A survey of commercially harvested North Island Oysters *(Crassostrea gigas)* for *Vibrio parahaemolyticus* and *Vibrio vulnificus*

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Scientific Interpretive Summary

A SURVEY OF COMMERCIALLY HARVESTED NORTH ISLAND OYSTERS (CRASSOSTREA GIGAS) FOR *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS*

NZFSA commissioned Cawthron Research Institute to conduct a microbiological survey to determine the levels of total and pathogenic *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in Pacific oysters (*Crassostrea gigas*) collected from commercial growing areas in the North Island of New Zealand.

Vibrios are a type of bacteria that cause foodborne illness. The types of illness range in severity from mild gastroenteritis, septicaemia (blood poisoning) or cholera. The Vibrio species most commonly associated with foodborne illness include *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. *V. cholarae* is associated with contaminated drinking water (not in New Zealand). Foodborne *V. vulnificus* illness is typically associated with underlying medical conditions, such as liver disease. *V. parahaemolyticus* usually causes mild gastrointestinal illness.

Vp and Vv are found in estuaries and coastal waters, including those around New Zealand. Past surveys have found Vp and Vv in New Zealand waters and shellfish harvested from them, although not all types of Vp and Vv are capable of causing illness. There have not been any reported cases of foodborne Vp or Vv illness associated with the consumption of commercially harvested shellfish.

During summer 2008/2009 (November to April), oyster samples and corresponding environmental data was collected from specific growing areas by oyster farmers once every 2 weeks. The laboratory determined the number of Vp and Vv present in the oysters using a method based on most probable number (MPN) and standard culture methods. Confirmation of the Vp and Vv isolates was conducted using real-time PCR.

Vp was detected in 94.8% of oyster samples examined with a mean density of 564 MPN g-1. Real-time PCR was used to determine the presence of the potentially pathogenic Vp strains (tdh and trh genes). While the frequency of Vp positive samples was 1.7 fold greater compared to a study conducted three decades ago in New Zealand, only two (3.4%) Vp samples were found to carry the tdh gene and no sample was positive for the trh gene. The densities of pathogenic Vp (tdh) could not be determined. Additionally, there was no evidence of the presence of pandemic Vp serotype O3:K6 which has been associated with outbreaks in Japan and other parts of the world.

Vv was detected in 17.2% of oyster samples examined with a mean concentration of 7.4 MPN g^{-1}. Using a 16S rRNA genotyping assay only 58.8% of Vv isolates could be typed. Of those, where a genotype could be assigned, the majority (80%) of the isolates were assigned to Type A and 10% were assigned to both Type B and Type AB.

Although there appears to be a seasonal component driving the distribution of Vp and Vv, this could not be confirmed as the study was conducted during a relatively short period (summer season). There was no significant correlation (link) between any of the environmental parameters tested and Vp or Vv concentrations, at any growing site or when sites were pooled; except for a weak correlation between precipitation and Vp titres (R^2=0.13, P=0.0113).

The results indicate that whilst Vp and Vv may be present in shellfish growing areas in the North Island of New Zealand, the levels of pathogenic Vp (trh and tdh) and Vv are very low. This suggests that consumer exposure from eating commercially grown and harvested oysters is low. This is supported by the very low number of reported cases of Vp and Vv in New Zealand.

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CAWTHRON

A Survey of Commercially Harvested North Island Oysters *(Crassostrea gigas)* for *Vibrio parahaemolyticus* and *Vibrio vulnificus*

RESEARCH BASED SOLUTIONS

A Survey of Commercially Harvested North Island Oysters *(Crassostrea gigas)* for *Vibrio parahaemolyticus* and *Vibrio vulnificus*

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EXECUTIVE SUMMARY

A microbiological survey was conducted to determine the levels of total and pathogenic *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in Pacific oysters (*Crassostrea gigas*) collected from commercial growing areas in the North Island of New Zealand. The study was limited to the region of New Zealand where the environmental conditions are most likely to sustain Vp and Vv populations and to the summer period when the highest risk for Vp and Vv infections exists based on the environmental data and accepted scientific evidence.

Vp and Vv were enumerated using a method based on most probable number (MPN) utilising standard culture methods. In parallel real-time PCR was used to confirm Vp and Vv positive isolates.

Vp was detected in 94.8% of oyster samples examined with a mean density of 564 MPN g^{-1}. While the frequency of positive samples was 1.7 fold greater compared to a study conducted three decades ago in New Zealand, the Vp levels were comparable to those reported in United States of America oysters at the time of harvest using identical tests.

Real-time PCR was used to determine the presence of the potentially pathogenic Vp strains (*tdh* and *trh* genes). The Vp isolates carrying the *tdh* gene were detected in two samples (3.4%) but densities could not be determined. No sample was positive for the *trh* gene. Additionally, there was no evidence of the presence of pandemic Vp serotype O3:K6.

There was a significant (R^2=0.95, P<0.001) linear relationship between the Vp MPN estimates determined by real-time PCR and by standard culture methods, indicating good agreement between molecular-based and cultivation-based tests. The real-time PCR method has the additional benefit that it can determine and quantify pathogenic fractions of Vp.

Vv was detected in 17.2% of oyster samples examined with a mean concentration of 7.4 MPN g^{-1}.

Using a 16S rRNA genotyping assay only 58.8% of Vv isolates could be typed. Of those, where a genotype could be assigned, the majority (80%) of the isolates belonged to Type A and 10% were assigned to both Type B and Type AB. These results are similar to those reported for *V. vulnificus* isolated from oysters harvested from the Pacific and Gulf States of the United States of America. The high level of isolates that could not be typed is interesting and warrants further investigation.

There was no significant correlation between Vp and Vv concentrations (R^2=0.055, P=0.4880). Vp densities exceeded those of Vv in all the oyster samples tested.

Although there appears to be a seasonal component driving the distribution of Vp and Vv, this could not be confirmed as the study was conducted during a relatively short period (summer season). There was no significant correlation between any of the environmental parameters tested and Vp or Vv concentrations, at any given site or when sites were pooled; except for a weak correlation between precipitation and Vp titres (R^2=0.13, P=0.0113).

Association with other factors could not be established due to the limited nature of the environmental dataset, and because there was little change in environmental parameters during the study.

Adequate control measures are currently in place to ensure the quality of the oysters harvested in New Zealand.

TABLE OF CONTENTS

EXECUTIVE SUMMARY III
1. BACKGROUND V
2. INTRODUCTION 2
2.1. Ecology 2
2.2. Virulence 2
3. STUDY OUTLINE 3
3.1. Sampling and environment 3
3.2. Laboratory procedures 6
3.2.1. Enrichment and selective plating 6
3.2.2. Molecular and immunological analyses 6
4. *VIBRIO PARAHEMOLYTICUS* IN NORTH ISLAND OYSTERS 7
5. *VIBRIO VULNIFICUS* IN NORTH ISLAND OYSTERS 11
6. SUMMARY 14
7. ACKNOWLEDGEMENTS 15
8. REFERENCES 16
9. APPENDICES 19

LIST OF FIGURES

Figure 1. Locations of oyster farms participating in the study. 4
Figure 2. Water temperature, salinity, turbidity and precipitations at the study sites. 5
Figure 3. Concentrations of *Vibrio parahaemolyticus* (Vp) in Pacific oyster samples studied. 8
Figure 4. Comparison of *Vibrio parahaemolyticus* most probable number (MPN) estimates achieved by real-time PCR and selective plating. 9
Figure 5. Linear regression analyses of *Vibrio parahaemolyticus* (Vp) data. 10
Figure 6. Concentrations of *Vibrio vulnificus* in Pacific oyster samples studied. 12
Figure 7. Linear regression analyses of *Vibrio vulnificus* concentration data. 13
Figure 8. Genotypes of *Vibrio vulnificus* isolates from New Zealand oysters. 14

LIST OF TABLES

Table 1. Distribution of *Vibrio parahaemolyticus* (Vp) in the study area. 7
Table 2. Production method and distribution of *Vibrio parahaemolyticus* positive samples 11
Table 3. Distribution of *Vibrio vulnificus* positive samples in the study area. 11
Table 4. Production method and distribution of *Vibrio vulnificus* positive samples. 13

LIST OF APPENDICES

Appendix 1. Environmental dataset and corresponding results for each sampling site and date. 19

1. BACKGROUND

The New Zealand Food Safety Authority (NZFSA) is committed to protecting consumers of New Zealand food worldwide. It is the role of NZFSA to ensure that food produced in New Zealand is safe, suitable and meets all national and export requirements.

