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Assessing the Occurrence of Pathogenic *Vibrio* Species in Oysters from Moulting Bay

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

Contents

Tables

Figures

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Executive Summary

Background

In January 2016 there was an outbreak of gastrointestinal illness that was confirmed through a health trace-back investigation to be associated with the marine bacterial pathogen *Vibrio parahaemolyticus* consumed via oysters harvested from the Moulting Bay region (St Helens) in Tasmania. A total of seven confirmed illnesses and a further three suspected cases were reported. *Vibrio* are naturally occurring bacteria that are found in marine environments and certain types, such as *V. parahaemolyticus* and *Vibrio vulnificus* are capable of causing illness in humans. Bivalve molluscs such as oysters are a known risk vector overseas as they are often consumed raw and their filter feeding action can concentrate bacteria within their tissues. Until now, *Vibrio*-related illnesses that have occurred in Australia have been limited and have mainly occurred as wound infections amongst susceptible populations. There have been large outbreaks *Vibrio* related gastrointestinal illnesses in Australia, but these were associated with imported seafood products. The 2016 outbreak is the first recorded outbreak of gastrointestinal illness associated with this pathogen to occur due to consumption of seafood harvested in Australia. The event coincided with unusually warm seawater temperatures in the growing region. There was an immediate need to collect information from the growing region that could underpin risk management considerations. This report describes the results of analysis undertaken by the South Australian Research Development Institute from February to May 2016 to rapidly collect information on the detection and enumeration of *V. parahaemolyticus* and *V. vulnificus* in the Moulting Bay Region.

O**bjectives**

1. Assess for the prevalence of pathogenic *Vibrio* species in the Moulting Bay harvesting area
2. Assess for the presence of genes associated with virulence in *V. parahaemolyticus*
3. Evaluate if a relationship exists between prevalence, sea water temperature and salinity

Methodology

Oysters were sampled from 5 sites in the Moulting Bay harvest region on six occasions from February to May 2016, aiming to be sampled on a fortnightly basis. Oysters were analysed for *V. parahaemolyticus* and *V. vulnificus* using a combination of traditional microbiological culture and DNA-based detection methods. The approach used differentiates between pathogenic and non-pathogenic strains of *V. parahaemolyticus* based on the presence of molecular markers for pathogenicity genes (*tdh* or *trh*). In addition, a clinical isolate of *V. parahaemolyticus* received from Launceston General Hospital from a

patient who was implicated in the 2016 outbreak was assessed to establish the profile of the strain implicated in the illnesses.

Results

V. parahaemolyticus was detected in 100% of samples (*n*=30) at levels higher than one-off testing that was undertaken in 2010. The proportion of samples that tested positive for *tdh* and *trh* were 20% and 16.6% respectively – only one sample tested positive for the presence of both genes. Samples were considered in comparison to the Food Standards Australia New Zealand (FSANZ) Guidelines for the Microbiological Examination of Ready-to-Eat Foods. Using the framework in the Guidelines, the *V. parahaemolyticus* levels in all samples collected in mid-February were considered unsatisfactory from a public health perspective and three of five (temperature abused) samples collected from the 7-9th of March were considered potentially hazardous. The clinical isolate received from Launceston General Hospital was confirmed as *V. parahaemolyticus* and this isolate carried both of the virulence associated genes *tdh* and *trh*. The proportion of samples that tested positive for *V. vulnificus* was 53.3% at levels that are concerning and warrant future attention. Counts of both *V. parahaemolyticus* and *V. vulnificus* declined with decreasing seawater temperatures.

Implications

There may be a regulatory impact associated with these illnesses as Australia can no longer claim an absence of risk due to a lack of epidemiology. Thus far, controls for these organisms in Australia have been limited to temperature control specifications. However, Codex Alimentarius Commission, an organisation that sets international standards, recommends that monitoring be undertaken to understand the seasonal and regional prevalence of these organisms. This information should be considered together with epidemiology to identify the need for controls that reduce risk. Yet, there is extremely scant information detailing the prevalence of these organisms in Australian growing regions to base these decisions on.

Recommendations

The occurrence of these illnesses have highlighted the paucity of information on the prevalence of these organisms in Australian bivalve harvest regions. The data collected in this study represents a short-term study that demonstrates notable levels in Moulting Bay harvesting region. More data is required to demonstrate if this is just a one-off event, or an ongoing risk that requires control mechanisms to be in place.

The prevalence of total and potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* should be assessed in the Moulting Bay harvesting region. This can be used to either demonstrate negligible risk (in light of the recent outbreak) or to determine if control mechanisms are warranted. The sampling

framework should capture increasing seawater temperatures through a high-risk summer sampling period and also capture the decreasing seawater temperatures. This data can then be used in any future management plans, as critical inputs into predictive models or as a point of comparison in the event of any future epidemiology.

There is little information on the prevalence of these organisms in the rest of Tasmania, and indeed Australia-wide. In light of the recommendations of the Codex Alimentarius Commission and the recent illnesses in Tasmania, a market survey of oysters from Australian growing regions should be undertaken during warmer months to assess the level of risk Australia-wide.

Keywords

***Vibrio parahaemolyticus*, *Vibrio vulnificus*, oyster, *Crassostrea gigas* and gastrointestinal illness.**

Introduction

Bacteria from the genus *Vibrio* are Gram-negative, facultative anaerobic rods that occur naturally in marine environments (Dalsgaard et al., 1998; Tantillo et al., 2004). Some members of the genus such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* have a well-known association with human illness world-wide and can result in distinct syndromes including diarrheal illness, wound infections and septicaemia. The highest risk period from these bacteria is during summer months as the number of vibrios usually correspond to increased water temperature; their prevalence is also impacted by salinity (Tantillo et al., 2004). Bivalve shellfish are considered to be a particular risk with regard to these pathogens as they pump large volumes of water across their gills and trapped food particles become highly concentrated in the digestive gland (Potasman et al., 2002). The risk is further increased due to the tendency for bivalve shellfish to be consumed raw or lightly cooked.

