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RESEARCH ARTICLE

Environmental influences on the seasonal distribution of *Vibrio parahaemolyticus* in the Pacific Northwest of the USA

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One sentence summary: Seasonal dynamics of *Vibrio parahaemolyticus* in the Pacific Northwest.

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ABSTRACT

Populations of *Vibrio parahaemolyticus* in the environment can be influenced by numerous factors. We assessed the correlation of total (*tl*+) and potentially virulent (*tdh*+) *V. parahaemolyticus* in water with three harmful algal bloom (HAB) genera (*Pseudo-nitzschia*, *Alexandrium* and *Dinophysis*), the abundance of diatoms and dinoflagellates, chlorophyll-*a* and temperature, salinity and macronutrients at five sites in Washington State from 2008–2009. The variability in *V. parahaemolyticus* density was explained predominantly by strong seasonal trends where maximum densities occurred in June, 2 months prior to the highest seasonal water temperature. In spite of large geographic differences in temperature, salinity and nutrients, there was little evidence of corresponding differences in *V. parahaemolyticus* density. In addition, there was no evident relationship between *V. parahaemolyticus* and indices of HAB genera, perhaps due to a lack of significant HAB events during the sampling period. The only nutrient significantly associated with *V. parahaemolyticus* density after accounting for the seasonal trend was silicate. This negative relationship may be caused by a shift in cell wall structure for some diatom species to a chitinous substrate preferred by *V. parahaemolyticus*. Results from our study differ from those in other regions corroborating previous findings that environmental factors that trigger vibrio and HAB events may differ depending on geographic locations. Therefore caution should be used when applying results from one region to another.

Keywords: pathogens; Vibrio; HABS; microbial ecology

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INTRODUCTION

Recent emergence of *Vibrio* illnesses in temperate regions have been linked to global warming and subsequent increases in water temperature (Martinez-Urtaza *et al.* 2010; Baker-Austin *et al.* 2013; Vezzulli, Colwell and Pruzzo 2013). In the USA, illnesses due to *Vibrio* spp., primarily *Vibrio parahaemolyticus* and *V. vulnificus*, have increased threefold over the past 17 years (1996–2012) with the majority of the increase attributed to *V. parahaemolyticus* (Newton *et al.* 2012). The first shellfish closures in the Pacific Northwest (PNW) related to *V. parahaemolyticus* occurred in 1997–1998 and they have also been increasing in recent years (Centers for Disease Control and Prevention 1998; Washington Department of Health, personal communication).

The majority of *V. parahaemolyticus* infections result in severe gastroenteritis that is usually self-limiting, while *V. vulnificus* can cause more severe infections including septicemia, with a 50% fatality rate (http://www.cdc.gov/vibrio/). Infections can occur with both species when open wounds are exposed to seawater harboring these vibrios; such infections have increased in recent years (Weis *et al.* 2011). While *V. vulnificus* has been isolated from environmental samples in the PNW of the USA (Kaysner *et al.* 1987), there are no recorded illnesses due to this bacterium in this region, presumably because *V. vulnificus* tends to thrive in regions with higher water temperatures such as the US Gulf Coast. *Vibrio parahaemolyticus* is considerably more tolerant of colder temperatures, and is commonly isolated from water, sediment and shellfish in the PNW (Baross and Liston 1970; Depaola *et al.* 2003a; Johnson *et al.* 2012).

At the same time, the frequency, intensity and duration of harmful algal bloom (HAB) events have also been increasing in the PNW (Trainer *et al.* 2013), with shellfish beds closed to commercial, recreational and tribal subsistence harvesting often for several weeks at a time. Presently, the toxin-producing HAB species of concern in the PNW are diatoms of the genus *Pseudo-nitzschia* that produce domoic acid (DA) (Taylor and Horner 1994; Horner, Garrison and Plumley 1997; Stehr *et al.* 2002; Trainer, Hickey and Horner 2002; Lewitus *et al.* 2012) and dinoflagellates of the genera *Alexandrium* and *Dinophysis* that produce paralytic shellfish toxins (saxitoxin and its congeners) and diarrhetic shellfish toxins (okadaic acid and its congeners), respectively (Horner, Garrison and Plumley 1997; Trainer, Hickey and Horner 2002; Trainer *et al.* 2013). The first confirmed human illness due to diarrhetic shellfish poisoning in the USA occurred in Washington State in 2011 (Trainer *et al.* 2013) and at least three severe cases of paralytic shellfish poisoning were reported in 2012 (Hurley *et al.* 2014).

In addition to the public health hazard caused by vibrios and HABs, the resulting closures of commercial and recreational shellfish harvesting also place a significant economic burden on the shellfish industry of Washington State, the largest on the West Coast of the USA, which generates over $110 million in revenue annually (http://pcsga.org/economics). Currently no predictive tools are available for shellfish growers or public health managers in the PNW to forecast potential illnesses due to vibrios or HABs.

Numerous studies have attempted to identify both biotic and abiotic factors that influence the abundance of *Vibrio* species in the environment at sites around the world. These studies, largely focused on species that represent a threat to human health, have been reviewed recently (Takemura, Chien and Polz 2014). Water temperature has been reported to be the primary driver of the abundance of *Vibrio* spp., including *V. parahaemolyticus* (Kaneko and Colwell 1973; Cook, Bowers and DePaola 2002; Duan and Su 2005; Froelich *et al.* 2015; Blackwell and Oliver 2008; Parveen *et al.* 2008; Johnson *et al.* 2010; Julie *et al.* 2010; Caburlotto *et al.* 2011; Johnson *et al.* 2012; Böer *et al.* 2013), with differences in the effect of salinity dependent on species and geographic conditions (Takemura, Chien and Polz 2014). It has long been recognized that *Vibrio* spp. (particularly *V. cholerae*) are often found associated with other inhabitants of the marine and estuarine communities such as copepods and crustaceans (Kaneko and Colwell 1975, Huq *et al.* 1983; Colwell 1996; Montanari *et al.* 1999; Rawlings, Ruiz and Colwell 2007) and a few studies have suggested direct and indirect associations of *V. cholerae* and other pathogenic *Vibrio* spp. with aquatic plants and phytoplankton (Asplund *et al.* 2011, Islam, Drasar and Bradley 1989; Huq *et al.* 1990; Tamplin *et al.* 1990; Islam *et al.* 2007; Turner *et al.* 2009, 2014) as well as with algal blooms (Romalde, Barja and Toranzo 1990, Romalde, Toranzo and Barja 1990; Epstein 1993; Mouriño-Pérez, Worden and Azam 2003; Main *et al.* 2015). Recent studies suggest that the association between *Vibrio* and phytoplankton may be species specific (Main *et al.* 2015) and specific genotypes may be distinctly affected by shifts in the composition of plankton reservoirs (Turner *et al.* 2014).