There has been an increase in the worldwide incidence of food-borne *Vibrio* disease (European Commission 2001) and, therefore, the health risk associated with virulent *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) is a major concern. These pathogens are usually associated with the consumption of raw or lightly cooked seafood from warm temperate waters. However, in the last few years there have been Vp-associated outbreaks reported at cooler water temperatures, *e.g.* in Alaska (Nart 2004) and southern Chile (Fuenzalida *et al.* 2006). A number of possible reasons for variations in geographical distribution have been proposed including; climate change, adaptation of pathogens to cooler waters, emerging strains and distribution via ballast water.

A New Zealand survey conducted 1982-1983 found low levels of Vp present in 57% of North Island Pacific oysters (*Crassostrea gigas*) sampled, but 95% of these contained less than 10 colony forming units (CFU) per gram (g) and only one contained levels greater than 1,000 CFU per g (Fletcher 1985). The study identified the food poisoning hazard from Vp as minimal. The proportion of virulent Vp in New Zealand remains unknown (Lake *et al.* 2003). Outbreaks traced back to Vp infections have been recorded in New South Wales (Kraa 1995), also a link between Vp-related gastroenteritis in New Zealand and imported seafood from Pacific Islands has been indicated (Thornton *et al.* 2002). It is estimated that the annual rate of Vp-related incidences is approximately 0.5 per 100,000 of population in New Zealand, although this could be an underestimation due to limited testing, and high dependency on ethnicity as well as geographic region (Lake *et al.* 2003). The recent death of a patient at Whangarei Hospital due to Vp wound infection caused by an anchor accident off the coast of northern New Zealand (Payinda 2008), and another fatal Vv wound infection (Upton & Taylor 2002), indicates that NZFSA should not be complacent on this issue.

Vp and Vv are not notifiable food-borne diseases in New Zealand, as there have not been any reports of Vp- or Vv-related illness associated with consumption of commercially harvested seafood in New Zealand (Lake *et al.* 2003). Currently, no statutory limits exist for Vp and Vv concentrations for seafood in New Zealand, although adequate control measures (NZFSA 2006) are in place to ensure the quality of the oysters produced in New Zealand, therefore health risk is limited. A risk assessment study of Vv infections from New Zealand oysters concluded that the commercial oyster fishing environment is not conducive to Vv mainly due to high salinity of the growing areas (McCoubrey 1996). However, given the recent lethal Vp and Vv infections off the coast of northern New Zealand (Upton & Taylor 2002; Payinda 2008), changes in the global geographical distribution of pathogenic Vp, the emergence of a highly virulent pandemic Vp strain O3:K6, environmental changes and current industry practices, it is timely to revisit and reassess the conclusions reached in 1996 and 2003.

Due to this concern the survey of oysters (*Crassostrea gigas*) harvested from North Island growing areas was conducted by Cawthron Institute, United States of America Food and Drug Administration (US FDA), Environmental Science and Research (ESR) and NZFSA to determine:

1. The total numbers of Vp and Vv present in oysters harvested from targeted North Island growing areas where environmental conditions were favourable for their presence and proliferation.
2. The proportion of pathogenic Vp present, relative to the total number of Vp, using molecular methods.

2. INTRODUCTION

2.1. Ecology

Vibrios are perhaps the most dominant heterotrophic bacteria in estuarine and marine waters. Vp and Vv are part of the natural flora of estuarine and coastal marine environments worldwide and have been isolated from water, sediments, and a variety of seafood (EC 2001; Lake *et al.* 2003; Jones & Oliver 2009).

Vp can grow at a wide range of temperatures (5-45ºC), while the optimum temperature is 37-38ºC. Vp tolerates a wide range (<1-96 parts per thousand (ppt)) of salinity with an optimum between 17-23 ppt. Likewise, Vv grows in a wide range of temperatures (9-40ºC) and salinity (5-35 ppt), but the optimal growth temperature and salinity range are somewhat lower, 30ºC and 5-10 ppt respectively (Lake *et al.* 2003). Water temperatures and salinities in most New Zealand oyster growing areas are not optimal for growth of Vv and Vp, however, both taxa would be able to survive and grow in New Zealand conditions.

2.2. Virulence

Vp is a leading cause of gastroenteritis linked to seafood consumption (Mead *et al.* 1999; EC 2001), although the vast majority of environmental Vp isolates are non-virulent (EC 2001). Molecular epidemiological studies have linked pathogenicity to strains mainly carrying *tdh* (thermostable direct hemolysin) (Nishibuchi *et al.* 1992) and/or *trh* (thermostable-related hemolysin) gene (Nishibuchi *et al.* 1989). Hemolysin is an enzyme that lyses (breaks down) red blood cells on Wagatsuma blood agar plates, which is referred to as the Kanagawa phenomenon. The expression mechanism of those genes is poorly understood. Based on the studies conducted in different regions of the world, roughly 0-3% of environmental Vp isolates are potentially pathogenic (Lake *et al.* 2003; Center for Food Safety and Applied Nutrition 2005; Nordstrom *et al.* 2007). Since 1995, a highly virulent pandemic *tdh* positive, but *tdr* negative serovar (group of bacteria grouped together based on the cell surface antigens) O3:K6

has emerged, causing large outbreaks in Africa, the Americas, Asia, and Europe (Boyd *et al.* 2008).
Human feeding trials have suggested that relatively high dosage (>10^5) of the virulent Vp strain are needed for onset of illness, and a lower infectious dose for O3:K6 serotype has been suggested (Daniels *et al.* 2000). In a relatively recent study (McLaughlin *et al.* 2005), an outbreak was linked to relatively low (2.1-3.5 MPN g^{-1}) Vp concentrations in raw oysters.

Vv is known to be a highly invasive bacteria, having the highest rate of mortality for any food-borne disease (Mead *et al.* 1999). Infections can occur via wound exposure and seafood consumption. They can demonstrate different clinical presentations, of which primary septicaemia, and gastroenteritis are the most important (EC 2001). Infections are severe (case to fatality rate approximately 50%), but rare, and usually limited to immunocompromised individuals or individuals with pre-existing chronic illnesses (EC 2001; WHO/FAO 2005).

The virulence is complex and not well understood and in addition several genes (*vvhA, vvpE, rtxA1*) and mechanisms (*e.g.* iron acquisition) have been indicated (Jones & Oliver 2009). None of the current detection methods are able to reliably distinguish between virulent and non-virulent strains of Vv (Campbell & Wright 2003) Type A and B sequence variants of the 16S rRNA gene of Vv have been identified (Aznar *et al.* 1994), as have isolates expressing both alleles (Type AB) (Vickery *et al.* 2007). Type B variants have been associated with higher potential virulence in the majority of Vv-related clinical fatalities (Nilsson *et al.* 2003; Vickery *et al.* 2007). However, another recent study has identified up to 53% of clinical isolates belonging to Type A in Florida (Gordon *et al.* 2008), indicating that 16S RNA type might be more related to geographical variation in the population structure, rather than a reliable virulence indicator.

3. STUDY OUTLINE

3.1. Sampling and environment

A total of 58 samples of Pacific oysters (*Crassostrea gigas*) were collected from six oyster growing areas (indicated as Sites A, B, C, D, E, F in this report) located in the North Island, New Zealand (Figure 1). Sites were predetermined by the NZFSA, based on the assessment of previous environmental data (McCoubrey 1996; Lake *et al.* 2003).

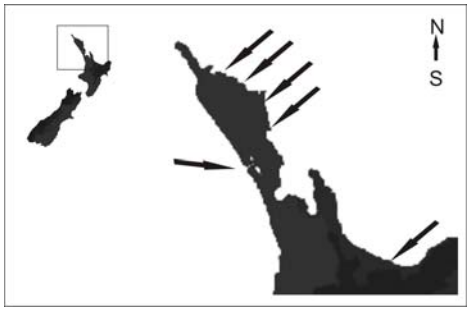

Figure 1. Locations of oyster farms participating in the study.

