Final Report

Fishery Resource Grant Program

Project Title: Analysis of short-term temporal variation in densities of pathogenic *Vibrio* species in Virginia oysters

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Summary

The objective of this project was to establish the baseline densities of the pathogenic *Vibrio parahaemolyticus* (*Vp*) and *Vibrio vulnificus* (*Vv*) strains in oysters from Virginia across seasons and over a short time scale. Oyster and water samples were collected on a biweekly basis from April through November 2014 and were analyzed for total *Vp*, pathogenic *Vp* (*Vp tdh* and *Vp trh*), total *Vv* and pathogenic *Vv* (*Vv pilF*). Concentration of these bacteria was evaluated following previously published protocols using a most-probable number approach followed by quantitative PCR. Total *Vp*, total *Vv* and *Vv pilF* showed a clear seasonal cycle but with different dynamics among sites. As shown in other studies, under high salinities (> 30 psu) lower total *Vv* densities were observed. Such association with the salinity regime was not as clear for total *Vp.* Under warm water temperatures (≥ 24˚C), salinity and turbidity were significant predictors of total *Vv* densities in oysters, while salinity and chlorophyll a were significant predictors of total *Vp* densities and *Vv pilF*. For these bacteria, densities measured in oysters were significantly correlated to densities measured in surrounding waters. Densities of *Vv pilF* were found to be significantly correlated to total *Vv*. Occurrence of *Vp tdh* and *Vp trh* in oysters did not appear to follow a clear seasonal cycle as observed for total *Vp*. Furthermore, densities of these pathogenic strains was not correlated to the density of total *Vp*. Temperature, however, was found to be a significant predictor of the occurrence of *Vp tdh* and *Vp trh* in oysters.

I. Project description

Problem to be addressed

During the summer of 2013, an increased number of *Vp* cases associated with the consumption of raw oysters or clams harvested from the Atlantic Coast of the United States was observed compared to the previous years (CDC 2013). The harvest states implicated in this "outbreak" were Connecticut, Massachusetts, New York and Virginia. The consequences were in some cases closure of the harvest area, recalls and all cases bad publicity and loss of revenue threatening coastal communities whose economies are tied to the shellfish industry.

Within the state of Virginia, the length of the closure of harvest area(s) may have been reduced if density data relative the pathogenic *Vp* strains had been available. Not all *Vp* strains are pathogenic and the detection of *Vp* pathogenic strains currently relies on the presence of the thermostable direct-hemolysin gene (*tdh*) and/or the detection of the thermostable direct hemolysin-related gene (*trh*) as these genes have been detected in more than 90% of the persons affected by the associated disease. The lack of data relative to the densities of these pathogenic strains in oysters documenting baseline levels and short time scale variability are lacking. This information, however, may help to design more sound responses to vibrio cases caused by either *Vp* or *Vv*, another *Vibrio* species of concern. Baseline levels are particularly relevant and needed in order to accurately assess risks and determine levels that may be safe or unsafe. Moreover, examining such densities over a short time scale may help to better understand density dynamics promoting more appropriate responses to outbreaks protecting both the oyster industry and human health.

Objective

The objective of this project was to establish the baseline densities of the pathogenic *Vp* and *Vv* strains in oysters from Virginia across seasons and over short time scale so that sound response measures can be implemented in the event of vibrio illness associated with consumption of oysters from this region.

Materials and methods

Study sites. Oysters provided by oyster growers, as well as the overlying water samples were collected biweekly from April through November 2014 from three sites in Virginia. Two of the sites were located on the Western shore of the lower Chesapeake Bay, with one site being exposed to low salinities (≤ 16 psu) and the other site being exposed to moderate salinities (13-24 psu). The third site was exposed to high salinities (> 30 psu) and was located on the seaside of the Eastern shore of Virginia. Oysters were grown intertidally, i.e. they were potentially exposed to air at low tide, at both the moderate and the high salinity sites. Oysters were grown under subtidal conditions at the low salinity site, i.e. they were not exposed to air even at low tide.
Additional water samples were collected for the analysis of turbidity and chlorophyll a and were analyzed by the VIMS analytical service center. Although we initially intended

to collect data regarding tidal conditions, as well as light and wind conditions these data could not be collected consistently across sites so they were not taken into account in this study.