Since the PNW region has experienced an increase in both *Vibrio* and HAB events, in this investigation, we examine the possible correlation with HABs, and nontoxic and toxin-producing diatoms and dinoflagellates, and abiotic conditions that may influence increases in concentrations of the potentially pathogenic *Vibrio*, *V. parahaemolyticus* in five geographically separate sites within the Puget Sound basin (Washington State, USA). We used models to quantify the relationship among *V. parahaemolyticus*, three HAB genera that are most prevalent in the PNW (*Pseudo-nitzschia* spp., *Alexandrium* spp. and *Dinophysis* spp.), concentration of the neurotoxin domoic acid produced by members of the genus *Pseudo-nitzschia*, the abundance of total diatoms and dinoflagellates, and the abiotic factors temperature, salinity and nutrient concentrations. A better understanding of possible linkages between the occurrence of *V. parahaemolyticus* and phytoplankton populations may lead to the development of improved predictive models for resource managers to reduce or mitigate shellfish-associated illnesses in coastal systems, or to issue public health warnings.

MATERIALS AND METHODS

Study Sites, sample collection and analyses

Five sampling sites were selected in Washington State for their proximity to shellfish growing areas, sites implicated as the source of vibriosis outbreaks and/or their historical prevalence of phytoplankton blooms (Fig. 1). These five sites are widely distributed throughout the Puget Sound region.

Site 1: Sequim Bay—Jamestown S'Klallam Tribe

This is the site of a commercial, recreational and tribal shellfish closure due to DA in September 2005 (Trainer *et al.* 2003). Historically, paralytic shellfish poisoning (PSP) cases have been recorded here since the 1950s when monitoring by the Washington State Department of Health (WDOH) began; however there have been limited *Vibrio*-related illnesses reported from this area.

Site 2: Northern Hood Canal, Quilcene Bay—Coast Seafoods Company

Coast Seafoods Company is the largest oyster hatchery on the US West Coast and the leading oyster and clam larvae

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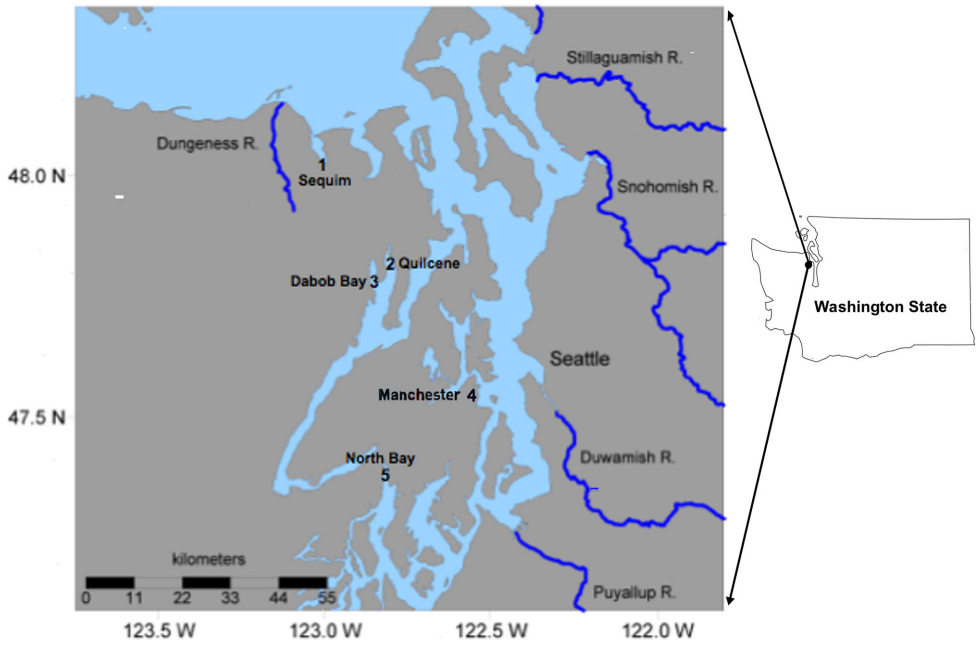

Figure 1. Location of the five sampling sites in Washington State.

vendor. PST closures do occur here, but are relatively infrequent. However, this site has been implicated in *V. parahaemolyticus*-related illnesses.

Site 3: Northern Hood Canal, Dabob Bay—Taylor Shellfish Farms
Taylor Shellfish Farms is the second largest company in the USA in terms of volume of oysters grown and sold. They distribute shellfish worldwide. PSP toxins have caused closures in Puget Sound since the early 1990s. In addition, oysters from this site have frequently reflected relatively high concentrations of *V. parahaemolyticus* during the warmest summer months.

Site 4: Northwest Fisheries Science Center, Manchester Research Station
Located in the Central Puget Sound, this site was chosen because it has a documented previous history of HABs, but not of vibrios.

Site 5: North Bay, Allyn
This site located in South Puget Sound has historically shown high concentrations of both PST and *V. parahaemolyticus*.

Sample Collection

Water samples were collected at a depth of 0.3 meters below the surface weekly at each of the five sites from March through October in 2008, and March 2009 through August 2009 and every 2 weeks during the interim colder months. At all sites, samples were collected on the same day of each week between 8 and 10 a.m. to ensure similar tidal stage. Samples for *Vibrio* spp. and phytoplankton analyses were collected in pre-sterilized 500 ml containers. Samples for chlorophyll-*a* analysis and phytoplankton identification and enumeration were collected in brown polyethylene bottles. All samples were kept cold (10–15°C) during transport to the laboratory. A total of 145 samples were collected and analysed in 2008 and 130 samples in 2009 (Table 1).

Phytoplankton analysis

Phytoplankton cells were preserved with buffered formalin (<1% final concentration) and samples (50 ml) were settled for 12–24 h before analysis for phytoplankton species. Cells were identified to the genus level and counted in a Palmer–Maloney counting chamber using a Zeiss Axiovert 135 inverted light microscope at 100× magnification.

Chlorophyll-*a* analysis

For chlorophyll-*a* analysis a known volume of water was filtered through a 25 mm GF/F glass fiber filter (Whatman, Int. Ltd, Maidstone, UK) until the filter showed a light green color. Filters were wrapped in aluminum foil to protect from light and stored at −20°C until analysis. Chlorophyll-*a* was analysed using a Turner Designs 10-AU fluorometer, following the non-acidification method of Welschmeyer (1994).

Nutrient analysis

Water samples (50 ml) were filtered through 25 mm glass microfiber filters (Whatman) and collected in acid-cleaned and distilled water-rinsed bottles for dissolved inorganic nutrient analyses. Samples were analysed for nitrate (NO_3^-), nitrite (NO_2^-), ortho-phosphate (PO_4^{3-}), silicic acid [$Si(OH)_4$], and ammonium (NH_4^+) with a Technicon Model AAII Segmented Flow Analysis System using the protocols of UNESCO (Knap, Michaels and Close 1996) and Valderrama (1981).

