Influence of shellfish bed substrate on rate of growth of *Vibrio parahaemolyticus* in oysters harvested from Washington State.

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Background

Bacteria in the genus *Vibrio* are responsible for the majority of bacterial infections from the consumption of seafood in the United States. One species, *Vibrio parahaemolyticus,* is responsible for the majority of illnesses in Washington State and the United States, usually from consumption of raw or undercooked oysters. Predicting increases in concentrations of *Vibrios* in oysters and water to optimize harvest conditions has been challenging. Current tools based on changes in air temperature offer some insights on the influence on the growth of *V. parahaemolyticus* in oysters. However, there is a strong need to evaluate and understand the role of additional environmental parameters on the concentrations of these bacteria in oysters and to improve the ability to predict such increases. Such information would assist public health managers and shellfish growers to take conservative measures to protect their shellfish harvest and to protect public health.

Washington State is the largest shellfish producing state in the U.S and has the largest burden of *V. parahaemolyticus*-related illnesses. Regional differences, based on variations in ecology, *V. parahaemolyticus* strains and shellfish growing areas and harvesting practices may ultimately affect the likelihood of illnesses. Since a significant proportion of the oysters in this region are harvested inter-tidally, differences in the type of substrate in intertidal shellfish growing areas (gravel, mud, etc) could potentially influence the growth of *V. parahaemolyticus* in oysters.

Approach

This study was conducted to evaluate the effect of substrate type on concentrations of *V. parahaemolyticus* in oysters. A second objective was to determine if modeled weather data is useful for prediction of *V. parahaemolyticus* in oysters.

The study was conducted in three distinct growing areas in Washington State; Oakland Bay, Samish Bay and Hood Canal 5, areas that have been historically associated with a significant number of *V. parahaemolyticus*-associated illnesses (Figure 1). In the first year of the study, 2015, *V. parahaemolyticus* concentrations were analyzed in oysters harvested from three different substrate types rocky (gravel), muddy and a mixture of sand and mud (regular) within each growing area. Oysters were collected and analyzed for *V. parahaemolyticus* concentrations (total*, tlh+* and *tdh+*) weekly from June through September 2015. Environmental parameters collected at time of sampling included, air and water temperature,

oyster tissue temperature, and salinity. Samples were collected immediately after low tide to avoid undue exposure to ambient air temperature. The study was repeated in 2016 at the same sampling locations with all the same parameters and analysis for both years.

Methods and analysis

In this follow up study, a total of 121 oyster samples were collected throughout fourteen weeks of sampling from June 20 through September 15, 2016. Oyster samples were analyzed for the species marker, *tlh* to determine the presence of total *Vibrio parahaemolyticus* and the pathogenicity marker *tdh* (thermostable direct hemolysin). The Washington Department of Public Health Lab (WA DOH) analyzed the routine environmental samples from mixed substrates at Hood Canal 5, Oakland Bay and Samish Bay while NOAA NWFSC analyzed the additional environmental samples from rocky and muddy substrates at each site weekly. Temperature dataloggers were deployed at each site for the season and collected at the end of the project. Additional climate data, wind, temperature and radiation was obtained from the North American Mesoscale Forecast System (NAM) site (https://www.ncdc.noaa.gov/data-access/model-data/model-datasets/north-american-mesoscale-forecast-system-nam) for each site.

Detection and enumeration of *V. parahaemolyticus*
V. parahaemolyticus were enumerated following the FDA, MPN (Most probable number) protocol
(http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm).
After 24 h of enrichment in Alkaline Peptone broth (APW), 1-ml aliquots of each enrichment were boiled for 10 mins, cooled immediately in ice-water and stored at -20°C for analysis. Samples were analyzed for *tlh* and *tdh* following the protocol by Nordstrom et al ( ).

Statistical analysis

Two general lines of questioning were pursued with the combined 2015-2016 data. First, we examined the influence of substrate type on *V. parahaemolyticus* concentration relative to the sampling location, water temperature, and year. This was conducted by coding site and substrate and using water temperature as a covariate in a general linear model (Proc GLM, SAS). Second, we examined the potential for modeled weather data to be used in predictive models for elevated concentration of *V. parahaemolyticus* (> 10,000/g) and the presence of virulence-associated genes (*tdh, trh*). Stepwise logistic regression was employed to choose candidate variables for the models (Proc Logistic, SAS). In addition, simple frequency analysis was used to describe the proportion of *tdh,* and *trh* positive samples by location and substrate type.

