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Long-Term Study of *Vibrio parahaemolyticus* Prevalence and Distribution in New Zealand Shellfish

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The food-borne pathogen *Vibrio parahaemolyticus* has been reported as being present in New Zealand (NZ) seawaters, but there have been no reported outbreaks of food-borne infection from commercially grown NZ seafood. Our study determined the current incidence of *V. parahaemolyticus* in NZ oysters and Greenshell mussels and the prevalence of *V. parahaemolyticus tdh* and *trh* strains. Pacific (235) and dredge (21) oyster samples and mussel samples (55) were obtained from commercial shellfish-growing areas between December 2009 and June 2012. Total *V. parahaemolyticus* numbers and the presence of pathogenic genes *tdh* and *trh* were determined using the FDA most-probable-number (MPN) method and confirmed using PCR analysis. In samples from the North Island of NZ, *V. parahaemolyticus* was detected in 81% of Pacific oysters and 34% of mussel samples, while the numbers of *V. parahaemolyticus tdh* and *trh* strains were low, with just 3/215 Pacific oyster samples carrying the *tdh* gene. *V. parahaemolyticus* organisms carrying *tdh* and *trh* were not detected in South Island samples, and *V. parahaemolyticus* was detected in just 1/21 dredge oyster and 2/16 mussel samples. Numbers of *V. parahaemolyticus* organisms increased when seawater temperatures were high, the season when most commercial shellfish-growing areas are not harvested. The numbers of *V. parahaemolyticus* organisms in samples exceeded 1,000 MPN/g only when the seawater temperatures exceeded 19°C, so this environmental parameter could be used as a trigger warning of potential hazard. There is some evidence that the total *V. parahaemolyticus* numbers increased compared with those reported from a previous 1981 to 1984 study, but the analytical methods differed significantly.

Because of the halophilic nature and marine habitat of *Vibrio parahaemolyticus*, raw seafood can naturally harbor this microorganism and is the main food source responsible for the gastroenteritis the microorganism causes (1). A recent report released by the U.S. Centers for Disease Control and Prevention estimated that the average annual incidence of *Vibrio* species infections in the United States increased by 43% from the 2006 to 2008 period to 2012 (2). The incidence rate of *V. parahaemolyticus* infection in New Zealand (NZ) is calculated to be 1.6/100,000, increasing to 15.3/100,000 in the Pacific Islander population, with most cases linked to imported seafood (3).

An international risk assessment of *V. parahaemolyticus* in raw seafood highlighted the importance of exposure to raw oysters based on the incidence of *V. parahaemolyticus* harboring the thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) genes at harvest (4).

NZ has two main islands (Fig. 1) that extend from latitudes 34° to 47° south. This means that there are diverse habitats and significant differences in water temperature along the length of the country, with cooler waters in the south. The first reported NZ isolation of *V. parahaemolyticus* was from Bay of Islands' shellfish (5). Subsequently, Fletcher (6) conducted a 3-year survey on the incidence of *V. parahaemolyticus* in NZ Pacific oysters (1981 to 1984), showing that high incidences of the microorganism were found when seawater temperatures were elevated in summer. In the 1990s, the NZ Ministry of Health's Domestic Food Monitoring program found *V. parahaemolyticus* present in 25% of oysters and 14% of cockles sampled from the Waikato region (7). None of these studies investigated the presence of *tdh* and *trh* pathogenic genes, and only one NZ study has investigated these (8). This was conducted in the summer of 2008 to 2009 in commercially grown NZ Pacific oysters and detected the *tdh* gene in 2 of 58 samples.

Concerns have been raised in recent years with *V. parahaemolyticus*-associated outbreaks in areas not previously considered to be hazardous because of their cooler water temperatures: Alaska (9) and Chile (10). The effects of climate change (i.e., increase in seawater temperature), adaptation of pathogens to cooler water, the emergence of new strains, and their distribution via ballast water have been suggested as reasons (11, 12).

A 1994 study by the National Institute for Water and Atmospheric Research (NIWA; NZ) concluded that the average annual NZ temperatures over the ocean surface have warmed by about 0.7°C since the beginning of the century, with a slightly smaller increase in the surface seawater temperature (SST) (13).

The current study sought to evaluate the effect of environmental parameters (temperature and salinity) on *V. parahaemolyticus* numbers in NZ shellfish, to compare current *V. parahaemolyticus* numbers with those found in the 1980s, and to address a knowledge gap on the incidence and prevalence of *V. parahaemolyticus* strains carrying *tdh* and *trh* in commercially grown NZ shellfish.