Sample processing using a MPN approach. Vibrio densities were measured using a most-probable number (MPN) approach followed by quantitative PCR (qPCR) as previously described (Audemard et al. 2011). Briefly, samples and decimal dilutions thereof were inoculated into an alkaline peptone water (APW) most-probable-number (MPN) series as described in Chapter 9, "Vibrio", of the most recent online version of the FDA Bacteriological Analytical Manual (U.S. FDA 1998). For the oyster samples, 4 replicate samples each consisting of homogenates prepared from the tissues and shell liquor of 10 oysters were analyzed at each time point. For each site a water sample was analyzed and decimal dilutions were prepared and inoculated as described above for the oyster homogenates.

Detection of Vibrio *spp. in enrichments by quantitative PCR.* Detection of total *Vp* in the enrichments was accomplished by targeting the thermolabile hemolysin gene (*tlh*) in a multiplex qPCR assay during which both *Vp tlh* and *Vp tdh* strains were targeted using the primers and probes designed by Nordstrom et al. (2007). Modifications to the published protocols were as described in Audemard et al. (2011) except the master mix used for this study was the TaqMan® Fast Advanced Master Mix (Life Technologies, Grand Island, NY) and the fast cycling conditions recommended with this mix were used (20 s at 95˚C followed by 40 cycles with each cycle consisting of 3 s at 95˚C and 30 s at 60˚C). Detection of the *Vp trh* strains was performed in a separate qPCR assay using the primers, probe and cycling conditions described by Nordstrom et al. (2007) with the following master mix final concentrations: bovine serum albumin at 0.4 µg/µl, 1X TaqMan® Fast Advanced Master Mix (Life Technologies, Grand Island, NY), 0.3 µM of primers and 0.15 µM of the probe, 1 µl of the template and a final reaction volume of 10 µl. Detection of total *Vv* in APW enrichment lysates was performed by targeting the hemolysin/cytolysin gene (*vvhA*) using the assay designed by Campbell and Wright (2003) as described in Audemard et al. (2011) except that TaqMan® Fast Advanced Master Mix was used. Finally detection of potentially pathogenic *Vv* strains (*Vv pilF*) was performed using the assay described by Baker Austin et al. (2012) by targeting the *pilF* gene following the published conditions except for the template and reaction volumes which were reduced to 1 µl and 10 µl, respectively. Polymorphism within this gene has previously being associated with pathogenicity in humans (Roig et al. 2010). Resulting MPN values were calculated using approved MPN tables (2008). Individual replicate samples for which there was no detection of a particular *Vibrio* spp., were given a < 3.0 MPN g^{-1} or < 0.3 MPN ml^{-1} value for an oyster and a water sample, respectively.

Data analyses. Prior to the analyses described below, the individual replicate oyster or water samples that were associated with a 'less than' value were transformed to the whole integer, i.e. 3.00×10^{0} MPN g^{-1} or 3.00×10^{-1} MPN ml^{-1}, respectively. For the graphical representations and statistical analysis, the *Vibrio* spp. abundance data were $\log_{10}$ transformed to reduce the variance inherent to the bacterial densities.

For the oyster samples, the geometric means for total *Vp*, the *Vp tdh* and *Vp trh* strains, total *Vv* and *Vv pilF* were calculated from the four replicate samples collected from each time point.

Multiple linear regressions were run on density data collected for total *Vv*, *Vv pilF* and total *Vp* under water temperatures ≥ 24˚C to assess the relationship between these densities and salinity, chlorophyll a and turbidity under warm water temperatures favoring high bacteria densities. The values measured in individual replicate samples and after grouping the data from the three sampled sites. A hierarchical approach was used with the full model including interactions between salinity and chlorophyll a or turbidity. If one of the interaction terms was not significant the model was run without it.