Samples were collected fortnightly by volunteering oyster farmers during the period 12 December 2008 to 15 April 2009. Salinity, tidal condition, wind direction, water and air temperature, oyster production method and distance from the sea floor were logged by volunteers during each sampling event (Figure 2, Appendix 1). Precipitation data were provided by Elizabeth Watts (Northland Health Board) and Cameron Ormsby (Auckland Regional Public Health Service). No precipitation data could be obtained for Site C. During the study period the water temperature varied from 16.0-31.5˚C, with a mean of 20.6˚C and all except one sample being below 24.8˚C. The salinity ranged from 13.4 to 36.5 ppt with a mean value of 34.1 ppt, and all except one sample being above 27.5 ppt. The low salinity outlier (13.4 ppt) appears to be a true result and due to a preceding rainfall event, while the cause of the temperature outlier (31.5ºC) is unknown. Turbidity and precipitation varied widely over the study period (1.1-590 NTU (nephelometric turbidity units) and 0-268 mm 14 day^{-1} respectively), with major rain events late February to early March. Collected oysters were grown in bags (31%), racks (14%) or on sticks (55%); 0.3-2.0 m from the sea floor (mean 0.7 m). Oysters were collected during different tidal regimes and shipped on ice to Cawthron's laboratory and analysed within 24-30 hours following collection. Temperature of the received samples was recorded to confirm that Vp and Vv levels would be reflective of those at harvest. All of the samples arrived at 4.8-14.3ºC temperature range, except for the three samples which arrived at 17.5, 18.1 and 19.0ºC. Oyster samples were analysed immediately after arrival as described in Section 3.2; samples were never frozen.

It should be noted that the study was conducted during the summer months and limited harvest was conducted in the farms selected for commercial use. In addition, the farms were regulated and did comply with regulations set by the NZFSA (Animal Products (Specifications for Bivalve Molluscan Shellfish) Notice 2006 (NZFSA 2006)).

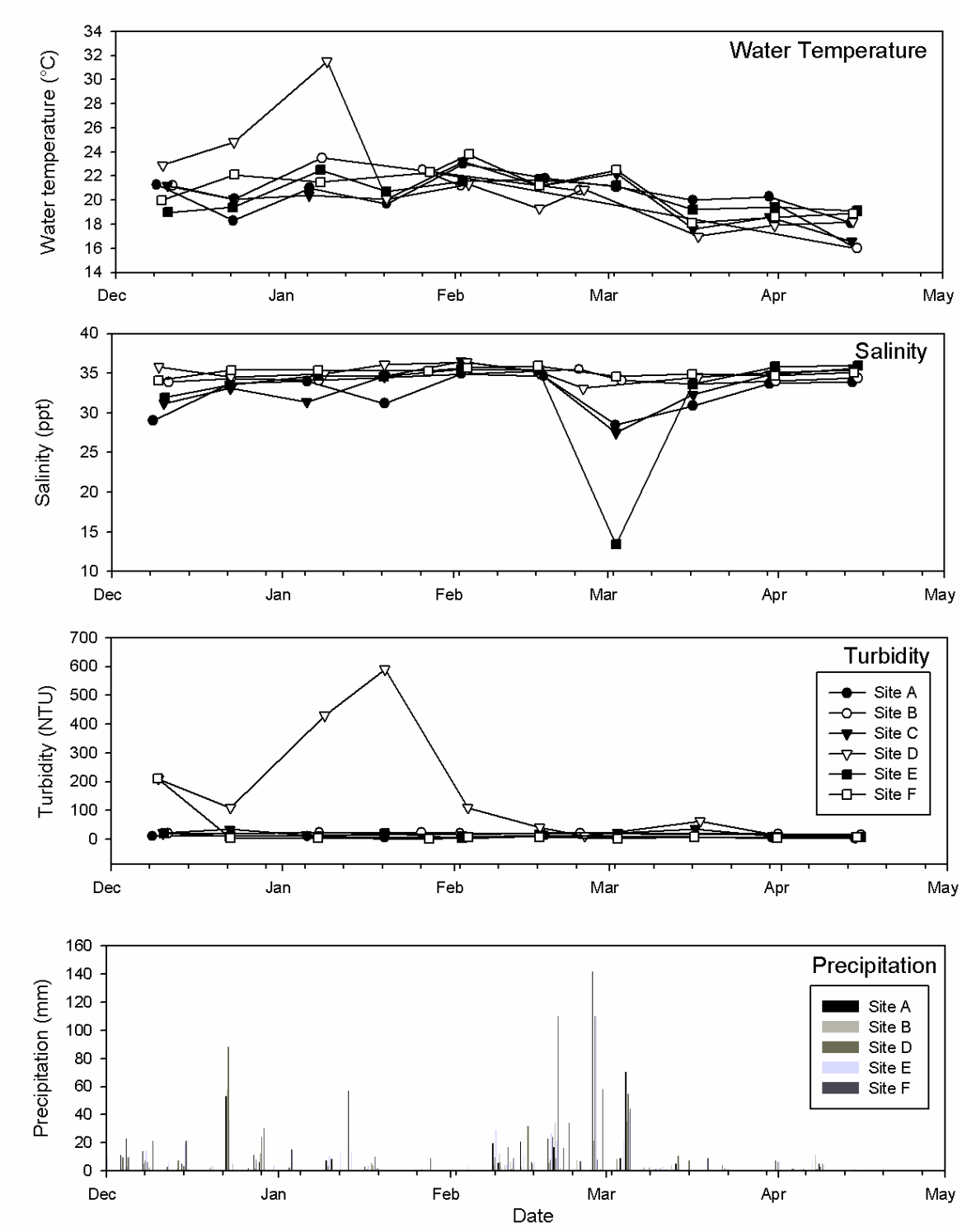

Figure 2. Water temperature, salinity, turbidity and precipitations at the study sites.

3.2. Laboratory procedures

3.2.1. Enrichment and selective plating

Oyster samples were analysed at Cawthron using ISO 17025-accredited method for Vp and Vv. Oysters (10-12) were scrubbed and rinsed under running tap water and shucked as described by American Public Health Association (1970). Samples were diluted (1:10) in phosphate buffered saline (PBS-vibrio) and blended at high speed for 1 minute and serially diluted in PBS-vibrio. Alkaline Peptone Water (APW) tubes were innoculated with 1 ml of each dilution in triplicate. The tubes were incubated for 18-24 hours at 35ºC. One loopful from the top 1 cm of each turbid broth tube was streaked onto TCBS agar, CHROMagar™ Vibrio (CHROMagar, Paris, France) and Modified Marine Agar (MCC), and incubated at 35ºC for 18-24 hours, except MCC plates which were incubated at 37ºC.

Vibro parahaemolyticus
When green colonies growing on TCBS and mauve colonies growing on CHROMagar™ Vibrio were observed, these were then streaked onto blood agar and incubated overnight. Pure cultures were tested for oxidase reactions (positive) and the ability to grow in the absence of NaCl (no growth). Presumptive Vp colonies were further confirmed using API 20 NE test strips.

Vibro vulnificus
When yellow colonies were observed growing on MCC they were presumptively identified as Vv, streaked onto blood agar, incubated, and further screened for Vv by performing an oxidase test (positive), the ability to grow in the absence of salt (no growth) and using API 20 NE test strips. The API results were, however, often inconclusive and inconsistent. The methodology was therefore modified by streaking suspicious colonies on MCC back onto TCBS (green colonies) and CHROMagar™Vibrio (aqua colonies) before any confirmation steps were performed, and the use of API 20 NE test strips was discontinued. Results were recorded as presumptive for Vv.

Tubes identified as presumptively positive were used to calculate the most probable number (MPN) for each sample by using a MPN chart and formulas (Swanson *et al.* 2001).

3.2.2. Molecular and immunological analyses

One Vp and one Vv isolate (identified as presumptive positives by selective plating) per turbid APW enrichment dilution series was re-innoculated into APW and incubated for 18-24 hours at 35ºC. Two millilitres of culture were boiled for 10 minutes, cooled and stored at -70ºC until being shipped to the United States of America Food and Drug Administration, Division of Seafood Science and Technology (Dauphin Island, AL, USA), where all molecular tests (real-time PCR analyses) were conducted. A total of 280 Vp and 18 Vv isolates were shipped and tested by the US FDA. In addition, 2 ml of each turbid APW enrichment culture collected

during the last three sampling occasions (18 samples in total), were treated and shipped as above.

Multiplex real-time PCR analyses were used to confirm Vp (*tlh* gene) and identify potentially pathogenic strains (*tdh* and *trh* genes) as described in Nordstrom *et al.* (2007). An unpublished multiplex real-time PCR assay based on Campbell & Wright (2003) and Vickery *et al.* (2007) was used for identification of 16S ribotypes of Vv. Internal amplification controls (IAC) were used in all tests to avoid reporting of false negatives due to inhibition from the sample matrix. Analyses were conducted on a SmartCycler II system (Cepheid, Sunnyvale, CA).