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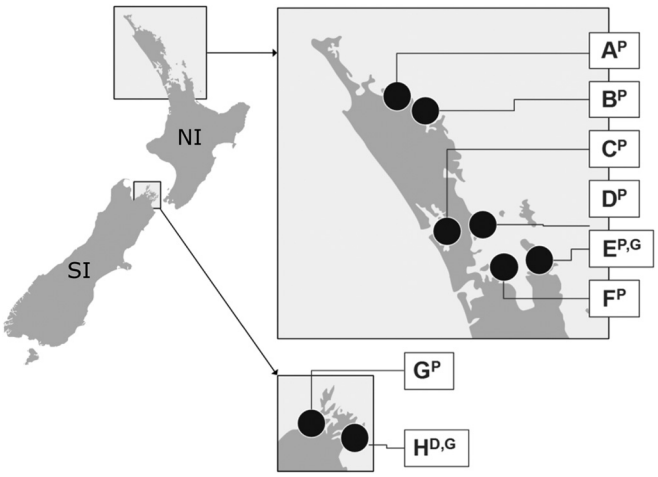

FIG 1 Location of oyster- and mussel-growing areas of the North (NI) and South Islands (SI) of New Zealand. A, Whangaroa Harbor; B, Bay of Islands; C, Kaipara Harbor; D, Mahurangi Harbor; E, Coromandel; F, Hauraki Gulf; G and H, Marlborough Sounds. Superscript letters: P, Pacific oysters; G, Greenshell mussels; D, dredge oysters.

The information generated by the study will be used by commercial shellfish growers to provide an enhanced picture of the distribution and ecology of *V. parahaemolyticus* across NZ seawaters and to help them to manage shellfish harvesting within safety limits.

MATERIALS AND METHODS

Shellfish harvest. All samples used in this study were harvested between December 2009 and May 2012, which included three complete southern summers. Commercial aquaculture areas for Pacific oyster (*Crassostrea gigas*) (seven areas; $n = 235$ samples), dredge oyster (*Ostrea chilenses*) (one area; $n = 21$ samples), and Greenshell mussel (*Perna canaliculus*) (two areas; $n = 55$ samples) were evaluated (Fig. 1). Shellfish were harvested by farm staff on a monthly basis (fortnightly for North Island samples from December to June, when higher numbers of *V. parahaemolyticus* were present). Toward the end of 2011, the NZ oyster industry was affected by the Pacific oyster mortality disease, resulting in fewer samples being available for analysis that season. Sites C, D, G, and H (Fig. 1) continued to provide samples until the end of the study (May 2012). Salinity and surface seawater temperature (SST) were measured using a refractometer (Atago S/Mill, Japan) and a conventional thermometer, respectively. Pacific oysters from the North Island (sites A to F) were grown in intertidal racks (exposed to air and sunlight at low tides), while dredge and Pacific oysters from South Island (sites G and H) and mussels (sites E and H) were grown subtidally (fully submerged at all times).

All samples, except those from site G, were shipped overnight to our laboratory in plastic bags and placed in a polystyrene box containing ice but not in direct contact with the samples. Transport temperature was monitored during shipment by using a stainless steel data logger device (iButton; Thermochron, NZ) or by measuring the meat temperature upon arrival using a digital probe thermometer (model TFX 410-1/TPX 400; Ebro, NZ). Microbiological analysis was initiated within 24 h of sample collection. Oyster samples from site G were analyzed by The Cawthron Institute (Nelson, NZ) because of shipment constraints. Open or cracked oysters were not used in the analysis.

Microbiological analysis. Samples were tested for numbers of total *V. parahaemolyticus* organisms, as well as numbers of *V. parahaemolyticus* organisms carrying *tdh* and *trh*, using the FDA most-probable-number (MPN) method (14), with minor modifications (15). Briefly, each shellfish was scrubbed with a sterile brush under running tap water to remove any mud. The meat and liquor from 12 shellfish were pooled to constitute one sample for the analysis. Samples were transferred to a sterile laboratory blender (Waring, United States) and processed at low speed for 1.5 min. One gram of each blended shellfish sample was weighed into each of three sterile tubes containing 10 ml of alkaline peptone water (APW; Difco). Decimal serial dilutions were prepared in *Vibrio* phosphate-buffered saline (*Vibrio*-PBS) composed of 7.650 g NaCl (AnalaR, England), 0.724 g Na_2HPO_4 (AnalaR), and 0.210 g KH_2PO_4 (Univar, Australia) per liter of distilled water, with pH adjusted to 7.4 using a 1N NaOH solution according to expected densities of *V. parahaemolyticus* in the sample. Triplicate APW tubes then were inoculated and incubated at 35°C for 24 h. To obtain a more efficient culture-based method, we included a chromogenic agar as well as the conventional (thiosulfate-citrate-bile-salt-sucrose [TCBS]) agar and a confirmatory selective cross-streaking step. After incubation in APW, one loopful from the surface of each turbid APW enrichment tube was streaked onto CHROMagar (CA; CHROMagar, Paris, France) and TCBS (Difco) agars for isolation. Four presumptive (based on color and colony morphology) *V. parahaemolyticus* colonies were selected from each agar and checked by cross-streaking on the other agar (i.e., blue colonies from CA were streaked onto TCBS agar and green colonies from TCBS agar were streaked onto CA). CA and TCBS agar plates were incubated at 35°C for 18 to 24 h (Fig. 2).

