-	Soft and less elastic	-	Slightly mushy	-	Mushy
Mantle	Strong colour Brown/black	-	Slightly fading	-	Mostly faded	-	Faded
Gill	Filaments well defined	-	Filaments less defined	-	Filaments poorly defined	-	Filaments undefined
Adductor muscle	Pale white, Translucent	-	Light-grey, Translucent	-	Light grey, Partially Opaque	-	White, Opaque

7.3.5 Seafood processor sensory evaluation

The quality of the oysters (peak-season trial) was also assessed by an independent seafood processor. Live oysters were randomly sampled (n=12) from each treatment per sampling day and delivered to The Fish Factory (Athol Park, South Australia) in insulated containers. Oysters were manually shucked before being placed without rinsing on oyster trays and assessed prior to and after turning. The assessment framework was an acceptable, marginal or unacceptable focussing on oyster odour, overall appearance and texture. The oysters were provided in a coded format.

7.3.6 Data analysis

All data were analysed using the statistical software package R. Analysis of variance was applied to the microbiological and sensory/quality characteristic data to assess whether there were statistically significant differences over time and between the different temperature conditions (P-value = 0.05). Tukey's test was also applied to determine pairwise comparisons when statistically significant differences were identified.

7.4 **Results**

7.4.1 Mid- and Peak- season size and weight

Shell length, total oyster weight, drained meat weight, dry meat weight and dry shell weight from oysters of both trials (mid- and peak-season) are reported in Table 7-2. Overall, the oysters harvested during the peak-season trial were slightly larger and had a higher overall total and meat weight than the mid-season oysters. However, there were no significant differences in the initial meat to shell ratio (P>0.072) and condition index (P>0.800) of the mid-season and peak-season oysters when assessed on day 2.

Table 7-2: Size and weight ranges of BROs (n = 252)

	Shell length (mm)	Total weight (g)	Wet meat weight (g)	Dry meat weight (g)	Dry shell weight (g)
Mid-season (Aug 2022)	69.4 ± 4.0 (min: 58.0; max: 82.0)	56.4 ± 8.6 (min: 37.4; max: 83.8)	7.4 ± 1.5 (min: 2.1; max: 11.7)	2.1 ± 0.4 (min: 0.6; max: 3.2)	43.3 ± 7.0 (min: 27.7; max: 65.2)
Peak season (Nov 2022)	70.3 ± 4.0 (min: 59.0; max: 80.0)	58.3 ± 9.5 (min: 35.8; max: 89.0)	8.2 ± 1.6 (min: 2.7; max: 13.8)	2.2 ± 0.5 (min: 0.6; max: 4.0)	44.7 ± 7.6 (min: 28.2; max: 68.0)

The meat to shell ratio of the mid- and peak-season BROs is shown in Figure 7-1. The meat to shell ratios were significantly affected by storage duration for mid- (P<0.004) and peak-season (P<0.011) but not temperature for mid-(P>0.8732) and peak season (P>0.531). The mean meat to shell ratio (c.f. mean 10-12) is lower than those specified in Sydney Rock (c.f. 14-17) and Pacific Oyster (c.f. 16-21) Grading Systems. However, the measurements in this study used the total shell length, not top shell length (i.e. total shell length – hinge length).

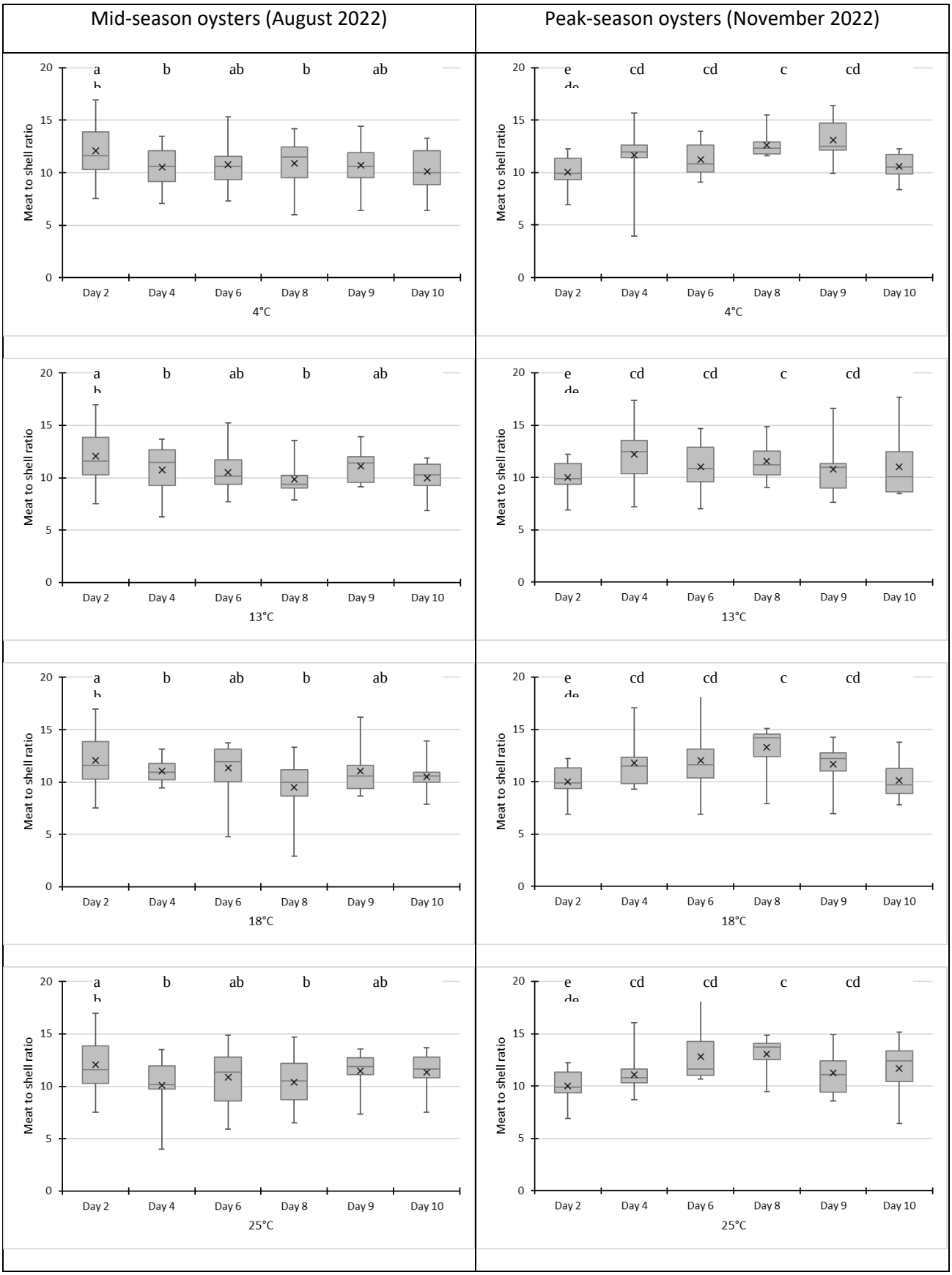


Figure 7-1: Meat-to-shell ratio of the oysters. The mean values between days with different letters (a-e) were significantly different (p<0.05) by a Tukey test.

7.4.2 Mid- and Peak-season condition index

The condition indices for mid- and peak-season BROs were not significantly affected by storage duration for mid- (P>0.240) and peak-season (P>0.156) or temperature for mid- (P>0.960) and peak season (P>0.834) (Figure 7-2).

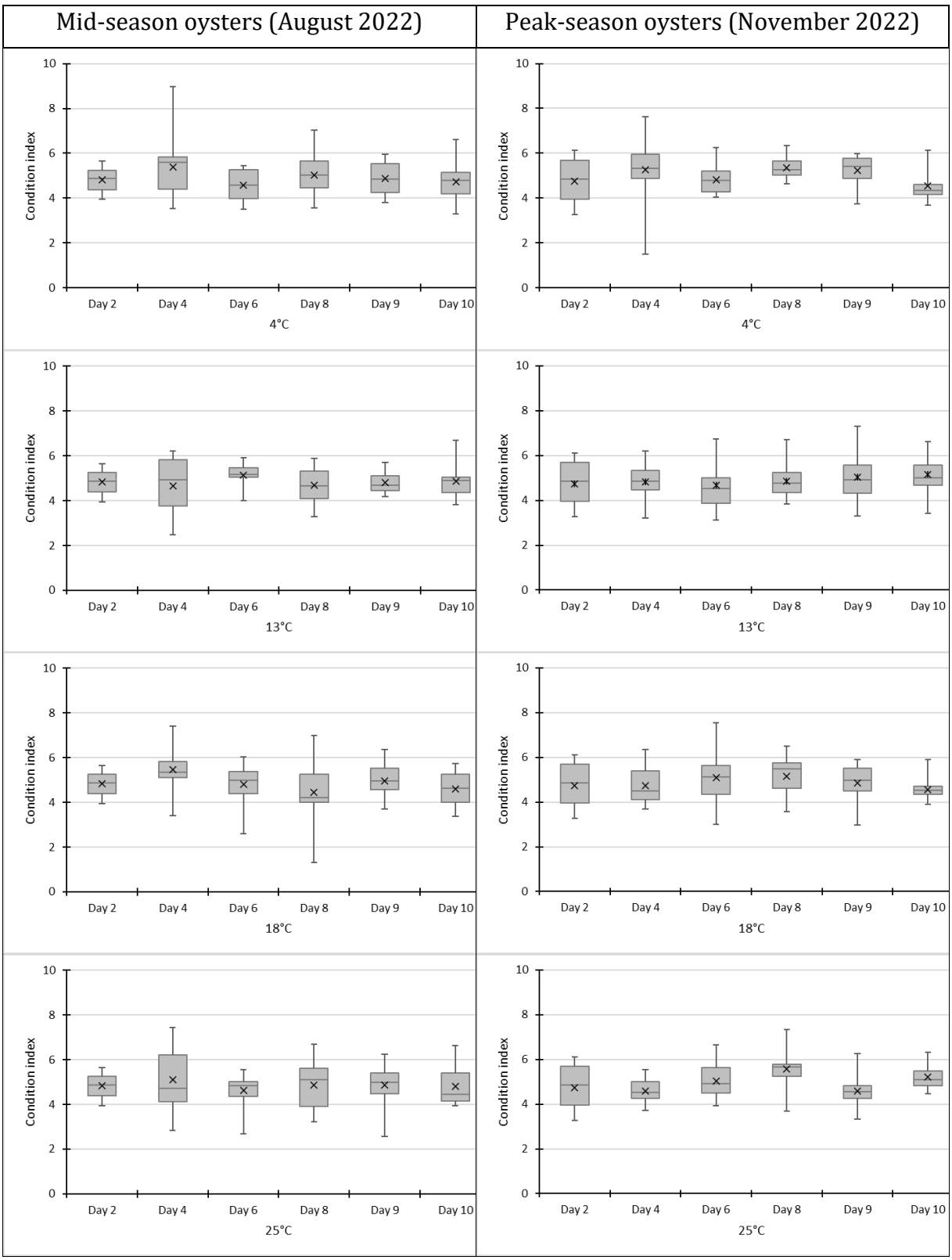


Figure 7-2: Condition index of BROs from mid- and peak-season harvest and storage trials.

7.4.3 Oyster gaping

Nearly all of the BROs assessed in this project remained tightly closed throughout the storage trials, however, those stored at 13°C had a greater tendency to gape. Whilst most of these gaping oysters were responsive to the percussion test, a few were not responsive and considered dead. Summaries of the gaping and mortality assessment results for the mid-season and peak-season harvest storage trials are reported in Tables 7-3 and 7-4 respectively.

Table 7-3: Gaping and mortality assessment for mid-season BROs harvest and storage trial. Only the number of responsive gaping and non-responsive gaping oysters are shown (n=12).

Storage temperature	Days					
	2	4	6	8	9	10
4°C	-	-	-	-	1 non-responsive	-
13°C	-	-	-	-	3 responsive 1 non-responsive	7 responsive
18°C	-	-	-	-	1 responsive 1 non-responsive	3 responsive
25°C	-	-	-	-	-	1 non-responsive

Table 7-4: Gaping and mortality assessment for peak-season BROs harvest and storage trial. Only the number of responsive gaping and non-responsive gaping oysters are shown (n=12).

Storage temperature	Days					
	2	4	6	8	9	10
4°C	-	-	-	-	-	-
13°C	-	-	5 responsive 1 non-responsive	5 responsive 2 non-responsive	5 responsive 2 non-responsive	8 responsive
18°C	-	-	-	1 responsive 1 non-responsive	-	-
25°C	-	-	-	-	1 responsive	-

7.4.4 Intravalvular liquor weight

The effect of storage duration on the intravalvular liquor weight at 4°C, 13°C, 18°C and 25°C for mid- and peak-season oysters is shown in Figure 7-3. For the mid-season BROs, whilst there was a decreasing trend of intravalvular liquor weights for those BROs held at 13°C and 25°C, the results were not significantly different with time ($P>0.258$). However, there was a significant lower ($P<0.007$) liquor weight in the BROs held at 25°C compared to those stored at 4°C and 18°C. For the peak-season BROs there was a significant difference ($P<0.014$) in liquor weights between 18°C and 25°C treatments (less liquid weight at 25°C). There was also a significant relationship ($P<0.001$) between liquor weights and time, i.e. as storage time progressed, liquor weights decreased.

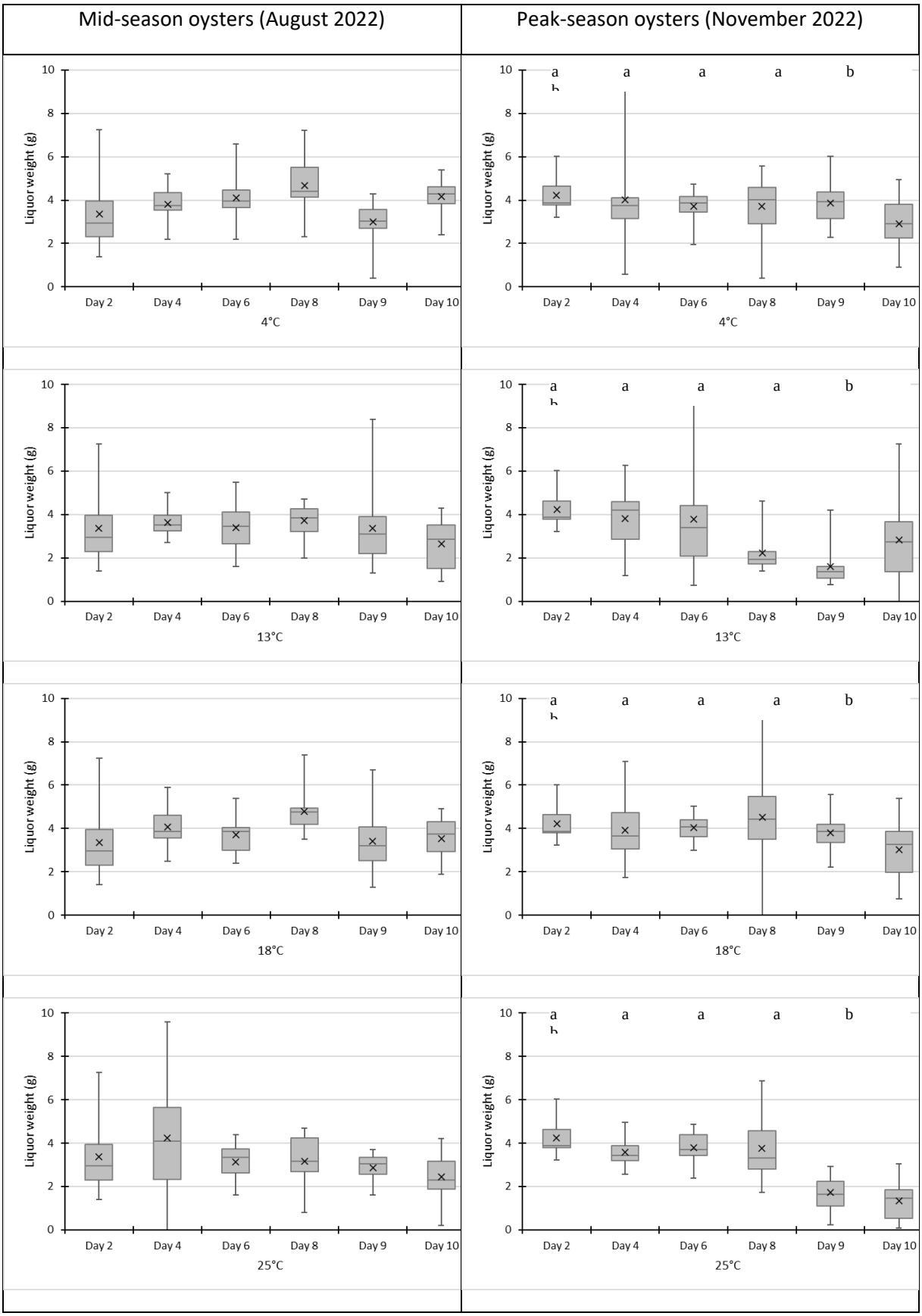


Figure 7-3: Intravalvular liquor weights of the BROs from mid- and peak-season harvest and storage trials. The mean values between days with different letters (a-b) were significantly different (p<0.05) by a Tukey test.

7.4.5 Microbiology - Total viable counts (TVCs)

TVCs of BROs from mid- and peak season harvest after being incubated on Plate Count Agar and on Marine Agar are shown in Figure 7-4. Plating on Marine Agar was undertaken in the second shipment (peak-season trial) as TVC on Plate Count Agar can underestimate counts from marine bivalves (Madigan 2013). Plating on Marine Agar gave TVC results that were consistently higher compared to standard Plate Count Agar. For mid-season BROs there was a significant decrease in TVC on Plate Count Agar with storage time ($P<0.002$) but not temperature ($P>0.914$). Samples were not plated on Marine Agar. For peak-season BROs there was no significant differences in TVC on Plate Count Agar with storage time ($P>0.184$) or temperature ($P>0.465$). However, when plated on Marine Agar there was a significant difference with storage time ($P<0.003$) and temperature ($P<0.001$). BROs stored at 13°C and 25°C had significantly higher TVCs on Marine Agar compared to those stored at 4°C or 18°C.

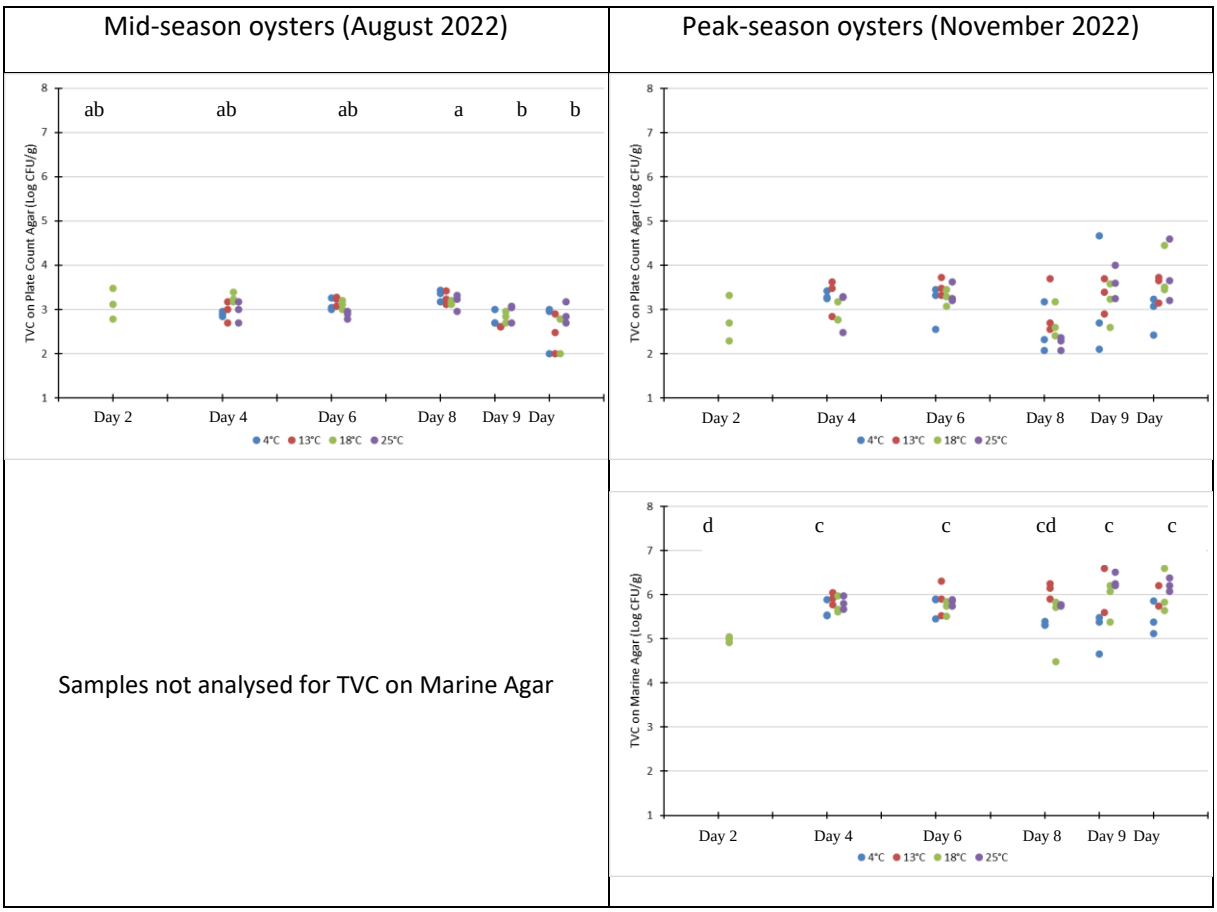


Figure 7-4: TVC of BROs on Plate Count Agar and Marine Agar from mid- and peak-season harvest and storage trials. Mean values between storage days with different letters (a-d) were significantly different ($p<0.05$) by a Tukey test.

7.4.6 Sensory trends

Odour, body colour, intravalvular liquor clarity, texture, mantle, gills and adductor attribute scores for the mid- and peak season trials are shown in Figures 7-5 to 7-11, respectively. In mid-season oysters, except for odour, sensory attributes had a significant relationship with time, but not temperature. Over time, the quality attributes decreased. Similar sensory attribute trends occurred with peak-season BROs. In the case of odour, there was also a significant difference ($P<0.023$) with storage temperature. BROs stored at 4°C had a significantly lower (higher quality) odour score compared to those stored at 25°C when there was a noticeable increase in odour from days 9-10 (Figure 7-5).