Toxin analysis

One liter of seawater was filtered through a 0.45 μm, 47 mm diameter nitrocellulose filter (Millipore) and the filter was stored at −20°C for toxin analysis. Particulate domoic acid (pDA) was determined using the receptor binding assay method outlined by Baugh *et al.* (2004) and using certified toxin standards (National Research Council, Canada).

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Table 1. Distribution of *V. parahaemolyticus*, phytoplankton, and abiotic parameters in 2008 and 2009.

	2008				2009			
Variable	Number positive/ total	Min	Max	Mean ± SD	Number positive/ total	Min	Max	Mean ± SD
V. parahaemolyticus (*tl*+)[a]	136/145	0	5.2×10^6	$5.6 \times 10^4 \pm 4.3 \times 10^5$	109/130	0	8.4×10^4	$1.1 \times 10^3 \pm 7.4 \times 10^3$
V. parahaemolyticus (*tdh*+)[a]	115/145	0	3.5×10^6	$3.7 \times 10^4 \pm 2.9 \times 10^5$	62/130	0	2.0×10^4	$1.9 \times 10^2 \pm 1.7 \times 10^3$
Pseudo-nitzschia spp.[b]	77/138	0	585	24 ± 76	37/126	0	390	10 ± 41
Alexandrium spp.[b]	14/139	0	108	2.0 ± 11	0/136	0	0	0
Dinophysis spp.[b]	21/136	0	17	0.3 ± 1.5	2/136	0	7	0.1 ± 0.7
Diatom spp.[c]	133/138	0	10+	na	118/126	0	10+	na
Dinoflagellate spp.[c]	53/138	0	4	na	34/126	0	7	na
Domoic acid[d]	4/122	0	630	8.8 ± 63.8	1/101	0	93	0.9 ± 9.3
Chlorophyll *a*[g]	116/119	0	105.36	4.9 ± 10.9	126/126	0.12	38.2	3.8 ± 4.98
Temperature[e]	na	7	24	12.9 ± 3.8	na	4.8	28	12.1 ± 4.4
Salinity[f]	na	24.0	36.5	30.6 ± 2.3	na	14	36	30.3 ± 3.0
Silicate[h]	110/110	2.34	95.87	39.8 ± 23.8	110/110	3.71	110.8	34.9 ± 21.1

[a]ge/100 ml; [b]cells/ml; [c]1–10+ relative abundance scale, cells/ml: 1–20 = 1, 21–40 = 2, etc. 181–200 = 10 and over 200 = 10+; [d]ng/L; [e]C; [f]PSU; [g]μg/L; [h]μM. na, not applicable.

DNA extraction and analyses for *Vibrio* spp.

Between 200 and 250 ml of seawater was filtered through a 0.45 μm, 47 mm diameter polyethersulfone membrane filter (Sterlitech Corp., Kent, WA, USA). Filters were stored in Kapak Pouches (Kapak Corporation, St Paul, MN, USA) at −70°C. Total DNA was extracted using an adaptation of the method by Boström *et al.* (2004). Cell recovery and lysis were accomplished as described in the DNA extraction protocol. After lysis the DNA was extracted with phenol (pH 7.9, Amresco, Solon, OH, USA) and chloroform (24:1 mix of chloroform:isoamyl alcohol, Fisher Scientific, Pittsburg, PA, USA) and precipitated with 0.1 volume of 3 M sodium acetate (pH 4.8) followed by 2.5 volumes of 95% ethanol with 2 μl of pellet paint (EMD Chemicals, Philadelphia, PA, USA) added as a carrier. The pellet was washed with 70% ethanol, followed by 95% ethanol, dried and resuspended in 400 μl of water. A second ethanol–sodium acetate precipitation was performed to maximize the removal of inhibitors, and the resultant pellet was resuspended in 50 μl of T low E buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). To evaluate extraction efficiency, several extraction sets included a positive control filter prepared separately by filtration of artificial seawater spiked with a known number of cells of *V. parahaemolyticus* (Strain RIMD2210633, genotype *tl*+, *tdh*+). Downstream analysis of this positive control filter gave an estimate of the recovery in those extractions. Limit of detection for both total (*tl*+) and potentially pathogenic (*tdh*+) *V. parahaemolyticus* was 160 genome equivalents (ge)/100 mls.

Quantification of *V. parahaemolyticus* and *V. vulnificus*

Water samples were analysed for total (*tlh*+) as well as the potentially pathogenic thermostable direct hemolysin (*tdh*+) *V. parahaemolyticus* and total *V. vulnificus* (*Vvu*) by quantitative PCR using the Mx3005P™ Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA). For *V. parahaemolyticus*, primers and probes were those previously published (Nordstrom *et al.* 2007). The *tl*, *tdh* and internal amplification control (IAC) primers and the *tl* and IAC probes were purchased from Integrated DNA Technologies (Coralville, IA, USA), while the *tdh* probe was obtained from Applied Biosystems (Foster City, CA, USA). Each 25 μl reaction consisted of 12.5 μl of 2× Brilliant Multiplex QPCR Master Mix (Agilent Technologies) and the following reaction components (final concentrations): all three probes at 150 nM, all six primers at 75 nM and BSA (New England Biolabs, Beverley, MA, USA) at 400 ng/μl (Kreader 1996). The remainder of the reaction consisted of nuclease-free water, 1 μl of IAC template, and 2 μl of template. The two-step thermal profile used throughout this work consisted of an initial 9.5 min denaturation step at 95°C followed by 40 cycles of 30 s denaturation at 95°C and a 45 s combined annealing/extension step at 59°C. Fluorescence data were collected at the end each amplification cycle. The IAC, developed by Nordstrom *et al.* (2007), was used to ensure absence of inhibition. The concentration the IAC was adjusted to give a cycle threshold (C_t) of around 22 if the sample contained no inhibitors. When the C_t was observed to be significantly greater than 22, indicating the presence of PCR inhibitors, samples were diluted (1:10) and reanalysed.

All samples were also analysed separately for *V. vulnificus* from the same extracts using a previously published assay (Panicker and Bej 2005) and the oligonucleotide primers F-vvh785 and R-vvh990 along with the probe P-vvh875 (Integrated DNA Technologies). This assay targets the *vvh*A gene of *V. vulnificus*.

Statistical analysis

Descriptive and analytic analyses were performed using R 3.03 (R Core Team 2014). We describe and summarize *Vibrio* spp., water quality characteristics, nutrients and phytoplankton evaluated among water samples collected from five sampling sites in the Puget Sound basin. Supplementary Table S1 summarizes variables that were examined in the statistical treatment.