The relationship between the abundance of total *Vv*, *Vv pilF* and total *Vp* in water samples and their abundances in oyster samples was investigated using simple linear regressions. The analysis of the relationship between the abundance of *Vp tdh* and *Vp trh* in water and oyster samples could not be evaluated due to the low number of positive samples for these strains in water samples.

The relationship between the densities in oysters of *Vv pilF* and the densities of total *Vv* was investigated using linear regression. Similarly, the relationship between both *Vp tdh* and *Vp trh* and the densities of total *Vp* was investigated using linear regression, however, in this case, due to the high number of *Vp tdh* and *Vp trh* values below the detection limit ($< 3.00 \times 10^0$ MPN g^{-1}), the analysis was conducted using only the data above the detection limit to reduce the large weighting of samples below the detection limit.

Finally, the relationship between the occurrence of both *Vp tdh* and *Vp trh* in oysters and the environmental parameters was investigated using logistic regression. For this analysis and for each gene, when the samples were positive for the gene of interest they were given a value of 1, and when the samples were negative for the gene they were given a value of 0. All statistical analyses were conducted using SPSS software (IBM SPSS Statistics 20; IBM, Armonk, NY). An alpha level of 0.05 was considered the minimum level for statistical significance.

II. Results

Environmental parameters

Water temperatures recorded at the three studied sites presented similar seasonal trends and ranges although the moderate salinity site differed from the other 2 sites by being associated with a wider range of recorded temperature (Fig. 1). Water temperature rose from ~ 15˚C in late April to ≥ 20˚C by mid-May at the 3 sites. In June, water temperatures at all the sites reached 24˚C and remained ≥ 24˚C until mid-September. Water temperature was above 20˚C till late October. The low, moderate and high salinity sites were associated with salinities ranging from 7 to 16 psu, 13 to 24 psu and 31 to 35 psu, respectively (Fig.1). For the chlorophyll a, the low, moderate and high salinity sites were associated with values ranging from 6.12 to 22.51 µg/l, 0.91 to 18.31 µg/l and 1.28 to 32.47 µg/l, respectively. Finally the turbidity measurements recorded at the low, moderate and high salinity site ranged from 0.5 to 18.1 NTU, 0.6 to 41.1 NTU and 3.2 to 160.0 NTU, respectively.

Total *Vv* and *Vv pilF*

Relationships with environmental parameters. Both total *Vv* and *Vv pilF* densities measured in oysters and water samples appeared to follow a seasonal cycle with the lowest values observed in spring and late fall and the highest values observed during the summer to early fall (Fig. 2 & 3). For total *Vv*, values as high as 1.10×10^6 MPN g^{-1} were observed in oysters at the low salinity site in early October. For *Vv pilF*, values as high as 2.14×10^4 MPN g^{-1} were observed at the low salinity site in early June. Overall higher values of total *Vv* and *Vv pilF* were observed at the low salinity site compared to the moderate and high salinity site. The analysis of data collected under water temperatures ≥ 24˚C using multiple liner regressions indicated that both total *Vv* and *Vv pilF* measured in oysters and water samples was negatively correlated to salinity. In addition to salinity, total *Vv* densities in oysters were positively correlated to turbidity and the interaction term salinity * turbidity was also significant (Table 1). For *Vv pilF*, chlorophyll a was found to be a significant predictor with the interaction term salinity * chlorophyll a being also significant.

Relationship of Vv *and* Vv pilF *abundance in oysters with abundance in water.* For both total *Vv* and *Vv pilF*, significant positive associations between the abundance of these bacteria in oysters and in surrounding waters were observed (Table 1).

Relationship of Vv pilF *with total* Vv. Significant positive association between the densities of *Vv pilF* in oysters and the densities of total *Vv* were observed (Table 1).