Samples which were identified as *tdh* positive, based on the real-time PCR, were sent to the Institute of Environmental Science and Research (Wellington, New Zealand) to determine whether they were pandemic strain O3:K6. This was undertaken using O and K antisera.

4. *VIBRIO PARAHEMOLYTICUS* IN NORTH ISLAND OYSTERS

A total of 55 (94.8%) oyster samples out of 58 samples collected were identified positive for Vp (Table 1). This is a very high portion of positive samples, similar to the concentrations found in mussels in Brazil (Matte *et al.* 1994), and is roughly a 1.7 fold increase compared to the earlier studies in 1982-1982, when 57% of oyster samples were identified positive for Vp in New Zealand (Fletcher 1985). Vp-positive samples were relatively evenly distributed among the sampling sites, with 87.5-100% of samples being positive at each location. The molecular analyses targeting *tlh* gene confirmed 274 isolates out of 280 tested (97.8%) as Vp. False positive isolates (2.2%) did not affect sample-based evaluation. Two consecutive samples collected on 3 and 16 February 2009 at Site F (Table 1), were identified positive for *tdh* gene (*tdh*+), known to be associated with the virulence of Vp. Therefore 3.4% of all samples collected were potentially pathogenic (Table 1). No sample was positive for the *trh* gene. The proportion of *tdh* positive isolates was comparable to concentrations found in oysters collected in the United States of America (WHO/FAO 2002), being intermediate between those found in United States of America Pacific and Atlantic coasts (Cook *et al.* 2002a; Center for Food Safety and Applied Nutrition 2005). None of the three samples which arrived at elevated temperatures was *tdh* positive.

Table 1. Distribution of *Vibrio parahaemolyticus* (Vp) in the study area.

	Location						Total
	A	**B**	**C**	**D**	**E**	**F**	
Number of samples analysed	10	8	10	10	10	10	58
Number of Vp positive samples	10 (100%)	7 (87.5%)	9 (90%)	10 (100%)	10 (100%)	9 (90%)	55 (94.8%)
Number of Vp (*tdh*+) positive samples	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (20%)	2 (3.4%)

Vp concentrations varied from below detection limit (<3 MPN g^{-1}) to 1500 MPN g^{-1} with an arithmetic mean concentration 564 MPN g^{-1} (Figure 3) in Pacific oysters. Vp concentrations exceeded 1000 MPN g^{-1} in 8.6% of samples collected. The range and mean of Vp concentrations was comparable to concentrations detected in the United States of America and Japan (DePaola *et al.* 2000; Cook *et al.* 2002b; Hara-Kudo *et al.* 2003). The three samples which arrived at elevated temperatures (17.5, 18.1 and 19.0ºC), contained moderate to high levels (460, 4600 and 460 MPN g^{-1} correspondingly) of Vp.

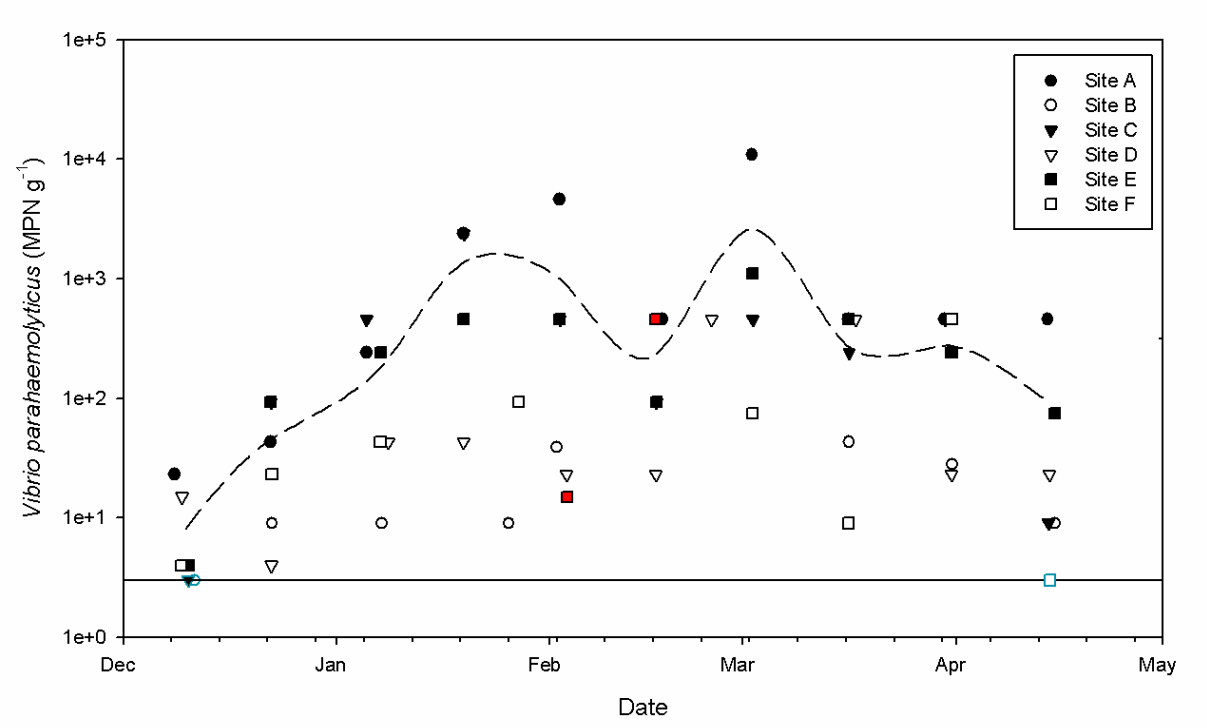

Figure 3. Concentrations of *Vibrio parahaemolyticus* (Vp) in Pacific oyster samples studied. Major rain events (50+ mm of precipitation in most of the locations) occurring 23/12/2008, 4/1/2009, 27/2/2009 and 3/5/2009.
Solid black line indicates the detection limit of the assay, dashed trend-line indicates average Vp concentrations pooled for all sites; red indicates *tdh*+ samples and blue edge color indicates samples with concentrations below detection limit.

Concentration of *tdh* positive Vp was equal or less than 15 or 460 MPN g^{-1} in both positive samples correspondingly. These concentrations are relatively low, as probability of illness is <0.001% when concentrations are 50 cells per gram of oysters, although probability of illness is about 50% when concentrations reach $5x10^5$ cells per gram of oyster (Center for Food Safety and Applied Nutrition 2005). However, much lower levels (<200 CFU g^{-1}) have been found in oysters linked with Vp disease outbreaks in the United States of America and Canada (Centers for Disease Control and Prevention 1999). As Vp concentrations as low as 2.1-3.5 MPN g^{-1} have been linked to a Vp outbreak (McLaughlin *et al.* 2005), concentrations detected in the current study could have been at levels sufficient to onset the disease.

The actual concentration for *tdh* positive fraction could not be determined as most of the APW enrichments in the MPN series were not analysed by real-time PCR. APW enrichments were analysed directly by real-time PCR for the last three consecutive sampling rounds for each site (18 samples in total) and corresponding MPN identified. There was a significant (P<0.001) linear relationship between the Vp MPN estimates by real-time PCR and selective plating, the values were identical in 72% of cases (Figure 4).