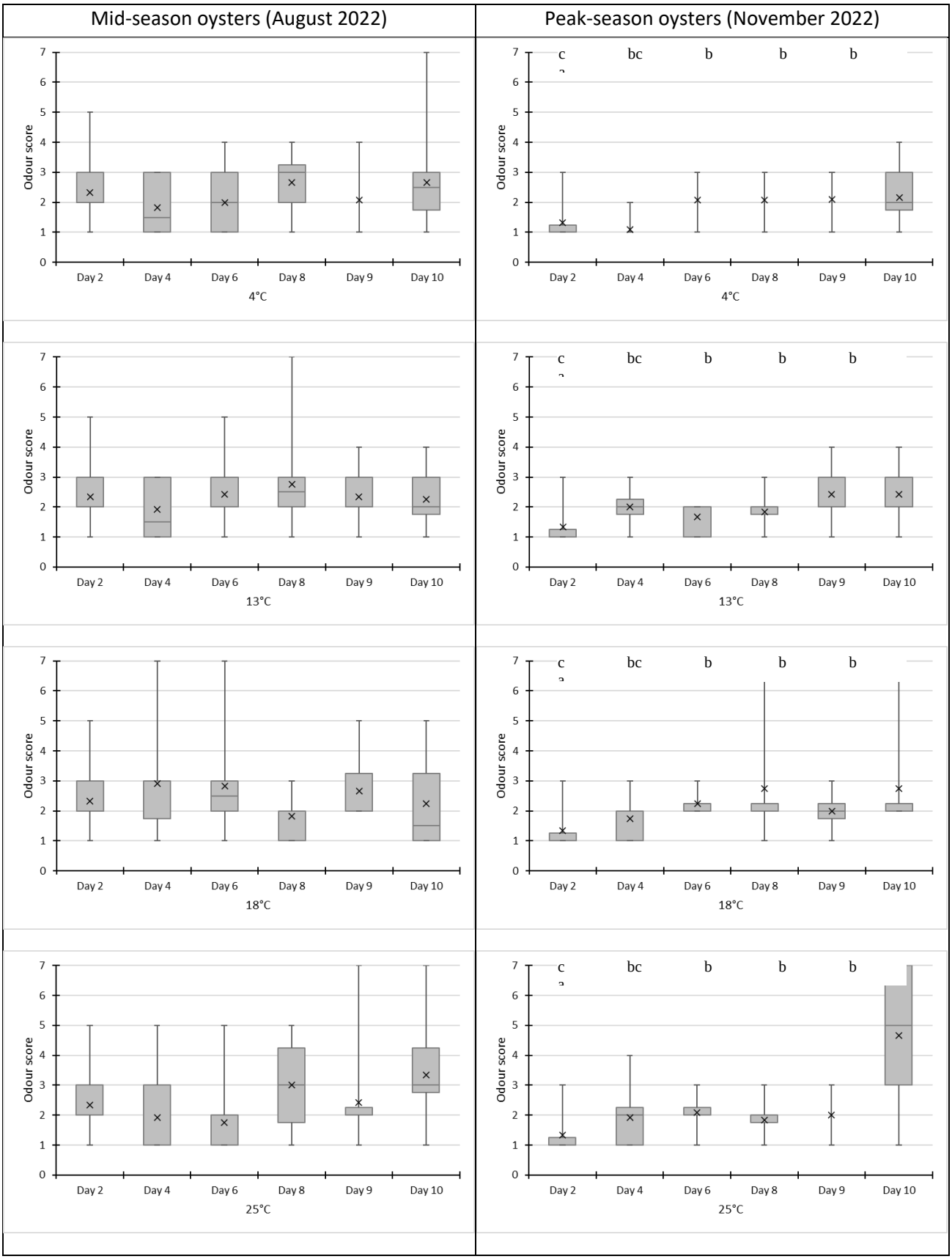


Figure 7-5: Odour score of BROs from mid- and peak-season harvests. Mid-season: no significant differences with storage duration (P>0.120) or temperature (P>0.719); peak-season: significant difference with storage duration (P<0.001) and temperature (P<0.023). The mean values between days with different letters (a-c) were significantly different (p<0.05) by a Tukey test.

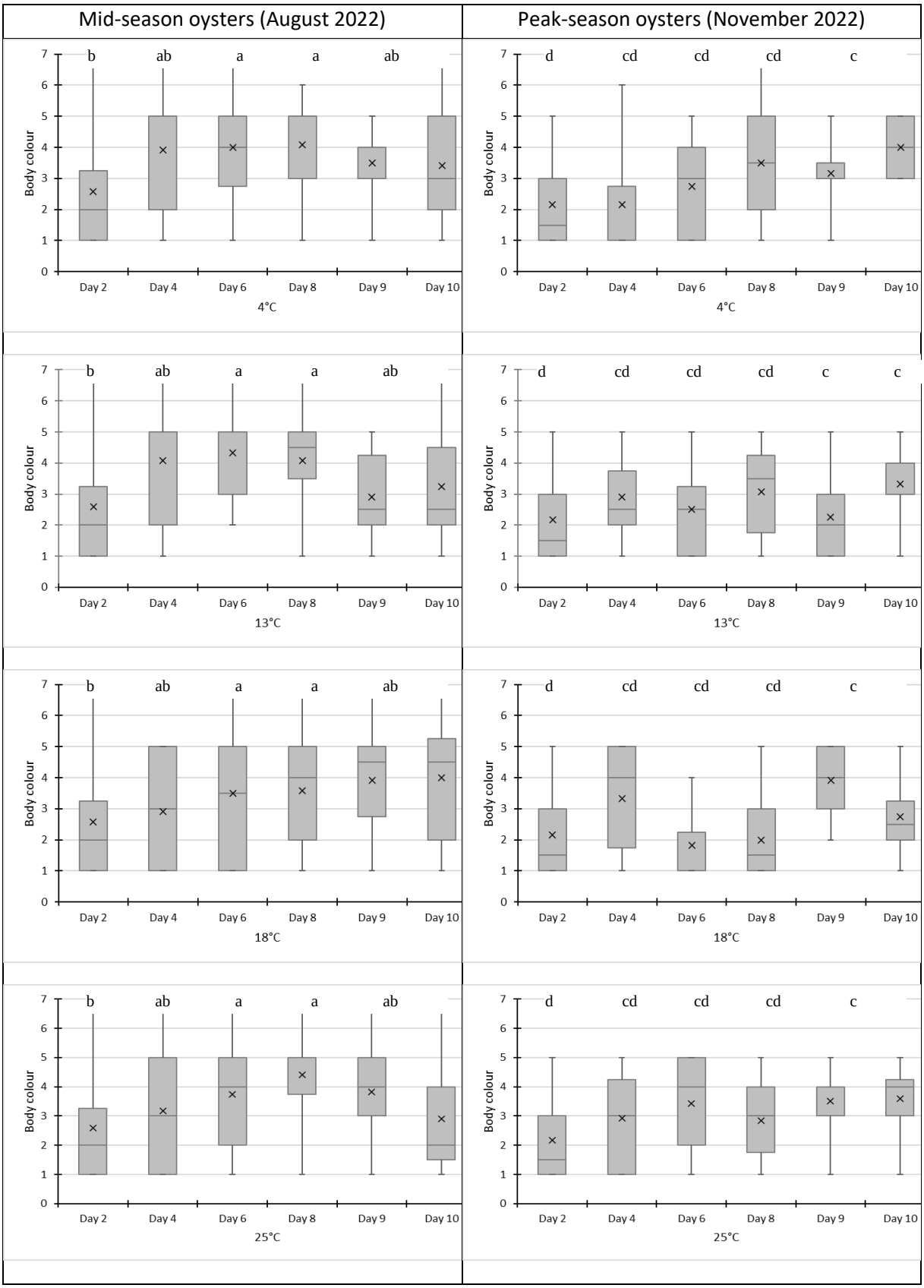


Figure 7-6: Body colour score of the BROs from mid- and peak-season harvest and storage trials. Mid-season: significant difference with storage duration (P<0.020) but not with temperature (P>0.951); peak-season: significant difference with storage duration (P<0.001) but not with temperature (P>0.271). The mean values between days with different letters (a-d) were significantly different (p<0.05) by a Tukey test.

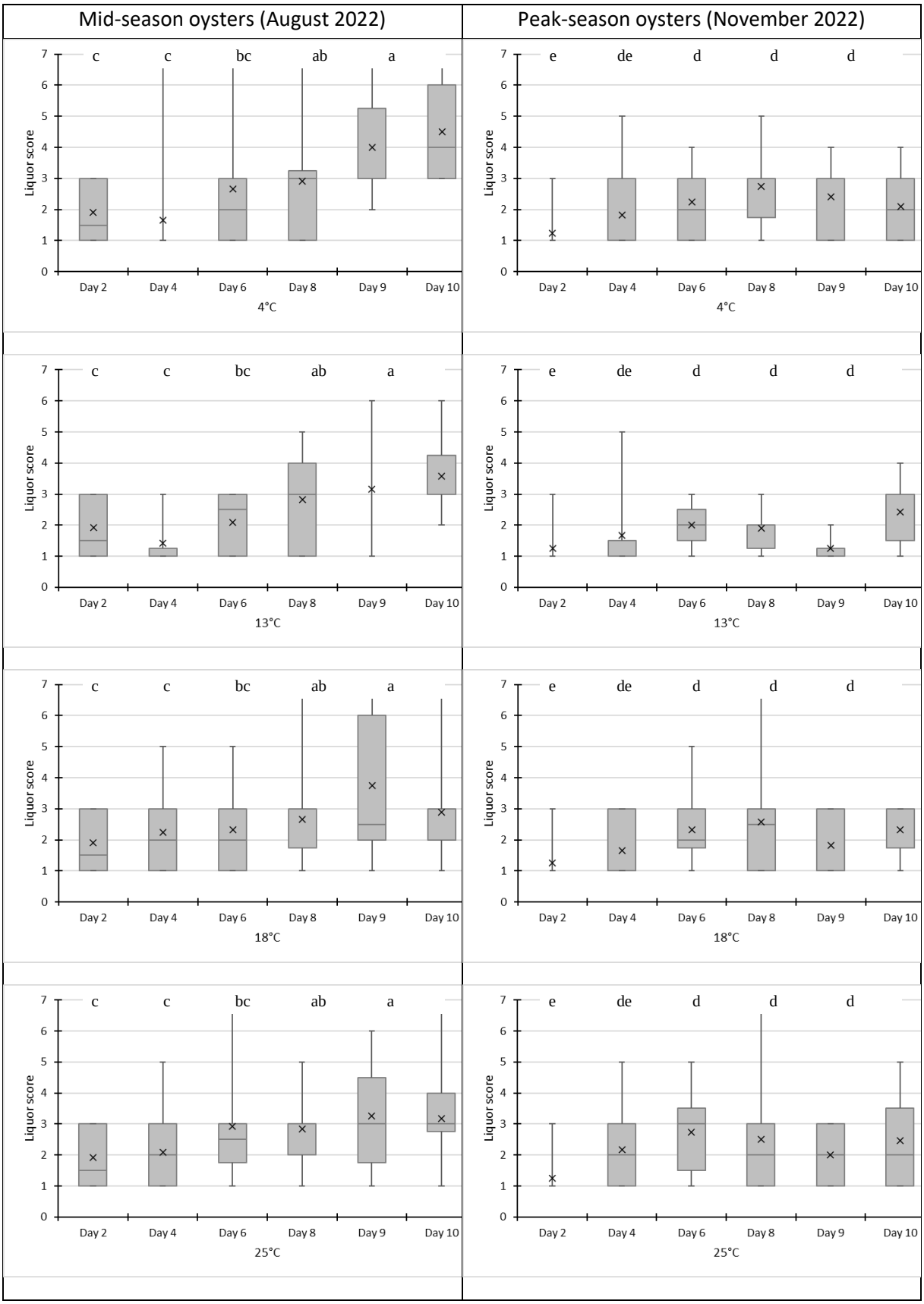


Figure 7-7:Intravalvular liquor score of BROs from mid- and peak-season harvests. Mid-season: significant differences with storage duration (P<0.001) but not temperature (P>0.367); peak-season: significant difference with storage duration (P<0.001) but not temperature (P>0.395). The mean values between days with different letters (a-e) were significantly different (p<0.05) by a Tukey test.

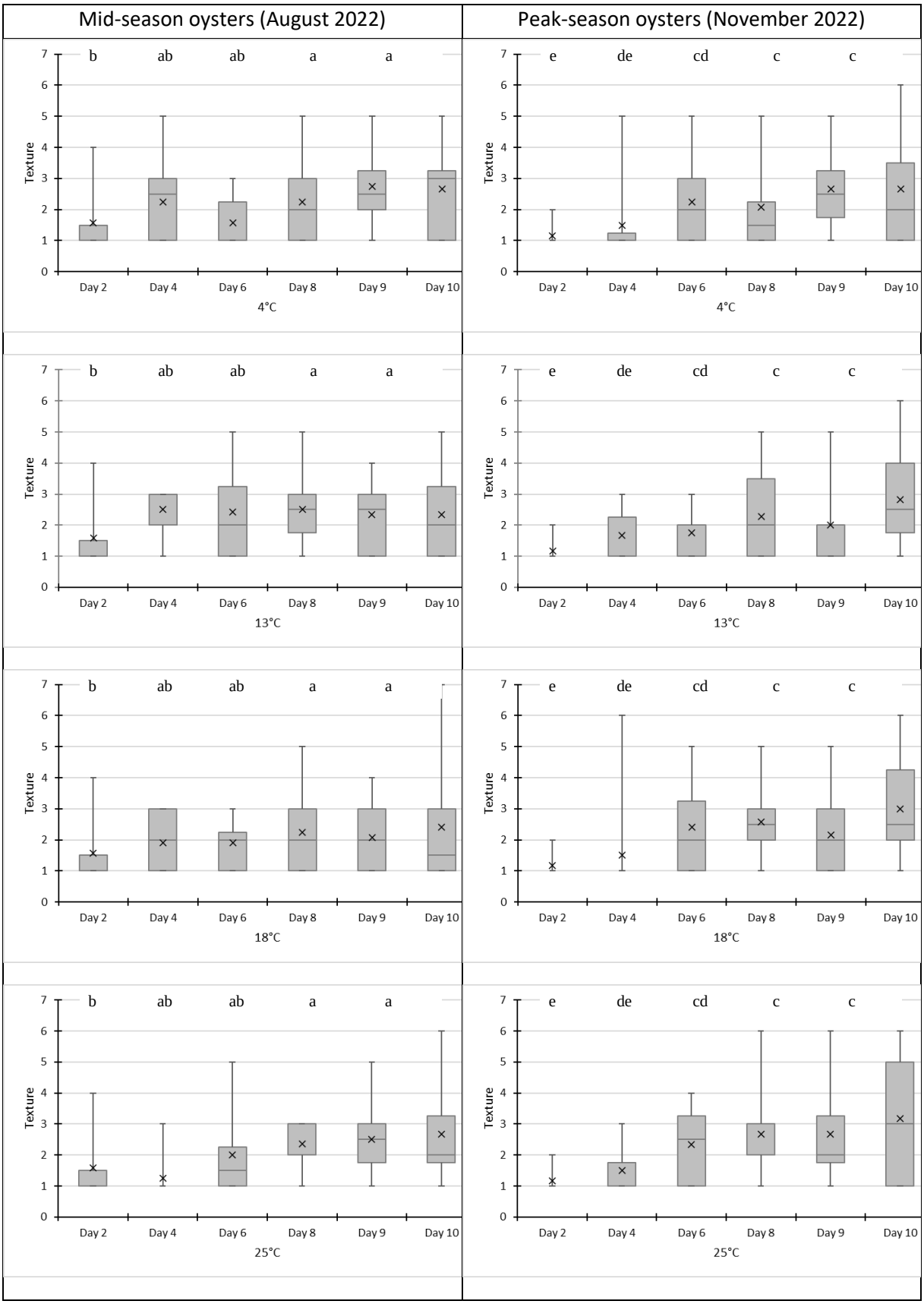


Figure 7-8:Texture score of BROs from mid- and peak-season harvests. Mid-season: significant differences with storage duration (P<0.001) but not with temperature (P>0.581); peak-season: significant difference with storage duration (P<0.001) but not with temperature (P>0.524). The mean values between days with different letters (a-e) were significantly different (p<0.05) by a Tukey test.

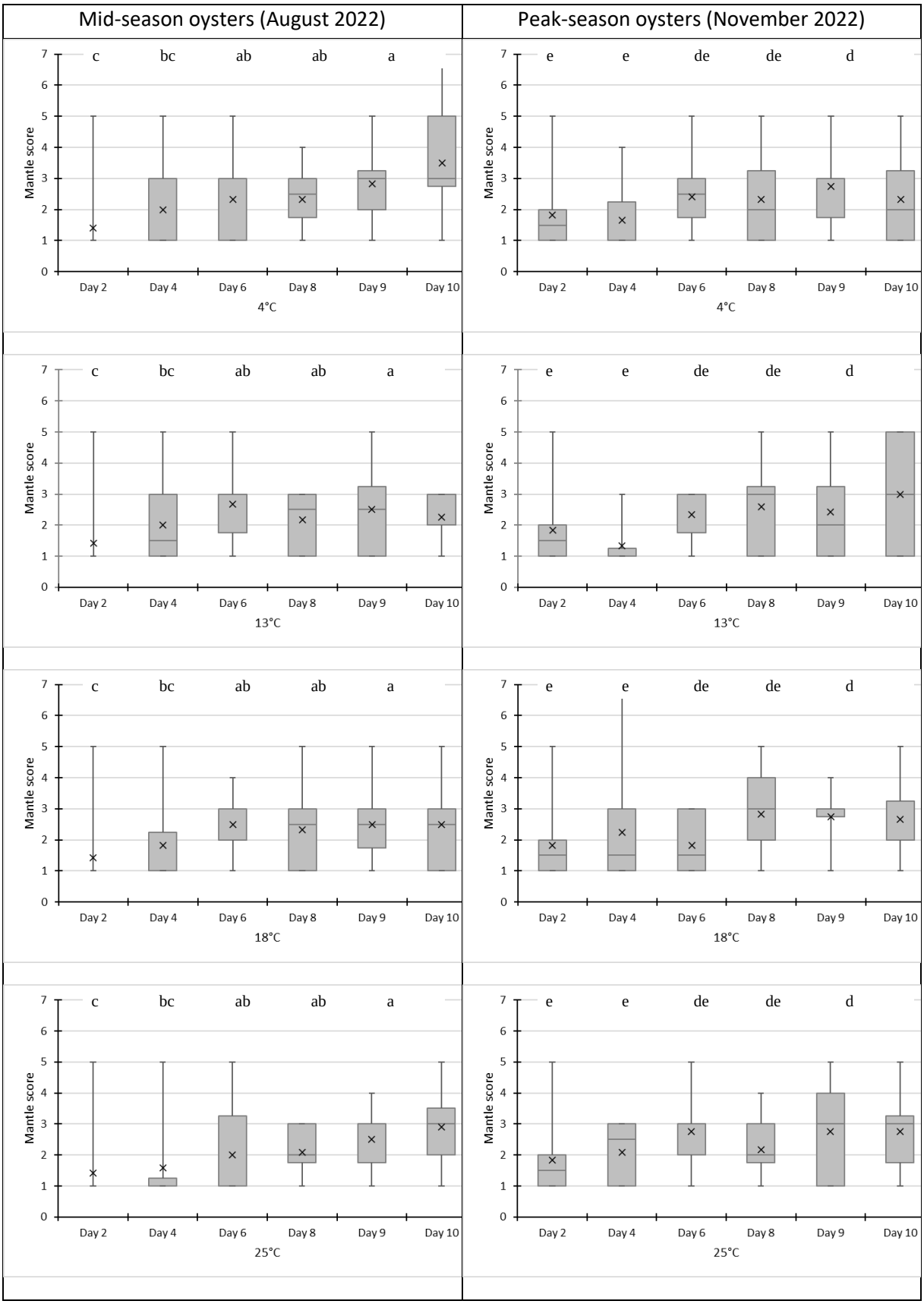


Figure 7-9:Mantle score of BROs from mid- and peak-seasons. Mid-season: significant differences with storage duration (P<0.001) but not with temperature (P>0.436); peak-season: significant difference with storage duration (P<0.001) but not with temperature (P>0.819). The mean values between days with different letters (a-e) were significantly different (p<0.05) by a Tukey test.

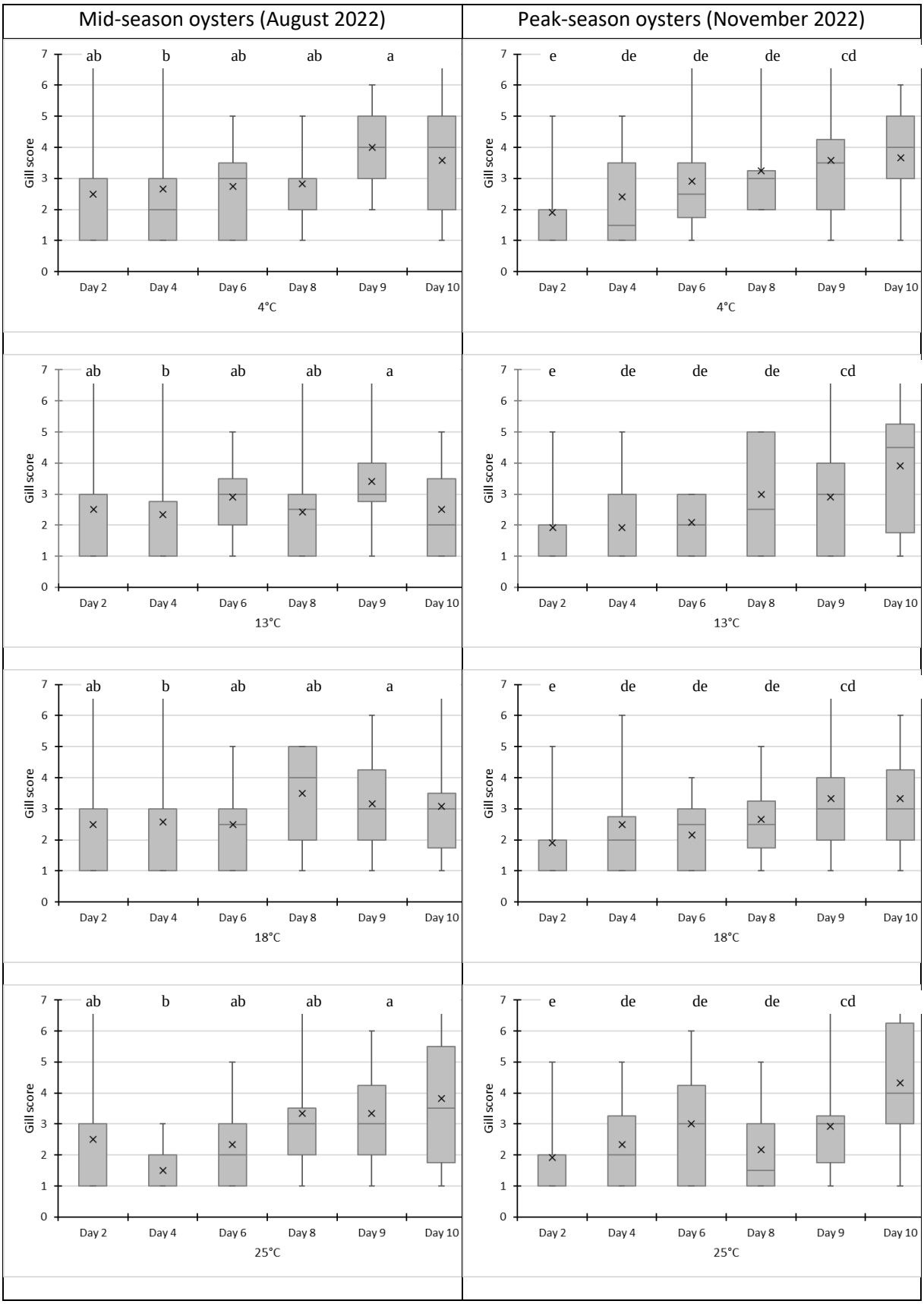


Figure 7-10:Gill score of BROs from mid- and peak-season harvests. Mid-season: significant differences with storage duration (P<0.001) but not with temperature (P>0.604); peak-season: significant difference with storage duration (P<0.001) but not with temperature (P>0.609). The mean values between days with different letters (a-e) were significantly different (p<0.05) by a Tukey test.