Total *Vp*, *Vp tdh* and *Vp trh*

Relationships with environmental parameters. As for *Vv*, densities of total *Vp* followed a seasonal cycle with the highest values of the order of 10^4 MPN g^{-1} being observed at each of the 3 sites during the summer (Fig. 4 & 5). Under water temperatures ≥ 24˚C, total *Vp* measured in oysters and in water samples was negatively correlated to salinity and chlorophyll a with the interaction term salinity * chlorophyll a also being significant (Table 2).

For both *Vp tdh* and *Vp trh*, highest densities in oysters were observed at the moderate salinity site from late May to early June (Fig. 4). At this site, levels of *Vp tdh* and *Vp trh* remained < 10 MPN/g during most of the sampling period except for 3 consecutive dates on May 20th, June 4th and June 18th during which both *Vp tdh* and *Vp trh* indicated elevated levels of the pathogenic strains. During this period, the observed levels of pathogenic *Vp* ranged from 30 to 86 MPN/g and 30 to 190 MPN/g for *Vp tdh* and *Vp trh*, respectively. Using logistic regression, temperature was found to be a significant predictor of the abundance of *Vp tdh* and *Vp trh* (Table 3). In the case of *Vp trh*, salinity was also found to be a significant predictor although the regression coefficient (-0.044) suggests a marginal association.

Relationship total Vp *abundance in oysters with abundance in water.* A significant positive association between the abundance of total *Vp* in oysters and in surrounding waters was observed (Table 2).

Relationship of Vp tdh *and* Vp trh *with total* Vp. Densities in oysters of both *Vp tdh* and *Vp trh* were not found to be significantly associated with the densities of total *Vp* (Table 3).

III. Conclusions

Analysis of the samples collected on a biweekly basis from April through November provides baseline data for Virginia waters regarding the concentration and dynamics of total and pathogenic *Vp* and *Vv*. Based on the biweekly sampling schedule, vibrio levels appeared to be significantly associated with changes in several environmental parameters. Conducting multiple regressions on the data collected under water temperatures ≥ 24˚C was very informative to explore which environmental factor may be predictor under temperature that are not limiting for the growth of these bacteria. This analysis indicated that total *Vv* in oysters and in water is negatively correlated to salinity. Prior studies have shown that salinities higher than 30 psu appear detrimental to the survival of *Vv* (Kaspar & Tamplin 1993; Motes et al. 1996; Motes et al. 1998; Audemard et al. 2011). Furthermore, turbidity was also a significant predictor of total *Vv* densities in oysters. For *Vv pilF* densities in oysters and water samples were also negatively correlated to salinity and chorophyll a was also found to be a predictor of the densities of these bacteria. For temperatures ≥ 24˚C densities of total *Vp* in oysters and in water samples were also negatively correlated to salinity, however for *Vp* turbidity was not a significant predictor while chlorophyll a was.

In this study, dynamics of potentially pathogenic *Vv* and *Vp* strains in oysters were of particular interest due to the potential human health concern associated with oyster consumption. In the case of *Vv pilF*, a strong correlation with total *Vv* levels was observed suggesting that these pathogenic strains respond to the same environmental cues as total *Vv*. Surprisingly, the multiple regression analysis suggested that turbidity is a stronger predictor of total *Vv* densities than chlorophyll a while the opposite was observed for *Vv pilF*. Such results may need to further investigated. Analysis of the samples collected in Virginia waters suggest that *Vv pilF* strains can represent a high percentage of the total *Vv* strains, in some cases accounting for 100% of them. This would imply that evaluating the risk of illness associated with *Vv* may be accomplished by assessing total *Vv* levels.

Densities in oysters of *Vp tdh* and *Vp trh* strains were not correlated with total *Vp*. As other studies (Zimmerman et al. 2007; FAO/WHO 2011), this study continues to highlight how total *Vp* levels are not a good indicator of human health risks. Levels of *Vp tdh* and *Vp trh* measured during this study were relatively low and based on logistic regressions analysis, water temperature was the main predictor of the occurrence of these strains in oysters. Interestingly, the highest levels of these 2 strains were measured concomitantly at the moderate salinity site during 3 consecutive sampling time point late spring. This seems to suggest that *Vp* related illness risks during this period may be higher than during the rest of the year. However, it needs to be reiterated that densities of these strains remains an indicator of risks but high densities may actually not be associated with elevated illness cases associated with *Vp*.