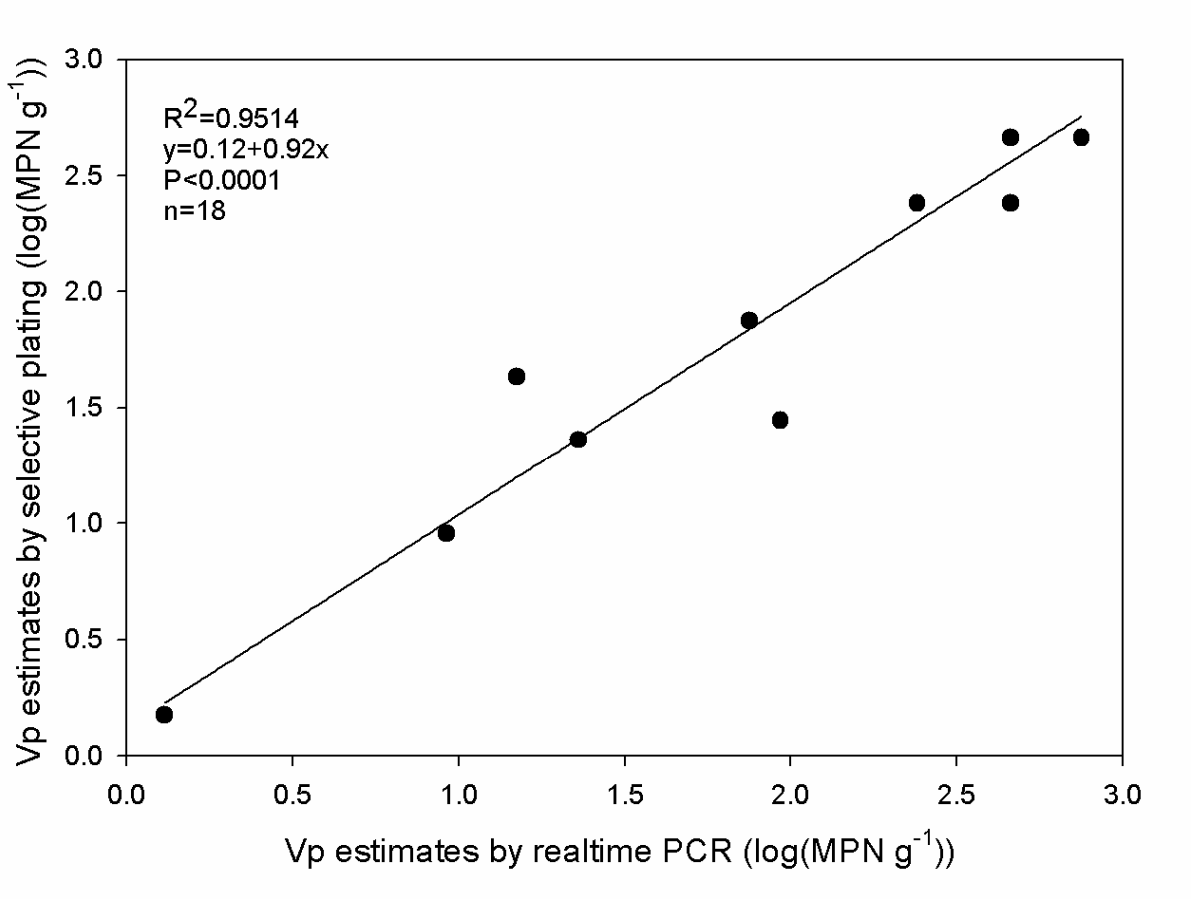

Figure 4. Comparison of *Vibrio parahaemolyticus* most probable number (MPN) estimates achieved by real-time PCR and selective plating.

While the seasonal trend in Vp densities, peaking late January to March, appears to be evident (Figure 3), there was no significant correlation between Vp concentrations and any of the environmental parameters studied when data from all sites were pooled (Figure 5) or analysed for each site independently. A weak correlation between precipitation and Vp MPN estimates (R^2=0.131, P=0.113) was observed. Rain may reduce salinities to ranges that are more optimal for Vp and Vv (especially Vv, which is often non-detectable at salinities >30 ppt). Additionally, this weak correlation might be coupled with other parameters not studied. For example, it has been shown that sediment re-suspension can be a significant source of *Vibrio sp.* in the water column and in oysters (Zimmerman *et al.* 2007; Fries *et al.* 2008). Therefore it is possible that strong wind patterns often associated with rain events might be contributing factors to high Vp concentrations during the summer season in New Zealand. Lack of correlation between Vp concentrations and salinity or temperature is somewhat surprising as both are the key elements controlling Vp distribution (WHO/FAO 2002). This might be because temperature and salinity varied little during the study period in the area. The majority of the samples were collected from the narrow range of temperature (18-24˚C) and salinity (31 ppt and above) (Figure 2). Furthermore, as bacteria are accumulated over time in oysters, in highly fluctuating environments such as coastal zones, use of continuous data loggers should be advocated as a single measure at a single time point has little value. Correlation between precipitation data which was pooled over 14 days and Vp concentrations appears to support this argument. It should be noted that the temperature and salinity of Vp *tdh* positive samples was above average (Figure 5).

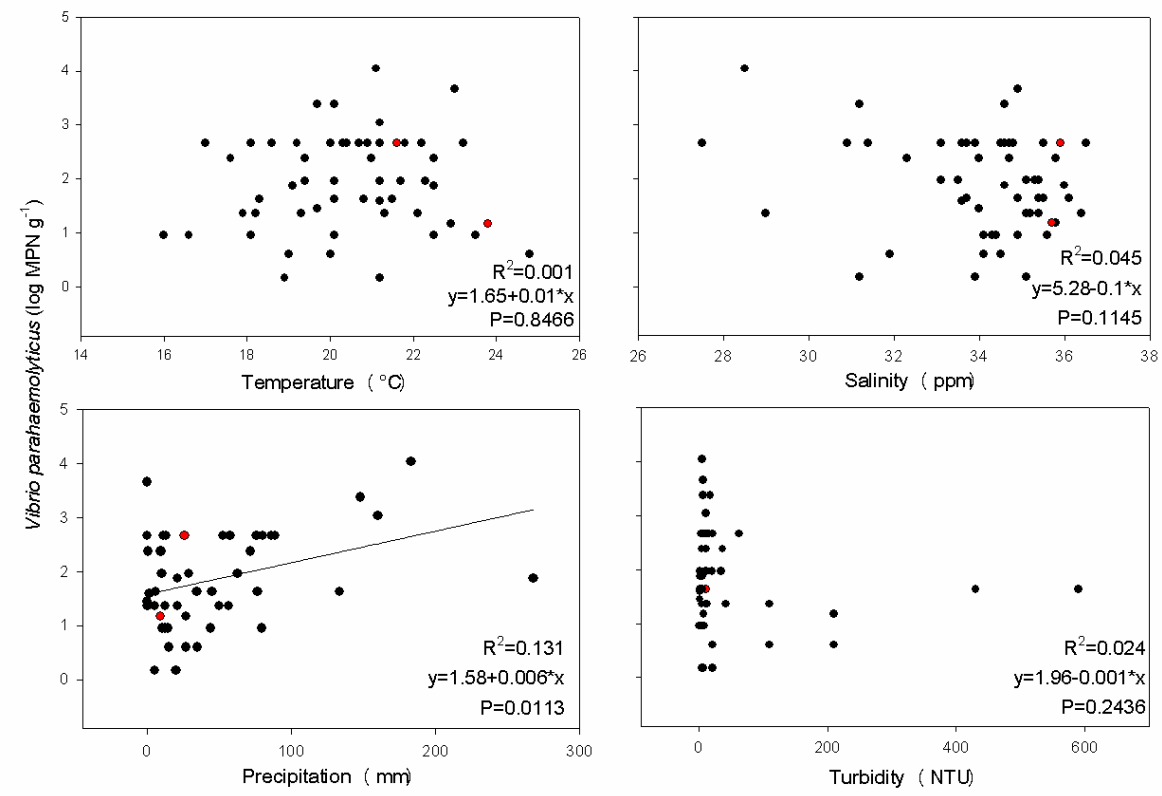

Figure 5. Linear regression analyses of *Vibrio parahaemolyticus* (Vp) data. Vp (*tdh*+) samples are indicated in red. Precipitation measurements were pooled over last 14 days before each sample collection.

There was also no significant correlation between the distance from the sea floor and Vp concentrations when sites were pooled (R^2=0.02, P=0.255) or analysed independently.

Production method appears to have little or no impact on Vp concentrations in Pacific oysters (Table 2). Vp was identified in 88%, 100% and 94% of samples collected from bags, racks or sticks respectively. Both Vp *tdh* positive isolates were isolated from oysters grown on sticks.

Table 2. Production method and distribution of *Vibrio parahaemolyticus* positive samples

	Production Method			Total
	Bag	**Rack**	**Stick**	
Number of samples analysed	18(31%)	8(14%)	32(55%)	58
Number of Vp positive samples	16(88.8%)	8(100%)	30(94%)	55(94.8%)
Number of Vp (*tdh*+) positive samples	0(0%)	0(0%)	2(6%)	2 (3.4%)

Two isolates which tested positive for the *tdh* gene, based on the real-time PCR, were serotyped by ESR (Wellington) using O and K antisera by ESR. The isolates did not agglutinate with O3 and K6 antisera. Therefore the presence of pandemic serotype O3:K6 was not confirmed in New Zealand coastal waters. However, Vp readily seroconverts (an array of clonally related serotypes O4:K68, O6:K18, O1:K25, O1:K41 or O1:Kuk have been identified) and therefore further molecular tests (Okura *et al.* 2004; Davis *et al.* 2007) are warranted.