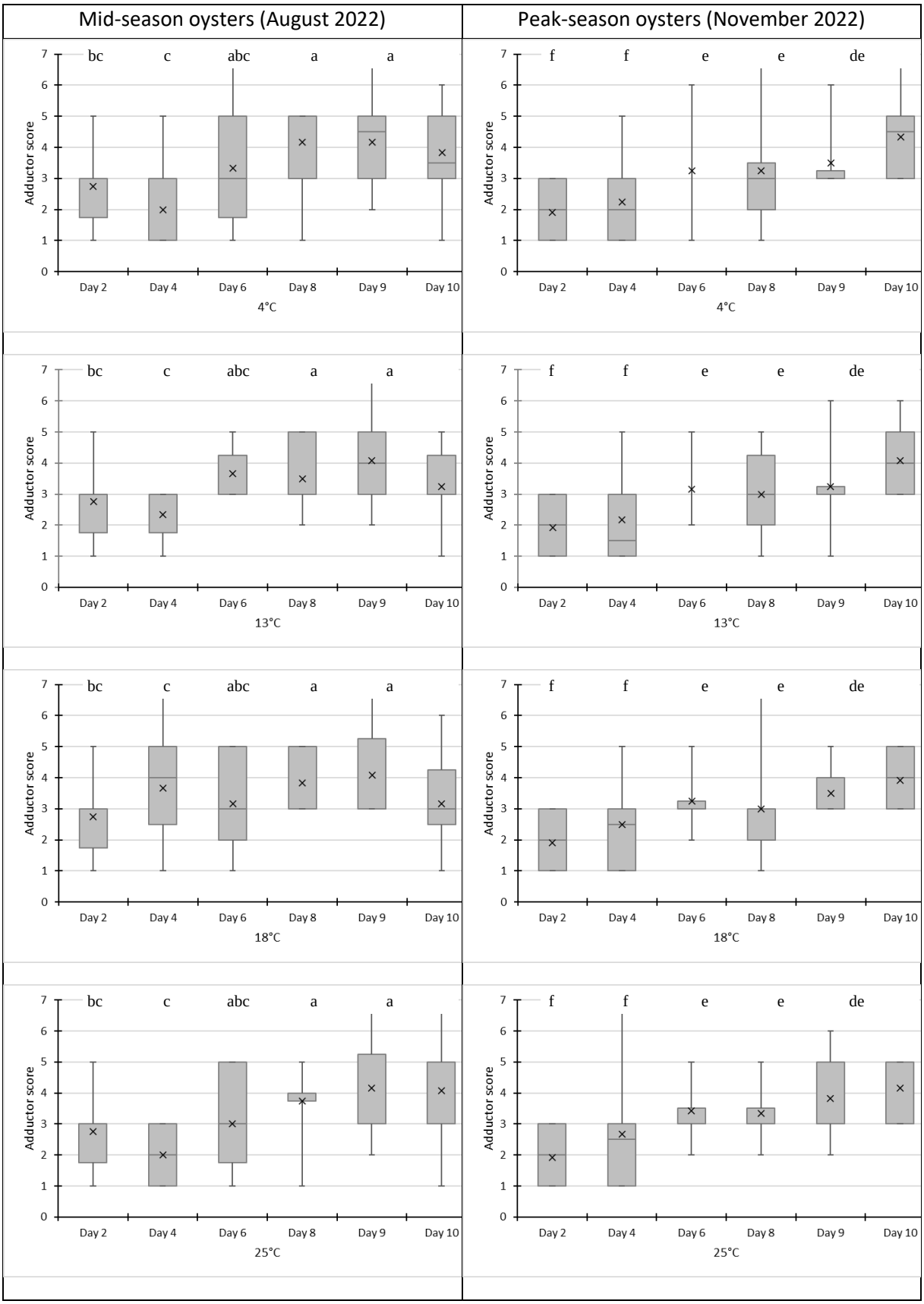


Figure 7-11:Adductor score of BROs from mid- and peak-season harvests. Mid-season: significant differences with storage duration (P<0.001) but not with temperature (P>0.877); peak-season: significant difference with storage duration (P<0.001) but not with temperature (P>0.525). The mean values between days with different letters (a-f) were significantly different (p<0.05) by a Tukey test.

7.4.7 Acceptability for sale

The percent of BROs that were considered acceptable for sale and/or consumption from mid- and peak-season harvest trials are shown in Figure 7-12. There is considerable variability in the percentage of oysters that the panellists considered to be acceptable. Panellists were not asked to justify why a particular oyster was considered unacceptable.

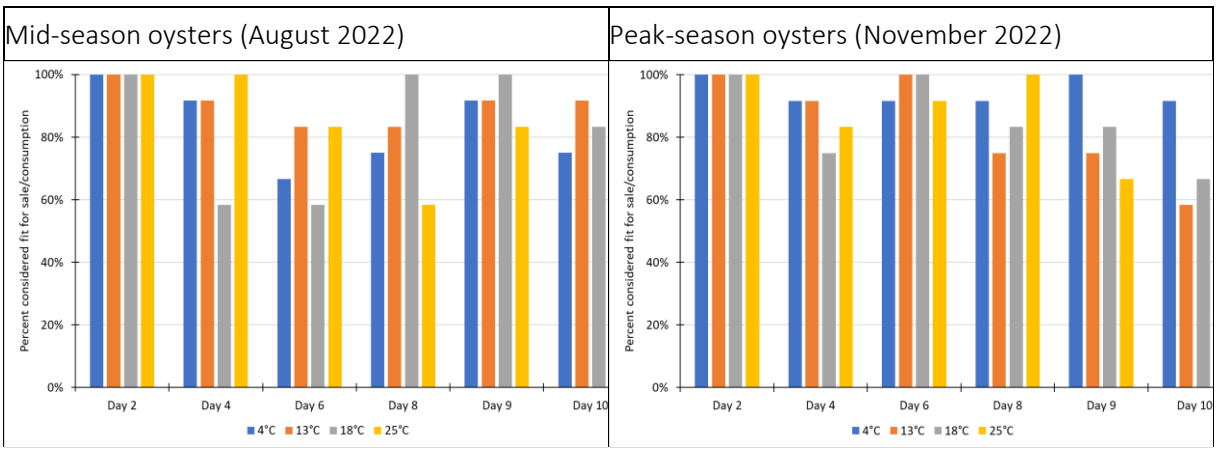


Figure 7-12: Fitness of sale/consumption from mid- and peak-season harvest and storage trials.

The seafood processor assessments for peak-season storage trial were performed on the same storage duration intervals as the consumer panel evaluation. The seafood processor was very familiar with Pacific Oysters, but not BROs. A summary of the results from this assessment is reported in Table 7-5. This assessment was not part of the original project plan, but was added to help distinguish between treatments. The same person assessed the oysters on all days except for day 9 when they were unavailable, and a different staff member assessed. The samples that corresponded to the 4°C storage condition were generally assessed as leading, but on day 9 (which corresponded to the different assessor) these oysters received the lowest preference. The seafood processor noted that the oysters were 'robust and resilient' and was surprised at the consistency over the range of storage temperatures. They determined that there was very little difference in the oysters between the treatment, and once turned/flipped all oysters would be considered acceptable for sale.

Table 7-5: Summary of seafood processor assessment

Storage temperature	Days					
	2	4	6	8	9	10
4°C		-	-	-	-	-
13°C	-	-	Less firm	Mantle becoming shrivelled	-	-
18°C	-	-	-	-	-	-
25°C	-	-	Very little liquor, less firm	-	-	-
Order of preference	-	-	-	4°C leading	25°C > 18°C > 13°C > 4°C (based on colour and texture) 4°C received lower preference due to some green tinge in gills	4°C > 18°C > 25°C > 13°C (based on colour and texture)

Photographs of the peak-season oysters from day 10 are shown in Figures 7-13 to 7-16.

Figure 7-13: Oysters stored at 4°C (day 10).

Figure 7-14: Oysters stored at 13°C (day 10).

Figure 7-15: Oysters stored at 18°C (day 10).

Figure 7-16: Oysters stored at 25°C (day 10).

7.5 **Discussion**

The objective of this study was to assess Blacklip Rock Oyster (BRO) shelf life at realistic storage temperatures to maximise product quality and inform cold supply chains. Oysters in mid-season and peak-season condition gave similar results. Temperatures used in this study were informed by results from the *Vibrio parahaemolyticus* growth study which showed no growth at 4°C, slow growth at 13°C and no difference at 18 and 25°C. Although the previous study focused on Vibrio spp. in the present study, assessment was based on food spoilage bacteria generally, assessed by total viable counts (TVCs). The TVCs of BROs from mid- and peak season harvest and storage trials were assessed after incubation on both Plate Count Agar and on Marine Agar. Plating on Marine Agar was undertaken in the second shipment (peak-season trial) as TVC on Plate Count Agar can underestimate microorganism counts from marine bivalves (Madigan 2013). In fact, plating on Marine Agar gave TVC results that were consistently higher compared to standard Plate Count Agar. For mid-season BROs, TVC from Plate Count Agar decreased significantly over time but not temperature, and for peak-season BROs there was no significant differences in TVC over time or temperature. However, when plated on Marine Agar there was a significant difference with time and temperature. BROs stored at 13°C and 25°C had significantly higher TVCs on Marine Agar compared to those stored at 4°C or 18°C. These results contradict those of the tropical oyster *M. belcheri* (formally *C. belcheri*), where TVCs on Plate Count Agar significantly increased when held at 4°C or 30°C (Songsaeng et al. 2010).

There are no microbiological limits of total viable counts (standard plate count) for bivalve molluscs in the Australia New Zealand Food Standards Code. The FSANZ Compendium of Microbiological Criteria for Foods (March 2022) noted that for raw commodities including fish, TVCs can be quite high due to the microbiota normally present (10^6-10^7 cfu/g) (Food Standards Australia New Zealand 2022). Whereas, in the UK for ready-to-eat meat and fish TVC are generally considered satisfactory when $<10^6$ and borderline when 10^6-$<10^7$ cfu/g (Health Protection Agency 2009). The range of TVCs (standard plate count) measured in the BROs irrespective of storage time or temperature were all below 10^5 cfu/g.

In the mid-season oysters, except for odour, the sensory attributes had a statistically significant relationship with time, but not temperature. As time progressed, the quality attributes decreased. Similar sensory attribute trends were observed with the BROs harvested during the peak-season. Although, in the case of odour, there was also a significant difference with storage temperature. BROs stored at 4°C had a significantly lower (higher quality) odour score compared to those stored at 25°C when there was a noticeable increase in odour from days 9-10. The resilience and robustness of the BROs is noticeably different from a similar study with the tropical *M. belcheri* (Songsaeng et al. 2010). These authors concluded that shell-on *M. belcheri* could be accepted at less than 3 days at ambient (30±2°C) temperature and 7-9 days under chilled storage (4±2°C).

Although BROs were robust and resilient to all storage temperatures, there was a greater tendency for BROs to gape (open) at 13°C leading to a loss of intravalvular liquor. There was no growth of microbiological indicator organisms at any time for each storage temperature. Low levels of mortality occurred from day 6 (peak-season trial) and from day 9 (mid-season trial). Most BROs had an acceptable oceanic through to a neutral smell throughout the trial, except for those held at 25°C where there was deterioration between days 9 and 10 (peak season trial). The occasional and random oyster from each treatment was considered spoilt, but there was no trend with storage temperature or time. Although quality attributes declined slightly throughout the storage trial at all storage temperatures, the independent seafood processor's assessment was that BROs held at all storage temperatures were suitable for sale/consumption. However, those stored at 4°C were considered more favorable based on texture and appearance.

Oyster gaping, particularly in elevated air temperatures, is believed to support an evaporative cooling mechanism in some species (Davenport and Wong 1992). Nearly all of the BROs assessed in this project remained tightly closed throughout the storage trials, however, those stored at 13°C had a greater tendency to gape. Whilst most of these gaping oysters were responsive to the percussion test, a few were not responsive and considered dead. Gaping occurred earlier in the peak-season oysters. The general lack of gaping supports findings from several other tropical oyster species. The tropical Belcher's cupped oyster *Magallana belcheri* (formally *Crassostrea belcheri*), Philippine cupped oyster *Magallana bilineata* (formally *Crassostrea iredalei*) and the Hooded oyster *Saccostrea cucullata* (misspelt *S. cucullata*) respond to emersion by tight shell valve closure (Davenport and Wong 1992). Gaping will also result in the loss of intravalvular liquor, which may cause rapid oyster death. Whilst this project did not set out to determine upper or lower lethal temperatures for the BROs, the lower mean lethal temperature (LC_{50}) in aerated seawater for *M. bilineata*, *S. cucullata* and *M. belcheri* were 1.4°C, 4.8°C and 5.0°C, respectively (Davenport and Wong 1992).

7.6 **Conclusions**

Both the mid-season and peak-season results were similar. Although BROs were robust and resilient to all storage temperatures, there was a greater tendency for BROs to gape (open) at 13°C leading to a loss of intravalvular liquor. There was no growth of microbiological indicator organisms at any time for each storage temperature. Low levels of mortality occurred from day 6 (peak-season trial) and from day 9 (mid-season trial). Most BROs had an acceptable oceanic through to a neutral smell throughout the trial, except for those held at 25°C where there was deterioration between days 9 and 10 (peak season trial). The occasional and random oyster from each treatment was considered spoilt, but there was no trend with storage temperature or time. Although quality attributes declined slightly throughout the storage trial at all storage temperatures, the independent seafood processor's assessment was that BROs held at all storage temperatures were suitable for sale/consumption. However, those stored at 4°C were considered more favorable based on texture and appearance.

8 Risk profile for *Vibrio* spp. in Tropical Rock Oysters

8.1 Introduction

Tropical Rock Oysters (TRO) have been harvested by Aboriginal people for food and trade for thousands of years. More recently TRO have been the focus of a developing commercial industry across Western Australia (WA), Northern Territory (NT) and Queensland (Qld). Government, businesses, and Indigenous communities are supporting this development through significant investment focused on the Blacklip Rock Oyster (BRO, *Saccostrea echinata*/ lineage J). The aims are to drive economic growth and diversification in these regions (Nowland et al., 2020, FRDC project 2020-043 unpublished data).

One of the risks for cultural harvest and a hurdle for commercial development is the appropriate management of food safety. Oysters are filter feeders, concentrating contaminants (natural or anthropogenic) in their tissues, and are therefore regarded as a high-risk food source if appropriate management regimes are not in place (FAO/WHO, 2018). The hazards of concern include marine biotoxins, faecal contamination, heavy metals and *Vibrio* bacteria (CAC, 2008; FAO/WHO, 2020). The latter are an emerging risk in Australia, with national awareness heightened due to recent outbreaks associated with Pacific Oysters from more southern states (DHW, 2022; Harlock et al., 2022). Vibrios are a known issue in oysters and studies have indicated a relationship between warmer temperatures and prevalence of vibrios in both seawater and in oysters (Baker-Austin et al., 2017; Green et al., 2019; Ndraha and Hsiao, 2022; Semenza et al., 2017; Vezzulli et al., 2016).

Risk commensurate management approaches to vibrio need to be developed for the developing industry in northern Australia. This requires an understanding of the level of risk and potential drivers that increase risk. However, as with most jurisdictions in Australia, little is known about the risk of vibriosis in northern Australian. In such scenarios, risk managers will commonly outsource a preliminary risk assessment, known as a risk profile.

Risk profiles are an important tool for risk managers and industry. They provide a summary of all information available on the specific hazard/food combination, in this case vibrios in TRO. The purpose of a risk profile is to assist initial risk management activities, such as identifying future actions required (if any), and the options for food safety management. They also inform the level of resourcing required to control the hazard/food pairing and highlight knowledge gaps that need to be filled to inform improved risk assessment and management. It is important to note that risk assessment is an iterative process that should be repeated on a regular basis as more information is collected.

This risk profile is supported by:

- Environmental surveys on vibrios *Vibrio* in water and shellfish in northern Australia
- Experimental determination of *Vibrio* growth rates in Blacklip Rock Oysters (BRO) at various temperatures
- Experimental studies on appropriate storage temperatures and shelf-life of TRO.

8.2 Objectives

This risk profile critically reviewed the information available on the human health or market access risk associated with *Vibrio* spp. in TRO and addressed the following questions:

1. What is the risk of vibriosis from Tropical Rock Oysters sourced from northern Australia?

2. What control measures are available?
3. What are the options for risk management in northern Australia?
4. What are the priorities for future research?

For the purpose of this risk profile, northern Australia is defined as north of the Tropic of Capricorn (latitude 23.5°S). This area includes parts of WA, NT, and Qld. Cholera infection caused by *Vibrio cholerae* O1 or O139 is not included in the scope of this document as it is not usually associated with seafood consumption and is not endemic in Australia.

8.3 Methods

This risk profile was compiled following CODEX risk assessment guidelines of hazard analysis, hazard characterisation, exposure assessment and evaluation of risk (CAC, 2023). The uncertainties associated with the information were carefully identified and considered during the evaluation of risk. Knowledge gaps that hinder further assessment and risk management actions were identified.

Standard web search engines were used to identify information on pathogenic *Vibrio* spp. globally and TRO production in Australia. A focus was given to tropical vibriosis cases, and scientific reports on vibrio communities in northern Australian environments.

Results from studies undertaken in this FRDC project 2020-043 (spatial and temporal surveys of vibrio communities in northern Australian waters and oysters, *Vibrio* growth rates in TRO, shelf-life studies for TRO) were used to inform this risk profile.

These studies used novel methods such as the *hsp60* sequencing assay which provides detailed information about the *Vibrio* community present in a sample. In this method, the *hsp60* gene is amplified using the *Vibrio*-centric *hsp60* primers Vib-hspF3-23 and Vib-hspR401-422 and then sequenced as previously described (King et al., 2019). Results are in the form of *Vibrio* species relative abundance within a sample. The *hsp60* assay contributes to our knowledge of the spatial distribution and temporal aspects of *Vibrio* communities, and how species richness and species relative abundance change within the *Vibrio* community over time, space and under a range of environmental conditions. Specific details of methods related to each study are detailed further in the hazard/food pairing section.

Records of illness from non-choleragenic vibriosis were requested from the health departments in WA, NT, and Qld, after obtaining the appropriate ethics approvals where necessary. Information was requested on the number of illnesses per year (including hospitalisations and deaths), causative species, wound or gastroenteritis infections, and any information on the source food for gastroenteritis cases. Epidemiological data were aggregated where necessary under state legislation to protect individuals' identities.

A literature search of case reports was conducted to seek additional data on *Vibrio* spp. infections occurring in WA, Qld, and the NT. The search was conducted using google scholar with the keywords "vibrio", "Queensland", "Western Australia" and "Northern Territory". Any case reports from between 2000 – 2023 were included in the dataset.

8.4 **Results and Discussion**

8.4.1 Hazard Identification

8.4.1.1 Pathogenic Vibrio

Vibrio bacteria are a diverse group of bacteria that are ubiquitous in the marine environment. They are found free floating in the water, or in association with sediments, phytoplankton, or biota. Some species can cause disease in marine animals, others are pathogenic to humans, causing either skin infections through contact with water, or gastroenteritis symptoms if consumed in seafood (Baker-Austin et al., 2018; FAO/WHO, 2020).

Non-choleragenic vibriosis associated with seafood consumption is a significant source of illness in Japan, USA and Asia, and is a growing issue in many other countries including Australia (Baker-Austin et al., 2018; FAO/WHO, 2020; Harlock et al., 2022; Raszl et al., 2016). The three main pathogenic species of concern are *Vibrio cholerae* (non-O1 or non-O139), *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Other species less frequently associated with illness include *Vibrio fluvialis, Vibrio hollisae*[1]*, Vibrio alginolyticus, Vibrio furnissii, Vibrio mimicus Vibrio metschnikovii* and *V. diabolicus*[2] (Baker-Austin et al., 2018; Cao et al., 2013).

Vibrio bacteria can be found in a wide range of environments, from temperate to tropical waters and across a wide range of salinities (Baker-Austin et al., 2010; Oberbeckmann et al., 2012). Temperature and salinity are often major drivers of abundance, with *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* all growing preferentially at temperatures between 20 and 30 °C, *V. cholerae* and *V. vulnificus* preferring salinities <30 ppt (Baker-Austin et al., 2010), and *V. cholerae* being found generally at lower salinities, including in freshwater (Bourke et al., 1986; Desmarchelier, 2003; Rogers et al., 1980). Species assemblages often show regional and seasonal variation (Oberbeckmann et al., 2012; WHO/FAO, 2021).

While levels of *Vibrio* in the temperate environment tend to be low (FAO/WHO, 2020), this is not usually the case in the tropics (Padovan et al., 2021). Water is warmer and temperature less variable in the tropics compared to temperate regions. Some studies indicate temperature has less influence on *Vibrio* abundance under these conditions (Deepanjali et al., 2005), however, other studies show temperature may still have a substantial influence (Machado and Bordalo, 2016; Padovan et al., 2021). Other factors such as turbidity, nutrients, phytoplankton, and zooplankton biomass may also influence abundance (Asplund et al., 2011; Gregoracci et al., 2012; Padovan et al., 2021; Rehnstam-Holm et al., 2014; Wong et al., 2019).

From a community perspective, *Vibrio* communities in the environment are highly dynamic, and environmental drivers are not the same at all locations or for all *Vibrio* species. Furthermore, different *V. parahaemolyticus* strains respond differently to changes in the environment (Liu et al., 2016; Ndraha et al., 2020) and some may pose greater health risks than others; hence, information about environmental drivers is necessary for risk assessment (Sampaio et al., 2022).