For the DNA extraction, up to four presumptive *V. parahaemolyticus* colonies isolated from the cross-streaking checking step were streaked onto tryptic soy agar (TSA; Difco) supplemented with 2% NaCl (Scharlau, Germany). TSA plates were incubated at 35°C for 18 to 24 h. Isolated colonies were resuspended in 250 μl of ultrapure water in a sterile tube and boiled at 100°C in a digital dry bath (Labnet, United States) for 10 min. The tubes then were placed on ice for at least 5 min. *V. parahaemolyticus* primers encoding the *Vibrio* regulatory protein *toxR* were used to confirm species (16), and primers for *tdh* or *trh* were used to identify pathogenic strains (17, 18). The final PCR mix consisted of 12.5 μl of BioMix Red (Bioline, NZ), 1 μM forward and reverse primers (IDT, NZ), 5 μl of extracted DNA, and 7.5 μl of ultrapure water. One negative and one positive control were prepared for each PCR assay. PCR cycle conditions were performed according to the methods of Tada et al. (18), Kim et al. (16), and Shirai et al. (17). *V. parahaemolyticus* strains ATCC 43996 (carrying *tdh*) and NZRM 4289 (carrying *trh*), as supplied by the Institute of Environmental Science and Research (ESR, NZ), were used as positive controls.

The MPN approach was used for enumeration, recording positive and negative results from a range of decimal serial dilutions of the triplicate set of APW tubes. Turbid APW tubes that were confirmed by conventional PCR for the presence of total *V. parahaemolyticus* organisms, as well as those carrying *tdh* and *trh* genes, were recorded as positive in the MPN spreadsheet developed by Blodgett (19), and the MPN/g value was calculated. The detection limit was 0.36 MPN/g, except for samples from site G that were analyzed by The Cawthron Institute with a detection limit of 3.0 MPN/g.

Statistical analyses. When *V. parahaemolyticus* was not detected, a value of half the detection limit (0.18 or 1.5 MPN/g) was used for statistical analyses. To test variations of $\log_{10}$ *V. parahaemolyticus* numbers between shellfish species and sites and over time, unbalanced analyses of variance (ANOVAs) were fitted, testing the effects of site and year against site-year interaction, and summer/winter differences were tested for consistency against site and site versus year interactions. To test the relationships of $\log_{10}$ *V. parahaemolyticus* counts with water temperature or salinity, random coefficient regression models were fitted. The models tested linear relationships between these and the bacterial populations reported, allowing for variations between harvest region and harvest period and interaction with shellfish species.

ANOVA and random coefficient regression models were fitted in Genstat (version 15; VSNi Ltd., Hemel Hempstead, United Kingdom). Graphics were produced in SigmaPlot (version 10; Systat Software Inc., San Jose, CA) and Microsoft Excel. The level of significance was set at a P value of <0.001.

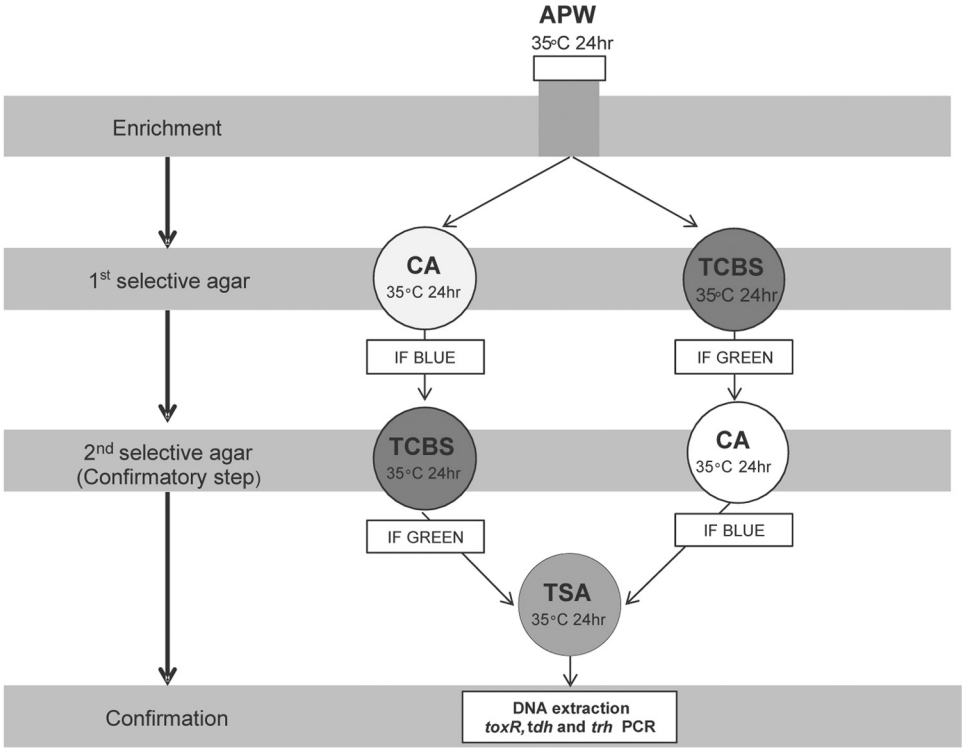

FIG 2 Flow chart of *Vibrio parahaemolyticus* most-probable-number detection method adapted from Kaysner and DePaola (14) and Cruz et al. (15) using a chromogenic agar and a two-step culture confirmation.