5. *VIBRIO VULNIFICUS* IN NORTH ISLAND OYSTERS

A total of 10 (17.2%), out of 58 oyster samples collected, were identified positive for Vv (Table 3). All sites were positive for Vv at some point during the study (~10-40% samples being positive) (Table 3). There was a good agreement between identification based on the selective plating and real-time PCR method, as only one isolated (5.5%) was not confirmed by the molecular test.

Table 3. Distribution of *Vibrio vulnificus* positive samples in the study area.

	Location						Total
	A	**B**	**C**	**D**	**E**	**F**	
Number of samples analysed	10	8	10	10	10	10	58
Number of Vv positive samples	2 (20%)	1 (12.5%)	1 (10%)	1 (10%)	4 (40%)	1 (10%)	10 (17.2%)

All positive samples confirmed by real-time PCR.

Vv concentrations were low and varied from below detection limit to 10 MPN g^{-1} in the oysters sampled (Figure 6). The three samples which arrived at elevated temperatures (17.5,

18.1 and 19.0ºC), contained no or low levels (3, 9 and <3 MPN g^{-1} correspondingly) of Vv. All oysters tested would meet "treated to reduce to non detectable levels" labelling definition (<30 MPN of Vp or Vv g^{-1}) (FDA USDoHaHSPHS 2007), approved in several states in the United States of America, without any further treatment.

There was no significant correlation between Vp and Vv concentrations (R^2=0.055, P=0.4880). Vp densities exceeded those of Vv in all oyster samples tested.

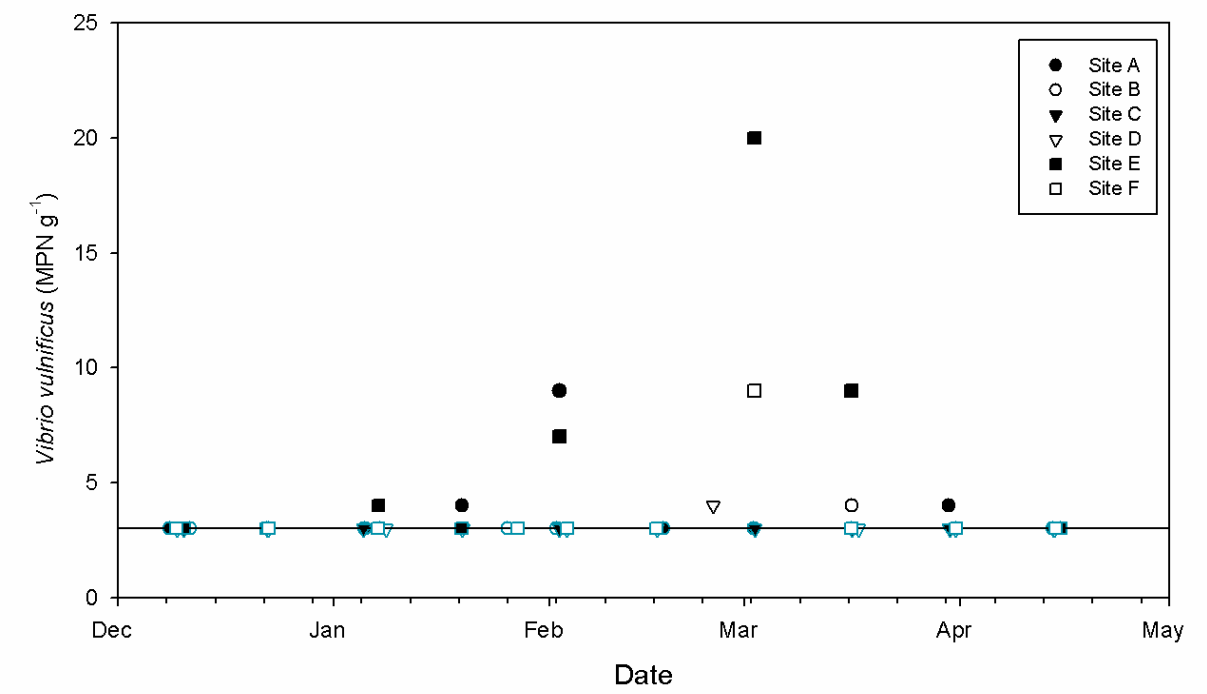

Figure 6. Concentrations of *Vibrio vulnificus* in Pacific oyster samples studied.
Solid black line indicates the detection limit of the assay; blue edge color indicates samples with concentrations below detection limit.

There was no significant correlation between Vv concentrations and any environmental parameter studied when data from all sites was pooled (Figure 7). This contradicts earlier findings (McCoubrey 1996), where temperature and salinity have been identified as key factors controlling Vv distribution. Temperature and salinity varied little throughout the study period and the majority of the samples were collected from a narrow range of temperatures (18-24˚C) and salinities (31 ppt and above) (Figure 2). Furthermore, in highly fluctuating environments such as coastal zones, use of continuous data loggers should be advocated as a single measure at a single time point has little value. There were too few Vv positive samples to enable a site by site comparison.

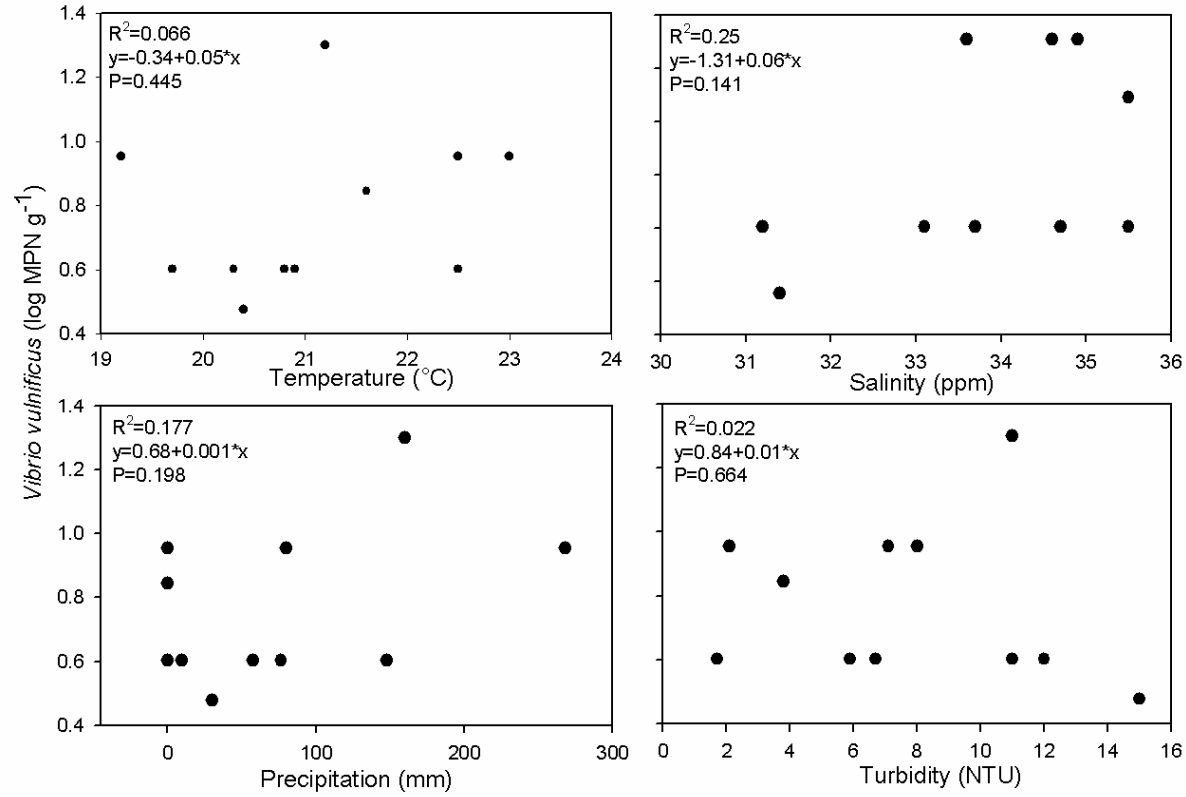

Figure 7. Linear regression analyses of *Vibrio vulnificus* concentration data. Precipitation measurements were pooled over the last 14 days before each sample collection

Vv positive oysters were grown on stick, bag and rack. Oysters grown on sticks were most often positive for Vv (21.8%), while bag- and rack-grown material was positive for Vv 11.1% and 12.5 % of cases respectively (Table 4).

Table 4. Production method and distribution of *Vibrio vulnificus* positive samples.