V. parahaemolyticus was first recognised as a foodborne pathogen following an outbreak of food poisoning in Japan in 1950. It is most commonly associated with gastroenteritis, however, on rare occasions has been associated with wound infections and primary septicaemia in immuno-compromised populations (Dalsgaard et al., 1998). The infectious dose was initially considered to be between 10^5 to 10^9 cells (Desmarchelier, 2003). However, in Canada and the United States illnesses have been reported when counts of *V. parahaemolyticus* were less than 200 cells per gram. *V. parahaemolyticus* illnesses are usually self-limiting and generally do not result in deaths; although, mortalities have occasionally been reported (Heitmann et al., 2005; Kraa, 1995). Haemolysins, which are a known virulence factor in pathogenic *Vibrio* species, are extracellular toxins that are released to attack cell membranes and rupture cells. The thermostable direct haemolysin (TDH) and the TDH related haemolysin (TRH) of *V. parahaemolyticus* have both been shown to be major virulence factors of *V. parahaemolyticus* (Okuda and Nishibuchi, 1998). These haemolysins are encoded by the *tdh* and *trh* genes, respectively and their presence is used as an indicator of likely capacity for pathogenesis in these bacteria. A third haemolysin of *V. parahaemolyticus* is described as a thermolabile haemolysin (TLH), is encoded by the *tlh* gene, and is not considered to be associated with virulence (Taniguchi et al., 1985). However, the *tlh* gene has been found in all isolates of *V. parahaemolyticus* that have been examined irrespective of whether they are from clinical or environmental sources and it is considered to be a species-specific gene that is ideal for the identification to species level (Zhang and Austin, 2005).

V. parahaemolyticus has been reported in Australian bivalve shellfish (Bird et al., 1992; Lewis et al., 2002; Madigan et al., 2007). Unpublished work undertaken by the South Australian Research and Development Institute in 2010 used a highly sensitive technique that combined a most probable

number (MPN) technique with a multiplex polymerase chain reaction (PCR) method. The results of this one off short survey (*n*=24) showed a low prevalence of *V. parahaemolyticus* in the sample areas in New South Wales, South Australia and Tasmania despite samples being collected in the warmer months. Pathogenic strains were only detected in one sample from New South Wales.

Despite the occurrence of these organisms in Australian shellfish, there has not been any significant epidemiology associated with this organism in Australia; when *Vibrio* associated illnesses have occurred they have been associated with imported seafood or occur in a person who has recently travelled overseas. There has been the occasional exception to this trend with two deaths being reported due to *V. parahaemolyticus*; one following consumption of oysters in 1992 and another in 2001 due to complications arising from a wound infection (Kraa, 1995; Ralph and Currie, 2007). However, during the summer of 2016, waters on the East coast of Tasmania were impacted by an El Nino weather pattern that resulted in seawater temperatures that were 4.5 °C above average. In January, an outbreak of severe gastrointestinal illness occurred and *V. parahaemolyticus* was identified as the causative organism by a Department of Health and Human Services investigation (Personal Communication Michelle Green). The investigation identified a total of seven cases that all traced back to oysters harvested from the St Helens harvesting region in Tasmania – also known as the Moulting Bay harvest area. There was also a further three probable cases and a trade level recall was instigated. These illnesses represented a shift in the epidemiology of *V. parahaemolyticus* in Australia as this represents the first reported occurrence of an outbreak associated with this organism and Australian seafood. There was an immediate need to collect information from the harvesting region to develop a prevalence profile that could be used to inform future work and risk management.

Objectives

1. Assess for the prevalence of pathogenic *Vibrio* species in the St Helens harvesting region
2. Assess for the presence of genes associated with virulence in *V. parahaemolyticus*
3. Evaluate if a relationship exists between prevalence, sea water temperature and salinity

Method

Oysters were collected from five locations with fortnightly sampling where possible from the St Helens harvesting region (Figure 1). These represented a combination of intertidal and sub-tidal leases sampled from March 2016 to May 2016. A total of 12 individual oysters were collected by a regional co-ordinator from each location, placed in a foam esky, with a frozen cold pack to maintain ambient temperature and sent overnight to the South Australian Research and Development Institute in Adelaide. The oysters were transported under a permit granting exemption of translocation of oysters from Tasmania to South Australia. All oysters arrived at the laboratory within 72 hours of harvest. The intent was to use data from fixed monitoring stations to estimate temperature and salinity in the growing region. Regrettably, these stations were not working at the time and limited data collected by the Tasmanian Shellfish Quality Assurance (TASQAP) has been used as a proxy. These measurements were not concurrent with samples collected for this study.