RESULTS

V. parahaemolyticus was detected in a total of 80.2% ($n = 174$) of Pacific oyster samples harvested from the North Island and was not detected in samples from the South Island ($n = 18$). The numbers of *V. parahaemolyticus* in Pacific oysters ranged from 0.36×10^4 to 2.4×10^4 MPN/g with a mean of 8.9×10^2 MPN/g, where detected. *V. parahaemolyticus* was detected in one dredge oyster sample harvested from the South Island ($n = 21$), with a count of 0.36 MPN/g. *V. parahaemolyticus* was observed in Greenshell mussels in 16/38 (42.1%) of the North Island samples and in 2/17 (11.8%) of the South Island samples. *V. parahaemolyticus* numbers in mussel samples ranged from 0.36 to 95.4 MPN/g, with a mean for positive samples from the North Island of 12.1 MPN/g and 0.36 MPN/g for South Island samples.

A seasonal distribution of the microorganism was observed (Fig. 3). The presence and numbers of *V. parahaemolyticus* organisms observed in summer months were significantly higher than in winter months (June-November versus December-May, $P < 0.001$). In February, the averages recorded for total *V. parahaemolyticus* numbers were 3.0×10^2 MPN/g (22.5°C), 2.0×10^3 MPN/g (23.7°C), and 7.0×10^3 MPN/g (21.4°C) in 2010, 2011, and 2012, respectively. The highest count of 2.4×10^4 MPN/g was recorded in three samples of Pacific oysters harvested in the North Island during the summer of 2012 (February and March). Conversely, during the winter (between June and November), all shellfish samples harvested had total *V. parahaemolyticus* numbers below 10 MPN/g or had nondetectable numbers (<0.36 MPN/g and <3 MPN/g for site G) (June-November versus December-May, $P < 0.001$), with SST ranging from 5.5 to 20.7°C.

The incidences of total *V. parahaemolyticus* observed in growing areas A, B, C, D, E, and F were 87.8%, 89.8%, 77.1%, 92.3%, 78.8%, and 71.6%, respectively. The shellfish-growing areas with the highest recorded incidence (areas B and D) also had the highest average numbers of *V. parahaemolyticus* across all samples (8.6×10^2 and 2.6×10^3 MPN/g, respectively). In contrast, shellfish-growing areas C and F had the lowest incidence and lower-than-average *V. parahaemolyticus* numbers (8.9×10^1 and 4.9×10^2 MPN/g, respectively).

There was no statistical difference in the numbers of total *V. parahaemolyticus* organisms detected each year across the two-and-a-half years of study ($P = 0.436$) (Fig. 3).

The SST and salinity of the seawater ranged from 7.9 to 25.5°C (mean, 18.7°C) and 8.5 to 40 ppt (mean, 33.1 ppt), respectively, for North Island harvesting areas, while in the South Island sites SST varied from 10.7 to 20.5°C (mean, 15.0°C) and salinity from 33.8 to 37.0 ppt (mean, 35.2 ppt) (Fig. 3).

V. parahaemolyticus numbers in Pacific oysters appeared to be sensitive to SST (Fig. 4), but we had no evidence of temperature dependence in dredge oysters or Greenshell mussels ($R^2 < 0.05$; data not shown; for shellfish species-SST interaction, $P = 0.009$; slope for Pacific oysters on a $\log_{10}$ scale, 0.229 [standard errors {SE}, 0.026]; slope for Greenshell mussels on a $\log_{10}$ scale, 0.048 [SE, 0.058]; slope for dredge oysters on a $\log_{10}$ scale, 0.000 [SE, 0.125]).

To assess the consistency of the SST influence on *V. parahaemolyticus* numbers in Pacific oysters observed in the pooled data, we analyzed the unpooled data of all the sampled North Island shellfish-growing areas (Fig. 4). Similar patterns were observed, with increasing SST leading to high *V. parahaemolyticus* populations in each growing area.