Results

Influence of substrate type on concentrations of total (*tlh*)*V. parahaemolyticus (Vp)*

The concentration of total *Vp* in oysters was significantly influenced by substrate type with highest concentrations found on gravel, followed by mud and regular substrates (P <.0001). While this trend was apparent in both years, *Vp* concentrations were higher overall and the differences in substrate more pronounced in 2015 than in 2016 (Figure 2). In both years, concentrations were significantly lower in Samish than the other sites. Both air and water temperatures were significantly higher overall in 2015 by over 2°C (P < .0001) which may have influenced results.

Logistic regression was employed to examine the potential power of NAM weather variables to predict when *Vp* concentrations would exceed the FDA risk criteria of 10,000 CFU/g. A model using only substrate type and upward long-wave radiation flux (ULWRF) demonstrated high concordance, correctly classifying high concentrations 92% of the time. ULWRF is the rate of energy flow from terrestrial emissions, or stored radiative energy being released. The relationship of ULWRF and elevated *Vp* concentrations is positive.

Virulence associated genes

The thermostable direct hemolysin gene (*tdh*) was more prevalent in 2015 than 2016 (42% vs 18% of all samples). Interestingly, *tdh+* strains were rarely identified when total concentrations exceeded 10,000/g. In fact, *tdh+* strains were found in only 7% of samples exceeding the FDA criteria of 10,000/g and thus absent 93% of the time. Similarly, *tdh+* strains were more prevalent on regular substrate (which is associated with the lowest *Vp* concentrations in this study) than on mud or gravel. The frequency of detection on regular substrate differed between years being 79% in 2015 and 31% in 2016, but was similar for mud and gravel (8-12% both years). While only *tdh* was examined in this study, WDOH also tested for *trh* in their routine monitoring throughout the Puget Sound in 2015 and 2016. To determine the relevance of using *tdh* only, we examined the frequency of occurrence of both virulence-correlated genes in the larger data set. In only one instance was *trh* detected in the absence of *tdh* (Table 1) that gives great confidence that *tdh* alone is a reliable indicator of the presence of both genes in this data set. However, *trh* was present in 75% of samples negative for *tdh* and in 87% of all samples from this monitoring effort.

Preliminary logistic regression models were also explored for the presence of *tdh*. A model using substrate type, ULWRF and VWIND (horizontal wind vector) demonstrated concordance of 86.6 percent. Parameter estimates for VWIND and ULWRF were positive suggesting elevated long-wave surface radiation and southerly winds were influential. However, the inclusion of VWIND in the model does not dramatically improve performance and requires further evaluation.

Discussion

The consistent relationship between substrate type and concentrations of V. parahaemolyticus in spite of significant variation in air temperature for the 2 years 2015 and 2016 offers

promise that differences in substrate concentration together with climate data could be used to predict concentrations of these bacteria in oysters from various shellfish growing areas. By using substrate type and NAM variables, the potential exists to provide short term (several day lead) forecasts of the probability of exceeding FDA criteria or the presence of virulence-associated genes. However, the annual variability noted in this study will require more data in subsequent years, or further exploration of the WDOH dataset to validate the model. WDOH has classified their growing areas based on substrate type, and the potential exists to use these models help tailor harvest during the growing season to minimize risk.
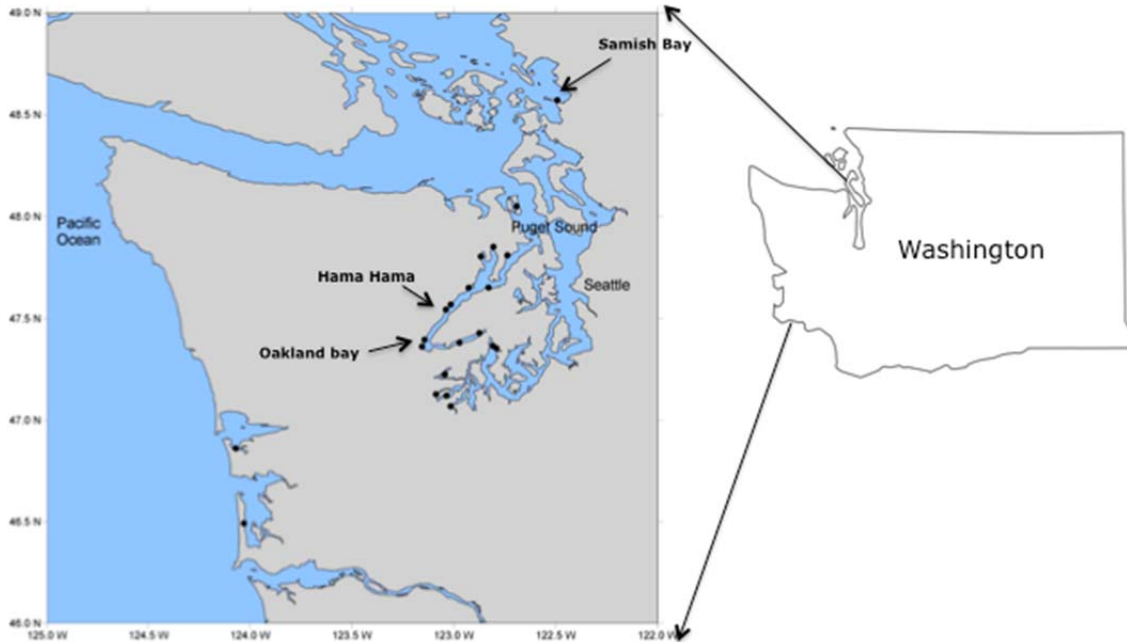