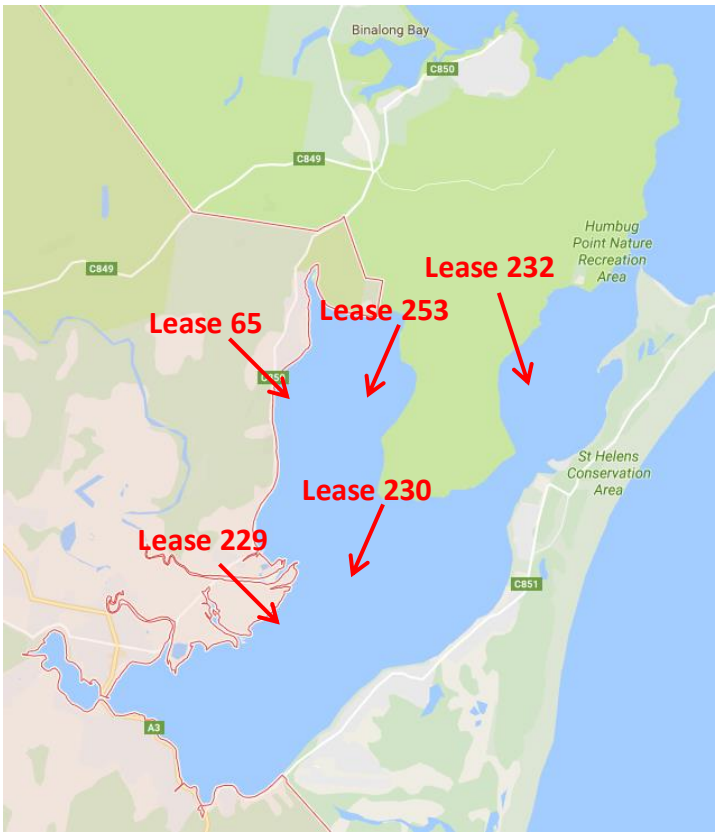

Figure 1: Map depicting sampling points in Moulting Bay growing region.

Immediately upon receipt at the laboratory, the surface temperature of the oysters was recorded and the animals were prepared for analysis. There is an Australian Standard method for the assessment of *V. parahaemolyticus* in foods (AS 5013.18—2010) that utilises a MPN technique. However, this

microbiological method can only provide an estimate of total *V. parahaemolyticus* and does not differentiate between pathogenic strains. Consequently, in-house unpublished methods established and used by the United States Food and Drug Administration were used. This method combines the MPN method with a real-time PCR technique and enables differentiation between pathogenic and non-pathogenic *V. parahaemolyticus* strains. Briefly, crude nucleic acid samples were prepared by taking 1 mL aliquots of the overnight MPN tubes and boiling for 15 minutes in 1.5 mL microcentrifuge tubes. DNA templates were immediately placed on ice for five minutes prior to centrifugation at 15,000 × g for 30 seconds. Samples were stored at -20 °C until used for analysis. PCR protocols used the primer sets described by Nordstrom et al., (2007) for *V. parahaemolyticus* and by Campbell and Wright (2003) for *V. vulnificus*. The exact PCR conditions differ slightly to the published method; however, exact methodologies are not described here as permission has not been granted to publish these methods. However, as a general overview, duplex PCR reactions were undertaken that targeted either the species-specific genes *tlh* (*V. parahaemolyticus*) or *vvhA* (*V. vulnificus*) together with an internal amplification control (IAC) that assessed the presence of any inhibition in PCR reactions. If samples tested positive for *tlh*, two more duplex PCR reactions were undertaken that separately targeted the virulence genes *tdh* and *trh* – each reaction included the IAC as a second PCR target. PCR reactions were undertaken using a QuantStudio™ 6 Flex Real-Time PCR System and analysed using the QuantStudio™ Real-Time PCR Software. The level of detection for this method was 0.3 per gram of shellfish.

A clinical isolate of *V. parahaemolyticus* was obtained from the Launceston General Hospital. This isolate had been isolated from a stool sample from one of the case reports for *V. parahaemolyticus* that was traced back to the Moulting Bay harvesting region. The isolate was grown overnight in alkaline peptone water, a crude DNA sample was prepared as above and the presence of the *tlh*, *tdh* and *trh* genes determined as per the method described above. A further five isolates isolated from MPN tubes from samples collected from Moulting Bay and isolated from the selective medium CHROMagar™ *Vibrio* were also assessed using the PCR method described above.

Results & Discussion

A total of 30 samples were collected from across the study. The initial aim was to collect 40 samples on a fortnightly basis spread across eight sampling occasions, but difficulties were encountered in collecting and couriering samples. All samples collected were positive for total *V. parahaemolyticus* with a lesser prevalence of pathogenic strains and *V. vulnificus.*

The results of sampling in the Moulting Bay harvest area are reported in Table 1. All samples tested (*n*=30) were positive for *V. parahaemolyticus* based on the presence of the *tlh* gene. The proportion of samples that tested positive for *tdh* and *trh* (implying that pathogenic strains were present in these samples) were 20% and 16.6% respectively – only one sample tested positive for the presence of both genes. Strains with indicative pathogenicity were detected across all sites. A typical duplex PCR profile is provided in Figure 2; this PCR targeted the *tlh* gene of *V. parahaemolyticus*.

The counts of *V. parahaemolyticus* were higher than those reported for Moulting Bay in 2010 (Table 2) and pathogenic strains were detected in the present work. Both studies have used a similar diagnostic approach with the same MPN/PCR technique and primer sets; indicating based on this limited information that a shift may have occurred in prevalence and pathogenicity profiles of *Vibrio*. Counts were notably elevated in samples harvested between the 7-9th of March in this study. It is probable that these elevated counts are due to temperature abuse, as these samples were received at approximately 20 °C. Transport duration and temperature control of samples is critical for accurate *V. parahaemolyticus* enumeration; if the samples become too cold for extended periods they may become non-culturable, alternatively if samples are too warm this will also impact vibrio enumeration. Ideally samples should be sent near10 °C and arrive in less than 24 hours. Some samples in this study took up to 72 hours to arrive at the laboratory due to the remoteness of the harvesting region, and samples being collected over two days on some occasions.