An additional level of unpredictability comes with climate change, and globally there is an increase in *Vibrio* spp. disease outbreaks with rising sea temperature (Froelich and Daines, 2020; Logar-Henderson et al., 2019; Semenza et al., 2017). While the *Vibrionaceae* is a metabolically and

[1] *V. hollisae* was reclassified as *Grimontia hollisae* in 2003 (Thompson et al. 2003).
[2] Strain E0666 which caused foodborne illness was initially classified as *V. alginolyticus* in the literature but has since been classified as *V. diabolicus* (Cao et al. 2013, Turner et al. 2018).

genetically diverse family, warming oceans have enabled two potential human pathogens, *V. parahaemolyticus* and *V. vulnificus,* to occupy an increasing range of environments for longer periods of the year in temperate climate zones (Baker-Austin et al., 2018). Extreme events, such as hurricanes and floods, increase the accumulation of *V. parahaemolyticus* in oysters (Ndraha et al., 2020), and in low latitudinal tropical regions that includes monsoonal north Australia, the number and severity of tropical storms and monsoonal events are steadily increasing as a result of climate change (Ebi et al., 2021).

To distinguish between pathogenic and non-pathogenic *Vibrio*, much attention has focused on comparing genetic variations between environmental and clinical strains. The virulence mechanisms for *Vibrio* normally associated with seafood infection (*V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* non-O1 and non-O139) are not well understood (Dziejman et al., 2005; FAO/WHO, 2020; WHO/FAO, 2011). Some genetic markers are often (but not always) associated with human infections, but reliable methods for identifying pathogenicity are still being researched (Jones et al., 2012; Ndraha et al., 2020).

For *V. parahaemolyticus*, commonly measured virulence factors are the *tdh* gene, required for production of thermostable direct haemolysin (TDH), and the *trh* gene, linked to TDH related haemolysin (TRH) (Baker-Austin et al., 2010; FAO/WHO, 2020; Sakazaki et al., 1968). Other genes sometimes examined include those linked to Type III secretion systems and/or urease production (FAO/WHO, 2020; Park et al., 2000; Wang et al., 2022). Examples of these genes are shown below in Table 8-1.

Table 8-1: Alternative examples of markers that can indicate virulence.

Gene	Gene Description	Reference
vtrB	T3SS2 transcriptional regulator	(Okada et al., 2017)
hlyB	Alpha hemolysin – Type 1 secretion system	(Zha et al., 2023)
vopP	T3SS2 effector	(Caburlotto et al., 2009)
vopC	T3SS2 effector	(Zhang et al., 2012)

However, not all clinical isolates include these genes (Okada et al., 2009), and furthermore, they may be present in isolates not known to cause illness (Klein and Lovell, 2017).

Pathogenic markers in *V. vulnificus* have been difficult to define as *V. vulnificus* strains are highly diverse and have a flexible gene pool with a propensity for this species to exchange DNA via horizontal gene transfer (Quirke et al., 2006). Virulence has been associated with the presence of a capsular polysaccharide (CPS) and a functional flagellar biogenesis system (Paranjpye and Strom, 2005). Virulence genotypes have been classified using both a 16S rRNA method (Aznar 1994) and a *V. cholerae* virulence marker gene (Warner and Oliver, 1999). A number of studies have shown that both methods provide similar results of separating *V. vulnificus* into two genomic types – either environmental or clinical strains (FAO/WHO, 2020). Due to the complexity in defining the mode of pathogenicity of *V. vulnificus* and the severity of illness caused by the bacteria, many consider all strains to be virulent (DePaola et al., 2003; FAO/WHO, 2005).

For other *Vibrio* spp. that are less commonly implicated in clinical infections, there is limited knowledge of the mode of pathogenesis, though virulence factors are often shared among species. For example, strains of *V. diabolicus* and *V. alginolyticus* have been noted to carry hemolysin genes homologous to *tdh* and *trh* in *V. parahaemolyticus*, and some strains of *V. mimicus* carry prophages with genes that contribute to the production of cholera toxin (Boyd et al., 2000; González-Escalona et al., 2006; Klein et al., 2014).

Serotyping and genotyping of *Vibrio* species are valuable epidemiologic tools that have been used during outbreak investigations (FAO/WHO, 2016). Multi-locus sequence typing (MLST) and whole genome sequencing (WGS) in particular have added to the knowledge and identification of enteropathogenic *Vibrio* (Baker-Austin et al., 2018; González-Escalona et al., 2008; Hernández-Cabanyero and Amaro, 2020; Jesser et al., 2019; Martinez-Urtaza et al., 2017), enabling investigation of source, geographic and temporal spread of these strains. If the serotype/genotype of an isolate can be determined, it is possible to examine international databases for molecular typing, such as PubMLST (Jolley et al., 2018) to determine if this isolate has previously been associated with clinical cases.

Serotyping and genotyping of *Vibrio* species has led to the identification of several pandemic strains of *Vibrio*. The first recorded pandemic of *V. parahaemolyticus* was of serotype O3:K6 (ST3), initially isolated in Calcutta, then rapidly spreading through southeast Asia and from there to the Americas, Europe and Africa (Abanto et al., 2020; Baker-Austin et al., 2018). Other pandemic strains have since emerged (e.g. ST36, ST120) and are concerning due to their increased virulence, meaning lower levels of bacteria could be sufficient to cause illness.

8.4.1.2 The food Tropical Rock Oysters

Bivalve molluscs are well-known vectors of pathogenic *Vibrio* spp. as they concentrate bacteria found in the environment through their filter feeding. In northern Australia, the species of concern for commercial production is the Blacklip Rock Oyster (BRO). The current production of BRO in northern Australia is low, with only one farm in Bowen, Qld, consistently supplying the market and four other aquaculture areas in development (**Error! Reference source not found.**). However, significant investment is occurring in northern Australia to support the development of a TRO industry. This includes investment by government, businesses, and Indigenous communities. Recent and current research projects to support the growth of this industry have totalled more than $2.7M, including:

- The Co-operative Research Centre for Northern Australia project A.2.1819053NT Northern Territory Tropical Rock Oyster research and development ($1.2M) – Species identification and biosecurity risks; hatchery research and development; grow-out method development
- FRDC project 2022-186 Supporting attendees at the international Tropical Rock Oyster Workshop & World Aquaculture Conference $37.8k
- FRDC project 2021-047 Harnessing the aquaculture potential of Queensland's native rock oysters $628.7k
- FRDC project 2020-043 Toxicogenic vibrio baselines and optimum storage, transport, and shelf-life conditions to inform cold supply chains in the north Australian Tropical Rock Oyster industry $199.3k
- FRDC project 2020-021 Contextualising shellfish food safety in Northern Australia $147k
- FRDC project 2018-115 National tropical oyster aquaculture workshop – Darwin 2018 $55k
- FRDC project 2018-005 Where should I farm my oysters? Does natural Cadmium distribution restrict oyster farm site selection in the Northern Territory $123.3k
- FRDC project 2017-061 Pilbara rock oyster research and development project $300k

- FRDC project 2012-223 Tactical research fund: assessment of heavy metals in tropical rock oysters (Blacklip and Milky) and implications for placement into the Australian seafood market and for Indigenous enterprise development in the NT. $37.7k

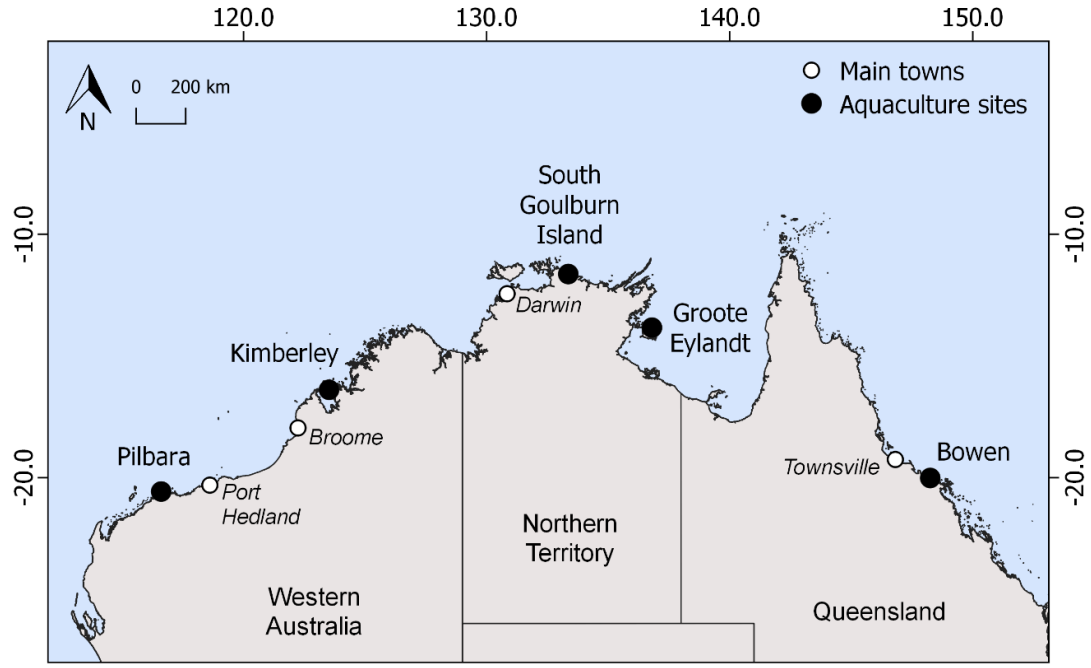


Figure 8-1: A map of Northern Australia indicating current Blacklip Rock Oyster aquaculture sites.

Investment is focused on the Blacklip Rock Oyster (*Saccostrea echinata*/ lineage J), a large oyster species that has been historically harvested by aboriginal communities and is found across northern Australia. The aims are to drive economic growth and diversification in these regions (Nowland et al., 2020). In the NT, TRO has been harvested by Aboriginal people for food and trade for thousands of years and Indigenous sea-based aquaculture is a culturally integrated form of work that aligns with customary practices on sea country (Fleming et al., 2015). On South Goulburn Island, cultural harvest at each site occurred sporadically, with communities revisiting sites approximately every two years (presentation by Yagbani elder Bunug Galaminda, World Aquaculture Conference).

More recently, trial commercial sites have been established at South Goulburn Island (Warruwi) by the Yagbani Aboriginal Corporation and at Groote Eylandt (Alyangula) by the Anindilyakwa Land Council (Figure 8-1). The first harvest of a small volume of oysters from South Goulburn Island (Warruwi) was released to market in June 2023. Other trial farms are yet to produce market-sized oysters in commercial quantities (Matt Osborne, Tropical Rock Oyster workshop, Darwin June 2023).

In WA, development is centred on sites in two aquaculture zones in the Kimberley near Derby and in the Pilbara near Karratha (DPIRD, 2020). Development is limited by suitable sites, remoteness and the associated high cost of development, regulatory challenges, acceptance from local communities and biosecurity risk. Development is supported by WA government to develop Indigenous and regional job opportunities, with production aims of $200M per annum (including finfish aquaculture).

There are 5 marine approvals for TRO production in Qld, with only one farm in northern Australia (defined here as above the tropic of Capricorn) in Bowen. This farm produced 1,000 dozen TRO in 2017 (Nowland et al., 2020). Several initiatives aim to support industry expansion, including Marine

Aquaculture Development Areas to identify new sites, and research at the Department of Agriculture and Fisheries Bribie Island Research Centre (pers. comm., Skye Lewis, Fisheries Queensland).

BRO grow quickly, reaching market size in two years or less. All areas across northern Australia are trialling both sub and inter-tidal systems. Oysters from Qld are generally available year-round, whereas the NT is expected to have a peak harvest season during the dry season and before first rains (August to December). Peak harvest season for WA is yet to be determined.

Aquaculture sites selected for development in the NT and WA are remote, with no sealed road access, and substantial distances from infrastructure such as telecommunications, laboratory facilities and regular transport routes (McCoubrey, 2021). In addition, hurdles for aquaculture development include a wet season (flooding, monsoons, and cyclones), high tidal energies and range, and crocodiles. The remoteness and lack of on-site expertise has hampered food safety classification of the growing areas, with timely access to laboratories and qualified personnel a key consideration. Aside from classification, access to refrigerated transport is potentially an issue (McCoubrey, 2021) and will be a key consideration to control the growth of *Vibrio*. The Qld growing area in Bowen has food safety classification and access to key infrastructure. Development of new growing areas in Qld is not restricted by existing aquaculture zones, so these issues will need to be assessed on a site-by-site basis for any future development.

Present trade for BRO is aimed at small volume, high value sales in the fresh product domestic market. As such there is no processing step to reduce *Vibrio* levels post-harvest, aside from cool storage.

*8.4.1.3 Hazard/food pairing (*Vibrio *in northern Australia)*

While it is well established that bivalves accumulate *Vibrio* spp., and oysters have been shown to host higher levels than water (Odeyemi, 2016), the relationship between *Vibrio* in water and shellfish is not linear and varies between regions (FAO/WHO, 2020). Environmental parameters have an important influence on levels of *Vibrio* spp. in waterways and importantly, levels of pathogenic species and incidences of illness (Brumfield et al., 2021). In this regard, Australia has been classified as a climate hotspot and a potential location for increased risk of *Vibrio* and human interactions is acknowledged (Hobday and Lough, 2011; Williams et al., 2022).

Climate change has implications for flood-prone regions and monsoonal northern Australia (Moisan et al., 2015). Because *V. parahaemolyticus* and *V. vulnificus* have high replication rates, they can quickly react to favorable environmental conditions resulting from these events (Froelich and Daines, 2020; Hackbusch et al., 2020; Williams et al., 2022), potentially forming blooms that pose a human health risk. This has implications for areas like Darwin Harbour in northern Australia, where there was a relatively high abundance of *V. parahaemolyticus* and *V. vulnificus* compared to the rest of the Australian east coast (Williams et al., 2022).

In the Williams et al. (2022) study, water samples were taken along Australia's east coast at 28 sites from Hobart to Darwin in December 2017 to January 2018. Four sites were in northern Australia (Darwin, Cooktown, Cairns, and Townsville). *V. cholerae* was not detected in any sample. *V. parahaemolyticus* levels were highest on average in the tropical zone, with Cooktown River showing the highest average levels of all sites, and Darwin Harbour showing the highest levels of total *Vibrio* (Error! Reference source not found.).

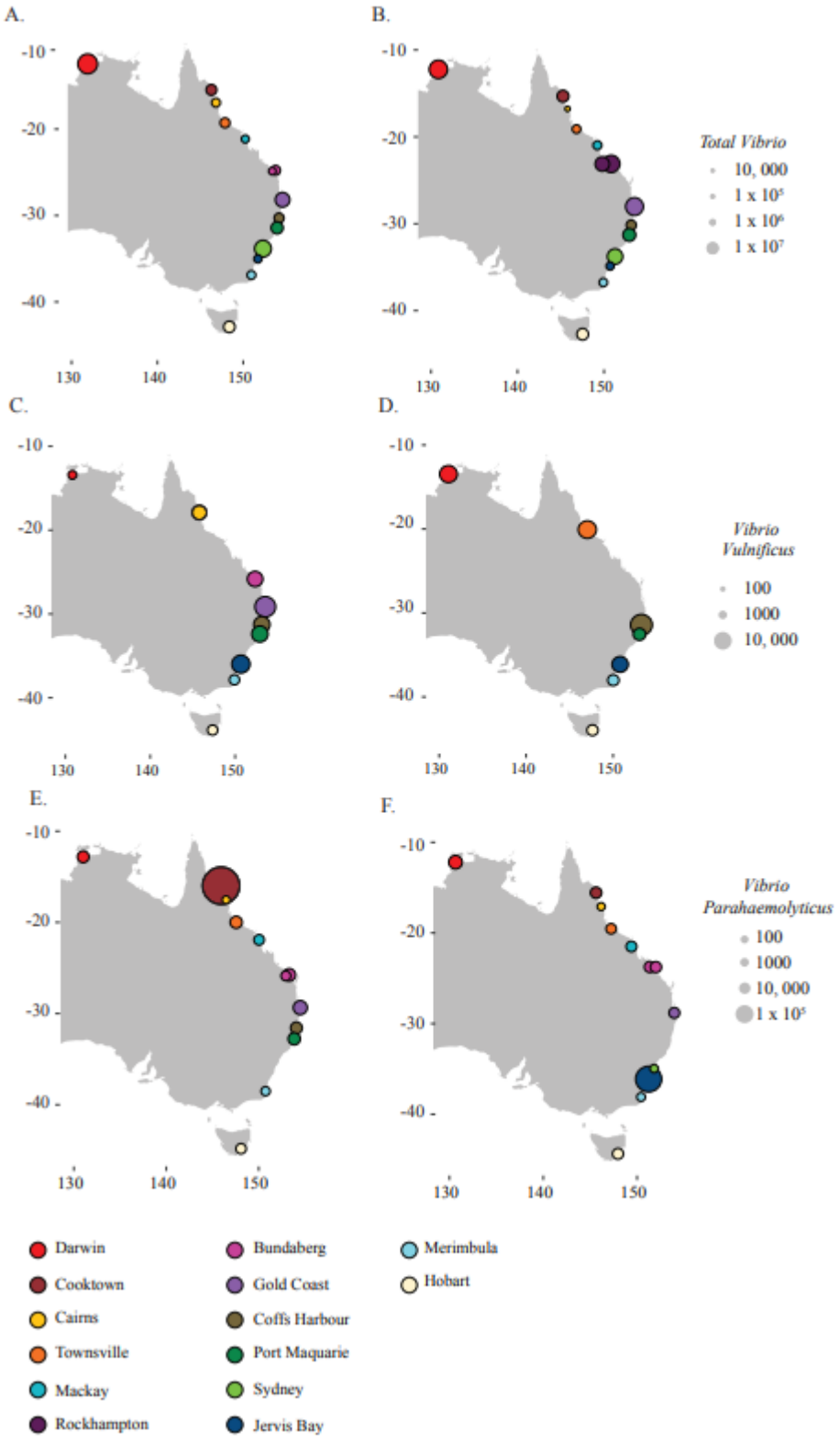


Figure 8-2: Results from (A,B) Total Vibrio qPCR, (D,D) Vibrio vulnificus ddPCR, (E,F) Vibrio parahaemolyticus ddPCR. (A,C,E) are rivers sites, while (B,D,F) are beach sites. Size scale represents copies/L of each assay. From Williams et al. (2022).

8.4.1.4 Darwin Harbour Studies

Darwin Harbour is a multi-use harbour, supporting recreational activities and artisanal food harvest including molluscs (Padovan et al., 2020). Remnant shells can often be found in campfires around the area, raw, lightly roasted, steamed, or boiled. Numerous *Vibrio* spp. were detected in shellfish, water, and sediment in Darwin Harbour, including virulent strains of *V. parahaemolyticus* in shellfish food collected in the mangroves (Padovan et al., 2020; Padovan et al., 2017).

In the Padovan et al. (2020) study of wild shellfish in a tropical estuary subject to treated effluent discharge, 13 sites were selected in Darwin Harbour based on a mix of reference sites, and sites in proximity to discharge locations. Field sampling occurred in 2015/16 in the dry and wet seasons. Molluscs typically eaten in Darwin Harbour and therefore targeted for this study were *Telescopium telescopium* (longbum or mud whelk), *Nerita balteata* (mangrove snail), *Saccostrea mordax*/lineage A (Milky oysters) as well as the crustacean *Scylla serrata* (giant mud crab). Oysters were limited to rocky areas, so they were only targeted along the rocky coastline at East Point. Shellfish are often eaten raw or lightly cooked. Uncooked shellfish were tested as this represents the worst-case scenario for potential pathogen transmission, however mud crabs are only eaten cooked and so were steamed prior to testing. Biota samples were tested for *V. parahaemolyticus* and *V. vulnificus* using a pre-enrichment PCR method. Samples positive for *V. parahaemolyticus* were further tested for potentially virulent strains by testing for *trh* (thermostable-related direct haemolysin).

V. parahaemolyticus was detected in 102 of 126 (81%) biota samples in the wet season and in 58 of 121 (48%) biota samples in the dry season. In *N. balteata*, *V. parahaemolyticus* was present in all samples across all sites in the wet season and absent in the dry season. In oysters, *V. parahaemolyticus* was only detected in the wet season at one discharge site and in one reference site in the dry season. *V. parahaemolyticus* was detected in cooked crab meat samples at three sites in the wet season, and once in the dry season. *V. parahaemolyticus* was prevalent in *T. telescopium*, detected at all sites in the wet season in nearly 100% of samples. In the dry season, *V. parahaemolyticus* in *T. telescopium* was less prevalent.

Virulent *V. parahaemolyticus* strains positive for *trh* were found in *T. telescopium* from 5 of the 13 sites, and in *N. balteata* from one site. Of the 14 samples that contained virulent *V. parahaemolyticus* strains, 12 occurred during the wet season and two in the dry season.

V. vulnificus was detected in 51% of samples in the wet season and in 24% of samples in the dry season. *V. vulnificus* was detected in *N. balteata* samples in both the dry and wet season including two reference sites. Only one cooked *S. serrata* flesh sample was positive for *V. vulnificus* and *V. vulnificus* was not detected in *S. cucullata* at any time. In *T. telescopium* samples, *V. vulnificus* was prevalent in the wet season occurring at all sites bar one reference site but was less prevalent in *T. telescopium* in the dry season.

Sites were grouped into zones for analyses to better understand the relationship between discharge, potential impact, and reference zones. *V. parahaemolyticus* was significantly more prevalent in molluscs (multiple species) during the wet season compared to the dry season ($P < 0.001$) but there was no difference in prevalence between zones. *V. parahaemolyticus* prevalence was significantly correlated to *E. coli* concentrations in biota ($P = 0.015$). *V. vulnificus* was significantly more frequent at the reference sites compared to potential impact and discharge sites in the wet season ($P = 0.0032$). There was also a significant relationship between *V. vulnificus* prevalence and *E. coli* concentrations in the different biota species ($P = 0.033$). *V. vulnificus* prevalence in the wet season was significantly greater than in the dry season ($P = 0.003$).

Interestingly, in April 2016 just after the wet season sampling when the highest incidence of vibrios in shellfish occurred, the NT Department of Health released a media alert "following a cluster of severe infections caused by *Vibrio* bacteria". These infections were not gastrointestinal from eating shellfish, but from invasive skin infections in people after entering coastal waters at Derby, Darwin Harbour, and the Gulf of Carpentaria (Peter Markey, pers. comm., NT Centre for Disease Control, Darwin). One isolate was identified as *V. vulnificus* and a second was identified as *V. cholerae* (Rob Baird pers. comm., Director Pathology, Territory Pathology, RDH). During 2016 the north of the NT showed the warmest January to June on record, and unseasonably low rainfall (BOM, 2023).

Padovan et al. (2021) studied the ecology of V*ibrio* in seawater in the wet-dry tropics of northern Australia. Three tidal creek sites in Darwin Harbour were selected as sampling sites because of their use for recreational fishing and boat launching, as well as seafood harvesting. Sites were potentially influenced by treated wastewater and/or wet season stormwater inputs. Samples were collected between January and August of 2018.