To analyze the influence of water salinity on the numbers of *V.*

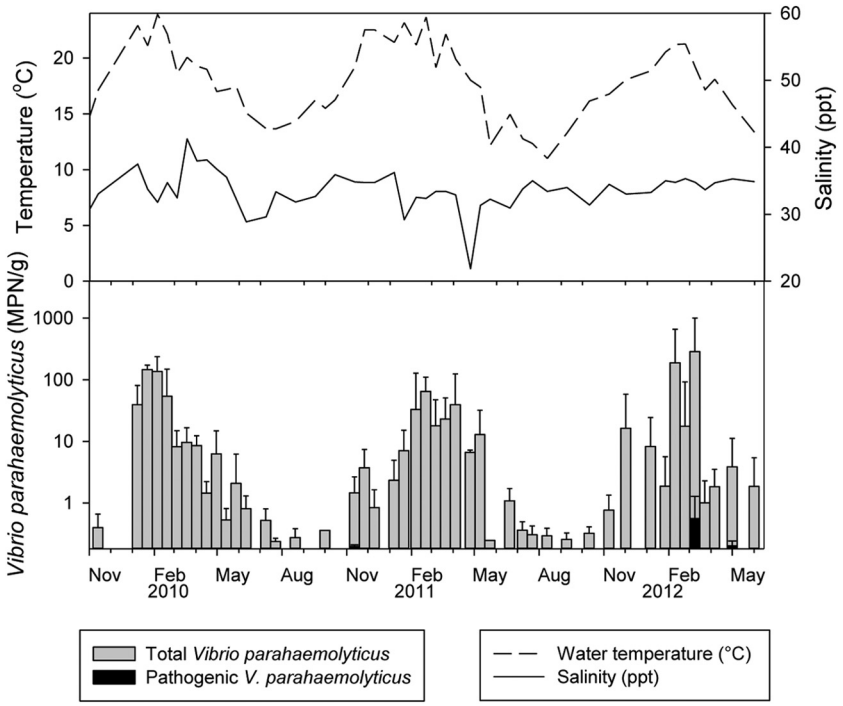

FIG 3 Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* numbers in New Zealand shellfish. Each line or bar represents the arithmetic mean of eight sites and three types of shellfish averaged over 14 days. □, total *V. parahaemolyticus*; ■, pathogenic *V. parahaemolyticus* (all *tdh* positive, no *trh* genes were detected); solid line, surface seawater temperature (°C); dashed line, salinity (ppt).

parahaemolyticus organisms present in shellfish samples, a model similar to that of SST was fitted, but no correlation was observed (for salinity main effect, $P = 0.212$; for salinity-shellfish species interaction, $P = 0.949$) (Fig. 5). However, adding salinity to the SST model gave a small but significant improvement to the model ($P = 0.031$), which translated to the fact that each additional unit of salinity would represent an average reduction of 7% total *V. parahaemolyticus* numbers. The relatively low R^2 values in Fig. 4

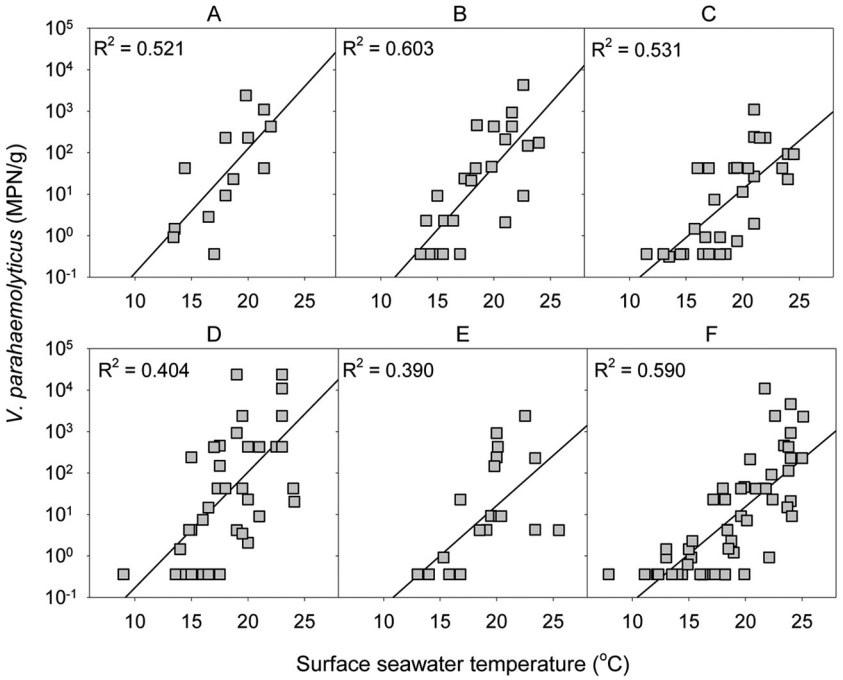

FIG 4 Numbers of total *Vibrio parahaemolyticus* in New Zealand Pacific oysters harvested from each of the North Island shellfish-growing areas (2009 to 2012). Capital letters above each plot indicate the sites of harvest according to Fig. 1.