There are no limits set for vibrios in bivalve shellfish under the Food Standards Code. However, the Food Standards Australia New Zealand (FSANZ) Guidelines for the Microbiological Examination of Ready-to-Eat Foods suggests that levels less than 3 per gram in a food product of *V. parahaemolyticus* are satisfactory, from 3 to 100 per gram are marginally acceptable, from 100 to 10,000 per gram is unsatisfactory and that over 10,000 per gram could be considered potentially hazardous. Using this system to characterise the results, all samples collected in mid-February were unsatisfactory and three of five (temperature abused) samples collected between the 7-9th of March could be considered potentially hazardous. For the remainder of the sampling occasions, samples were in the marginally acceptable or satisfactory classifications with the exception of two samples from lease 253 (6th April; potentially hazardous) and lease 232 (19th April; unsatisfactory). Oysters from lease 253 also contained the highest counts amongst the temperature abused samples collected between 7-9th of March. It should be noted that the classification of samples under the FSANZ Guideline only considers the total number of *V. parahaemolyticus* and not pathogenic strains, so this classification uses a conservative approach.

The counts reported here for *V. parahaemolyticus* are similar to a study undertaken in New Zealand by Kirs et al., (2011), who reported that for their sampling locations in the upper part of the North

Island the percentage of positive samples ranged from 87.5 to 100 %. The approach used in the present study is more sensitive than that of the Kirs et al., study where they confirmed presence by isolation rather than using a molecular technique directly on the enriched MPN tubes. The counts of total *V. parahaemolyticus* found in the New Zealand study were similar to the current study. However, a smaller percentage of pathogenic strains were detected with only two samples positive for *tdh*. This may be due to sensitivity differences in methodologies; however, the authors did use a similar approach to the one used here on a limited number of samples (18 of their 58 samples) and still did not detect either the *tdh* or the *trh* genes.

The proportion of samples that tested positive for *V. vulnificus* was 53.3%. The levels of *V. vulnificus* detected in this study ranged from 0.4 to 600 MPN per gram of shellfish. The two highest results from samples collected on the 18 and 19th of April exhibited a slightly unusual MPN profile; however, good amplification was noted in the positive tubes. The National Shellfish Sanitation Program in the United States sets criteria that if oysters are contaminated with *V. vulnificus* at levels over 30 MPN pergram then they should be subject to post-harvest processing to reduce the counts. In light of this the counts detected in this study are of concern and warrant further investigation. The percentage of positive samples for *V. vulnificus* was higher in this study in comparison to the Kirs et al. (2011) study, where samples ranged from 10 to 40 % positive. The highest counts of *V. vulnificus* in the Kirs et al. (2011) study were below 30 MPN per gram. However, as per their *V. parahaemolyticus* sampling, their methods were likely less sensitive and they did not employ an MPN/PCR technique for *V. vulnificus* in any of their samples. To the author's knowledge, there is no comparable data on the prevalence of *V. vulnificus* in Australia, although, several cases of wound infections and deaths have been reported (Maxwell et al., 1991; Ralph and Currie, 2007).

Table 1: Results of testing by a combined most probable number and polymerase chain reaction technique for the *tlh*, *tdh*, *trh* genes from *V. parahaemolyticus* and *vvhA* gene from *V. vulnificus* from oysters harvested from the Moulting Bay harvesting region.

Date Collected	Lease No.	*Vibrio* MPN			
		tlh MPN	*tdh* MPN	*trh* MPN	*vvhA*MPN
15/02/2016	229	430	<0.3	<0.3	<0.3
15/02/2016	230	230	<0.3	<0.3	0.4
15/02/2016	65	230	<0.3	<0.3	1.5
15/02/2016	253	150	0.4	<0.3	<0.3
15/02/2016	232	150	<0.3	4	0.4
7/03/2016	229	**750**	<0.3	<0.3	<0.3
7/03/2016	230	**4300**	**4**	<0.3	<0.3
7/06/2016	65	**350**	**4**	<0.3	<0.3
9/03/2016	253	**16,000**	<0.3	**4**	**0.4**
9/03/2016	232	**4300**	<0.3	<0.3	**0.4**
Not provided	229	43	<0.3	<0.3	<0.3
Not provided	230	93	<0.3	4	<0.3
Not provided	65	23	<0.3	<0.3	<0.3
Not provided	253	7.5	<0.3	<0.3	<0.3
Not provided	232	70	<0.3	<0.3	140
4/04/2016	229	7.5	<0.3	<0.3	15
4/04/2016	230	3.8	<0.3	<0.3	9
4/04/2016	65	2.3	<0.3	<0.3	11
6/04/2016	253	1110	<0.3	<0.3	<0.3
6/04/2016	232	4.3	4	<0.3	9
18/04/2016	229	93	4	<0.3	<0.3
18/04/2016	230	4.3	<0.3	<0.3	300
18/04/2016	65	43	<0.3	<0.3	<0.3
19/04/2016	253	0.9	<0.3	<0.3	600
19/04/2016	232	150	<0.3	9	9
5/05/2016	229	23	0.7	4	0.9
5/05/2016	230	4.3	<0.3	<0.3	0.9
5/05/2016	65	0.9	<0.3	<0.3	<0.3
5/05/2016	253	21	<0.3	<0.3	<0.3
5/05/2016	232	3.8	<0.3	<0.3	0.4

Note: Samples highlighted in bold were likely subjected to temperature abuse.