Total (*tl*+) *V. parahaemolyticus* was modeled using abiotic and biotic variables. A model for the *tdh*+ *V. parahaemolyticus* was not constructed because it was highly correlated with total (*tl*+) *V. parahaemolyticus*. Abiotic explanatory variables included temperature, salinity, silicate, nitrite and ammonium. The biotic predictors included, chlorophyll-*a*, *Pseudo-nitzschia* cells, the diatom cell index and presence/absence of *Alexandrium* spp. The variables nitrite, ammonium and chlorophyll-*a* were log transformed (base e) due to skewed distributions. Because there were some values of 0 for chlorophyll-*a* and nitrate, we added 0.01 before log transformation. To account for unexplained seasonal patterns, we added a natural cubic spline (Harrell 2001) with five knots spaced evenly across the two years (1 January, 1 July

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2008 and 1 January, 1 July and 31 December 2009). This temporal spline changes smoothly at a seasonal scale allowing it to explain large scale and annual differences while only adding four degrees of freedom to the model. To account for values below the detection limits in the outcome variable (*V. parahaemolyticus*), Tobit models (Tobin 1958) were used. The outcome variable was also log transformed to achieve a more bell-shaped distribution and stabilize the variance. We considered a number of different combinations of explanatory variables. The abiotic group of variables included temperature, salinity, silicate, log nitrite and log ammonium, while the biotic variables included log chlorophyll-*a*, log *Pseudo-nitzschia* spp. cells, *Alexandrium* spp. and presence/absence of diatom cells. Observations with missing values for any of the explanatory variables were excluded for all analyses. Relative model performance was assessed using Akiake's information criteria (AIC) and absolute explanatory power was approximated using a pseudo R^2 metric (McKelvey and Zavoina 1975). Model fit was assessed with QQ plots of the residuals, and plots of residuals vs location, date and other explanatory variables. All explanatory variables were standardized to allow for comparison between coefficients.

RESULTS

Correlation of *V. parahaemolyticus* with phytoplankton and abiotic factors in the Puget Sound Basin

Although we detected little variability in the abiotic parameters between the two years, both mean and maximum concentrations of the total (*tl*+) and *tdh*+ *V. parahaemolyticus* were considerably higher in 2008 than in 2009 (Fig. 2 and Table 1). In addition, the temperature profile for both years was not consistent with concentrations of *V. parahaemolyticus* (Fig 2). The maximum temperature in water was measured in July 2008 (24°C) and August 2009 (28°C); while the highest concentrations of both total (*tl*+) *V. parahaemolyticus* (5.2×10^6 ge in 2008 and 8.4×10^4 ge in 2009), and *tdh*+ *V. parahaemolyticus*, (3.5×10^6 ge in 2008 and 2.0×10^4 ge in 2009) were observed in June of both years, well before the maximum water temperature was reached (Table 1 and Fig. 2). Salinity showed little variation between the two years throughout the sampling period (Table 1 and Fig. 2). Water samples were also analysed for chlorophyll-*a*, nitrate, nitrite, silicate, phosphate and ammonium. The relationship between the nutrients and the biotic variables was examined through correlations (Fig. 3), and plots vs time (Fig. 2). For both years we observed a strong correlation between the nutrients phosphate, nitrate, nitrite and silicate. Notably, concentrations of silicate were inversely proportional to levels of both *tl*+ and *tdh*+ *V. parahaemolyticus* (Fig. 3). The decline in concentrations of silicate was observed between mid-May and June in both years, corresponding with the highest concentrations of *V. parahaemolyticus* (Fig. 2), while the highest concentrations of silicate were observed in November (2008) and January (2009) when the *V. parahaemolyticus* abundance was the lowest (Fig. 2).

Surprisingly 85% (115/136) of *tl*+ *V. parahaemolyticus* detected in 2008 were also *tdh*+, with the proportion dropping only slightly to 57% (62/109) in 2009 (Table 1). These data suggest that during both years, the proportion of *tdh*+ *V. parahaemolyticus* detected in our study sites was substantially higher than populations of *tdh*+ *V. parahaemolyticus* reported from most geographic locations (Robert-Pillot *et al.* 2004; Ottaviani *et al.* 2005; Johnson *et al.* 2010; Julie *et al.* 2010). Two more recent studies, one from the coast of Georgia in the USA and another from the northwest coast of Mexico (Turner *et al.* 2014; De Jesus Hernandez-Diaz *et al.* 2015), also report higher concentrations similar to those detected in our study. However, another study where the dynamics between HABs and *V. parahaemolyticus/vulnificus* was examined in a very different environment, retention ponds and tidal creeks in South Carolina, *tdh*+ *V. parahaemolyticus* was not detected (Greenfield *et al.* manuscript submitted). Since *V. vulnificus* was detected in only one sample at a low level (179 ge/100 ml) the analyses focused only on *V. parahaemolyticus*. It is likely that the higher salinities at the sampling sites together with generally lower temperatures are not optimal for proliferation of this pathogen, which has historically been detected rarely and only at low concentrations in this region (Kaysner *et al.* 1987).

We did not observe any significant HAB events during the sampling period although periods of elevated domoic acid levels were observed in four samples from Sequim Bay in 2008 (17–30 June and 22–30 September) at high concentrations between 55 and 630 ng/L and in one sample from North Bay in 2009 (March) again at a high concentration of 93 ng/L (Table 1). Cells of *Pseudo-nitzschia* spp. were detected in 56% (77/138) of the samples in the first year, and in 29% (37/126) of the samples in the second year. Maximum concentrations of 585 cells/ml in 2008 and 394 cells/ml for 2009 were detected at the Sequim Bay site around the beginning of July in both years. Low concentrations (1–108 cells/ml) of *Alexandrium* spp. were detected in 10% of samples in 2008. *Alexandrium* spp. were not detected in in 2009 (Table 1).

The distribution of *Dinophysis* spp. followed a similar pattern, with a low abundance (1–17 cells/ml) detected in 15% (21/136) of the samples in 2008 and less than 2% (2/136) of the samples in the following year. In addition to the harmful algal species, water samples were also evaluated for abundance of total diatoms and dinoflagellates. The incidence and abundance of total diatoms were higher than that of dinoflagellates in both years studied. We detected diatoms in >95% of the samples in both years with abundances ranging from 10 to >200 cells/ml (Table 1). Dinoflagellates were detected only in 38% (53/138) of the samples in 2008 and 27% (34/126) of samples in 2009 and at significantly lower concentrations than diatoms (2–80 cells/ml). The distribution of diatoms showed a seasonal pattern with higher abundances between spring and summer than in the winter months (Fig. 2).