Water samples were screened for *V. parahaemolyticus, V. vulnificus* and *V. cholerae* by qPCR. *V. parahaemolyticus* was detected most frequently (98%; 62/63 samples), *V. vulnificus* was detected in 65% (41/63) of samples and *V. cholerae* was the least frequently detected (14%; 9/63 samples). *V. parahaemolyticus* and *V. cholerae* were more abundant in the wet season compared to the dry season ($P < 0.0001$ for *V. parahaemolyticus*; $P = 0.0444$ for *V. cholerae*), but no seasonal difference in abundance was observed for *V. vulnificus* ($P = 0.387$). Buffalo Creek had the highest concentrations of all three potentially pathogenic *Vibrio* species. *V. parahaemolyticus* and *V. cholerae* were screened for virulence genes (*trh* and *tdh* for *V. parahaemolyticu*s and *ctxA* for *V. cholerae*) but none were detected by qPCR.

Analysis of the relationship between *V. vulnificus* and measured environmental variables accounting for sites and month indicated positive associations for total nitrogen (TN) ($P = 0.006$), total phosphorus (TP) ($P = 0.007$), and turbidity ($P = 0.006$). *V. parahaemolyticus* abundance was positively associated with conductivity ($P = 0.003$) and temperature ($P < 0.001$). There was a significant interaction between temperature and dissolved oxygen (DO) i.e. the higher the temperature and the less DO in the water, the higher the *V. parahaemolyticus* levels.

Forty two *Vibrio* species were identified using amplicon sequencing targeting the *hsp60* gene. Amplicon sequence variants (ASVs) classified the potential human pathogens *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The relative abundances of *V. alginolyticus* (22%), *V. cholerae* (38%) and *V. parahaemolyticus* (30%) were highest in Buffalo Creek in the wet season. The highest relative abundance of *V. vulnificus* (54%) was observed at Rapid Creek in January. Across the entire data set, the relative abundance of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* was 9.1, 8.3 and 1.6% respectively. Several other species that have been isolated from patients infected in northern Australia were also identified, namely *V. fluvialis*, *V. alginolyticus* and *V. mimicus*.

Analysis of the association of the *hsp60 Vibrio* community with environmental variables showed that the seasonal variations were more pronounced than the spatial variations. A positive correlation between TN and *V. vulnificus*, and *V. cholerae* was observed, while *V. parahaemolyticus* was positively correlated with water temperature and wet season samples, and *V. campbellii* were more abundant in the dry season. Moreover, the *Vibrio* community was more diverse in the wet season compared to the dry season for all three creeks.

Although the abovementioned published work reaffirmed the higher abundance of *V. parahaemolyticus* and *V. vulnificus* in seawater during the wet season, until recently there was no substantive data indicating whether this results in a higher load of these species in sediment and biota

during the wet season. To fill this gap, a study was conducted of water, sediment and the sediment-grazing *T. telescopium* (Simma 2023). This gastropod was chosen as overharvesting of oysters in Darwin Harbour means these are scarce. Simma (2023) showed that *V. parahaemolyticus* and *V. vulnificus* were more abundant in water, sediment, and snails during the wet season across three tidal creeks, and interestingly, the abundance of *V. parahaemolyticus* in *T. telescopium* was correlated to concentrations in sediment rather than water. Overall, *V. parahaemolyticus* was detected in 91% of wet season samples (April 2021, January 2022) and in 78.8% of dry season samples (July 2021, August 2021). The occurrence of high and medium *V. parahaemolyticus* concentrations was more frequent in the wet than in the dry season. The addition of another most probable number (MPN) dilution step for sediment in the dry season (August 2021) and the wet season (January 2022) allowed a better estimation of *Vibrio* concentrations. In Rapid Creek, water samples had lower concentrations of *V. parahaemolyticus* in the wet season (<0.1 – 4.3 MPN.mL^{-1}) compared to the dry season (2.1 – 110 MPN.mL^{-1}) while the opposite trend was observed in sediment and biota, with *V. parahaemolyticus* concentrations ranging from <3 - >1,100 MPN.g^{-1} in the dry season and 93 - >11,000 MPN.g^{-1} in the wet season.

During the period of this study by Simma (2023), *V. parahaemolyticus* and *V. vulnificus* abundance spiked in January 2022 during a monsoonal event. Of the biota samples taken during this period, the *tlh* gene (indicating *V. parahaemolyticus*) was present 90% of the time, the pathogenicity gene *tdh* was not detected, but 6.7% of samples were positive for the *trh* gene. DNA extracts (n=1287) of the 122 samples showed only one sediment sample from the wet season was positive for the virulence gene *vopP.* Eleven DNA extracts of 3 sediment and 8 biota that were *toxR*$^{+}$, were also positive for *vopC* (63.6%; 2 sediment and 5 biota), and *trh* (45.5%; 1 sediment and all 8 biota).

8.4.1.5 Geographical study across northern Australia

As part of this FRDC project, the *Vibrio* community of two species of tropical rock oysters was assessed from 12 locations across northern Australia (Error! Reference source not found.A). Samples were taken in various seasons between March 2019 and July 2021. Three oysters were collected in triplicate from four sites within a location. Except for Goulburn Island, the NT oysters were wild harvested from intertidal zones. Farmed oysters were also collected from Flying Foam Passage, Cossack and West Lewis in the Pilbara, WA (Error! Reference source not found.B) and from Bowen, Qld (Error! Reference source not found.C).

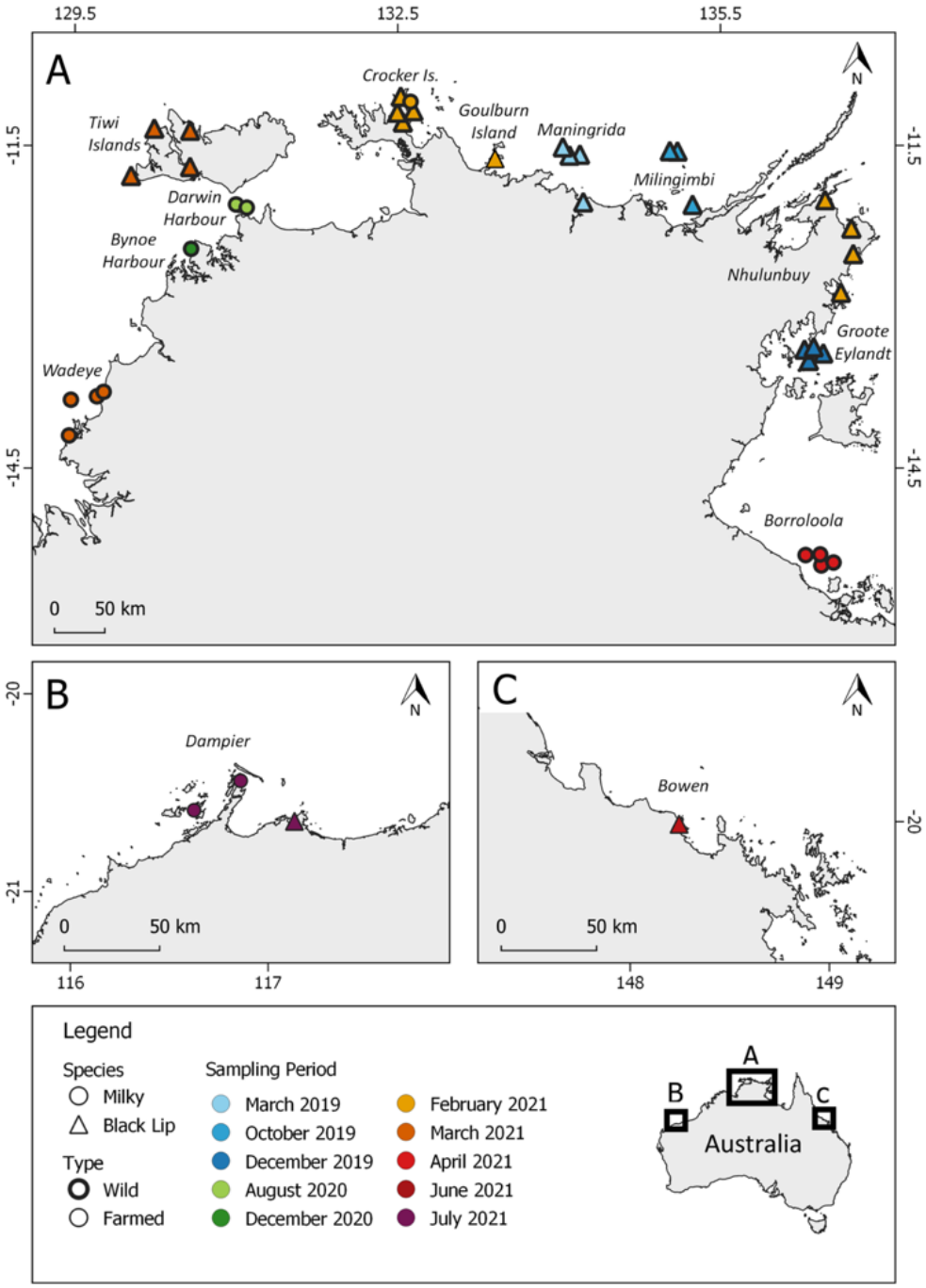


Figure 8-3: Tropical rock oyster sampling locations across northern Australia; A. Northern Territory; B. Western Australia; C. Queensland.

Three individual oysters were pooled to form one sample. For 78 samples, fresh homogenate was used to enumerate *V. parahaemolyticus* and *V. vulnificus* using an MPN-qPCR method and samples positive for *V. parahaemolyticus* were also tested for *trh* and *tdh* virulence genes (Jones et al., 2016; Kaysner et al., 2004). Of the 78 oyster samples tested, 60 samples were positive for *V. parahaemolyticus,* and 23 samples were positive for *V. vulnificus*, with concentrations ranging from 3.6 to >1,100 MPN/g for both species. Neither virulence gene *trh* or *tdh* was detected in any oyster samples.

To characterise the composition and diversity of the *Vibrio* community, *Vibrio* were amplified from oyster DNA extractions using the *Vibrio* - centric hsp60 assay (King et al., 2019). To provide a measure

of *Vibrio* abundance, a quantitative PCR (qPCR) assay based on SYBR Green was used to quantify *Vibrio*-specific 16S rRNA gene copies in each sample as previously described (Thompson et al., 2004; Vezzulli et al., 2012). Forty-eight *Vibrio* species were identified in oysters, with 10 of these making up 90% of the total *hsp60* sequences. The 10 most abundant *Vibrio* species (and relative abundance) across all samples and sites were *V. parahaemolyticus* (24%), *V. harveyi* (12%), *V. kanaloae* (11%), *V. campbellii* (10%), *V. diabolicus* (9%), *V. mediterranei (6%)*, *V. toranzoniae* (5%), *V. shilonii* (4%), *V. alginolyticus (4%)* and *V. rotiferianus* (3%). Approximately 3% of sequences could not be resolved to species.

The relative abundance of total *Vibrio* varied across sites as indicated by the qPCR values. Of the *Vibrio* species comprising more than 5% total relative abundance, *V. parahaemolyticus* occurred in most samples and was often the most abundant *Vibrio* species, except for oysters from the Tiwi Islands and Bowen. The latter had a distinctive *Vibrio* community composition compared to all other samples, with *V. kanaloe*, *V. toranzoniae* and *V. diabolicus* making up most of the *Vibrio* community. There was no clear clustering by oyster species or by farmed versus wild but when comparing sites, the Bowen samples formed a separate cluster.

Oyster samples contained the following potential human pathogenic species associated with gastroenteritis: *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis* and *V. diabolicus*. *V. cholerae* or other species isolated from clinical cases in the Northern Territory, *V. metschnikovii* and *V. mimicus* (McAuliffe et al., 2015) were not detected.

Using the enumeration MPN-qPCR method, 24 of the 78 samples (31%) were positive for *V. vulnificus* at concentrations ranging from 3.6 to >1,100 MPN/g. 16 (21%) of these were in the range 3-100 MPN/g and only 1 (1%) was >1100 MPN/g. However, using the hsp60 method, only 2 samples contained *hsp60 V. vulnificus* reads[3] - these were both samples from Wadeye in large Milky oysters, and both of these samples also had the highest MPN *V. vulnificus* values of 1,100 and 2,000 MPN/g. A third Wadeye replicate sample had 460 MPN/g *V. vulnificus,* but this sample did not produce any *hsp60* sequences. There were too few data points for any statistical analyses.

8.4.1.6 One year study at South Goulburn Island (Warruwi)

To examine the changes in *Vibrio* communities over the course of one year, a second study was undertaken over the course of a year at one Blacklip Rock Oyster lease on South Goulburn Island (Warruwi). Oysters were collected on six occasions during 2021/22. The three sample times designated 'dry season' were April 2021, August 2022, and September 2022. The three sample times designated 'wet season' and which did occur after rain in the preceding 40 days were December 2021 (29 mm rainfall), January 2022 (342 mm rainfall) and April 2022 (116 mm rainfall). At each time oysters were collected in triplicate from 3 randomly chosen baskets within the farm (27 samples in each season). Seawater (1 L) was also collected in triplicate at each site for each of the 6 sampling events.

DNA was extracted from homogenised oysters (sample = 3 pooled oysters) and 500 mL filtered seawater. PCR was performed on oyster and water DNA using the *Vibrio*-centric *hsp60* assay (King et al., 2019). To provide a measure of *Vibrio* abundance, a quantitative PCR (qPCR) assay based on SYBR

[3] The detection of *V. vulnificus* in 23 of 78 oyster samples by MPN-qPCR and only 2 samples by hsp60 highlights the difference in sensitivities of the two methods used. The first method focuses on identifying pathogenic species and is relatively sensitive, whereas the latter method is used to determining dominant species in the *Vibrio* community. Both methods give valuable information but are used for different purposes.

Green was used to quantify *Vibrio*-specific 16S rRNA gene copies in each sample as previously described (Thompson et al., 2004; Vezzulli et al., 2012).

Across both water and oyster samples, 763 sequence variants (SVs) were detected, which spanned 35 *Vibrio* species. In contrast to previous results, total *Vibrio* levels, measured by *Vibrio* 16S qPCR, were low in the wet season (December, January) in both oysters and water. The *Vibrio* community was influenced by water temperature and salinity in the dry season and turbidity in the wet season. The number of *Vibrio* 'species' (richness) in oysters was generally higher than in water. But within oysters, the number of *Vibrio* 'species' (richness) was lower in the wet season compared to the dry season. The *Vibrio* 'species' numbers varied widely between water samples, much more so than they did in oysters, particularly in the dry season.

Of the 35 *Vibrio* species identified in the South Goulburn Island oyster and water communities, the dominant identifiable species did not include known human pathogens, although *V. alginolyticus* was detected at >5% relative abundance in September 2021 and September 2022 in both water and oysters, and also occurred in oysters in April 2022 and August 2022. The most abundant species were *V. owensii*, *V. harveyi*, *V. brasiliensis*, *V. coralliilyticus* and *V. campbellii*. The relative abundance of these species changed with time and sample type (**Error! Reference source not found.**).

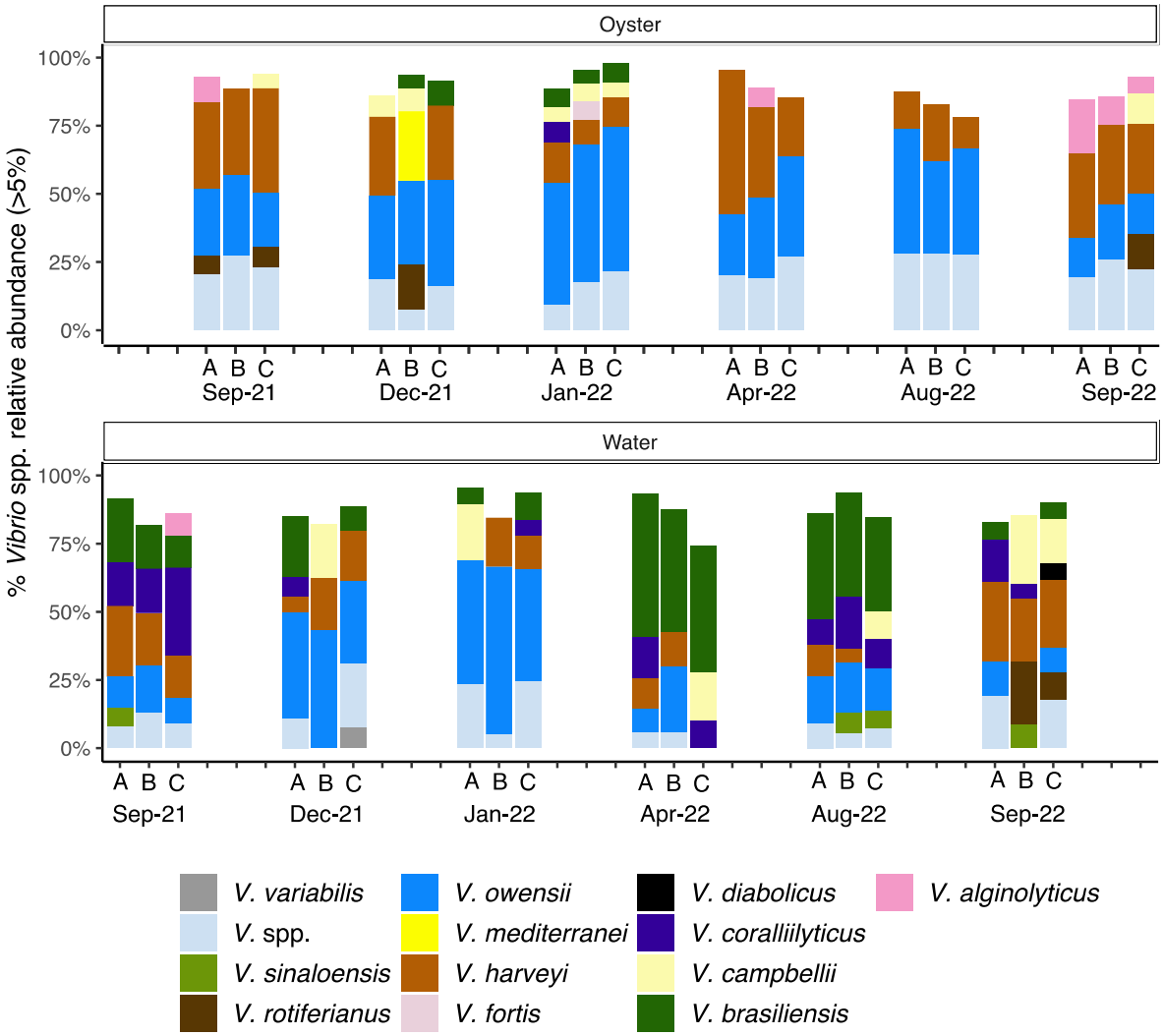


Figure 8-4: Taxa plots showing dominant (>5%) Vibrio species in oyster and seawater samples at each site A, B, C.

In the spatial study reported above, also using *hsp60* amplicon sequencing, *V. parahaemolyticus* was dominant (>5% relative abundance). However, this was not the case in the year-long study, although *V. parahaemolyticus* was detected in oysters in the January and April 2022 wet season sample times, coinciding with high rainfall in the preceding days before sampling. *V. parahaemolyticus* was not detected in water at any time except at <0.01% relative abundance at one site in April. Neither *V. cholerae* nor *V. vulnificus* were detected in the sequence dataset. *V. alginolyticus* (a potential human pathogen) occurred more frequently than *V. parahaemolyticus* in both oysters and water and was more abundant in the dry season samples.

8.4.1.7 Whole genome sequencing of Northern Territory isolates

In addition to the studies reported here, whole genome sequences (WGS) were obtained from 34 isolates of *V. parahaemolyticus*, 6 from the snail *T. telescopium* from Rapid Creek, 5 clinical samples from wounds and 2 clinical samples from faeces (Royal Darwin Hospital), 9 from Milky oysters (Wadeye and Buffalo Creek), 2 from Blacklip Rock Oysters (Tiwi Islands), 1 from a symptomatic Barramundi (Humpy Doo Barra Farm) and 4 seawater samples from Rapid Creek. None of these were positive for the *V. parahaemolyticus* virulence genes *tdh*/*trh* by qPCR, including the clinical samples (Padovan, unpublished). This was confirmed by WGS (Kaestli et al (2023) unpublished data), however, one of the clinical faecal *V. parahaemolyticus* isolates had three virulence genes which did not occur in the other isolates: *hlyB* (Zha et al 2023), *trhX* (homolog of *trh*; (Xu et al., 1994) and *vtrB* (Okada et al., 2017)

Multi Locus Sequence Type (MLST) data on these 34 isolates showed that the vast majority were new and the few MLSTs which have been previously assigned are shown in Error! Reference source not found.. Only one MLST has been previously assigned in Australia and this is the clinical faecal sample where oysters were consumed interstate prior to arrival in Darwin. But the other MLSTs are from across the world which presumably reflects the vast reach of marine organisms.

Table 8-2: V. parahaemolyticus sequence types from a range of environmental and clinical.

Source of Isolate from Kaestli 2023 Study	ST	Details from MLST Database (number of occurrences, country in which identified, environmental or clinical sample and year of identification)
Barramundi	2013	8x, Malaysia, environmental, 2017
Clinical - wound	2014	2x, Venezuela, environmental, 2018
Milky oyster	2058	1x, France, environmental, 2011
Telescopium	2249	1x, Thailand, environmental, 2017
Clinical - faecal	2901*	1x, Australia, clinical, 2022

* (Jolley et al., 2018)

8.4.2 Hazard characterisation

Vibriosis can manifest as gastroenteritis or wound infection. Both types of infection can progress to septicaemia, particularly in immunocompromised patients. Seafood associated illnesses of relevance to this risk profile are foodborne gastroenteritis, however, wound infections are indicative of pathogenic *Vibrio* spp. in the environment and are considered below as pertinent to inform risk.

8.4.2.1 Vibrio parahaemolyticus

V. parahaemolyticus infection is most commonly foodborne and usually causes sporadic illness (Bell and Bott, 2021) although occasionally outbreaks may occur (2 or more illnesses traced back to the same food source). Foodborne vibriosis results in gastroenteritis. Symptoms include watery diarrhoea, abdominal cramps, nausea, vomiting, fever, and chills (Barker and Gangarosa, 1974; Hlady and Klontz, 1996). Symptoms emerge 12-96 hours after consumption of the contaminated food, and last for an average of 3 days, but can range from 2 hours to 10 days. The disease is normally self-limiting and of short duration, but on occasion septicaemia may result (blood infection resulting in fever, hypotension and swelling of extremities, and/or haemorrhagic bullae), requiring treatment with antibiotics. Septicaemia is more common in immunocompromised patients and patients with liver disease. The disease does not normally result in chronic symptoms, although long-term reactive arthritis has been reported (Tamura et al., 1993). The United States of America (USA) has been undertaking long term monitoring of vibriosis; mortality rates associated with *V. parahaemolyticus* infection in the USA are 1-2% (USFDA, 2005).