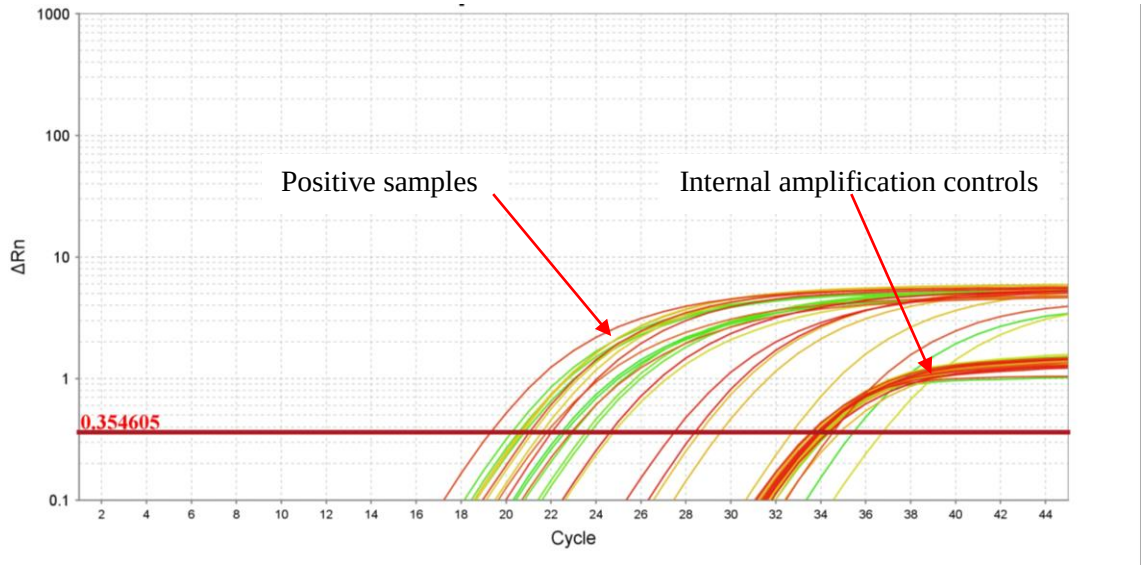

Figure 2: Typical duplex PCR profiles. This profile is from a reaction targeting the *tlh* gene of *Vibrio parahaemolyticus*.

Table 2: *Vibrio parahaemolyticus* in Australian oysters. Results from 2010 South Australian Research and Development Institute survey.

Location	Date	Water temp (°C)	Salinity (‰)	Sample	*tlh* (MPN/gram)	*tdh* (MPN/gram)	*trh* (MPN/gram)
Manning River, NSW	5/5/2010	20	22.4	1	60	<0.3	<0.3
				2	46	<0.3	<0.3
				3	46	0.4	<0.3
				4	75	<0.3	<0.3
Port Stephens, NSW	5/5/2010	19.6	31.4	1	4.3	<0.3	<0.3
				2	38	<0.3	<0.3
				3	23	<0.3	4
				4	9	<0.3	<0.3
Wallis Lakes, NSW	5/5/2010	19.5	33.6	1	2.3	<0.3	<0.3
				2	2.3	<0.3	<0.3
				3	24	0.4	<0.3
				4	24	<0.3	<0.3
Little Swanport, TAS	23/2/2010	18-26	34-36	1	46	<0.3	<0.3
				2	9.3	<0.3	<0.3
				3	9.3	<0.3	<0.3
				4	24	<0.3	<0.3
Moulting Bay, TAS	23/2/2010	15-23	31-35	1	1.5	<0.3	<0.3
				2	4.3	<0.3	<0.3
				3	1.5	<0.3	<0.3
				4	1.5	<0.3	<0.3
Pipeclay Lagoon, TAS	23/2/2010	17-26	34-36	1	0.4	<0.3	<0.3
				2	0.4	<0.3	<0.3
				3	<0.3	<0.3	<0.3
Franklin Harbour, SA	27/1/2010	18.9	38.8	1	<0.3	<0.3	<0.3
				2	0.3	<0.3	<0.3
				3	<0.3	<0.3	<0.3
				4	<0.3	<0.3	<0.3
				5	<0.3	<0.3	<0.3
				6	<0.3	<0.3	<0.3
				7	0.4	<0.3	<0.3
				8	0.4	<0.3	<0.3

The clinical isolate received from Launceston General Hospital was confirmed as *V. parahaemolyticus* based on the presence of the *tlh* gene. This isolate carried both of the virulence associated genes *tdh* and *trh*. However, this profile is distinct to the profile seen in the MPN/PCR results, with only one sample being positive for both virulence genes. The five isolates sourced from the MPN tubes all carried the species-specific gene *tlh* confirming that these strains were *V. parahaemolyticus*. However, none of these strains carried either of the virulence genes. In light of the low levels of potentially pathogenic strains, it is likely that a large number of strains would need to be collected to identify *tdh*/*trh* positive strains. This study did not carry a large focus on collecting strains for analysis. However, this should be included as a focus of further work to allow comparison to strains collected in any future epidemiological investigations. It would also be beneficial to identify strains of *V. vulnificus* by culture to confirm the results achieved in the MPN/PCR analysis. Purple colonies indicative of *V. parahaemolyticus* were easily cultured from CHROMagar™ *Vibrio* and to a lesser extent turquoise colonies indicative of *V. vulnificus* or *V. cholerae*; although, any identification from this medium can only be considered as presumptive (May et al., 2013)..

Consideration was given to the environmental parameters that could have resulted in the illnesses and impacted on the longitudinal testing undertaken in this study. The permanent sampling probes in Moulting Bay were not operational during the time of the study; however, limited environmental parameters from periodic sampling for temperature and salinity were collected by TASQAP, these are reported in Figure 3. Temperatures ranged from 11.6 to 26.1 °C and the salinity ranged from 9.2 to 33.8 parts per thousand (PPT). The salinity results should be considered as indicative as they represent individual sampling events and salinity can change on a daily basis whereas seawater temperature is more stable. Daily rainfall data is provided in Figures 4. The seawater temperatures and salinity were conducive to the growth of *V. parahaemolyticus* and *V. vulnificus*. The optimum salinity for *V. parahaemolyticus* is 30 parts per thousand (PPT) and the temperature growth range is 5-43 °C. For *V. vulnificus*, the optimum salinity is 25 PPT and the growth range is 5-43 °C. Counts of *V. parahaemolyticus* declined in line with seawater temperatures and by the 5th May, all counts were in the generally acceptable range. Aside from a drop in salinity at the end of January, associated with a large rainfall event (Figure 4), the salinity in the growing area was around the optimum for *V. parahaemolyticus*. The illnesses occurred prior to this rainfall event. Daily temperatures on the 8th and 9th of January were the hottest days recorded for the month, with a maximum of 29.4 °C on both days. Harvesting during intertidal exposure has been shown to elevate levels of *V. parahaemolyticus* (Nordstrom et al., 2004); however, the oysters implicated in the investigation all came from a subtidal lease indicating that ambient temperature at harvest was unlikely to be a factor that increased risk of illness. Although, the ambient temperatures may have resulted in increased growth of these organisms post-harvest if the product was not refrigerated as soon as possible. In any future control plan for

high-risk periods, consideration should be given to the time to refrigeration for harvested oysters and the length of intertidal exposure prior to harvest.