Comparison of biotic and abiotic factors between sampling sites

Figure 4 shows a comparison of the biotic and abiotic variables measured at three sites (Sequim Bay, Manchester and Dabob Bay) in 2008 and 2009 between early May and late August for both years. Since some data on a few of the variables measured were missing from Quilcene Bay and North Bay, these sites were not included in this comparison.

The prevalence of both *tl*+ and *tdh*+ *V. parahaemolyticus* was lower (~56-fold and ~195-fold, respectively) in 2009 as compared to 2008 at all sites studied. As noted earlier, however, the ratios of *tl*+ and *tdh*+ *V. parahaemolyticus* were comparable between the sites for both years (Table 1 and Fig. 4). There were notable differences in the abundance and distribution of the three HAB species among sites in both years. *Pseudo-nitzschia* spp. was most abundant in Sequim Bay in both years while slightly higher concentrations of *Alexandrium* spp. and *Dinophysis* spp. were detected in Manchester in 2008 (Fig. 4). However, in general concentrations of both *Alexandrium* spp. and *Dinophysis* spp. were low for both years with no cells of *Alexandrium* spp. detected in 2009 (Table 1). Concentrations of total diatoms were higher at Sequim Bay for both years. Chlorophyll-*a* concentrations were reflective of the concentrations of total phytoplankton, with the average

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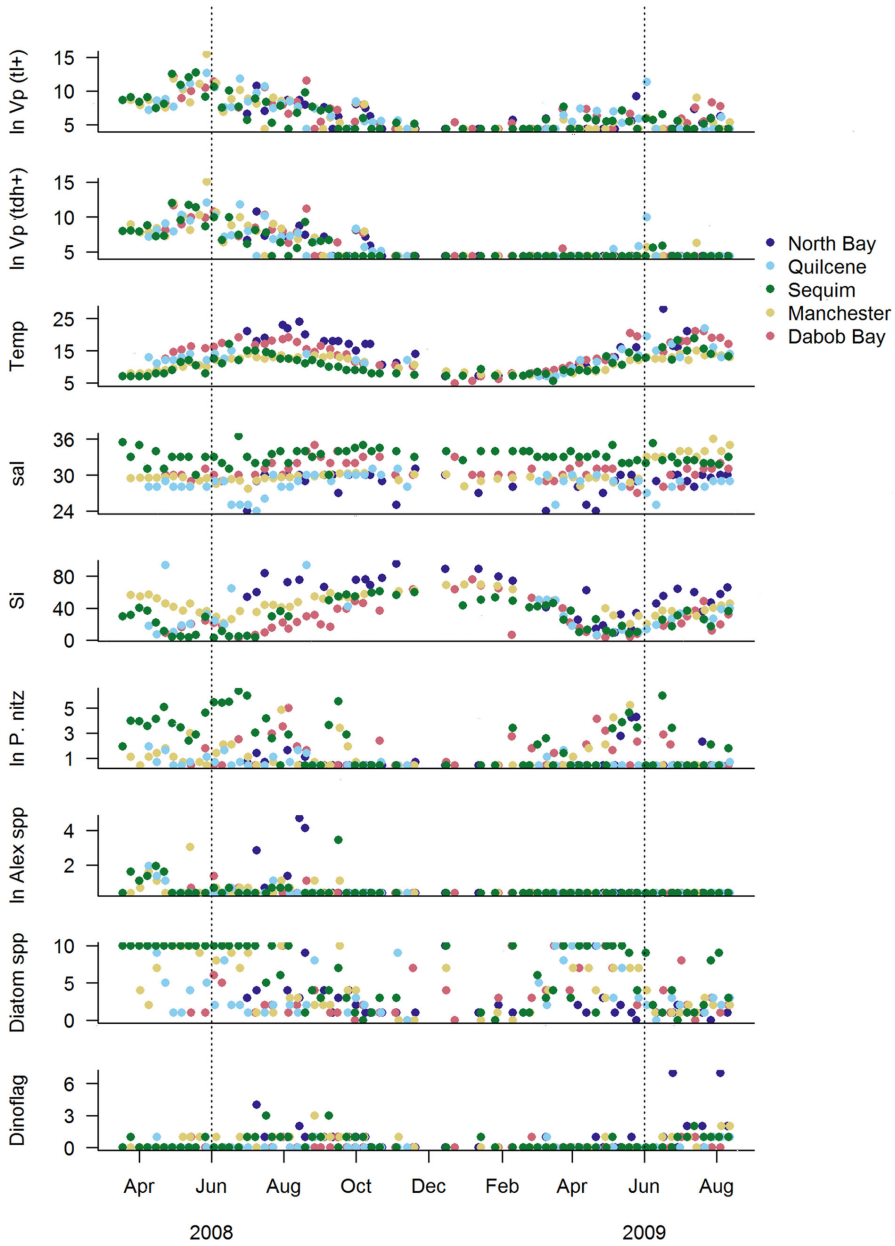

Figure 2. Comparison of the significant biotic (total *V. parahaemolyticus* ge/100 ml; *Pseudo-nitzschia* spp. and *Alexandrium* spp., cells/ml; diatom and dinoflagellate spp., relative abundance) and abiotic variables (temperature, °C; salinity, PSU; and silicate, μM) over the sampling period (April 2008–August 2009). The dashed vertical lines represent the maximum *V. parahaemolyticus* density for each year.

concentration highest at Sequim Bay and lowest at Dabob Bay (Fig. 4).

The abiotic parameters also showed between-site differences. Water temperatures were higher at Dabob Bay, while salinity was higher at Sequim Bay (33.1 ± 3.3 PSU) than that at the other locations (30.5 ± 1.9 PSU) for both years. However, concentrations of all macronutrients (phosphate, silicate, nitrate, nitrite and ammonium) were higher at Manchester than Sequim Bay or Dabob Bay (Fig. 4).

Modeling the relationship between *V. parahaemolyticus*, phytoplankton and abiotic parameters

We used models to investigate the predictive potential of the abiotic and biotic variables on the density of *V. parahaemolyticus* (see Materials and Methods). To limit the number of models considered, the abiotic and biotic variables were included in the models as groups; we looked at the importance of each of these groups as a whole instead of considering individual variables within groups. The abiotic variable group included temperature, salinity, silicate, ln nitrite, and ln ammonium, while the biotic group included ln chlorophyll-*a*, ln *Pseudo-nitzschia* spp., *Alexandrium* spp., and the index of diatom cells (Table 2).