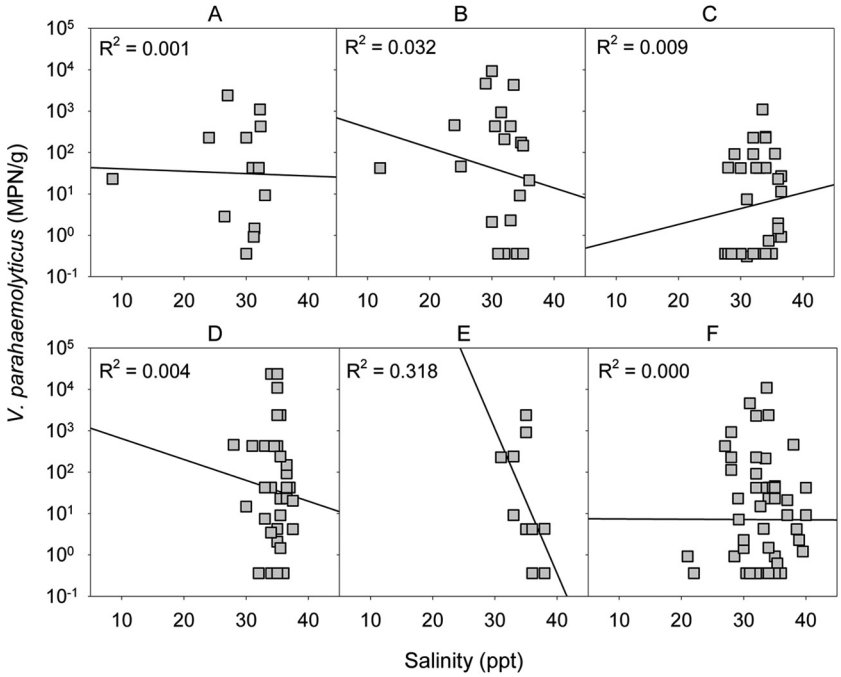

FIG 5 Numbers of total *Vibrio parahaemolyticus* in New Zealand Pacific oysters harvested from each of the North Island shellfish-growing areas (2009 to 2012). Capital letters above each plot indicate the sites of harvest according to Fig. 1.

indicate that other factors have a significant bearing on *V. parahaemolyticus* numbers as well as SST and salinity.

Although *V. parahaemolyticus* was detected at all North Island sampling sites included in the study, the distribution showed a distinctive pattern for some shellfish-growing areas. Figure 6 shows data from the same growing areas covered by our study (2009 to 2012), by the survey conducted in the summer of 2008 to 2009 (8), and by the one conducted in the 1980s (6). An increase in the *V. parahaemolyticus* numbers was observed compared with data from the 1981 to 1984 study, although methods differed in the three studies

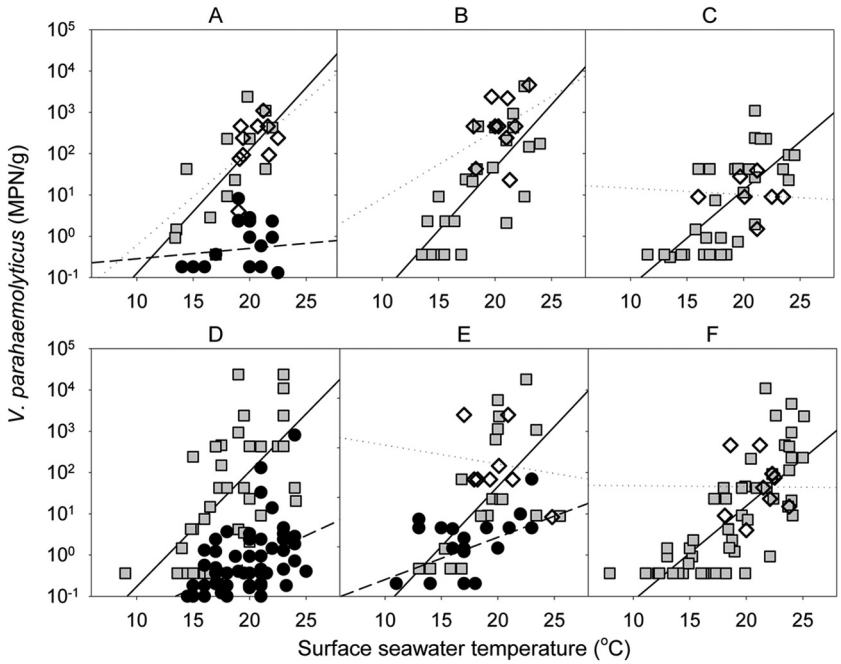

FIG 6 Numbers of total *Vibrio parahaemolyticus* in New Zealand Pacific oysters harvested in different shellfish-growing areas of the North Island (1981 to 2012). The capital letter above each plot indicates the shellfish-growing area according to Fig. 1. Grey boxes, harvests of 2009 to 2012; grey diamonds, harvests of 2008 to 2009; black circles, harvests of 1981 to 1984; solid line, regression line for 2009 to 2012; dotted line, regression line for 2008; dashed line, regression line for 1981 to 1984.

TABLE 1 Comparison of the different New Zealand studies on the detection and enumeration of *Vibrio parahaemolyticus* in different shellfish-growing areas

Parameter	Value from:		
	Fletcher (6)	Kirs et al. (8)	Current study
Period of study	1981–1984	2008–2009	2009–2012
No. of mo/yr	7 (summer)	5 (summer)	12 (fortnightly for 7)
No. of growing areas sampled	1–4	6	6–9
Shellfish species	Pacific oysters	Pacific oysters	Pacific oysters, dredge oysters, Greenshell mussels
Total no. of samples	194	58	311
Primary enrichment	Salt polymyxin broth	Alkaline peptone water	Alkaline peptone water
Selective agar(s)	TCBS	TCBS, CA	TCBS, CHROMAgar Vibrio
Confirmation	VP medium	API 20NE (quantitative PCR)	PCR
Positive samples (%)	57	95	73 (Pacific oysters)
Geometric mean (MPN/g)	1.6	77.4	25.5 (Pacific oysters)
Seawater temp (°C)	11–25	18–24	5.5–25.5
Salinity (ppt)	32–35.5	≥31	21–40