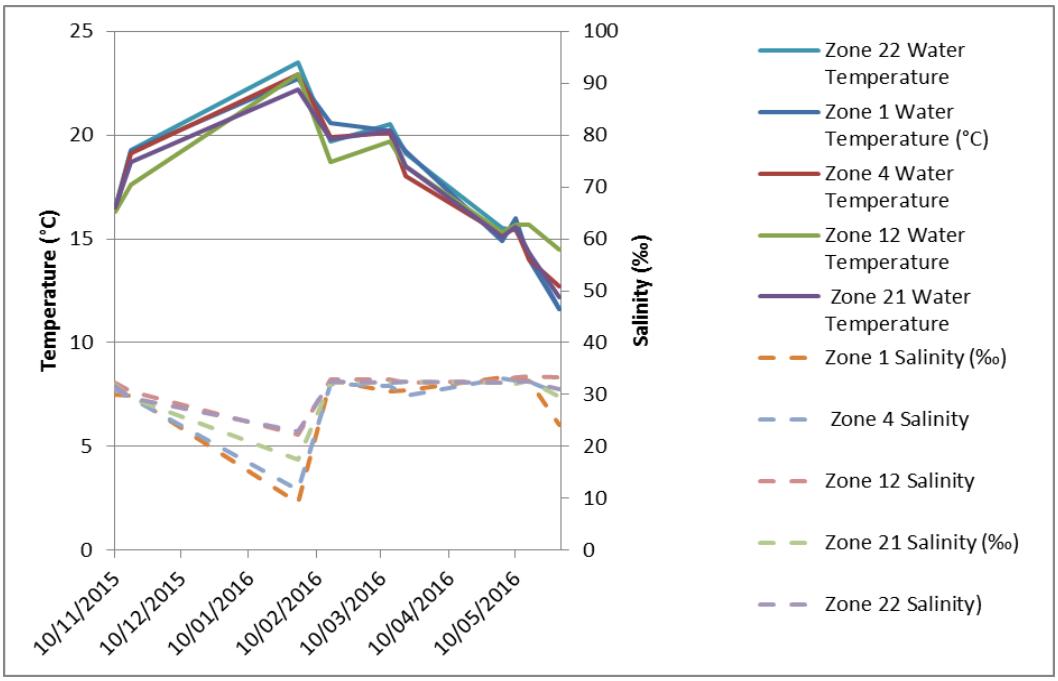

Figure 3: Temperature and salinity profiles collected in the Moulting Bay harvesting area from 10th November 2015 to the 30th May 2016.

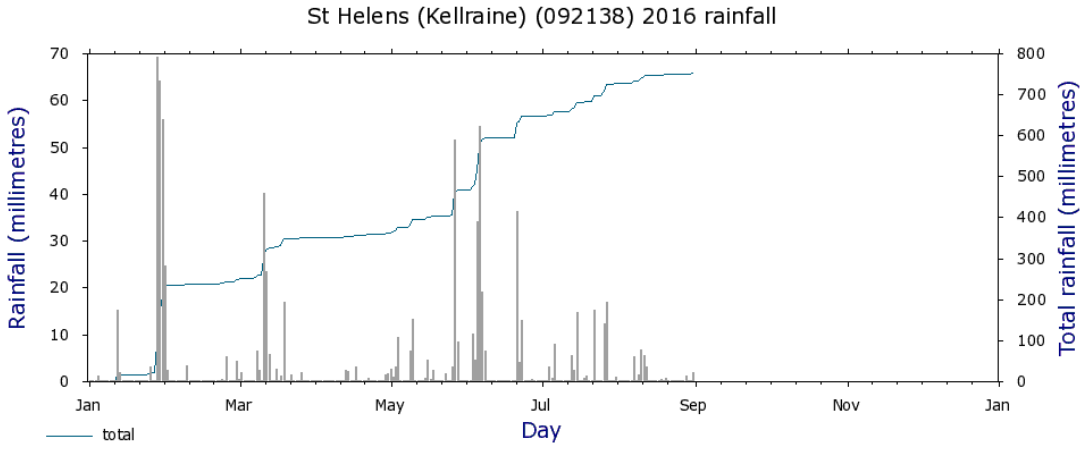

Figure 4: Daily and total rainfall for the St Helens region in 2016.