The USA has developed dose response models to estimate the amount of *V. parahaemolyticus* required to cause illness (USFDA, 2005). This model is based on three limited clinical feeding trials and adapted to align with illness rates recorded in the USA. The dose response model predicted a <0.001% probability of illness following consumption of 10,000 *V. parahaemolyticus* cells, increasing to a 50% chance of illness following a dose of 10^8 cells. However, more recent outbreak data by King et al. (2018), suggests that some outbreak strains such as O3:K6 may require fewer cells to cause illness.

8.4.2.2 Vibrio vulnificus

Vibrio vulnificus vibriosis is not as common as *V. parahaemolyticus* infections but progression to lethal septicaemia is more common (FAO/WHO, 2005; Newton et al., 2012). As a result, *V. vulnificus* is responsible for the majority of seafood-related deaths in the USA (Jones and Oliver, 2009), with hospitalisation rates of 91.3% and death rates of 34.8% (Scallan et al., 2011). Risk factors for *V. vulnificus* infection include patients with pre-existing medical conditions such as liver disease, alcoholism, diabetes mellitus, gastro-intestinal surgery, ulcers, and immune disorders.

A dose-response model for *V. vulnificus* was developed by FAO/WHO based on data from shellfish production, consumption and illness in the Gulf of Mexico (FAO/WHO, 2005). The model estimated that consumption of high numbers of *V. vulnificus* were necessary to cause illness, with a 0.001% chance of illness from 2.4 x 10^5 cells, increasing to 0.003% illness from 10^7 cells (50 x10^3 cells/g shellfish in a 200g meal).

8.4.2.3 Vibrio cholerae *non-O1 and non-O139*

Vibriosis from non-O1, non-O139 or non-toxigenic strains of *V. cholerae* is generally mild, self-limiting diarrhoeic illness. As with other *Vibrio* infections, immunocompromised people may develop sepsis, with associated high mortality rates (Chen et al., 2015; Finch et al., 1987). Dose response studies for this group of *Vibrio* have not been conducted.

8.4.2.4 Other Vibrio *spp.*

Other *Vibrio* spp. have been implicated in cases of foodborne illness both in Australia and globally, though to a far lesser extent than *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*. Of these species *V. alginolyticus* is concerning due to its prevalence in marine and estuarine environments, and detections in association with non-foodborne clinical cases in Australia (Harlock et al., 2022). There are also sporadic cases noted in the literature of *V. albensis*[4] causing infection, including an illness in NSW associated with the bacteria (OzfoodNet, 2022). In addition to causing gastroenteritis, a number of *Vibrio* spp. have also been implicated in causing skin infections, including the three major pathogenic species (*V. parahaemolyticus, V. cholerae* (non-O1, non-O139) and *V. vulnificus)* as well as *V. alginolyticus, V. fluvialis,* and *V. harveyi* (Akram et al., 2015; Daniels and Shafaie, 2000).

8.4.3 Exposure assessment

8.4.3.1 Likelihood of pathogenic Vibrio *present in the environment*

The studies conducted thus far have revealed important findings about the presence of potentially pathogenic species in northern Australia, particularly in the Northern Territory. Potential human pathogenic species *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. fluvialis* and *V. diabolicus* were detected in oysters. *V. cholerae* or other species isolated from clinical cases in the Northern Territory, *V. metschnikovii* and *V. mimicus* (McAuliffe et al., 2015) were not detected.

The studies also reveal information about environmental drivers contributing to the prevalence of these potentially pathogenic species. In general, increased prevalence and levels of the potential human pathogens *V. parahaemolyticus* and *V. vulnificus* is noted in the wet season, with *V. parahaemolyticus* abundance correlated to temperature and conductivity/salinity, whereas nutrient concentrations and turbidity best explained *V. vulnificus* abundance (Padovan et al., 2020).

In environmental samples detailed in hazard/food pairings above, virulence genes (*tdh*/*trh*) were rarely detected in isolates from water and biota, but when present they were more often associated with the wet season (Padovan et al., 2020; Padovan et al., 2021; Simma, 2023, FRDC project 2020-043 unpublished data).

The bulk of these studies have been carried out in the Northern Territory with a strong focus on Darwin Harbour. Limited information is available regarding potentially pathogenic vibrios from WA and Qld. The geographic survey conducted by Padovan during 2019-2021 (FRDC 2020-043 in preparation) found no difference between the *Vibrio* communities in WA and NT, but the Bowen *Vibrio* community was significantly different to both. Relatively high levels of *V. parahaemolyticus* and *V. vulnificus* were found in tropical waters from Qld (Williams et al., 2022) demonstrating that a potential risk for oysters does exist. Importantly, as *V. vulnificus* prefers lower salinities (15-20ppt), prevalence of this bacteria may be reduced in oyster aquaculture zones that are located outside of the proximity of a fresh water source (Oliver, 2015).

[4] *V. albensis* is a heterotypic synonym of *V. cholerae* and is referred to as Non-O1 Serovar *V. cholerae (V. albensis)* (Hada et al. 2023, Oren et al. 2023).

8.4.3.2 Likelihood of pathogenic Vibrio *uptake by TRO*

It is likely that the pathogenic species found in water and sediment are taken up by grazers and by filter feeders. And indeed, the results described in the hazard/food pairings section above demonstrate this, as *V. parahaemolyticus* (9% containing pathogenic markers *tdh* and/or *trh*) and *V. vulnificus* have been detected in gastropod snails in Darwin Harbour.

In the geographic survey across north Australia, 60 of the 78 oyster samples tested were positive for *V. parahaemolyticus,* and 23 samples were positive for *V. vulnificus*, with concentrations ranging from 3.6 to >1,100 MPN/g for both species. Neither virulence gene *trh* nor *tdh* was detected in any oyster samples. These findings reaffirm that potentially pathogenic species are being taken up by TRO.

A consistent theme in the hazard/food pairings studies is the link between pathogenic *Vibrio* spp. particularly *V. parahaemolyticus*, and the wet season. In all studies to date, rains are associated with either an increase in levels of *V. parahaemolyticus* and *V. vulnificus* in biota, or an increase in the number of biota positive for these pathogenic *Vibrio* spp. and where virulence genes are detected, incidence is significantly greater in the wet season. This has a significant bearing on risk, increasing the risk of vibriosis from oysters consumed during the wet season.

In oysters, the abovementioned hazards/food pairing section detailed environmental drivers. These studies indicate a role for temperature, salinity, and turbidity in contributing to the likelihood and level of *Vibrio* contamination in TRO.

8.4.3.3 Likelihood of growth in TRO post-harvest

Growth or decline of *Vibrio* spp. in oysters post-harvest is highly dependent on the temperature of storage. Padovan et al. (2023) investigated the growth of *V. parahaemolyticus* isolates from northern Australia injected into BRO and held under four different post-harvest temperature regimes (4 °C, 13 °C, 18 °C and 25 °C). *V. parahaemolyticus* did not grow at 4 °C, grew slowly at 13 °C, and growth rate increased at temperatures above this. The authors concluded that results support the safe storage of BROs at both 4 °C and 13 °C. In comparison to *V. parahaemolyticus* growth in other oyster species, growth in TRO was slower than in Pacific Oysters (*Magallana gigas*), Eastern Oysters (*Crassostrea virginica*), and Asian oysters (*Crassostrea ariakensis*). Sydney Rock Oysters (*Saccostrea glomerata)* kept at similar temperatures had no growth of *V. parahaemolyticus* at all temperatures tested (Bird et al., 1992; Eyles et al., 1985; Fernandez-Piquer et al., 2011).

Supply chains for the NT and WA are currently undefined, however, it is acknowledged that there are risk factors such as distance, availability of refrigerated transport, low harvest volumes (affecting viability of options), high air temperatures, and infrastructure challenges (McCoubrey, 2021).

The air temperature in northern Australia is a contributing factor to the likelihood of *Vibrio* growth post-harvest. Across the five aquaculture sites noted in this risk profile, the average minimum temperature ranges from 19.8 °C to 24.4 °C. A summary table of air temperatures for these aquaculture sites is included in Appendix 1. Error! Reference source not found. below provides examples of two scenarios of growth of *V. parahaemolyticus* in BRO. These growth estimates have been conducted using the *V. parahaemolyticus* growth rate in injected BRO at 25 °C (Padovan et al., 2023). This temperature was selected as it is representative of the average annual mean minimum temperature recorded at South Goulburn Island (Warruwi) (24.4 °C) as shown in Supplement 8-1. The scenarios include a low (100 MPN/g) and high (1,000 MPN/g) initial load of *V. parahaemolyticus.*

Table 8-3: Estimated growth of V. parahaemolyticus at air temperature (25 °C) within Blacklip Rock Oysters with a low (100 MPN/g) and high (1,000 MPN/g) initial load. Numbers per time point are MPN/g.

Time (hours)	Initial *V. parahaemolyticus* Load = 100 MPN/g	Initial *V. parahaemolyticus* Load = 1,000 MPN/g
T = 0	100	1,000
T = 6	190	1,904
T = 12	363	3,626
T = 24	1,315	13,151
T = 48	17,296	172,955

As shown in Error! Reference source not found. the amount of time in ambient northern Australia air temperature can have significant impact on growth of *V. parahaemolyticus* post-harvest. This is particularly important where the initial load of *V. parahaemolyticus* in BRO is elevated.

8.4.3.4 Consumption of TRO

The current production levels of TRO in northern Australia are low, and for commercial harvest are presently only coming from one oyster farm in Qld. Recreational and cultural harvest of TRO is also likely to be low due to the low populations in this extensive region. From this we assume the levels of consumption of TRO are very low and well below the levels of consumption of other oyster species in Australia.

Shellfish businesses in all areas have plans for expansion/development. The ultimate scale of production is currently unknown. In terms of harvest, oysters from Qld are generally available year around, but peak in the dry (see Chapter 7), whereas the NT is expected to have a peak harvest season during the dry season and before first rains (August to December). Peak harvest season for WA is yet to be determined, although trials have indicated high gonad index and good condition in the wet season (S. Gill, personal comment).

8.4.3.5 Surveillance systems for vibriosis in northern Australia and level of reporting of illness

Vibriosis is an emerging concern in Australia, however there is no state that conducts regular surveillance for *Vibrio* spp. in seafood and at this point it is uncertain whether there would be value in doing so. Illness reports are the best source of on-going information available to inform human health risk levels. *Vibrio* food poisoning assessment is from stool samples and invasive *Vibrio* are typically identified from blood cultures and those who have become particularly unwell after wounds have become infected. There are many factors that influence the level of reporting. Gastroenteritis cases are often minor and may not be reported to a medical practitioner; medical practitioners may not be available in the local community (particularly true of remote communities); if a patient presents to a medical practitioner, they may not identify vibriosis as a potential cause or confirm vibriosis via pathology testing. In some states/laboratories the standard pathology tests may not include *Vibrio* spp. in the clinical screen, or the practitioner might not report the case to the state health department. All foodborne and invasive vibriosis infections are notifiable in the NT, however only cholera is notifiable in Qld and only cholera and vibriosis from *V. parahaemolyticus* is notifiable in WA (Error! Reference source not found.). In any case, an outbreak (2 or more illnesses) if recognised is notifiable in all states. *V. cholerae* is notifiable in all states.

Table 8-4: Notifiable status of non-choleragenic vibriosis infections in northern Australia.

State	Notifiable disease status (year introduced[1])	Years *V. parahaemolyticus* reported in Notifiable Disease Database 1917-1991
Northern Territory	All *Vibrio* infections (sometime prior to 2012)	1985-1991
Queensland	Not notifiable	
Western Australia	*V. parahaemolyticus* (1985)	1984-1991

Under-reporting of illness is acknowledged world-wide. In the United States, it is estimated that 1 in every 20 cases of *V. parahaemolyticus* illness is reported (Mead et al., 1999).

At present there is no NATA registered laboratory offering *Vibrio* testing in foods in the region (NATA website, accessed 23/08/2023), although Charles Darwin University does offer commercial testing. *Vibrio* spp. is not a notifiable contaminant in food in Qld. The lack of local testing facilities could impede timely analysis of vibrios for growers if it is required and delay outbreak investigation.

8.4.3.6 Reported linkages with illness (domestic)

The reports of illness are heavily influenced by the notifiable disease status in each state (Error! Reference source not found. Thus, there are fewer notifications in Qld where non-choleragenic vibriosis is not notifiable unless there is an outbreak, and a wider range of causative organisms in the Northern Territory where all types of invasive and foodborne vibriosis are notifiable.

Vibrio spp. reported as causing illness across WA, Qld and the NT are: *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* (non-O1, non-O139), *V. alginolyticus*, *V. fluvialis*, *V. harveyi*, *V. metschnikovii,* and *V. mimicus.*

Epidemiological data for non-choleragenic vibriosis in northern Australia are presented in Error! Reference source not found. During the period 2000-2023 the only reported illnesses to Qld Health were associated with an outbreak from South Australian oysters (Error! Reference source not found.). No locally acquired outbreaks were reported. Literature searches provided additional information on vibriosis in Qld during this time period, with 100 bloodstream infections and an additional (1) locally acquired wound infection (Davidson et al.; Norton et al., 2001). The source of illness was not provided for the bloodstream infections. No information is available on the proportion of cases that occurred in northern Qld (i.e. above the Tropic of Capricorn).

In Western Australia, where *V. parahaemolyticus* is the only notifiable non-choleragenic vibriosis, there were 100 illnesses of *V. parahaemolyticus* recorded by OzFoodNet, 14 of which required hospitalisation (Error! Reference source not found.). Of these infections, 38 were wound infections, 7 cases noted as 'unknown', and the remaining 55 cases were gastroenteritis. Commercial oysters sourced from interstate accounted for 38 of the gastroenteritis cases (5 linked with oysters produced in Tasmania in 2016 and 33 linked with oysters produced in South Australia in 2021). Only 1 case was definitively linked with local seafood (recreationally caught crab), although the source state for many of the gastroenteritis cases is unknown (n=15). Literature searches also indicated a *V. cholerae* (non-O1, non-O139) infection as well as skin infections from other *Vibrio* spp. acquired while fishing in marine environments (Foote et al., 2017; Heath et al., 2001). No information is available on the proportion of cases that occurred in northern WA (i.e. above the Tropic of Capricorn).

Within the NT during the time period analysed, there were 21 cases from the notifiable disease database and 18 of these were acquired within the state (Error! Reference source not found.). Ten of these cases were reported as gastroenteritis from an NT source, one from oysters from South Australia, and one gastroenteritis case from an unknown source. Local oysters, *T. telescopium* and mangrove worms were specifically named in one case each. In addition to this data, there are 65 incidences of illness noted in case reports within the literature. Four of these cases were locally acquired and information on source of infection was not provided for 61 cases. Some of these illnesses may be duplicated across datasets, however, have been included as separate lines due to the additional detail they provide.

Table 8-5: Notifiable Summary of domestically acquired non-choleragenic vibriosis illnesses reported in WA, NT, and Qld from January 2000 – September 2023.

Pathogen	**Year**	**State/ Territory reported**	**Source state**	**Gastro (source)/ skin infection**	**Sporadic/ outbreak**	**Illnesses (hospitalis-ation/ death)**	**Setting**	**Comment**	**Information source**
V. vulnificus x 59, *V. cholerae* (non-O1, non-O139 x 15), *V. parahaemolyticus* x 13, *V. alginolyticus* x 7, *V. fluvialis* x 1, *V. harveyi* x 1, *V. metschnikovii* x 1	2000 – 2019	Qld	Unknown	Gastro x 10, Other x 90	Sporadic	100 (unknown /19)		Data from a retrospective study on bloodstream infections in QLD from 2000 – 2019. 71 patients noted as having comorbid illnesses.	Davidson et al. (2023)
V. cholerae non O1, non O139	2001	Qld	Qld	Wound	Sporadic	1 (1/0)	Contact with seawater <24h before symptom onset	This case is potentially reflected above	Norton et al. (2001)
V. parahaemolyticus	2021	Qld	SA	Gastro (Pacific Oysters)	outbreak	59			Russel Stafford, Qld Health, pers. comm
V. cholerae non O1, non O139	2001	WA	WA	gastro and bacteraemia		2 (2/2)	Community	Hep B, evidence of cirrhosis	Heath et al. (2001)
V. parahaemolyticus	2001	WA	Not stated	Unknown	Sporadic	1 (0/0)			DoH (2023b)
V. parahaemolyticus	2002	WA	Not stated	Unknown	Sporadic	1 (0/0)			DoH (2023b)

Pathogen	Year	State/ Territory reported	Source state	Gastro (source)/ skin infection	Sporadic/ outbreak	Illnesses (hospitalis-ation/ death)	Setting	Comment	Information source
V. parahaemolyticus	2006	WA	Not stated	Gastro (commercial oysters)	Sporadic	1 (0/0)			DoH (2023b)
V. parahaemolyticus	2007	WA	Not stated	Gastro x1 (seafood), wound x1 and unknown x1	Sporadic	3 (1/0)			DoH (2023b)
V. parahaemolyticus	2008	WA	Not stated	Wound	Sporadic	2 (1/0)			DoH (2023b)
V. parahaemolyticus	2009	WA	Not stated	Wound x 1, unknown x 1	Sporadic	2 (0/0)			DoH (2023b)
V. parahaemolyticus	2010	WA	Not stated	Wound	Sporadic	1 (0/0)			DoH (2023b)
V. parahaemolyticus	2011	WA	WA x 2, Interstate x 1	Wound	Sporadic	3 (1/0)			DoH (2023b), DoH (2011)
V. parahaemolyticus	2012	WA	Not stated	Wound	Sporadic	3 (0/0)			DoH (2023b)
Photobacterium damselae and *V. harveyi* (co-infection)	2013	International (Germany)	WA	Wound	Sporadic	1 (unknown /0)	Laceration while boating in the Murchison River		Hundenborn et al. (2013)
V. alginolyticus x 7 (co-infections x 6), *V. fluvialis* x 1 (co-infection)	2010-2013	WA	WA	Wound	Sporadic	8 (unknown)	Marine wound injuries, associated with fishing in the Midwest	3 cases had comorbidities	Foote et al. (2017)

Pathogen	Year	State/ Territory reported	Source state	Gastro (source)/ skin infection	Sporadic/ outbreak	Illnesses (hospitalis-ation/ death)	Setting	Comment	Information source
V. parahaemolyticus	2014	WA	Not stated	Gastro (seafood x 1, commercial oysters x 1), Wound x 2 Unknown x 1	Sporadic	5 (2/0)			DoH (2023b)
V. parahaemolyticus	2015	WA	Not stated	Gastro (seafood x 1), Wound x3	Sporadic	4 (1/0)			DoH (2023b)
V. parahaemolyticus	2016	WA	Tasmania (5 illnesses)	Gastro (commercial oysters x 6, 5 from Tasmanian oysters), Wound x 2	Outbreak & Sporadic	8 (0/0)			DoH (2023b), DoH (2016)
V. parahaemolyticus	2017	WA	Not stated	Gastro (commercial oysters x 2), Wound x 6	Sporadic	8 (1/0)			DoH (2023b)
V. parahaemolyticus	2018	WA	Not stated	Gastro (commercial oysters x 2, 1 x linked to South Australian oysters), unknown x 1	Sporadic	3 (0/0)			DoH (2023b), DoH (2018)

Pathogen	Year	State/ Territory reported	Source state	Gastro (source)/ skin infection	Sporadic/ outbreak	Illnesses (hospitalis-ation/ death)	Setting	Comment	Information source
V. parahaemolyticus	2019	WA	Not stated	Gastro (commercial oysters x 2, commercial prawns/mussels x 1, recreational crab x 1), Wound x 4	Sporadic	8 (0/0)			DoH (2023b)
V. parahaemolyticus	2020	WA	Not stated	Gastro (seafood x 1), Wound x 1	Sporadic	2 (0/0)			DoH (2023b)
V. parahaemolyticus	2021	WA	SA and WA	Gastro (commercial oysters x 35, 33 linked to an outbreak from South Australian oysters, 2 unknown source state), Wound x 5	Outbreak & Sporadic	40 (6/0)			DoH (2023b), DoH (2021)
V. parahaemolyticus	2022	WA	Not stated	Wound x 3	Sporadic	3 (1/0)			DoH (2023b)
V. parahaemolyticus	2023	WA	Not stated	Wound x1, Unknown x1	Sporadic	2 (0/0)			DoH (2023b)
V. vulnificus x 2, *V. parahaemolyticus* x 1	2000 – 2003	NT	NT	Wound x 3	Sporadic	3 (3/1)	Fishing around the	2 of the 3 cases presented with comorbidities	Ralph and Currie (2007)

Pathogen	Year	State/ Territory reported	Source state	Gastro (source)/ skin infection	Sporadic/ outbreak	Illnesses (hospitalis-ation/ death)	Setting	Comment	Information source
							Borroloola township		
V. parahaemolyticus x 25, *V. alginolyticus* x 23, *V. vulnificus* x 11, *V. cholerae* x 4, *V. metschnikovii* x 1 and *V. mimicus* x 1	2000 – 2013	NT	Not stated	Majority of isolations from skin and soft tissue x 52 followed by blood x 7, faeces x 6, respiratory x 2 and peritoneal, eye, urine, ear x 1	Sporadic	61(Not stated)		71 isolations of bacteria from 61 episodes of illness	McAuliffe et al. (2015)
V. cholerae (non-toxigenic)	2005	NT	NT	Gastro x 1 (from swimming)	Sporadic	1 (1/1)	History of recent swimming in tidal river, remove gulf country	immunocompromised	Markey (2005)
V. vulnificus x 1, *V. parahaemolyticus* x 1	2011	NT	Not stated	Gastro x 1, Invasive x 1	Sporadic	2 (2/0)			DoH (2023a)
V. fluvialis x 1, *V. vulnificus* x 1	2014	NT	NT	Gastro x 2	Sporadic	2 (2/0)		Mangrove worms, local oysters	DoH (2023a)

Pathogen	Year	State/ Territory reported	Source state	Gastro (source)/ skin infection	Sporadic/ outbreak	Illnesses (hospitalis-ation/ death)	Setting	Comment	Information source
V. vulnificus x 1, *V. parahaemolyticus* x 1	2015	NT	NT	Gastro x 1, Invasive x 1	Sporadic	2 (2/0)			DoH (2023a)
V. vulnificus x 1, *V. parahaemolyticus* x 1, *V. cholerae* (non cholera strain) x 1	2016	NT	NT	Invasive x 3	Sporadic	3 (2/0)			DoH (2023a)
V. vulnificus	2017	NT	NT	Invasive	Sporadic	1(1/0)			DoH (2023a)
V. parahaemolyticus x 2, *V. cholerae* (non cholera strain) x 1, *V. vulnificus* x 1	2018	NT	NT	Gastro x 2, Invasive x 2	Sporadic	4 (3/0)			DoH (2023a)
V. spp., *V. cholerae (non-O1/non-O139), V. vulnificus*	2020	NT	NT	Gastro	Sporadic	3 (3/0)			DoH (2023a)
V. parahaemolyticus	2021	NT	NT	Gastro	Sporadic	1 (0/0)			DoH (2023a)
V. vulnificus x 1, *V. parahaemolyticus* x 1	2022	NT	SA & NT	Gastro (commercial oysters from SA, *V. parahaemolyticus*) x 1, Invasive x 1	Sporadic	2 (1/0)			DoH (2023a)
V. spp. (PCR)	2023	NT	NT	Gastro (long bum molluscs)	Sporadic	1 (1/0)		Long bum mollusc *(T. telescopium)*	DoH (2023a)

Internationally *V. parahaemolyticus* is the most common source of vibriosis (Bell and Bott, 2021). NT is the only jurisdiction in northern Australia where all vibriosis is notifiable. In the NT, the causative agent was most commonly *V. vulnificus*, followed by *V. parahaemolyticus*, *V. cholerae* and *V. fluvialis* (n=8, 6, 3, 1 respectively) with two illnesses not identified to species level. At least 2 of the *V. vulnificus* cases were gastroenteritis.