(Table 1). While samples harvested in the 1980s from the common shellfish-growing areas A, D, and E showed *V. parahaemolyticus* incidences of 47, 64, and 73%, respectively, the current study found 92, 88, and 79% incidence, respectively, at the same locations. The bacterial populations showed similar differences, with mean counts of 2.2, 4.9×10^1, and 3.1 MPN/g versus 3.8×10^2, 2.6×10^3, and 4.2×10^2 MPN/g, respectively. For those samples, the average reported SSTs were 19.9, 19.7, and 17.3°C for the 1980s data and 18.0, 18.5, and 18.9°C, respectively, for the current study.

In contrast, the study conducted in the summer of 2008 to 2009 and our survey found similar incidences of total *V. parahaemolyticus* levels. Over the summer periods, *V. parahaemolyticus* was present in 100% of Pacific oysters from both studies. The numbers did not vary much between growing areas analyzed in the two most recent studies, with area B having the highest numbers of total *V. parahaemolyticus* and shellfish-growing area C the lowest. *V. parahaemolyticus* numbers ranged from 1.8×10^1 MPN/g to 1.1×10^3 MPN/g and 8.9×10^1 MPN/g to 7.7×10^2 MPN/g for the periods of 2008 to 2009 and 2009 to 2012, respectively (*P* values comparing the two studies adjusted for seasonality ranged from 0.221 to 0.772 at different sites) (Fig. 6).

V. parahaemolyticus carrying the *tdh* virulence gene was present in only 3/217 (1.4%) of the North Island Pacific oyster samples. The method used in this study did not detect *trh* genes in any samples, which is in agreement with the previous study of Kirs et al. (8). Shellfish samples harboring *V. parahaemolyticus* carrying the *tdh* gene were harvested only in the last two seasons of the survey (Fig. 4). Some PCR gels showed bands with molecular weights similar to those of the *trh* control (see Fig. S1 in the supplemental material). PCR products, amplified using the primers described above, were purified using a QIAquick PCR purification kit (Qiagen) and sequenced by Macrogen Inc. (Seoul, South Korea) in order to confirm the presence of *trh*. With the sequence results obtained, a comparative BLAST search was conducted against other bacterial DNA sequences present in GenBank (www.ncbi.nlm.nih.gov/GenBank/). None of these PCR products from NZ isolates matched the *trh* gene, whereas PCR products from a control strain gave a good match.

DISCUSSION

Currently, there are no regulatory limits for *Vibrio* spp. in seafood in NZ. However, appropriate control measures (e.g., time to chilled storage temperatures) are in place to ensure quality and safety (20), and no outbreaks of food-borne illness have ever been attributed to *V. parahaemolyticus* in seafood produced in NZ.

A seasonal trend was observed, with *V. parahaemolyticus* numbers in Pacific oysters increasing with increasing seawater temperatures. Numbers peaked in late summer, when most oyster farmers are not harvesting commercially. The seawater temperature may be used as a strong predictor of maximum incidence and numbers of total *V. parahaemolyticus* organisms in the aquatic environment and, consequently, in shellfish; this has been shown by other researchers worldwide and is reaffirmed in our study (21–30). Despite this, some of the samples harvested at warm temperatures (>20°C) had low numbers of *V. parahaemolyticus* organisms, clearly showing that temperature is not the only factor that influences the bacterium's abundance and distribution. For example, shellfish-growing areas C and D are located in similar latitudes but on opposite coasts. The average SST between these two locations differed by only 0.1°C, but *V. parahaemolyticus* numbers were very different (numbers in area C averaged 1.5 $\log_{10}$ MPN/g less than those in area D). The average salinity value also was similar (33 and 34.5 ppt for areas C and D, respectively). The differences in *V. parahaemolyticus* numbers between the two harbors might be related to differences in nutrient concentration (plankton composition and oxygen and particulate organic matter availability), as well as to levels of freshwater flows and depth of the harvesting area. Area C is located on a larger and more open harbor on the northwestern side toward Tasman sea, while area D is a more enclosed harbor, fed by a river that passes close to a township. Area D oysters also are grown at a slightly closer distance to the seafloor (1.0 m compared with 1.4 m in area C). Interestingly, Moore et al. (31) showed that two U.S. coast sites, with different mean salinities due to more fresh water input in one harvest area, had almost identical mean numbers of *V. parahaemolyticus* in oysters. They also showed the variety of association between bacterial abundance and either salinity or temperatures depending on the harbor studied.