The Codex Alimentarius Commission has set Guidelines for the Application of General Principles of Food Hygiene to the Control of Pathogenic *Vibrio* (CAC/GL 73-2010) and includes a separate Annex that relates directly to control of these species in bivalve shellfish. The document recommends that

monitoring be undertaken in harvest areas for levels of total and potentially pathogenic *V. parahaemolyticus* (based on the presence of the *tdh* and *trh* genes) and *V. vulnificus* to determine the level of regional and seasonal variation. These data can be useful in application of controls and can also be used to refine models that predict likely levels of these organisms based on temperature and salinity. They list the following factors (quoted from CAC/GL 73-2010) that should be considered in determining need for harvest controls:

- *"The number of sporadic illnesses and outbreaks of V. parahaemolyticus and V. vulnificus associated with bivalve molluscs harvested from a distinct hydrographic area, and whether these illnesses are indicative of an annual reoccurrence or an unusual increase of Vibrio spp. illnesses is reported;*
- *Water temperatures representative of harvesting conditions. Water temperatures below 15ºC for V. parahaemolyticus and below 20ºC for V. vulnificus have generally not been historically associated with illnesses;*
- *Time period to first refrigeration and post-harvest air temperatures above the minimum growth temperatures for V. parahaemolyticus (10ºC) and V. vulnificus (13ºC), which may increase risk regardless of harvest water temperature;*
- *Harvest practices that allow radiant solar heating to raise temperatures of bivalve molluscs to temperatures above ambient air temperatures prior to harvest (i.e. intertidal harvest) and exposure time;*
- *Salinity ranges and optima are different for V. parahaemolyticus and V. vulnificus. Environmental and epidemiological data indicate low V. parahaemolyticus and V. vulnificus levels and few cases of illnesses are associated with bivalve molluscs when salinity exceeds 35 ppt (g/l) and 30 ppt (g/l), respectively."*

Conclusion

Confirmed illnesses from *V. parahaemolyticus* in January 2016 resulted in an immediate need to understand the prevalence and pathogenicity of these organisms in the Moulting Bay harvest region during this period of unusually seawater temperatures. This project has collected samples from February through to May 2016 and has demonstrated an increased prevalence of both total and pathogenic *V. parahaemolyticus* in comparison to one-off sampling undertaken in in Australia during 2010. Counts of these organisms declined in-line with decreasing seawater temperatures. Genes indicative of *V. vulnificus* were also detected in the oyster samples. Considerations for determining the need for control mechanisms have also been provided.

Implications

The illnesses that occurred in January 2016, that were confirmed and reported as part of a trace-back investigation as being associated with *V. parahaemolyticus* and oysters represent a shift in the epidemiology of these organisms in Australia. Prior to this no multiple case diarrheal illnesses have been reported from Australian harvested seafood and Australia can no longer claim an absence of risk due to a lack of epidemiology. At the time the illnesses occurred, they passed unnoticed by the media; possibly due to the extensive media regarding a mass oyster mortality event that occurred at the time. Media associated with these types of events are well known to be detrimental to the wider seafood industry as consumers decrease their consumption of seafood overall.

There may be a regulatory impact associated with these illnesses. Due to the lack of previous epidemiology, controls for these organisms in Australia have been limited to temperature control specifications where product must be placed under 10 °C within 24 hours of harvest (for Pacific Oysters). The Codex Alimentarius Commission, an organisation that sets international standards, recommends that monitoring be undertaken using techniques that possess sufficient sensitivity and to identify pathogenic strains to understand the seasonal and regional prevalence of these organisms when considering the need for regulatory controls and refining models that predict risk. There is extremely scant information detailing the prevalence of these organisms in Australia. Such studies have been conducted in New Zealand, such as the recent study that assessed prevalence in their Northern growing areas and further work assessing *V. parahameoluyticus* and *V. vulnificus* in bivalves including mussels (Cruz et al., 2016; Cruz et al., 2015; Kirs et al., 2011). Ongoing routine testing for these species would represent a significant cost-burden to the Australian bivalve industry, as specialised expensive methods are required. However, to avoid this in the longer term, information on the prevalence of these organisms in Australian growing areas are needed to supplement the case that the illnesses in Tasmania are solely related to the extreme weather event and not likely to be an ongoing concern.

Recommendations

The occurrence of these illnesses have highlighted the paucity of information on the prevalence of these organisms in Australian bivalve harvest regions. The data collected in this study represents a short-term study that demonstrates notable levels in Moulting Bay harvesting region. More data is

required to demonstrate if this is just a unique event in one growing area, or an ongoing risk that requires control mechanisms to be in place.

The following recommendations are made:

1. The prevalence of total and potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* should be assessed in the Moulting Bay harvesting region. This can be used to establish if control mechanisms are warranted. The sampling framework should capture increasing seawater temperatures through a high-risk summer sampling period and also capture the decreasing seawater temperatures. Focus should be given to ensuring that samples are collected in a timely manner with supporting environmental information from the exact sample location and transported appropriately. This data can then be used in any future management plans, so as critical inputs into predictive models or as a point of comparison in the event of any future epidemiology.

2. There is little information on the prevalence of these organisms in the rest of Tasmania, and indeed Australia-wide. In light of the recommendations of the Codex Alimentarius Commission and the recent illnesses in Tasmania, a market survey of oysters from Australian growing regions should be undertaken during warmer months to assess the level of risk.

3. Isolates should be collected from samples to confirm the PCR-based identification of genes indicative of *V. parahaemolyticus* and *V. vulnificus*. Molecular based identification, serotyping and whole of genotyping should be undertaken for use in comparing to any future outbreaks of illness. This can be also be used to assess whether the profile of pathogenic strains is changing.

Extension and Adoption

This report will be provided to the Tasmanian Shellfish Quality Assurance Program. Results were presented in October at the Shellfish Futures 2016 annual industry conference and the Australian Shellfish Quality Assurance Program's Biennial Conference in November 2016.

References

Bird, P., Arnold, G., Holliday, J., Boronovshy, A., 1992. Effect of storage on the quality of purified live Pacific and Sydney rock oysters, Conference Internationale sur la Purification des Coquillages, Rennes (France).

Campbell, M.S., Wright, A.C., 2003. Real-time PCR analysis of Vibrio vulnificus from oysters. Applied Environmental Microbiology 69, 7137-7144.