The rates of vibriosis from *V. parahaemolyticus* in the NT and WA are similar (Error! Reference source not found.), and reflect those published for Australian states (Hall, 1993) based on data from 1984 to 1991 when vibriosis was previously notifiable. These rates are also similar to the infection rates reported for the USA (CDC, 2023; Newton et al., 2012). However, the NT *V. vulnificus* infection rate of 0.16 per 100,000 is higher than that noted for the USA (0.04 or 0.05 in 2010 depending on the information source; Newton et al., 2012).

Table 8-6: Average rate of vibriosis for WA and NT from 2000 – 2022 inclusive (determined from Australian Bureau of Statistics population data[1] and WA/NT Health data[2]) compared to average rates of vibriosis in USA[3].

	Sum population inclusive ('000)*	Total *V. parahaemolyticus* illness	Total *V. vulnificus* illness	*V. parahaemolyticus* illness rate (/100,000)	*V. vulnificus* illness rate (/100,000)
WA	53,982.3	96	NA	0.18[4]	NA
NT	4,990.3	6	8	0.12[4]	0.16[5]
USA (2010)				0.13 (COVIS[6]) 0.23 (FoodNet[7])	0.04 (COVIS) 0.05 (FoodNet)

1. https://www.abs.gov.au/statistics/people/population/national-state-and-territory-population
2. Listed in Table 8-5
3. Newton et al. (2012)
4. total *V. parahaemolyticus* illnesses reported per state 2000-2022/sum of annual population per state 2000-2022) x 100,000
5. total *V. vulnificus* illnesses reported per state 2000-2022/sum of annual population in per state 2000-2022) x 100,000
6. The Cholera and Other Vibrio Illness Surveillance (COVIS) system operated by the USA CDC (https://www.cdc.gov/vibrio/surveillance.html#:~:text=The%20Cholera%20and%20Other%20Vibrio,CDC%20maintains%20this%20surveillance%20system.)
7. FoodNet is the USA CDC's Foodborne Diseases Active Surveillance Network, a data tool for foodborne infections

8.4.4 Uncertainty and knowledge gaps

There are key knowledge gaps which contribute to the uncertainty of profiling the risk of vibrios in TRO. Many of these knowledge gaps persist worldwide which speaks to the complexity of the bacteria and the need for new technologies that will increase our understanding of pathogenicity triggers and virulence gene detection. In addition to these globally recognised data gaps, there are additional local knowledge gaps to consider when assessing TRO production in northern Australia for *Vibrio* risk.

As Australia has only recently restarted reporting foodborne illnesses related to vibrios in locally produced food products, there has been limited surveillance and reporting requirements for *Vibrio*. This reduces the level of information available on *Vibrio* related illness, foodborne or otherwise, that can support this review.

The knowledge gaps identified have been detailed below in Error! Reference source not found..

Table 8-7: A summary of the data gaps encountered during the preparation of this risk profile.

Data Gap	Summary	Relevance to this risk assessment	Reference
Viable but non-culturable cells (VBNC)	The ability of vibrios to enter a VBNC state under unfavourable conditions complicates analysis of vibrios as the bacteria may be present and may present a risk though will not be detected using standard microbiological methods	Surveys that have used culture-based techniques may underestimate the true number or presence of *Vibrio* species of interest due to these VBNC cells.	(Baffone et al., 2003; Li et al., 2014)
Measurement Techniques	Testing for vibrios can be achieved using a variety of different methods and there is a lack of consensus on the most suitable technique for each testing purpose. Culture based methods enable strain characterisation, though are unable to detect VBNC cells. Molecular methods that rely on genetic material, can detect VBNC cells but have other limitations, including matrix inhibition of PCR assays and assay specificity and sensitivity. Furthermore, ecological assays focus on identifying the predominant *Vibrio* species present but lack sensitivity to determine if pathogenic species are present in low numbers. Food safety assays are more focused on identifying specific known pathogens but lack the ability to identify other species present.	The studies supporting this risk profile had varying objectives (for example ecological community focused studies as opposed to food safety pathogen focused studies) and thus used different test methods. Subsequently different types of data were produced. There are also unknowns relating to limits of detection (sensitivity) and measurement uncertainties. PCR assays may be affected by matrix inhibition which can affect the performance of the assay. Defining these will improve the applicability of the data to definition of risk.	
Commercial Laboratory Capability	In Australia, commercial requirements for testing of *Vibrio* spp. have been quite low and there is reduced commercial testing capability. This is compounded by the remoteness of much of	Ease of access to testing facilities for analysis of *Vibrio* spp. in TRO is a significant gap highlighted during this review. It is unknown whether this	

Data Gap	Summary	Relevance to this risk assessment	Reference
	northern Australia, where there are currently no accredited facilities.	gap has affected investigations into the sources of illnesses reported.	
'Hot Oysters'	A study by Klein and Lovell demonstrated that *V. parahaemolyticus* is not uniformly distributed among samples, noting the possibility of 'Hot Oysters' containing greater densities than neighbouring oysters. A significant number of replicate samples would need to be taken on each sampling occasion to adequately address this phenomenon where it existed.	It is difficult to assess whether the results from the studies cited herein may have been affected by a highly variable distribution of *Vibrio*.	Klein and Lovell (2017)
Distribution, prevalence, and levels of pathogenic vibrios across northern Australia	*Vibrio* ecology is diverse and species prevalence and levels are acknowledged to be highly spatially variable, even within close proximities. A key pathogen, *V. vulnificus,* prefers moderate salinity and has an optimal salinity range of 5-20 ppt and this will have bearing on its presence at aquaculture sites if these are located away from fresh water sources.	The coastline of northern Australia is vast and presents a challenge for *Vibrio* characterisation. The current data relating to the distribution, prevalence and levels of potentially pathogenic *Vibrios* may not be representative of the whole of northern Australia, particularly within current and proposed oyster aquaculture sites. Further data collection is required at oyster aquaculture sites in conjunction with environmental parameters to aid in profiling risk.	(Deeb et al., 2018; Oliver, 2015)
Environmental Drivers	The current data relating to *Vibrio* spp. in northern Australia has limited multi-year data and weather events. Environmental drivers have been characterised within Darwin Harbour, but there are unknowns about how this applies to other aquaculture sites across northern Australia.	A multi-year study on both the *Vibrio* community and target species e.g. *V. parahaemolyticus*, would add weight to our current knowledge of the environmental factors driving prevalence of pathogenic *Vibrio* spp. The extension of this work would be beneficial in filling this knowledge gap, particularly in	Baker-Austin et al. (2010); Padovan et al. (2020); Padovan et al. (2021); Sampaio et al. (2022)

Data Gap	Summary	Relevance to this risk assessment	Reference
		Queensland where harvest is occurring independently of seasons.	
Virulence between different strains	Not all strains of *V. parahaemolyticus* are pathogenic. Furthermore, studies indicate that some clinically isolated strains of *V. parahaemolyticus* may be more virulent than others. Like *V. parahaemolyticus*, the pathogenesis of *V. vulnificus* is not fully understood and while not all strains are pathogenic, the severity of illness caused by *V. vulnificus* means that presence of this species is of concern.	The minimal data available about clinical isolates from Australia and their virulence adds to the complexity of translating presence and levels of *V. parahaemolyticus* into risk. The high levels of *V. vulnificus* noted in some samples analysed during the studies conducted in northern Australia cannot be translated into risk without understanding their virulence.	Baker-Austin et al. (2018); King et al. (2018)
Genes associated with virulence	The mode of pathogenesis of many human pathogenic *Vibrio* spp. is yet to be fully elucidated. For *V. parahaemolyticus,* it is widely accepted that two hemolysin genes, *tdh* and *trh*, contribute to a strains' virulence due to their detection, either in combination or separately, in clinical isolates. There has, however, been a notable percentage of clinical isolates that lack either of these genes. There are a number of additional genes recognised as being involved in contributing to virulence of strains. The work presented here has used novel approaches to assess presence of other factors that may indicate virulence, including WGS and PCR targeting other potential virulence factors. In addition to this, the	There is a lack of data available for clinical isolates from Australia. Characterisation of more clinical isolates and sharing of WGS data would improve knowledge of virulence genes present in endemic strains.	González-Escalona et al. (2006); Jones et al. (2012); Klein et al. (2014); Okada et al. (2017); Xu et al. (1994); Zha et al. (2023)

Data Gap	Summary	Relevance to this risk assessment	Reference
	literature reveals the presence of virulence markers for *V. parahaemolyticus* in other *Vibrio* spp. which complicates interpretation of PCR detections.		
Dose-response	In addition to varying levels of virulence among strains, there is a growing body of evidence that some virulent *V. parahaemolyticus* strains can cause illness in much lower doses than others. There is limited knowledge about dose-response of *Vibrio*, the only formally published dose-response curve is based on three limited clinical feeding trials.	Levels of pathogenic *Vibrio* in shellfish cannot be converted into a probability of illness estimate.	USFDA (2005)
Strain growth rates	Following an increase in infections in the Pacific Northwest of America, a study was conducted to review the growth rate of pandemic strain ST36 which is typically found in cooler waters. This study suggests the growth rate of ST36 is slightly higher at lower temperatures than other strains tested.	It is possible that endemic strains have different growth rates to the ones measured in these studies.	Ellett et al. (2022)
Differences between TRO species in accumulation of *Vibrio*	Different oyster species may accumulate and retain *Vibrio* spp. at different rates	We have drawn on information on pathogenic *Vibrio* prevalence and abundance from two species of TRO but differences in accumulation may occur between the species	
Growth rate of *V. vulnificus* in BRO	The *V. parahaemolyticus* growth rate study was the first *Vibrio* growth rate research conducted on BRO. As yet, the growth rate of *V. vulnificus* in BRO is yet to be determined.	Understanding the growth rate of *V. vulnificus* in BRO would help in informing the likelihood of growth post-harvest and related control plans. This is particularly	

Data Gap	Summary	Relevance to this risk assessment	Reference
		important when taking into account the detection of *V. vulnificus* in northern Australia and the reported illness rates associated with *V. vulnificus* infections in the NT.	
Harvest season	The harvest season of BRO is currently not defined but is likely to be pre-monsoon in the NT, whilst it is currently year around in Qld. Trials have indicated wet season could be the preferred harvest season in WA. *Vibrio* communities varied significantly in the surveys thus far conducted, with prevalence and levels of *V. parahaemolyticus* and *V. vulnificus* higher in the wet season.	Risk at harvest will change with the seasonal timing of the harvest.	
Harvest volumes	Shellfish businesses in all areas have plans for expansion/development. The ultimate scale of production is currently unknown. The timeframes to market are unknown for northern WA, NT is close to market pending a shellfish QA program, and Qld is already in the market.	Volumes of TRO consumption are currently low but it is unknown if this will continue to be the case.	
Supply Chains / Cool Management	Much of northern Australia and indeed, the current TRO aquaculture sites in WA and NT, are remote. For many of the sites, multiple modes of transport may be required from point of harvest to the marketplace, including by air and road. To mitigate post-harvest growth within oysters, refrigeration infrastructure would be required onsite at each location as well as throughout	There are gaps in our knowledge about the physical capacity for northern Australia to respond to the cool chain requirements to mitigate risk.	

Data Gap	Summary	Relevance to this risk assessment	Reference
	logistics to achieve effective cool-chain management.		
Under-reporting of Illness	As non-choleragenic vibriosis is often self-limiting, cases often resolve without the need for medical intervention and these cases are not recorded. This is exacerbated by a lack of notifiable status for some *Vibrio* infections in some states, potential lack of recognition by medical practitioners of vibriosis as a potential cause and difficulty in accessing medical attention or pathology testing,	This leads to gaps in our knowledge related to illness. We cannot estimate the level of under-reporting that exists in northern Australia but it is likely to vary between states.	(Mead et al., 1999)

8.4.5 Evaluation of risk (i.e. risk characterisation)

Although this report highlights important considerations about risk in this emerging industry, we cannot at present quantitatively evaluate risk to vibriosis associated with TRO from northern Australia due to key knowledge gaps and the difficulty in extrapolating NT-centric findings to sites outside of the NT.

Key considerations include:

- There is a low level of current commercial production and therefore little epidemiological data associated with oysters grown in aquaculture systems.
- There is a high prevalence and high levels of vibrios found in the majority of studies on water, sediment and biota from northern Australia.
- There is a high prevalence of *V. parahaemolyticus* and *V. vulnificus* in water, sediment, and biota from northern Australia, alongside detections of other potentially pathogenic species such as *V. alginolyticus*.
- An increase in prevalence and levels of *V. parahaemolyticus* (and pathogenic markers in isolates) and *V. vulnificus* has been observed during the wet season.
- Qld expects to harvest TRO year around (peaking in the dry season), and the NT is expected to have a peak harvest during the dry season and before first rains (August to December), i.e. prior to peak *Vibrio* prevalence and abundance. The harvest season for WA is currently unknown, but trials indicate harvest is likely to be in the wet season.
- There is a paucity of data on virulence genes in multiple aquaculture sites.
- Internationally there is a lack of data on dose response and variability of this with different serotypes.
- Local illnesses (wound and food) are key information informing risk, albeit illness is likely to be unreported, the level of under-reporting is unknown and is likely to be different between states for a variety of reasons.

    - WA acquired wound infections and at least two cases of WA acquired gastroenteritis from local oysters and recreational crab fishing indicates *V. parahaemolyticus* is potentially an issue in WA. Unfortunately, the spatial resolution of the data supplied by WA Health is not sufficient to draw more specific conclusions for the risk of *Vibrio* spp. infections in northern WA. Furthermore, data on illness from species other than *V. parahaemolyticus* is limited due to the reporting requirements within WA.
    - The available data for *Vibrio* spp. infections in Queensland is scarce, with no outbreaks involving locally produced seafood products reported from 2000 -2023. However, the identification of 100 x bloodstream infections within Qld is indicative of the presence of pathogenic *Vibrio* spp. in local seawater. Again, spatial resolution of the data prohibits commentary on *Vibrio* spp. infections in northern Qld.
    - The notifiable disease database In the NT identifies numerous cases of locally acquired gastroenteritis and although the source of infection was only definitively named as oysters in one case, the data demonstrate the presence of pathogenic *Vibrio* in NT coastal areas. As all *Vibrio* spp. implicated in foodborne and invasive infections are notifiable, the data from the NT indicates a range of species causing infections, including *V. cholerae* (non-O1, non-O139), *V. vulnificus, V. parahaemolyticus* and *V. fluvialis*. This is indicative of the risk posed by multiple species within the genus, however the high prevalence of infection by *V. vulnificus* is concerning due to the serious nature of illness arising from this species. It is unknown whether this risk would be the same in aquacultured oysters in the NT.

- In WA and NT rates of vibriosis were similar to those found in the USA where vibriosis is a serious concern for public health officials.

The above data lead us to conclude that there is a credible risk for aquaculture in northern Australia and that risk management actions should be taken.

8.4.6 Current risk management approaches

8.4.6.1 Risk Management in Australia

There is no specific guidance for *Vibrio* risk management in the Australian Shellfish Quality Assurance Manual (ASQAAC, 2022). However, Section 7 in the Manual does stipulate temperature requirements for post-harvest shellstock which are designed to control *Vibrio* growth. Specifically, the Manual states:

- Shellstock that are harvested and transported on a vessel/vehicle for more than four hours must be kept cool via shading, water sprays, covering with wet sacks or icing to prevent unacceptable increases in temperature and/or bacterial levels.
- Shellstock harvested for consumption must be under refrigeration at 10 ^{o}C or less within 24 hours of harvest[5].
- Once in refrigeration transported shellstock are not permitted to be outside temperature control for more than 2 hours.

Small scale studies on *Vibrio* growth rates in live Sydney Rock Oysters (SRO)[6] have demonstrated that *V. parahaemolyticus* does not grow in SRO at 23 ^{o}C (Bird et al., 1992) but showed low or no growth at storage temperatures of 30 ^{o}C or above (Eyles et al., 1985; Fernandez-Piquer et al., 2011). As a result, the temperature requirements for live SRO differ from the ASQAP Manual. The New South Wales Shellfish Industry Manual (NSWFA, 2018) states: "Sydney Rock Oysters must be placed under temperature control as follows:

(a) at 25°C or less within 24 hours of harvest; and

(b) at 21°C or less within 72 hours of harvest; or

(c) if harvested for depuration, after depuration is completed."

[5] Higher temperatures are considered acceptable if demonstrated by scientifically robust evidence that such temperatures will not support unacceptable growth of human pathogens in the product.
[6] These studies used natural populations present in depurated and un-depurated oysters as opposed to the injection trials used for BRO growth rates by Padovan et al. (2023).

Two states in Australia have implemented additional *Vibrio* risk management following recent *Vibrio* outbreaks (Tasmania in 2016 and 2017 (Harlock et al., 2022), and South Australia in 2021 (DHW, 2022)). Both of these states have mandatory reporting of *V. parahaemolyticus* illnesses and have adopted additional management approaches as they found the ASQAP Manual requirements were not effectively mitigating risk in some circumstances.

In South Australia, shellfish from all areas implicated previously as the source of illness are tested for *V. parahaemolyticus* on a fortnightly basis from September to March (PIRSA, 2022b). All results >3 MPN/g result in a precautionary closure and increased testing. Businesses also need to comply with Section 1.7 of the state HACCP plan (PIRSA, 2022a), which states the following control points for management of *Vibrio* risk:

- Once oysters are harvested, they must be under shade within 4 hours and under active refrigeration within 7 hours
- Internal oyster temperature is to be less than or equal to 10 °C at point of dispatch or within 24 hours from harvest (critical control point)
- Oysters are to be under active refrigeration (less than or equal to 5 °C) within 7 hours of harvest
- If the oyster internal muscle temperature is greater than 10 °C after 24 hours, do not dispatch, return product to harvest area. Returned product cannot be harvested for 48 hours (critical control point).

Note that times commence as soon as the first oyster comes out of the water, regardless of if this is due to the tide receding or harvesting.

Additional guidance in the South Australian HACCP plan to reduce risk includes:

- Harvesting at hours in the day when the air temperature is the lowest
- Reducing the number of harvest hours
- Shading the product.

In addition, the *Vibrio parahaemolyticus* Harvest Area Detection Protocol (PIRSA, 2022b) sets out the planned management response to illness reports, defining responses to sporadic and outbreak reports that arise over set periods of time. Planned management responses range from investigations of compliance with the state *Food Safety Arrangement* (PIRSA, 2022a) and increased sampling, to closures of individual business and/or closures of whole harvest/growing areas. The complexity of the response to illness reports reflects the difficulties in managing both delayed reporting of illness and the practicalities of trace back of the implicated oysters to a specific growing area. The planned management responses are based on the US National Shellfish Sanitation Program (USFDA, 2019). Importantly, the mechanisms to re-open closed harvest areas are also clearly stated in the Protocol.

Tasmania trialled monitoring of shellfish in growing areas for two summers following the 2016 outbreak, however, found minimal detections despite continued sporadic illness. A *Vibrio* Control Plan was added to the Tasmanian Food Safety Management System for oyster growers (Oysters Tasmania, 2019a), stipulating minimum time to cool chain at various water and air temperatures from November to April inclusive. It is mandated that all growing areas implicated previously in illness adhere to the Control Plan:

- Except where noted below, from November 1st – April 30th the time from harvest to cool chain must not be greater than 12 hours.
- When ambient air temperature is greater than 30 °C the time from harvest to cool chain must not be greater than 7 hours.
- When water temperature at the depth where oysters are harvested is greater than 19 °C the time from harvest to cool chain must not be greater than 7 hours.
- Unrefrigerated transport can only be used if it is within the designated time into the cool chain
- The time listed above is to be taken from the first basket/oyster removed from the water.
- For oysters harvested from intertidal areas during a low tide event, the time of harvest commences when the shellfish first come out of the water as the water is receding during a tide event. As this time may vary from site to site the approximate time of the oysters emerging from the water must be recorded and will be taken as the commencement time of the harvest.
- Oysters harvested the day prior to pack out must be maintained below 10 °C.
- Care must be taken to reduce the disturbance of bottom sediments when work with the oysters.
- Producers must ensure that baskets and racks do not come into contact with the bottom sediments.
- If you sell oysters directly to the general public or local businesses from your farm, you must ensure that the oysters have been refrigerated and less than 10 °C when sold.

In both states, oyster businesses have taken a pro-active approach to managing *Vibrio* risk. Many businesses have had to adapt their harvest practices and invest in upgrading on-site cooling systems. Oysters Tasmania produced a growers' best practice guide for managing *V. parahaemolyticus* (Oysters Tasmania, 2019b) that focuses strongly on temperature control at and post-harvest. Recommended practices are:

- Returning oysters back to the water before harvest for two cycles after handling, using deep cooler water, or relaying to lower risk areas for 7 days prior to harvest
- Harvesting earlier in the morning, as soon as possible after oysters are exposed and keeping them cool on boats during transport to and from the lease
- Getting stock to below 10 °C as soon as possible after harvest
- Maintaining the cool chain throughout the supply chain
- Keeping shucked oysters under 4 °C
- Cooking to over 65°C for elderly and immunocompromised individuals.

This guide was adapted for SA growers by the South Australian Oyster Growers Association in 2022 (SAOGA, 2022). The adaptions included aligning the time/temperature requirements with the SA government requirements listed above and included information to improve cooling practices, recommending hessian sacks rather than poly bags and stacking formats.

Food Standards Australia New Zealand has some guidance on acceptable levels of *V. parahaemolyticus* in ready-to-eat food (Error! Reference source not found.) in the Compendium of Microbiological Criteria for Food (FSANZ, 2022).