IV. Recommendations

Use of total *Vp* values for assessing human health risks is not warranted in light of data from this study (Zimmerman et al. 2007; FAO/WHO 2011). Therefore, risk criteria for *Vp* may need to be reevaluated. Better characterization of *Vp* strains for many locations, including Virginia, is needed. Identification of specific strains associated with outbreaks will provide information to develop better monitoring tools. For example, the recent development of a new tool that specifically identifies a pathogenic strain of *Vp*, specifically Atlantic strain ST36, that has been found associated with many of the *Vp* outbreaks (Whistler et al. 2015) is an important step in allowing more accurate risk assessment.

References

Audemard C, Kator HI, Rhodes MW, Gallivan T, Erskine AJ, Leggett TA, Reece KS. 2011. High salinity relay as a post-harvest processing strategy to reduce *Vibrio vulnificus* levels in Chesapeake Bay oysters (*Crassostrea virginica*). J. Food Prot. 74:1902–1907.

Baker-Austin C, Lemm E, Hartnell R, Lowther J, Onley R, Amaro C, Oliver JD, Lees D. 2012. *pilF* polymorphism-based real-time PCR to distinguish *Vibrio vulnificus* strains of human health relevance. Food Microbiol. 30:17-23.

Campbell MS, Wright AC. 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl. Environ. Microbiol. 69:7137–7144.

Centers for Disease Control and Prevention (CDC). 2013. Increase in *Vibrio parahaemolyticus* illnesses associated with consumption of shellfish from several Atlantic coast harvest areas, United States, 2013 http://www.cdc.gov/vibrio/investigations/index.html

FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization]. 2011. Risk assessment of Vibrio parahaemolyticus in seafood: Interpretative summary and Technical report. Microbiological Risk Assessment Series No. 16. Rome. 193pp.

Kaspar CW, Tamplin ML. 1993. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl. Environ. Microbiol. 59:2425–2429.

Motes ML, DePaola A. 1996. Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). Appl. Environ. Microbiol. 62:3875–3877.

Motes ML, DePaola A, Cook DW, Veazey JE, Hunsucker JC, Garthright WE, Blodgett RJ, Chirtel SJ. 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Appl. Environ. Microbiol. 64:1459–1465.

Nordstrom JL, Vickery MCL, Blackstone GM, Murray SL, DePaola A. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl. Environ. Microbiol. 73:5840–5847.

Roig FJ, Sanjuan E, Llorens A, Amaro C. 2010. *pilF* Polymorphism-Based PCR To Distinguish Vibrio vulnificus Strains Potentially Dangerous to Public Health. Appl. Environ. Microbiol. 76:1328-1333.

U.S. Food and Drug Administration. 2008. Most probable number procedures and tables. Available at: www.fsis.usda.gov/PDF/MLG_Appendix_2_03.pdf

Whistler CA, Hall JA, Xu F, Ilyas S, Siwakoti P, Cooper VS, Jones SH. 2015. Use of Whole Genome Phylogeny and Comparisons in the Development of a Multiplex-PCR Assay to Identify Sequence Type 36 *Vibrio parahaemolyticus***.** J. Clinical Microbiol., JCM.00034-15 DOI: 10.1128/JCM.00034-15

Zimmerman AM, DePaola A, Bowers JC, Krantz JA, Nordstrom JL, Johnson CN, Grimes DJ. 2007. Variability of total and pathogenic Vibrio parahaemolyticus densities in Northern Gulf of Mexico water and oysters. Appl. Environ. Microbiol. 73:7589–7596.