Cruz, C.D., Chycka, M., Hedderley, D., Fletcher, G.C., 2016. Prevalence, characteristics and ecology of Vibrio vulnificus found in New Zealand shellfish. Journal of Applied Microbiology 120, 1100-1107.

Cruz, C.D., Hedderley, D., Fletcher, G.C., 2015. Long-term study of vibrio parahaemolyticus prevalence and distribution in New Zealand shellfish. Applied and Environmental Microbiology 81, 2320-2327.

Dalsgaard, A., Serichantalergs, O., Forslund, A., Pitarangsi, C., Echeverria, P., 1998. Phenotypic and molecular characterization of Vibrio cholerae O1 isolated in Samutsakorn, Thailand before, during and after the emergence of V. cholerae O139. Epidemiol Infect 121, 259-268.

Desmarchelier, T., 2003. Pathogenic Vibrios, in: Hocking, A. (Ed.), Foodborne microorganisms of public health significance 6th ed. Australian Institute of Food Science and Technology, NSW Branch Food Microbiology Group Waterloo, New South Wales.

Heitmann, I., Jofre, L., Hormazabal, J.C., Olea, A., Vallebuona, C., Valdes, C., 2005. [Review and guidelines for treatment of diarrhea caused by Vibrio parahaemolyticus]. Rev Chilena Infectol 22, 131-140.

Kirs, M., DePaola, A., Fyfe, R., Jones, J.L., Krantz, J., Van Laanen, A., Cotton, D., Castle, M., 2011. A survey of oysters (Crassostrea gigas) in New Zealand for Vibrio parahaemolyticus and Vibrio vulnificus. International Journal of Food Microbiology 147, 149-153.

Kraa, E., 1995. Surveillance and epidemiology of foodborne illness in NSW, Australia. Food Australia 47, 6.

Lewis, T., Brown, M., Abell, G., McMeekin, T., Sumner, J., 2002. Pathogenic vibrio parahaemolyticus in Australian oysters.

Madigan, T., Lee, K., Pointon, A., Thomas, C., 2007. A supply-chain assessment of marine Vibrios in Pacific oysters in South Australia: prevalence, quantification and public health risk. Fisheries Research and Development Corporation.

Maxwell, E.L., Mayall, B.C., Pearson, S.R., Stanley, P.A., 1991. A case of Vibrio vulnificus septicaemia acquired in Victoria. Med J Aust 154, 214-215.

May, D., Madigan, T., Landinez, L., Tan, J., McLeod, C., 2013. Assessment of Methodologies for the Enumeration of Pathogenic Vibrio species in Australian Prawns, in: Corporation, F.R.a.D. (Ed.).

Nordstrom, J.L., Kaysner, C.A., Blackstone, G.M., Vickery, M.C.L., Bowers, J.C., DePaola, A., 2004. Effect of intertidal exposure on Vibrio parahaemolyticus levels in Pacific Northwest oysters. Journal of Food Protection 67, 2178-2182.

Nordstrom, J.L., Vickery, M.C.L., Blackstone, G.M., Murray, S.L., DePaola, A., 2007. 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.

Okuda, J., Nishibuchi, M., 1998. Manifestation of the Kanagawa phenomenon, the virulence-associated phenotype, of Vibrio parahaemolyticus depends on a particular single base change in the promoter of the thermostable direct haemolysin gene. Mol Microbiol 30, 499-511.

Potasman, I., Paz, A., Odeh, M., 2002. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. Clin Infect Dis 35, 921-928.

Ralph, A., Currie, B.J., 2007. Vibrio vulnificus and V. parahaemolyticus necrotising fasciitis in fishermen visiting an estuarine tropical northern Australian location. Journal of Infection 54.

Taniguchi, H., Ohta, H., Ogawa, M., Mizuguchi, Y., 1985. Cloning and expression in Escherichia coli of Vibrio parahaemolyticus thermostable direct hemolysin and thermolabile hemolysin genes. J Bacteriol 162, 510-515.

Tantillo, G.M., Fontanarosa, M., Di Pinto, A., Musti, M., 2004. Updated perspectives on emerging vibrios associated with human infections. Lett Appl Microbiol 39, 117-126.

Zhang, X.H., Austin, B., 2005. Haemolysins in Vibrio species. J Appl Microbiol 98, 1011-1019.

Appendices

List of researchers and project staff

- Thomas Madigan, SARDI
- Gayle Smith, SARDI
- Alison Turnbull, SARDI
- Geoffrey Holds, SARDI
- Kate Wilson, Tasmanian Shellfish Quality Assurance Program

FRDC FINAL REPORT CHECKLIST

Project Title:	**Assessing occurrence of pathogenic species of the marine bacteria Vibrio in Tasmanian oysters from St Helens.**		
Principal Investigators:	Tom Madigan, Kate Wilson, Gayle Smith and Alison Turnbull		
Project Number:	2015-042		
Description:	This project was undertaken in response to illnesses that occurred in association with *Vibrio parahaemolyticus* and oysters harvested from the Moulting Bay harvest region (St Helens), Tasmania. Counts of *Vibrio parahaemolyticus* and *Vibrio vulnificus* were estimated in 30 samples spread across 6 sampling occasions from February to May 2016.		
Published Date:	XX/XX/XXXX (if applicable)	**Year:**	2016
ISBN:	XXXXX (if applicable)	**ISSN:**	XXXXXXXXXXXXX (if applicable)
Key Words:	Needs to include key subject areas and species name (see www.fishnames.com.au)		

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

	Is it included (Y/N)	**Comments**
Acknowledgments	Y	
Executive Summary		
- **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/discussion	Y	

Conclusion	Y	
Implications	Y	
Recommendations and Further development	Y	
Extension and Adoption	Y	
Project coverage	NA	
Glossary	NA	
Project materials developed	NA	
Appendices	Y	