Hazard	Result (cfu/g)	Interpretation	Likely cause	Recommended actions
Vibrio parahaemolyticus	$>10^4$	Potentially hazardous	Poor temperature control (rapid chilling and storage at < 5°C), inadequate processing, cross contamination or high contamination levels in harvested seafood.	• Product disposition action to assess safety and determine if disposal or product recall is needed. May need confirmation to determine whether the genetic markers of virulence are present and the *V. parahaemolyticus* are able to cause disease. • An investigation should assess: ○ the source of raw product and potential for high levels of contamination (e.g. harvest water temperature and water salinity) ○ the adequacy of the time and temperature controls (chilling and storage) implemented post- harvest ○ the adequacy of the processing used (e.g. adequate cooking) ○ likelihood of cross contamination • Confirmation of identity and typing may be required where cases of foodborne illness are suspected.
	$10^2 – 10^4$	Unsatisfactory	As above.	• An investigation should be done, as above.
	$<3 – 10^2$	Marginal	Indication that temperature control or food handling controls are not fully achieved. It may be expected that naturally contaminated raw seafood may have low levels present (<100 cfu/g).	• Proactive investigation to ensure temperature and food handling controls are effectively implemented.
	<3	Satisfactory		

RTE = ready-to-eat, cfu/g = colony forming units per gram.

Figure 8-5: Guidance taken from the FSANZ Compendium of Microbiological Criteria for Food (FSANZ, 2022).

8.4.6.2 Risk Management approaches internationally

Approaches to *Vibrio* risk management vary between countries. Many countries, such as New Zealand, USA, and Hong Kong do not have regulations on maximum levels in food, relying instead on guidelines and best practice guides. Others, such as Canada, Japan, China, and Singapore do have regulations (Error! Reference source not found.), which may apply to processed and/or raw seafood.

Table 8-8: International food standards for Vibrio in seafood.

Country	Food Standard	Reference
Canada	<100 *V. parahaemolyticus* per g shellfish in 5 samples. *V. cholerae* absent in 5 x 25 g samples or a pooled 125 g sample.	Government of Canada (2019, 2020)
Japan	<100 *V. parahaemolyticus* per g fresh fish and shellfish for raw consumption and raw oysters. Not detected in boiled octopus, boiled crab	Japan External Trade Organisation (2011)
China	100 MPN/g acceptable limit, 1,000 MPN/g highest safety limit (contains sampling plan)	National Health Commission of the People's Republic of China (2021)
Singapore	100 CFU/g for ready to eat food	Singapore Statutes Online (2023)

CODEX Alimentarius has also published guidance for the control of pathogenic *Vibrio* spp. in seafood products, with an additional annex describing controls for *V. parahaemolyticus* and *V. vulnificus* in bivalve molluscs. While this guidance does not stipulate acceptable limits, it outlines the factors which determine the need for control and practical controls that can be applied for safe production of bivalves. These controls include use of clean water for cleaning shell stock and limiting time from harvest to refrigeration (CAC, 2010).

Growing area specific modelling of *V. parahaemolyticus* and *V. vulnificus* in the American Oyster (*Crassostrea virginica*) has proven to be valuable in estimating risk in this species in relation to environmental factors (FAO/WHO, 2020), however, it is acknowledged that these growth models may not apply to other species of shellfish or to other regions of the world. The European Centre for Disease Prevention and Control publishes a Daily *Vibrio* Risk map (ECDC, 2023), based on remote sensing data of sea-surface temperature. This model is calibrated to the Baltic Region in Northern Europe and ECDC acknowledges that it may not apply to other worldwide settings.

8.4.6.3 Post-harvest control measures

In general *Vibrio* spp. are sensitive to low pH but grow well at high pH, so infections caused by *Vibrio* spp. are often associated with low-acid foods (Rabbani and Greenough, 1999). Inactivation of *Vibrio* spp. in food can be achieved through post-harvest technologies such as high temperatures (cooking), drying, irradiation, and high hydrostatic pressure but most of these will change the organoleptic properties of the shellfish, rendering them less palatable for consumers (FAO/WHO, 2020).

Procedures that may be applicable in Australia that allow immediate access to oysters over a prolonged period include freezing with extended storage, cool pasteurisation, and relaying. Flash freezing followed by long-term frozen storage at -20 ^{o}C reduced *V. parahaemolyticus* in Pacific Oysters by greater than 3.52 log (MPN/g) (Liu et al., 2009). Low temperature pasteurization (e.g. 55 ^{o}C for 5 min, or 50 ^{o}C for 10 min) has been shown to reduce *Vibrio* levels in oysters (Cook and Ruple, 1992; WHO/FAO, 2021). Along with hydrostatic pressure processing and irradiation, these methods are US FDA approved to reduce *V. parahaemolyticus* in shellfish to acceptable levels (USFDA, 2019). Relaying is the translocation of contaminated stock from one growing area to another growing area free of the contaminant to allow natural purification. Relaying procedures and demonstration of the efficacy of *Vibrio* reduction would need to be developed in consultation with the state/territory regulator.

Depuration is a post-harvest processing procedure used to reduce levels of other undesirable bacteria in oysters, such as faecal coliforms, however, depuration studies on the reduction of *V. parahaemolyticus* and *V. vulnificus* in oysters have had mixed results (Campbell et al., 2022). Before these methods are deemed acceptable in Australia, they would need to be validated for each processing premise. The National Shellfish Sanitation Program (USFDA, 2019) contains validation guidelines.

Lowering and maintaining the temperature of the oyster-stock to below 10 °C as soon as is possible following harvest is a practical control measure proven to prevent post-harvest growth of vibrios. This temperature can be achieved by mechanical refrigeration, and the NSSP also includes provision for use of ice and ice slurries to reduce temperature (Lydon et al., 2015; USFDA, 2019). A recent study by Neil et al. (2023) demonstrated notable growth of *V. parahaemolyticus* in Pacific Oysters held in ambient temperatures for 5 hours and also compared the used of mechanical refrigeration versus icing. The study showed levels of *V. parahaemolyticus* were significantly higher in refrigerated oysters than those that had been iced prior to refrigeration. It is noted that rapid icing through use of an ice-slurry (20 minutes) as opposed to longer term storage on ice may reduce the likelihood of oyster contamination (Lydon et al., 2015).

8.4.6.4 Potential risk management options for northern Australia

The most cost effective post-harvest control measure to reduce *Vibrio* growth in oysters (excluding SRO) is rapid cooling of product post-harvest (CAC, 2010; Fernandez-Piquer et al., 2011; Lydon et al., 2015; Neil et al., 2023; USFDA, 2019). This method is the primary risk control implemented in South Australia and Tasmania, along with growing area closures following reports of illness. The storage trial by Padovan et al. (2023) indicates a temperature of less than 13 °C is appropriate to reduce *Vibrio* growth in BRO. Given the average temperatures in northern Australia, new businesses in this region should consider cool chain requirements early in their development plans. To meet ASQAP guidelines, this would mean refrigeration at 10 ^{o}C or less within 24 hours of harvest, which will likely require use of refrigeration facilities and refrigerated transportation of stock.

One concern expressed by the TRO industry was maintaining a live product at refrigeration temperatures. A live product is desirable both from a market acceptability perspective and to reduce spoilage organism growth, thus maintaining a high-quality product throughout shelf-life (Cao et al., 2009). As part of this FRDC study, S. Pahl assessed BRO shelf-life at different storage temperatures to maximise product quality and inform cool chain requirements. BROs were sourced from Bowen on two occasions. He found that BROs were robust and resilient at all storage temperatures, however there was a greater tendency for BROs to gape (open) at 13°C leading to a loss of internal liquor. There was no growth of microbiological indicator organisms at any time for each storage temperature and mortality levels were low and occurred from day 6 (peak-season/November harvest) and from day 9 (mid-season/August harvest). Most BROs had an acceptable oceanic through to a neutral smell throughout the trial, except for those held at 25°C when there was deterioration between days 9 and 10 (peak-season trial). The occasional oyster from each treatment was considered spoilt, but there was no trend with storage temperature or time. He concluded that although quality attributes declined slightly throughout the trial at all storage temperatures, the independent seafood processor's assessment was that BROs held at all storage temperatures were suitable for sale/consumption. However, those stored at 4°C were considered more favourable based on texture and appearance.

8.5 **Conclusions**

This risk profile is the first assessment of the risk of vibriosis associated with TRO in northern Australia. The conclusions below are based on the data presented in this report. We acknowledge the uncertainties and knowledge gaps are substantial and have a large bearing on the final assessment. More information is required before a quantitative assessment of risk can be made. This risk profile highlights the following:

- Vibriosis is a common risk associated with bivalve shellfish world-wide.
- Potentially pathogenic *Vibrio* spp. occur in northern Australia in sediments, water and biota (including seafood).
- Locally acquired illnesses (wound and food) have been reported from WA, NT, and Qld. Where illness rates could be calculated they were similar to those found in the USA, where vibriosis is a serious consideration for public health officials.
- The above data lead us to conclude that there is a credible risk for aquaculture across northern Australia.

The TRO industry should undertake active risk management to mitigate the risk of human illness and market incidents. Post-harvest cooling and maintenance of the cool chain during transport is the most effective critical control points that if managed, will avoid exposing stock to temperatures that may favour growth of vibrios. This method is the primary risk control implemented in many countries and followed in South Australia and Tasmania, along with growing area closures following reports of illness.

8.6 **Supplementary information**

Supplement 8-1: Mean minimum and maximum air temperatures near BRO aquaculture sites. Data from the Australian Bureau of Meteorology http://www.bom.gov.au/

Location		**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Annual Average**
Groote Eylandt	Mean Max.	33.4	33.1	32.8	32.6	30.9	29.0	28.8	30.2	32.6	34.2	34.6	34.4	32.2
	Mean Min.	25.3	25.0	24.0	21.9	19.3	17.2	15.7	15.3	18.0	21.2	23.7	25.0	21.0
South Goulburn Island (Warruwi Airport)	Mean Max.	32.1	31.5	31.6	31.7	30.8	29.4	28.8	29.3	31.0	32.6	33.7	33.2	31.3
	Mean Min.	25.6	25.3	25.3	25.1	24.1	22.1	21.4	21.8	23.7	25.4	26.4	26.3	24.4
Dampier	Mean Max.	35.9	36.0	36.2	34.4	29.9	26.6	26.2	27.7	30.5	32.7	34.3	35.7	32.2
	Mean Min.	26.1	26.5	25.5	22.8	18.2	15.1	13.4	14.6	16.8	19.7	22.2	24.6	24.6
Derby	Mean Max.	35.2	34.6	35.4	35.7	33.0	30.7	30.9	32.8	35.6	37.3	38.2	37.3	34.7
	Mean Min.	25.7	25.5	25.1	22.8	18.7	15.8	14.6	15.8	19.3	23.2	25.5	26.3	21.5
Bowen	Mean Max.	31.5	31.3	30.6	29.2	26.8	24.7	24.3	25.5	27.3	29.2	30.6	31.5	28.5
	Mean Min.	24.0	23.7	22.9	20.9	17.9	15.4	14.2	15.1	17.3	20.2	22.3	23.5	19.8

9 Implications

In this study there was excellent complementarity between experiments, and results from each contributed information that will inform TRO management decisions. The work on *Vibrio* growth behaviour showed that in injected BROs, growth did not occur at 4 °C but did occur at 13°C. Not surprisingly, growth rates were higher at higher temperatures, but what was surprising was that there was no difference between 18°C and 25°C.

Using these same temperatures in shelf life trials, BROs were robust and resilient to all storage temperatures, but there was a greater tendency for BROs to gape (open) at 13°C. Most BROs had an acceptable oceanic through to a neutral smell throughout the trial, except for those held at 25°C where there was deterioration between days 9 and 10 (peak season trial). Although quality attributes declined slightly throughout the storage trial at all storage temperatures, the independent seafood processor's assessment was that BROs held at all storage temperatures were suitable for sale/consumption. However, those stored at 4°C were considered more favorable based on texture and appearance. This was an important finding because there was uncertainty at the start of this project as to whether a tropical oyster species could be held at 4°C and still retain product quality. We note however that these oysters were held at 18°C for travel from Qld to SA prior to placing them at 4°C. Industry feedback during the review of this final report also indicated that oyster survival is closely linked to both the speed at which oysters are dropped to the low temperate and consistency of the temperature. Our results plus the feedback suggest there might still be some work needed on cold shock to provide the industry with guidance on best practice.

This project provided the first spatial and temporal *Vibrio* baselines in TROs in northern Australia. Potentially pathogenic *Vibrio* spp. were detectable, both in the present baseline studies, and also in previous analyses of sediments, water, and biota (including seafood) from northern Australia. While this may be of concern to the industry, the knowledge gained from these studies shows that these potential pathogens are more prevalent in the wet season, and thus their seeming predictability could be exploited favourably by choosing not to harvest in the wet season. Furthermore, virulence markers were rarely detected in potential pathogens such as *Vibrio parahaemolyticus*, and this was supported by both published and unpublished data from other studies that showed low levels of virulent strains, and when they did occur it was associated with the wet season and particularly monsoons. These emerging patterns provide insights that can be used to manage this potential risk - particularly if further multi-year data also show that the high risk seasons are confined to the wet season when TROs spawn and are generally not in condition suitable for sale.

In terms of risk, locally acquired illnesses (wound and food) have been reported from WA, NT, and Qld. Taken together these data represent a credible risk for aquaculture across northern Australia and potentially impacts end users including management, industry and consumers in Australia. Within that context, the results from this study do have implications for the TRO industry. Particularly the recommendation that active risk management is required to mitigate the risk of human illness and market incidents.

10 Recommendations

The *Vibrio* ecological and health data presented in this report indicate that post-harvest cooling and maintenance of the cool chain during transport represent the most effective critical control points that if managed, will avoid exposing stock to temperatures that may favour growth of vibrios.

Site specific *Vibrio* control plans should be developed based on the knowledge of growth rates in BRO to ensure temperature of harvest stock is brought to, and maintained, under temperature control in a timely manner, following ASQAP requirements. This would mean refrigeration at 10 ^{o}C or less within 24 hours of harvest, which may require use of refrigeration facilities and refrigerated trucks for transportation of stock. Templates are available from South Australia and Tasmania that could be adapted to suit the industries in each state.

Therefore, the ability to control temperature post-harvest is a key consideration for businesses and should be considered in the development phase when selecting and developing aquaculture sites.

The risk profile presented in this report was hindered by several knowledge gaps. Further studies are required to properly characterise *Vibrio* risk.

These studies should include:

- Surveys on *Vibrio* spp. in oysters in aquaculture zones in northern Australia across extended time periods and multiple weather events.
- Identification, quantification, and isolation of local *Vibrio* strains with a focus on comparing clinical and environmental isolates to determine pathogenicity markers.
- Investigations of potential food sources (using a quantitative test method and targeting virulence genes) associated with gastroenteritis vibriosis.
- The risk profile should be updated as more information comes to light.

Finally, we understand that our recommendations that post-harvest cooling and maintenance of the cool chain during transport will avoid exposing stock to temperatures that may favour growth of vibrios, has implications for end users. However, it also provides guidance and legitimacy to establish a credible and evidence-based post-harvest strategy. This establishes the TRO industry as best practice from the outset - a defendable and appropriate position for an emerging industry that is potentially high-risk in terms of food safety.

11 Extension and Adoption

11.1 **Overview**

The four project flyers produced during this project are available on public websites. In addition, there was significant media coverage during the life of the project. The two workshops on country were well received and these along with the multi-organisational/sectoral steering committee meetings represent significant extension during the life of this project.

One chapter in this report has been published (see 12.1 below), two submissions are in final preparation for submission and two are being prepared.

The information we have presented here represents the work of all partner investigators from four organisations. The methods development, partnerships formed and the resulting data put the Tropical

Rock Oyster industry in a very strong place for making determinations about ideal harvest times, SQAP compliance and post-harvest handling and storage.

The *Vibrio* qPCR tests optimised as part of this study are already being applied in larval and spat survivorship studies (Zarah Tinning PhD funded by the CRCNA and CDU).

This project provides valuable data on *Vibrio* baselines and risk, both in space and time, and pathogen patterns, including toxin gene behaviour. This information will inform the development of jurisdictional SQAPs for north Australia's Tropical Rock Oyster industry that are currently in the planning stage.

The *Vibrio* growth profiles and shelf life results will guide postharvest handling and provided some really important breakthroughs – for example the resilience of the product at 4°C.

The risk profile was carefully crafted based on existing data and amidst significant data gaps – some of which are international data gaps. This situation has occurred partly because of the complexity of technology lagging behind a naturally occurring pathogen that is taking advantage of a changing climate in ways we are still discovering. The data gaps reported here were well justified and will provide important justification for future grant applications.

As with regulatory bodies internationally, ASQAP *vibrio* guidelines are rapidly evolving. The industry is in the early stages of coming to terms with the threat of vibrio and the potential compounding impacts of marine heat waves, cyanobacterial blooms and turbulence/freshwater inflows from storms on vibrio abundance. It is very timely to have these data for a warm water oyster species, both to inform ASQAP regulations and to compare with cold water oyster species. Detailed datasets like these important because this is not a pollution source issue – but an incredibly complex ecological problem in a dynamic changing environment.

11.2 **Project coverage**

- https://www.safefish.com.au/technical-program/vibrio-science-day
- https://www.frdc.com.au/fish-vol-29-4/talking-oysters-northern-australia
- https://www.youtube.com/watch?v=6EXDK0F9zfg
- https://www.cdu.edu.au/news/cdu-researchers-highlight-emerging-climate-change-risk-seafood-industry
- Presentation: Vibrio *bacteria as an emerging world-wide threat in a changing environment – understanding tropical rock oysters and* Vibrios *for food safety and human health,* International Tropical Rock Oyster Workshop May 28-9, 2023, Darwin Convention Centre
- Presentation: *Growth of* Vibrio parahaemolyticus *in Blacklip Rock Oysters stored at different temperatures,* World Aquaculture May 29 – June 1, 2023, Darwin Convention Centre
- https://www.cdu.edu.au/files/2023-10/RIEL-Annual-Report-2022-WEB.pdf (pages 8-9)

12 Project materials developed

See file '171123 Final report 2020-043 Part 2 Project materials' for project materials comprising:

12.1 **Scientific papers**

Padovan, A.C.; Turnbull, A.R.; Nowland, S.J.; Osborne, M.W.J.; Kaestli, M.; Seymour, J.R.; Gibb, K.S. Growth of *V. parahaemolyticus* in Tropical Blacklip Rock Oysters. *Pathogens* 2023, *12*, 834. https://www.mdpi.com/2076-0817/12/6/834

12.2 **Factsheets**

12.2.1 Project flyer 1

12.2.2 Project flyer 2

12.2.3 Project flyer 3

12.2.4 Project flyer 4

12.3 **Workshop reports**

12.3.1 Workshop report 1

12.3.2 Workshop report 2

13 Appendices

13.1 **Project staff**

Table 13-1: List of researchers and project staff.

Position	Name	Organisation
Principal Investigator	Karen Gibb	Charles Darwin University (CDU)
Co-Investigators	Anna Padovan	Charles Darwin University
	Alison Turnbull	Institute for Marine and Antarctic Studies (IMAS) UTas
	Stephen Pahl	South Australian Research and Development Institute
	Matthew Osborne	Department of Primary Industry and Resources (NT)
	Samantha Nowland	Department of Primary Industry and Resources (NT)
	Justin Seymour	University of Technology Sydney (UTS)
Researchers	Zarah Tinning	CDU
	Cynthia Coyne	CDU
	Nachshon Siboni	UTS
	Claire Hedges	IMAS
Financial Admin	Kazi Bari	CDU
	Dylan Campbell	CDU

13.2 **Intellectual Property**

None – publications open access

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15 FRDC FINAL REPORT CHECKLIST

The final report checklist can now be filled in when submitting your final report deliverable in FishNet.

Project Title:	Toxigenic vibrio baselines and optimum storage, transport and shelf-life conditions to inform cold supply chains in the north Australian Tropical Rock Oyster industry		
Principal Investigators:	**Karen Gibb, Anna Padovan, Alison Turnbull, Stephen Pahl, Samantha Nowland, Matthew Osborne, Justin Seymour**		
Project Number:	2020/043		
Description:	Results from this project have led us to recommend that active risk management is required to mitigate the risk of human illness and market incidents. Specifically, we recommend that post-harvest cooling and maintenance of the cool chain during transport represent the most effective critical control points that if managed, will avoid exposing stock to temperatures that may favour growth of vibrios. While this has implications for end users – it also provides guidance and legitimacy to establish a credible and evidence-based post-harvest strategy. This establishes the TRO industry as best practice from the outset - a defendable and appropriate position for an emerging industry that is known to be high-risk in terms of food safety. The *Vibrio* baseline temporal study was intended to determine whether *Vibrio* pathogens are associated with particular times of the year, important data needed to inform risk-centric surveillance. Although more multi-year data are needed, *V. parahaemolyticus* in oysters was associated with the wet season, and this will also be pursued in further studies, particularly associations with first big rains and monsoon events. The ability to use reliable ecological data to inform food safety considerations is going to be increasingly important in the uncertainty associated with our changing climate.		
Published Date:	XX/XX/XXXX (if applicable)	**Year:**	XXXX
ISBN:	XXXXX (if applicable)	**ISSN:**	XXXXXXXXXXXXX (if applicable)
Key Words:	Tropical Rock Oysters, northern Australia, Blacklip Rock Oysters (BROs) (*Saccostrea echinata*/lineage J), Milky oysters (*Saccostrea mordax*/lineage A), *Vibrio parahaemolyticus*, *V. vulnificus*, amplicon sequencing, *hsp60,* vibriosis*, Vibrio* community diversity*,* shelf life, cold supply chains, *Vibrio* risk profile, food safety.		

Please use this checklist to self-assess your report before submitting to FRDC. Checklist should accompany the report.

	Is it included (Y/N)	**Comments**
Foreword (optional)	N	
Acknowledgments	Y	
Abbreviations	Y	
Executive Summary	Y	
– **What the report is about**	Y	
– **Background – why project was undertaken**	Y	

– **Aims/objectives – what you wanted to achieve at the beginning**	Y	
– **Methodology – outline how you did the project**	Y	
– **Results/key findings – this should outline what you found or key results**	Y	
– **Implications for relevant stakeholders**	Y	
– **Recommendations**	Y	
Introduction	Y	
Objectives	Y	
Methodology	Y	
Results	Y	
Discussion	Y	
Conclusion	Y	
Implications	Y	
Recommendations	Y	
Further development	Y	
Extension and Adoption	Y	
Project coverage	Y	
Glossary	N	
Project materials developed	Y	
Appendices	Y	
EXTENSION		
Extension plan developed?	**Y**	
Extension undertaken?	**Y**	
If extension was undertaken, who was it undertaken with and was it successful? **(Detail answer in comments section)**	**Y**	Multi-sector steering committee (membership listed in acknowledgements) and Yagbani Aboriginal Corporation – two on country workshops. Feedback from these workshops was very successful as indicated in the workshops reports provided in the accompanying file '*Final report draft 2020-043 Section 2 Project materials'.*
If No, then is further extension necessary? **With who?** **How? (detail answer in comments section)**		