In relation to *V. parahaemolyticus* strains harboring the *tdh* gene, one of three *tdh*-positive samples was isolated when SSTs were as low as 17.5°C in November 2010. These findings suggest that environmental factors interact differently for each subpopulation. Remote sensing of SST rise could be a valuable tool for a

risk assessment framework covering this pathogen in oysters at harvest (32), as well as plankton composition (32) and water turbidity (33). Other studies have shown that numbers of *V. parahaemolyticus* containing the *tdh* or *trh* gene were variable and sometimes inversely related to temperature (34, 35), whereas our limited *tdh* data did not support the latter observation. However, our data do support other studies where levels of *V. parahaemolyticus* harboring *tdh* and *trh* were not necessarily proportionate to the total *V. parahaemolyticus* population (25, 34–36). Although peak numbers of *V. parahaemolyticus* strains carrying *tdh* or *trh* occurred at a time point similar to that for total *V. parahaemolyticus* (Fig. 4), high total counts often were observed when no *tdh* or *trh V. parahaemolyticus* strains were detected. In 2010, despite seeing the normal summer peak in the total population, no *tdh* or *trh V. parahaemolyticus* strains were detected for the whole season.

In another as-yet unpublished study, the authors detected *trh* genes in *V. parahaemolyticus* isolated from NZ Pacific oysters using real-time PCR. The numbers of *V. parahaemolyticus* organisms carrying the *trh* gene were low; the maximum numbers recorded during the study were 2 MPN/g.

The literature reports contradictory conclusions on the association between salinity and *Vibrio* spp. Some studies have shown a linear relationship, similar to that of SST, when testing water samples (25, 28, 31, 33). However, the data from our study did not show a particular correlation between salinity and *V. parahaemolyticus* in oyster samples, and these are results that are similar to those from some other studies (8, 27, 30, 31, 37). This could be related to the narrow range of salinity in our shellfish-growing areas.

Martinez-Urtaza et al. (12) estimated that the maximum probability of *V. parahaemolyticus* detection was around salinity of 25 ppt, similar to the findings of optimal salinity for *V. parahaemolyticus* presence in oysters of 23 ppt (14). NZ seawaters are more saline. In the present study, most of our samples (71%) were harvested in areas with ≥30 ppt, with only 4.5% of them obtained from areas with ≤25 ppt water salinity. The samples harboring $>10^4$ *V. parahaemolyticus*/g were found in the salinity range of 33 to 35 ppt, while *V. parahaemolyticus* organisms carrying *tdh* were isolated from shellfish harvested in seawater with salinity measurements of 35 to 36.5 ppt.

Comparing the results from the three NZ *V. parahaemolyticus* surveys (Fig. 6 and Table 1), higher numbers were recorded in 2008 to 2013 than in the 1980s, and SST had a larger influence on the numbers (i.e., steeper slope). In previous NZ studies, the lack of relationship between SST and *V. parahaemolyticus* was assumed to be due to the relatively few samples analyzed and the narrow range of temperature where samples were collected (8). It is not clear if the differences between studies were due to the different methods used, which included the use of different enrichment broths (i.e., salt polymyxin broth and APW) and confirmatory tests (i.e., VP medium, API 20NE, and PCR), or whether they represent a real increase in numbers over the 30-year period, which could be related to higher average SST.

All shellfish from the South Island (areas G and H) had a low incidence and numbers of *V. parahaemolyticus*. In these areas, all of the shellfish were grown subtidally, whereas Pacific oysters were grown intertidally in all other areas. However, the effect of production method on *V. parahaemolyticus* numbers could not be evaluated in this study, as no samples of the same species were harvested from similar shellfish-growing areas while being grown under different conditions (i.e., tide exposure or distance from the sea floor). Previous NZ work did not show any impact on *V. parahaemolyticus* concentration when intertidal oyster samples were collected from bags, racks, or sticks or at different distances from the sea floor (8), but a survey carried out in the Pacific Northwest and Atlantic regions (38) showed that oysters harvested from intertidal sites harbored higher numbers of *V. parahaemolyticus* strains carrying *tdh* or *trh* as well as total *V. parahaemolyticus* than those harvested by dredging.

Conclusions. This study increases the knowledge of *V. parahaemolyticus* ecology and distribution in NZ shellfish, identifying conditions (SST) and times (December to May) where high numbers might be present.

By extending the length of the study, the number of harvest areas, and the shellfish species included, we could identify the influence of specific environmental parameters on the abundance of the microorganism. The results indicate that the incidence of *V. parahaemolyticus* has increased over a 30-year period. However, considerably more research is required to be able to predict when *V. parahaemolyticus* numbers will be high or low. The study shows that the environmental factors that influence the numbers of total *V. parahaemolyticus* and *tdh* or *trh* strains of *V. parahaemolyticus* are complex and may be site specific.

Furthermore, we have produced data that the presence of *V. parahaemolyticus* in NZ Pacific oysters harvested in North Island areas can be high when SSTs are elevated, but that *V. parahaemolyticus* strains harboring *tdh* or *trh* are rarely present. There is no evidence of human health risk from *tdh* or *trh* strains of *V. parahaemolyticus* in commercially grown NZ shellfish.

A seasonal correlation for North Island shellfish-growing areas was observed. Using a temperature of 19°C as a risk trigger parameter might be useful should growers wish to harvest oysters in the summer months in the different locations, but they must be sure to comply with U.S. regulatory limits of 10,000/g. The results presented here also provide information that can help the seafood industry to tailor risk management measures for specific shellfish-growing areas if the needs arise.

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