	Production Method			Total
	Bag	Rack	Stick	
Number of samples analysed	18 (31%)	8 (14%)	32 (55%)	58
Number of Vv positive samples	2 (11.1%)	1 (12.5%)	7 (21.8%)	10 (17.2%)

16S rRNA gene typing could not be identified for eight Vv isolates (41.2%) originating from seven samples (Figure 8). This is intriguing as these isolates might belong to a yet unknown type of Vv. When only isolates that could be typed were compared (n=10), the majority of the isolates (80%) belonged to Type A, one isolate was identified as Type B and one as Type AB (8:1:1 A:B:AB ratio). This is in agreement with other studies (Vickery *et al.* 2007; Gordon *et al.* 2008), where 92% of Vp isolates from oysters grown at sites characterised by good water quality have been reported to belong to Type A or Type AB.

As discussed in Section 2.2, dominance of Type A could indicate lower potential virulence of Vv in the area and associated health risk.

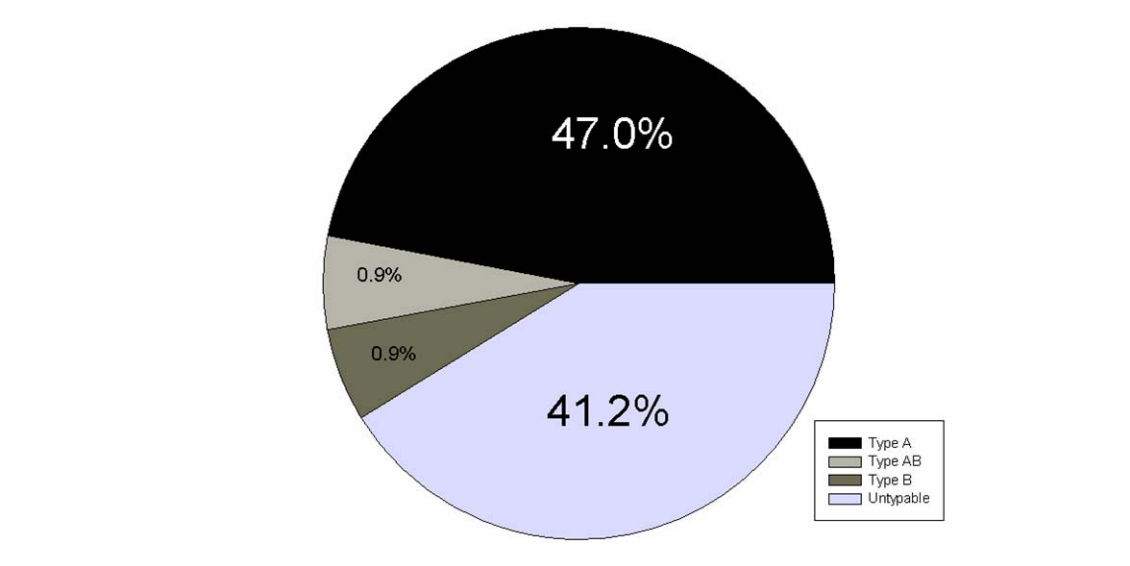

Figure 8. Genotypes of *Vibrio vulnificus* isolates from New Zealand oysters.

6. SUMMARY

This study identified high prevalence of Vp (94.8% positive samples) in Pacific oysters collected in the North Island of New Zealand. However, the proportion of potentially pathogenic samples (3.4%) was comparable to estimates in the United States of America and less than identified in Asian countries. It should be noted that potentially pathogenic Vp isolates were identified only at one site, and the study was conducted during the summer months and in selected regions where the environmental conditions are associated with the highest risk of Vp infections.

Pandemic serotype O3:K6 was not identified among the *tdh* positive Vp isolates. The pandemic strain is known to readily seroconvert and thus *tdh* positive isolates should be further investigated using molecular methods.

Vv was detected in 17.2% of oyster samples studied and the prevalence of Type A among Vv isolates in New Zealand could indicate lower virulence and associated human health risk, however the predominant genotype in New Zealand's clinical cases is unknown.

Although there appears to be a seasonal component driving the distribution of Vp and Vv, no significant correlation was detected between any of the environmental parameters tested and Vp and Vv concentration at any given site, or when sites were pooled, except for a weak correlation between precipitation and Vp titres (R^2=0.13, P=0.113). Association with other

factors could not be established as environmental parameters changed little during the study and the environmental dataset was limited. Further studies are needed to identify relevant factors determining the distribution of Vp and Vv populations in New Zealand.

There was a significant (R^2=0.95, P<0.001) linear relationship between the Vp MPN estimates by real-time PCR and selective plating, indicating good agreement between molecular- and cultivation-based techniques. In addition, 98.2% of Vv and 94.5 % of Vv isolates were confirmed by molecular tests. This indicates that molecular techniques (such as real-time PCR) should be used to complement culture-based techniques as they offer cost-effective rapid means to confirm cultivation-based identification and quantify potentially pathogenic fractions of Vibrios.

NZFSA receives information on all laboratory-confirmed Vp or Vv incidents in New Zealand and there has not been Vp or Vv illness related to the consumption of seafood produced in New Zealand (NZFSA, personal communication). Although no statutory limits exist for Vp and Vv concentrations in seafood, adequate control measures (NZFSA 2006) are in place to ensure the quality of the oysters produced in New Zealand. Therefore health risk associated with the consumption of raw oysters produced in New Zealand is limited.

7. ACKNOWLEDGEMENTS

We are very grateful to Dr. Dorothy-Jean McCoubrey for her work with the oyster industry, both in approaching the growing sites to participate and in assisting with the co-ordination of sampling. The authors thank Chris Cornelisen (Cawthron Institute) for valuable comments and discussions. We are very grateful to the anonymous oyster farmers for providing the samples and environmental data.

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9. APPENDICES

Appendix 1. Environmental dataset and corresponding results for each sampling site and date. *Vibrio parahaemolyticus tdh* positive samples are indicated in red.

Site	Status	Date	Salinity	Turbidity	Water Temp.[3]	Air Temp.	Tide	Distance from seafloor	Method for oyster growth	Vv[1]					Vp[2]					
										MPN[4]			Real-time PCR-Vv			MPN			Real-time PCR-Vp		
										95% confidence intervals						95% confidence intervals					
ID			ppt	NTU	˚C	˚C		m			Lower	Upper		16S type		Lower	Upper	*tdl*	*tdh*	trh
A	Auth.[5]	08-Dec-08	29	11	21.3	20.1	Incoming	0.8	Stick	<3	-	-	NA[6]	NA	23	9	130	-	-	-
A	Auth.	22-Dec-08	33.7	11	18.3	23.6	low	0.6	Stick	<3	-	-	NA	NA	43	10	210	+	-	-
A	Auth.	05-Jan-09	34	11	21	20	Incoming	0.6	Stick	<3	-	-	NA	NA	240	90	1400	+	-	-
A	Auth.	19-Jan-09	31.2	6.7	19.7	19.1	incoming	0.6	Stick	4	<1	21	+	a	2400	800	6400	+	-	-
A	Auth.	02-Feb-09	34.9	7.1	23	22	incoming	0.6	Stick	9	2	38	+	a	4600	1000	48000	+	-	-
A	Auth.	17-Feb-09	34.6	14	21.8	20.3	incoming	1.2	Stick	<3	-	-	NA	NA	460	100	2400	+	-	-
A	Auth.	02-Mar-09	28.5	5.3	21.1	22	incoming	0.6	Stick	<3	-	-	NA	NA	>11000	-	-	+	-	-
A	Auth.	16-Mar-09	30.9	7.3	20	14.2	incoming	0.6	Stick	<3	-	-	NA	NA	460	100	2400	+	-	-
A	Auth.	30-Mar-09	33.7	5.9	20.3	13.2	incoming	0.6	Stick	4	<1	21	+		460	100	2400	+	-	-
A	Auth.	14-Apr-09	33.9	5.1	18.1	11.3	incoming	1	Stick	<3	-	-	NA	NA	460	100	2400	+	-	-
B	Auth.	11-Dec-08	33.9	4.8	21.2	18	incoming	2	Bag	<3	-	-	NA	NA	<3	-	-	NA		
B	Auth.	22-Dec-08	34.3	1.1	20.1	-	incoming	0.9	Bag	<3	-	-	NA	NA	9	2	38	+	-	-
B	Auth.	07-Jan-09	34.1	3.8	23.5	n/a	incoming	0.9	Bag	<3	-	-	NA	NA	9	2	38	+	-	-
B	Auth.	23-Feb-09	35.5	1.7	20.8	22	low	1	Bag	NA	NA	NA	NA	NA	NA	NA	NA			
B	Auth.	03-Mar-09	34.1	1.1						NA	NA	NA	NA	NA	NA	NA	NA			
B	Auth.	16-Mar-09	33.6	1.7			incoming	0.9	Bag	4	<1	21	+		43	10	210	+	-	-
B	Auth.	31-Mar-09	34	1.9	19.7		incoming	1	Bag	<3	-	-	NA	NA	28	9	150			
B	Auth.	15-Apr-09	34.4	1.1	16	n/a	incoming	1.5	Bag	<3	-	-	NA	NA	9	2	38			
B	Auth.	26-Jan-09			22.5		outgoing	0.6	Bag	<3	-	-	NA	NA	9	2	38	+	-	-
B	Auth.	02-Feb-09			21.2		incoming	1.2	Bag	<3	-	-	NA	NA	39	10	180	+	-	-
C	Auth.	10-Dec-08	31.2	22	21.2	20.9	outgoing	0.7	Stick	<3	-	-	NA	NA	<3	-	-	NA	NA	NA
C	Auth.	22-Dec-08	33.1	35	20.1	18.6		0.7	Stick	<3	-	-	NA	NA	93	30	380	+	-	-
C	Auth.	05-Jan-09	31.4	15	20.4	12.2	outgoing	0.7	Stick	3	<1	17	-		460	100	2400	+	-	-