Figure 1. Sampling sites in Washington State.

Table 1. Frequency of occurrence of *tdh* and *trh* genes in WDOH routine monitoring stations for 2015-2016.

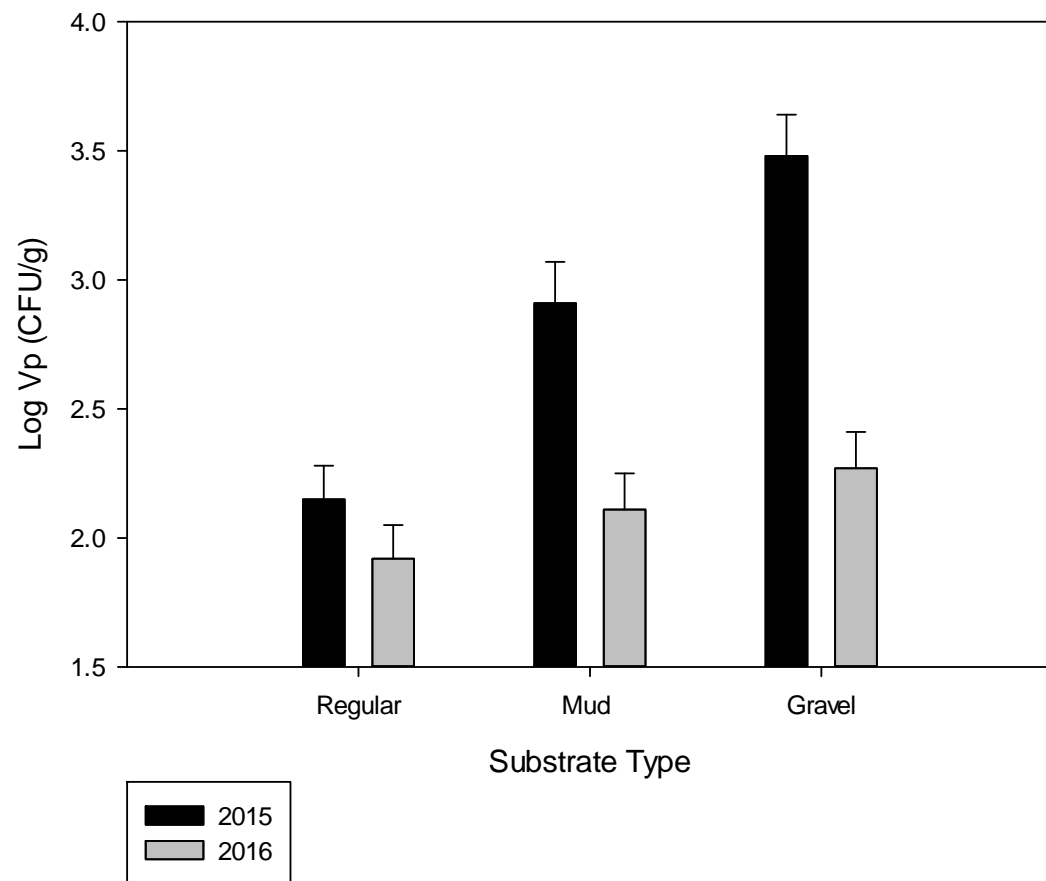| Frequency Percent Row Pct Col Pct	Table of tdhp by trhp		
		trhp	
tdhp	P	X	Total
P	225 48.49 99.56 55.56	1 0.22 0.44 1.69	226 48.71
X	180 38.79 75.63 44.44	58 12.50 24.37 98.31	238 51.29
Total	405 87.28	59 12.72	464 100.00

Figure 2 - Log Vp concentration by year and substrate type