Since the total (*tl*+) and *tdh*+ *V. parahaemolyticus* were significantly related, we only modeled the outcome of total *V. parahaemolyticus*. The abiotic variables provided explanatory power producing models that performed better (i.e. lower ΔAIC) than the intercept-only model (Table 2). The biotic variables (ln chlorophyll-*a*, *Pseudo-nitzschia* spp., *Alexandrium* spp. and abundance of diatom cells) further improved the fit. However, the

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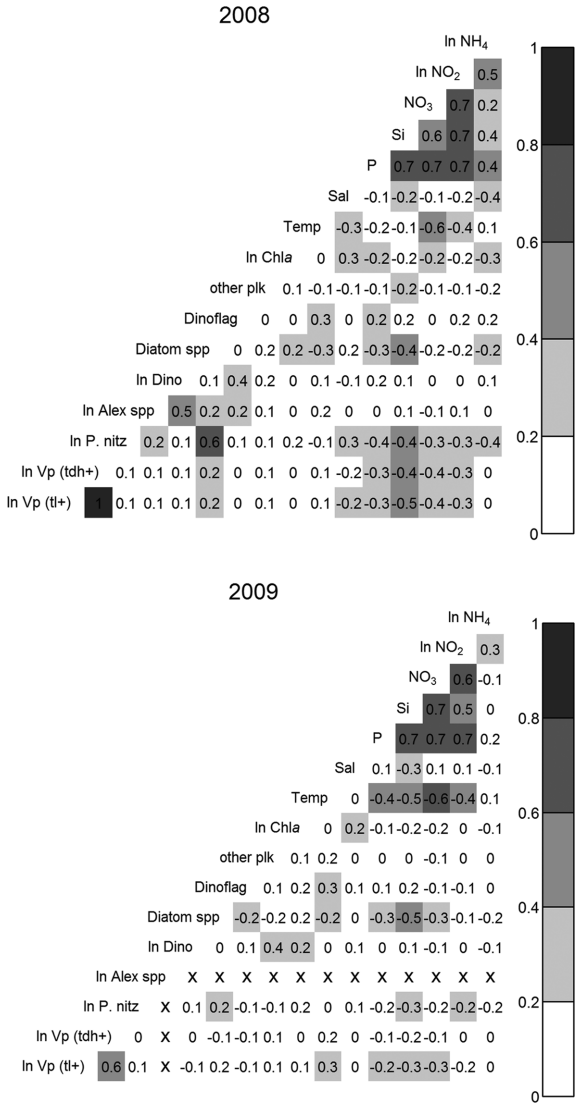

Figure 3. The correlation between the different biotic and abiotic variables examined in 2008 and 2009. The gray scale represents the magnitude of the correlation (independent of sign).

best model (with the lowest AIC) included the temporal spline (seasonal trend) alone confirming the seasonal pattern and suggesting that most of the variability explained by the biotic and abiotic covariates was seasonal. This is further illustrated by the estimated sizes of the individual model coefficients with and without the temporal spline included (Fig. 5). While silicate, temperature, nitrite and *Alexandrium* spp. all show predictive promise, when the temporal spline is added these results are largely erased (Fig. 5 and Table 2). In addition, any inference based on models without the temporal spline is likely invalid due to substantial temporal correlation in the residuals. In spite of clear differences between sites in the abiotic variables (Fig. 2), there was little support for corresponding between-site differences in *V. parahaemolyticus* (Table 2).

By including the temporal spline in the model we are removing large-scale temporal patterns in the outcome variables that could be explained by the biotic and abiotic explanatory variables. The spline represents the abundance of biotic and abiotic processes that occur seasonally where inferring specific causal links would be precarious (especially with only two years of data). However, there were some suggestive relationships between the explanatory and outcome variables. In particular, silicate was still significant in the model of *V. parahaemolyticus* that included the spline (Fig. 5). The importance of a number of other explanatory variables that were significant in the models without the spline and location is uncertain.

DISCUSSION

The environmental drivers that affect populations of *V. parahaemolyticus* are complex. Although the abiotic parameters such as temperature, salinity and nutrients can be measured relatively easily, assessment of the biotic factors such as differences in plankton taxa and abundance can be more challenging. In studies that have examined abiotic and biotic factors, higher *V. parahaemolyticus* densities have been shown to correspond with warmer summer months, especially in temperate regions (Kaneko and Colwell 1973; Cook, Bowers and DePaola 2002; Duan and Su 2005; Froelich *et al.* 2015; Blackwell and Oliver 2008; Parveen *et al.* 2008; Johnson *et al.* 2010; Julie *et al.* 2010; Caburlotto *et al.* 2011; Johnson *et al.* 2012; Böer *et al.* 2013). However other studies have indicated that the effect of temperature is less consistent depending on geographic location (Martinez-Urtaza *et al.* 2012; Moore *et al.* 2014; Ramos *et al.* 2014; Turner *et al.* 2014). A recent review synthesizing findings from several studies found that while temperature and salinity show the strongest correlation with populations of *Vibrio* spp., the significance of these and other abiotic and biotic factors, such as nutrients, chlorophyll-*a*, plankton, dissolved organic carbon and turbidity, have varying effects depending on species, geographic location or environmental niche (Takemura, Chien and Polz 2014). In addition, the correlation of temperature and salinity with total *Vibrio* populations appears to be less significant in areas or during seasons where there is limited variability in these factors (Takemura, Chien and Polz 2014). In our study the marginal effect of temperature and some of the other abiotic variables (nitrate, salinity) was overwhelmed after adding the effect of the seasonal component (temporal spline) to the model. *Vibrio parahaemolyticus* concentrations started increasing in early spring as temperatures increased, but the bacteria reached their maximum concentration much earlier in the summer (mid June) than the peak in water temperature (early August). This suggests that along with temperature other biotic and abiotic factors may have acted in concert to promote a flux in density of *V. parahaemolyticus* within the water column that was not perfectly correlated with temperature. It is possible that there is a threshold temperature (~12–15°C) that triggers increases in concentrations of these bacteria.

In our model, silicate was the strongest predictor of *V. parahaemolyticus* density among the biotic and abiotic variables although this predictive power was reduced when the seasonal component was added to the model, suggesting that seasonality is a key parameter in predicting trends of *V. parahaemolyticus* in the PNW. Interestingly the lowest concentrations of silicate coincided with maximum concentrations of *V. parahaemolyticus* for both years. This negative correlation of silicate concentration with *V. parahaemolyticus* density (albeit weaker when the seasonal trend was added) is intriguing in the context of our previous study that described the interaction of *V. parahaemolyticus* with the chitin-producing diatom *Thalassiosira weissflogii* (Frischkorn, Stojanovski and Paranjpye 2013). The mesocosm study by Frischkorn *et al.* reports that when starved of silicic acid, the diatom *T. weissflogii*, responds by increasing chitin