Site	Status	Date	Salinity	Turbidity	Water Temp.[3]	Air Temp.	Tide	Distance from seafloor	Method for oyster growth	Vv[1]				Vp[2]					
										MPN[4]		Real-time PCR-Vv		MPN			Real-time PCR-Vp		
										95% confidence intervals				95% confidence intervals					
ID			ppt	NTU	˚C	˚C		m		Lower	Upper		16S type		Lower	Upper	*tdl*	*tdh*	trh
C	Auth.	19-Jan-09	34.6	18	20.1	20.8	incoming	0.7	Stick	<3	-	-	NA	NA	2400	800	6400	+	-	-
C	Auth.	02-Feb-09	36.5	16	23.2	21.9	incoming	0.7	Stick	<3	-	-	NA	NA	460	100	2400	+	-	-
C	Auth.	16-Feb-09	35.1	21	21.2	17.8	incoming	0.7	Stick	<3	-	-	NA	NA	93	30	380	+	-	-
C	Auth.	02-Mar-09	27.5	22	22.2	21.5	incoming	0.7	Stick	<3	-	-	NA	NA	460	100	2400	+	-	-
C	Auth.	16-Mar-09	32.3	37	17.6	18	incoming	0.7	Stick	<3	-	-	NA	NA	240	90	1400	+	-	-
C	Auth.	30-Mar-09	34.8	11	18.6	15.8	incoming	0.7	Stick	<3	-	-	NA	NA	460	100	2400	+	-	-
C	Auth.	14-Apr-09	35.6	6.6	16.6	14.1	incoming	0.7	Stick	<3	-	-	NA	NA	9	2	38	+	-	-
D	Auth.	09-Dec-08	35.8	210	22.9	19.8	incoming	0.4	Bag	<3	-	-	NA	NA	15	5	50	+	-	-
D	Auth.	22-Dec-08	34.5	110	24.8	21.1	Incoming	0.4	Bag	<3	-	-	NA	NA	4	<1	21	+	-	-
D	Auth.	08-Jan-09	34.9	430	31.5	25.1	incoming	0.6	Bag	<3	-	-	NA	NA	43	10	210	+	-	-
D	Auth.	19-Jan-09	36.1	590	20.1	19	incoming	0.6	Bag	<3	-	-	NA	NA	43	10	210	+	-	-
D	Auth.	03-Feb-09	36.4	110	21.3	24.5	incoming	0.4	Bag	<3	-	-	NA	NA	23	9	130	+	-	-
D	Auth.	16-Feb-09	35.2	42	19.3	18.9	incoming	0.6	Bag	<3			NA	NA	23	9	130	+	-	-
D	Auth.	24-Feb-09	33.1	12	20.9	19.2	outgoing	0.6	Bag	4	<1	21	+	a	460	100	2400	+	-	-
D	Auth.	17-Mar-09	34.5	63	17	17.5	incoming	0.3	Bag	<3	-	-	NA	NA	460	100	2400	+	-	-
D	Auth.	31-Mar-09	35.1	13	17.9	14.7	incoming	0.6	Bag	<3	-	-	NA	NA	23	9	130	+	-	-
D	Auth.	14-Apr-09	35.4	12	18.2	17.8	outgoing	0.6	Bag	<3	-	-	NA	NA	23	9	130	+	-	-
E	Auth.	10-Dec-08	31.9	22	19	24.1	incoming	0.6	Rack	<3	-	-	NA	NA	4	<1	21	+	-	-
E	Auth.	22-Dec-08	33.5	11	19.4	20.5	outgoing	0.6	Rack	<3	-	-	NA	NA	93	30	380	+	-	-
E	Auth.	07-Jan-09	34.7	11	22.5	24.5	outgoing	0.6	Rack	4	<1	21	+	a	240	90	1400	+	-	-
E	Auth.	19-Jan-09	34.6	21	20.7	21.7	low tide	0.6	Rack	<3	-	-	NA	NA	460	100	2400	+	-	-
E	Auth.	02-Feb-09	35.5	3.8	21.6	21.3	incoming	0.6	Stick	7	2	28	+	a	460	100	2400	+	-	-
E	Auth.	16-Feb-09	35.4	11	21.7	21.8	incoming	1.2	Stick	<3	-	-	NA	NA	93	30	380	+	-	-
E	Auth.	02-Mar-09	13.4	11	21.2		incoming	1	Stick	20	7	60	+	a	1100	300	4800	+	-	-
E	Auth.	16-Mar-09	33.6	8	19.2	15.4	incoming	0.6	Stick	9	2	38	+	a	460	100	2400	+	-	-
E	Auth.	31-Mar-09	35.8	4.6	19.4	15.2	incoming	0.6	Stick	<3	-	-	NA	NA	240	90	1400	+	-	-
E	Auth.	15-Apr-09	36	6.2	19.1	15.3	incoming	0.6	Stick	<3	-	-	NA	NA	75	20	280	+	-	-
F	Auth.	09-Dec-08	34.1	210	20	17.5	incoming	0.6	Rack	<3	-	-	NA	NA	4	<1	21	+	-	-

Site	Status	Date	Salinity	Turbidity	Water Temp.[3]	Air Temp.	Tide	Distance from seafloor	Method for oyster growth	Vv[1]				Vp[2]					
										MPN[4]		Real-time PCR-Vv		MPN			Real-time PCR-Vp		
										95% confidence intervals				95% confidence intervals					
ID			ppt	NTU	˚C	˚C		m		Lower	Upper		16S type		Lower	Upper	*tdl*	*tdh*	trh
F	Auth.	22-Dec-08	35.4	4.5	22.1	22.7	outgoing	0.6	Rack	<3	-	-	NA	NA	23	9	130	+	-	-
F	Auth.	07-Jan-09	35.4	5.7	21.5	18	outgoing	0.6	Rack	<3	-	-	NA	NA	43	10	210	+	-	-
F	Auth.	27-Jan-09	35.3	1.5	22.3	21.8	low	0.6	Rack	<3	-	-	NA	NA	93	30	380	+	-	-
F	Auth.	03-Feb-09	35.7	7.8	23.8	23	incoming	0.6	Stick	<3	-	-	NA	NA	15	5	50	+	+	-
F	Auth.	16-Feb-09	35.9	8	21.2	19.3	outgoing	1.2	Stick	<3	-	-	NA	NA	460	100	2400	+	+	-
F	Auth.	02-Mar-09	34.6	2.1	22.5	21.5	incoming	1	Stick	9	2	38	+	a, b	75	20	280	+	-	-
F	Auth.	16-Mar-09	34.9	8.3	18.1	17	incoming	0.6	Stick	<3	-	-	NA	NA	9	2	38	+	-	-
F	Auth.	31-Mar-09	34.7	3.7	18.6	15.7	incoming	0.6	Stick	<3	-	-	NA	NA	460	100	2400	+	-	-
F	Auth.	14-Apr-09	35.1	7.4	18.9	18.8	Incoming	0.6	Stick	<3	-	-	NA	NA	<3	-	-	NA	-	-

1Vp – Vibrio parahaemolyticus, 2Vv – Vibrio vulnificus, 3Temp. – temperature, 4MPN- most probable number, 5Auth. – authorised, 6NA- not available)