Table 1: Results of multiple and simple linear regression analysis conducted during this study for *V. vulnificus*. Water temperature was not taken into account as an independent variable and the influence of environmental parameters on densities of total and pathogenic *Vv* was assessed under water temperatures ≥ 24˚C. Analyses were conducted using values from individual replicate samples for the oyster samples and after grouping the data from all sites. In the case of multiple regressions, non-significant parameters ($P < 0.05$) were not listed unless they were significant when interacting with another parameter.

Dependent variable$_{\text{sample type}}$	Independent variable	F_{df}= value	R^2	Regression coefficient	*P*-value
$Log_{10}Vv_{oys.}$	y-intercept	$F_{3,87}$= 248.986	0.899	5.104	< 0.0001
	Sal.			-0.088	0.008
	Log_{10}Turb.			1.655	0.018
	Sal.*Log_{10}Turb.			-0.076	0.007
$Log_{10}VvpilF_{oys.}$	y-intercept	$F_{3,91}$= 208.159	0.876	8.146	< 0.0001
	Sal.			-0.233	< 0.0001
	Log_{10}Chloa			-2.357	0.002
	Sal.*Log_{10}Chloa			0.077	0.006
$Log_{10}Vv_{wat.}$	y-intercept	$F_{1,87}$= 266.452	0.756	4.174	< 0.0001
	Sal.			-0.128	< 0.0001
$Log_{10}VvpilF_{wat.}$	y-intercept	$F_{3,91}$= 53.375	0.645	6.712	< 0.0001
	Sal.			-0.229	< 0.0001
	Log_{10}Chloa			-3.323	0.001
	Sal.*Log_{10}Chloa			0.114	0.002
$Log_{10}Vv_{oys.}$	y-intercept	$F_{1,187}$= 811.203	0.812	1.517	< 0.0001
	$Log_{10}Vv_{wat.}$			1.085	< 0.0001
$Log_{10}VvpilF_{oys.}$	y-intercept	$F_{1,191}$= 462.306	0.709	1.458	< 0.0001
	$Log_{10}VvpilF_{wat.}$			1.034	< 0.0001
$Log_{10}VvpilF_{oys.}$	y-intercept	$F_{1,191}$= 2152.907	0.959	0.011	0.817
	$Log_{10}Vv_{oys.}$			0.74	< 0.0001

Table 2: Results of multiple and simple linear regression analysis conducted during this study for V. *parahaemolyticus*. Water temperature was not taken into account as an independent variable and the influence of environmental parameters on densities of total *Vp* was assessed under water temperatures ≥ 24˚C. Analyses were conducted using values from individual replicate samples for the oyster samples and after grouping the data from all sites. In the case of multiple regressions, non-significant parameters or interactions were not listed.

Dependent variable$_{\text{sample type}}$	Independent variable	F_{df}= value	R^2	Regression coefficient	*P*-value
$Log_{10}Vp_{oys.}$	y-intercept	$F_{3,87}$= 6.267	0.183	6.823	< 0.0001
	Sal.			-0.119	0.002
	Sal.*Log_{10}Chloa			0.106	0.001
$Log_{10}Vp_{wat.}$	y-intercept	$F_{3,87}$= 20.334	0.177	5.728	< 0.0001
	Sal.			-0.155	< 0.0001
	Log_{10}Chloa			-3.710	< 0.0001
	Sal.*Log_{10}Chloa			0.139	< 0.0001
$Log_{10}Vp_{oys.}$	y-intercept	$F_{1,191}$= 416.905	0.685	1.735	< 0.0001
	$Log_{10}Vp_{wat.}$			0.882	< 0.0001
$Log_{10}Vptdh_{oys.}$	y-intercept	$F_{1,56}$= 3.481	0.060	0.547	0.016
	$Log_{10}Vp_{oys.}$			0.122	0.067
$Log_{10}Vptrh_{oys.}$	y-intercept	$F_{1,42}$= 0.088	0.002	0.870	0.088
	$Log_{10}Vp_{oys.}$			0.040	0.768

Table 3: Results of logistic regression analysis.