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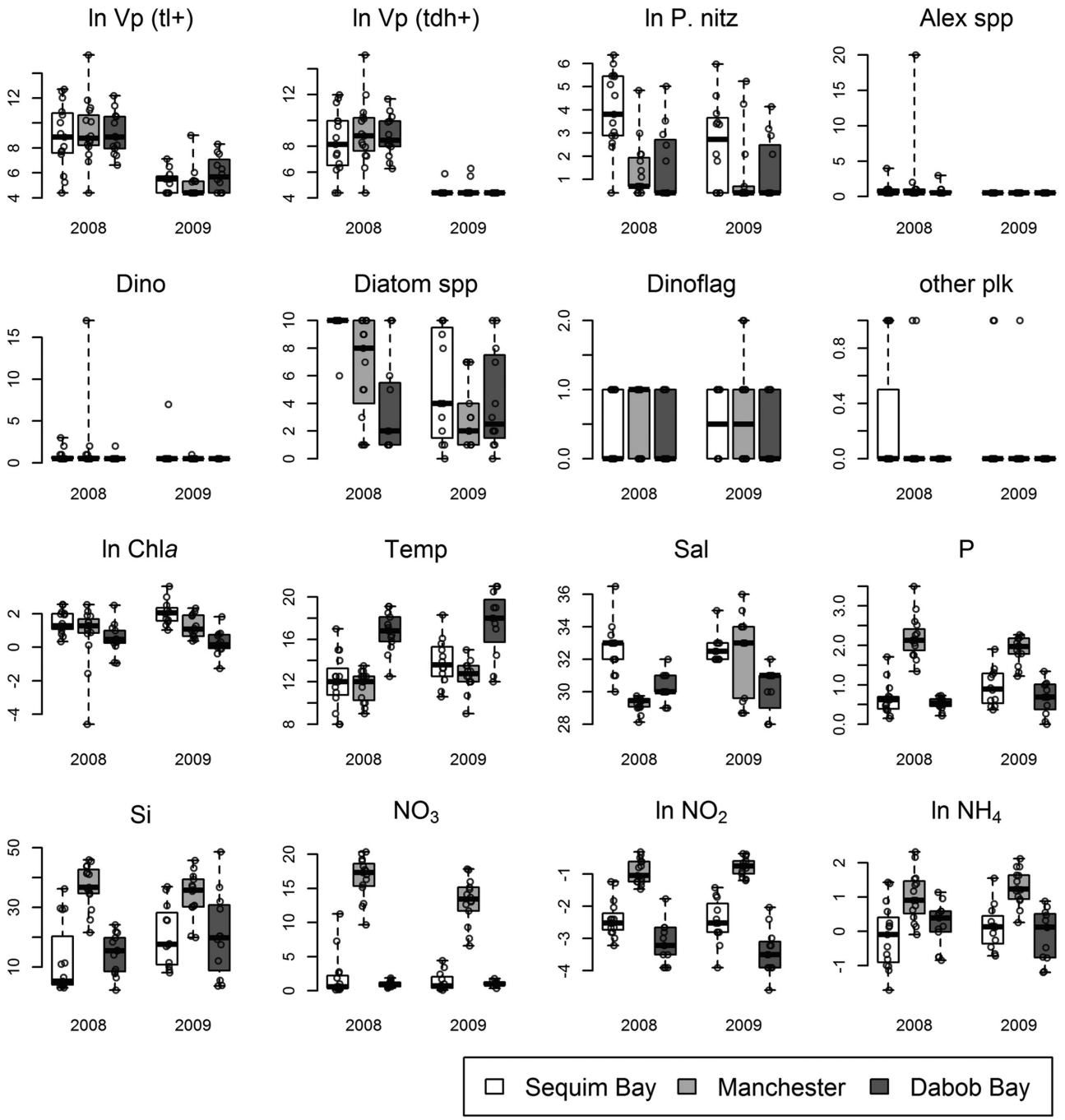

Figure 4. Comparison of the biotic [*V. parahaemolyticus* (Vp), *Pseudo-nitzschia* spp. (P. nitz), *Alexandrium* spp. (Alex), *Dinophsis* spp. (Dino), total diatoms, dinoflagellates, other plankton and chlorophyll-*a*] and abiotic variables [temperature (°C), Salinity (PSU), phosphorus (P), silicate (Si), nitrate (NO_3), nitrite (NO_2) and ammonium (NH_4)] between early May and late August across three sites for two years (2008–2009).

production (Durkin, Mock and Armbrust 2009), which in turn results in increased density of *V. parahaemolyticus* due to adherence to chitin (Frischkorn, Stojanovski and Paranjpye 2013). Like most vibrios, *V. parahaemolyticus* has been shown to adhere to and utilize chitin (Kaneko and Colwell 1975; Chiavelli, Marsh and Taylor 2001; Grimes *et al.* 2009). Another recent study also verified the increased detection of all *Vibrio* spp. with higher abundances of chitinous zooplankton and chitin-producing diatoms (Turner et al 2014). Decreasing environmental concentrations of silicate that induce increases in chitin production may therefore be one indicator of increased vibrio abundance in the Pacific Northwest. Future studies that examine the dynamics between silicate concentrations, populations of chitin-producing diatoms and densities of vibrios in the environment would be necessary to shed more light on these observations.

The effect of salinity was not significant and shifted from a slight negative effect without the seasonal component to no effect when the seasonal trend was included. Salinities at all locations in our study were relatively high and showed little variation during the sampling period. In other studies, salinity

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Table 2. A summary of fit for the different models of *V. parahaemolyticus* (ln Vp *tl*) concentrations. The term abiotic refers to the group of variables temperature, salinity, silicate, log nitrite and log ammonium, while biotic refers to log chlorophyll-*a*, log *Pseudo-nitzschia* spp. cells, *Alexandrium* spp. and presence/absence of diatom cells.

Outcome	Variables	ΔAIC[a]	R^{2b}	p^c	nllk[d]
ln Vp *tl*	1	141.2	0	1	370.3
	abiotic	119.7	0.17	6	354.6
	biotic	116.3	0.16	5	353.9
	abiotic + biotic	96.7	0.3	10	339.1
	temporal spline	0	0.56	5	295.8
	abiotic + biotic + temporal spline	5.7	0.6	14	289.6
	temporal spline + location	4.7	0.57	9	294.1
	abiotic +biotic + temporal spline + location	9.6	0.61	18	287.6

[a]Akaike information criterion (AIC) approximates the relative evidence for the different models with lower values indicating a better fit; [b]The pseudo R^2 metric describing absolute fit (McKelvey and Zavoina 1975); [c]Number of parameters in the model; [d]The negative log likelihood.

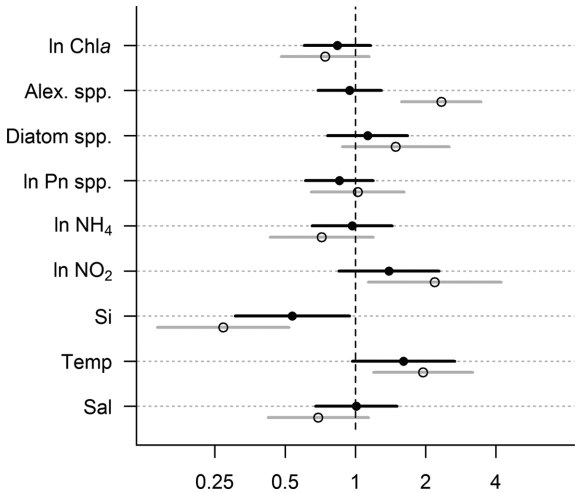

Figure 5. Coefficient plots showing the coefficient values for the dependence of *V. parahaemolyticus* density on selected variables and their approximate 95% confidence intervals. The predictor variables were standardized by dividing by their standard deviation so the relative magnitude of the coefficient values can be viewed as the approximate contribution/importance of the predictor. The gray line represents the model with biotic and abiotic variables alone while the dark line represent the addition of the temporal spline to these variables.

appears to be more significantly correlated with the incidence of *V. parahaemolyticus* when salinities vary over a wide range (Zimmerman *et al.* 2007; Johnson *et al.* 2010) while weak or no correlation has been reported in areas with little variation in salinity (Cook, Bowers and DePaola 2002; DePaola *et al.* 2003b; Duan and Su 2005; Baffone *et al.* 2006; Parveen *et al.* 2008). It is therefore not surprising that we did not see an effect of salinity on *V. parahaemolyticus* concentrations in our study since salinity did not vary significantly.

It is difficult to assess the optimal effects of the HABs, total diatoms and dinoflagellates, on *V. parahaemolyticus* populations during our study period since we did not observe any significant HAB blooms during 2008–2009 and concentrations of all the phytoplankton were relatively low during both years. *Alexandrium* spp. and to a lesser extent diatom spp. showed some correlation with concentrations of total *V. parahaemolyticus*. However as with the abiotic factors, this correlation was not significant after accounting for the seasonal influence.

The geographic locations of the sampling sites in our study varied from estuarine to coastal (Sequim Bay). This variation in the geographic location was reflected in the differences in the salinity, nutrients and phytoplankton populations in the areas sampled. However these differences did not affect the concentrations of either total or *tdh*+ *V. parahaemolyticus* between the sampling sites suggesting that the environmental drivers for *V. parahaemolyticus* and HABS may not be interdependent. Like all vibrios, *V. parahaemolyticus* can proliferate both as free living cells and attached to particles or substrates in the environment and the effects of HAB blooms may provide nutrients or substrates for increased concentrations of these bacteria. The lack of variation in vibrio densities between sites suggests that although we detected variations in concentrations of HAB species between sites, the generally low total concentrations of HABs likely had little impact on concentrations of vibrios.

Very low concentrations of *V. parahaemolyticus* were detected in the water during the colder months. The detection method we used did not rely on culturing the bacteria, and is therefore less biased. It is also conceivable that we detected viable but not culturable (VBNC) bacteria and/or dead cells in both early spring and late fall at colder temperatures that would not be detected by culture-based methods, similar to observations by Vezzulli *et al.* (2009). *Vibrio* spp. can persist in the aquatic environment in the VBNC state (Colwell *et al.* 1985). Detection of these bacteria is essential since they can be pathogenic under the right conditions and pose a threat to human health (Jiang and Chai 1996; Colwell *et al.* 1996; Baffone *et al.* 2003).

The high incidence of *tdh*+ *V. parahaemolyticus* in our study was verified using traditional culture methods to isolate the bacteria and subsequent endpoint PCR (data not shown). We have previously reported higher incidences of *tdh*+ and *trh*+ *V. parahaemolyticus* isolates from Washington State as compared to areas in the Gulf Coast and Maryland (Johnson *et al.* 2012). Studies reporting higher incidences of *tdh*+ environmental isolates suggest that shifts in genotypes may be influenced by increases in temperature and changes in plankton composition (Turner *et al.* 2014) or improved detection methods (Klein *et al.* 2014). Other factors may be in play. Matz *et al.* (2011) showed that *V. parahaemolyticus* strains carrying genes encoding the type 3 secretion system-2 (TTSS-2) are more resistant to grazing by a diverse list of protists including flagellates, ciliates and amoebae, suggesting that the presence of cytotoxins may offer a fitness advantage in the environment (Matz *et al.* 2011). Since *tdh* is genetically linked to TTSS-2 (Makino *et al.* 2003), it may be possible that the presence of TTSS-2 and/or the secretion of thermostable direct hemolysin offers a competitive advantage for these strains in the PNW. A detailed study of grazers unique to the Puget Sound region would be required to provide support for such a hypothesis.

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The higher abundance of both total (∼56-fold higher) and *tdh*+ (∼195-fold higher) *V. parahaemolyticus* in 2008 compared to 2009 was not related to the biotic and abiotic parameters examined in this study. A review of the large-scale patterns of climate variability in the Pacific Northwest suggested slightly delayed upwelling on the Washington Coast in 2009 compared to 2008 with a corresponding delay in chlorophyll-*a* concentrations (http://www.pfeg.noaa.gov/products/PFEL/modeled/indices/upwelling/upwelling.html). This may have resulted in an uncoupling of the timing of the spring diatom bloom from the window of optimal temperature for vibrios resulting in a lower density. However there were no differences in other indices of climate variations such as El Nino–Southern Oscillation (ENSO), the Pacific Decadal Oscillation (PDO), the North Pacific Gyre Oscillation (NPGO), or seasonal upwelling winds that might explain the variation between the two years (http://www.psp.wa.gov/downloads/psemp/PSmarinewaters_2011_overview.pdf). In 2009, the Puget Sound area did experience an unusually wet spring with heavy rainfall in the months of April–May that may have influenced the nutrient loads (http://cdiac.ornl.gov/epubs/ndp/ushcn/ushcn.html). However we did not detect any significant differences in the salinity at our sampling sites in 2009 that may reflect the effect of heavy rainfall or fresh water input.

In our study, concentrations of *V. parahaemolyticus* and the environmental variables show a strong temporal pattern that includes both a seasonal and annual component. Temperature by itself was not a strong predictor of *V. parahaemolyticus* densities in the PNW, but a threshold temperature (∼12–15°C) along with levels of nutrients, especially reduced levels of silicate, is more likely to trigger increases in *V. parahaemolyticus* concentrations. Future studies conducted over a longer period incorporating additional abiotic and biotic variables as well as hydrodynamic differences might provide additional insight into conditions that impact both *V. parahaemolyticus* and species of harmful algae in the Pacific Northwest. The complex correlation with temperature in our study strongly suggests that differences in geographic locations will need to be taken account when predicting increases of *V. parahaemolyticus* in the environment.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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