Dependent variable$_{\text{sample type}}$	Predictor	Regression coefficient	Wald's X^2 (df = 1)	*P*-value
$Log_{10}Vptdh_{oys.}$	y-intercept	-4.012	22.431	< 0.0001
	Temp.	0.143	15.572	< 0.0001
	Sal.	_		NS
	$Log_{10}Chloa$	_		NS
	$Log_{10}Turb.$	_		NS
$Log_{10}Vptrh_{oys.}$	y-intercept	-2.398	6.270	0.012
	Temp.	0.094	6.341	0.012
	Sal.	-0.044	4.816	0.028
	$Log_{10}Chloa$	_		NS
	$Log_{10}Turb.$	_		NS

NS: no significant effect for the independent variable tested.

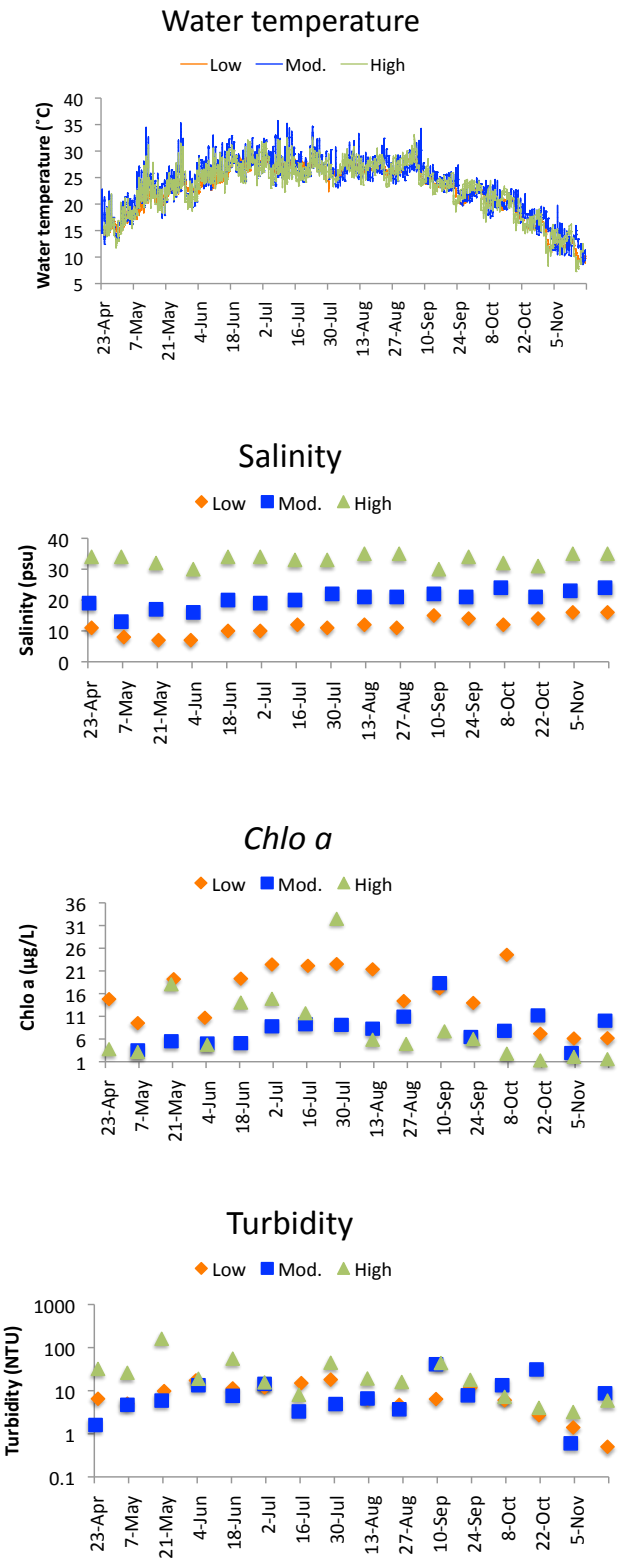

Fig. 1: Environmental parameters measured at the low, moderate and high salinity site during the course of the study.

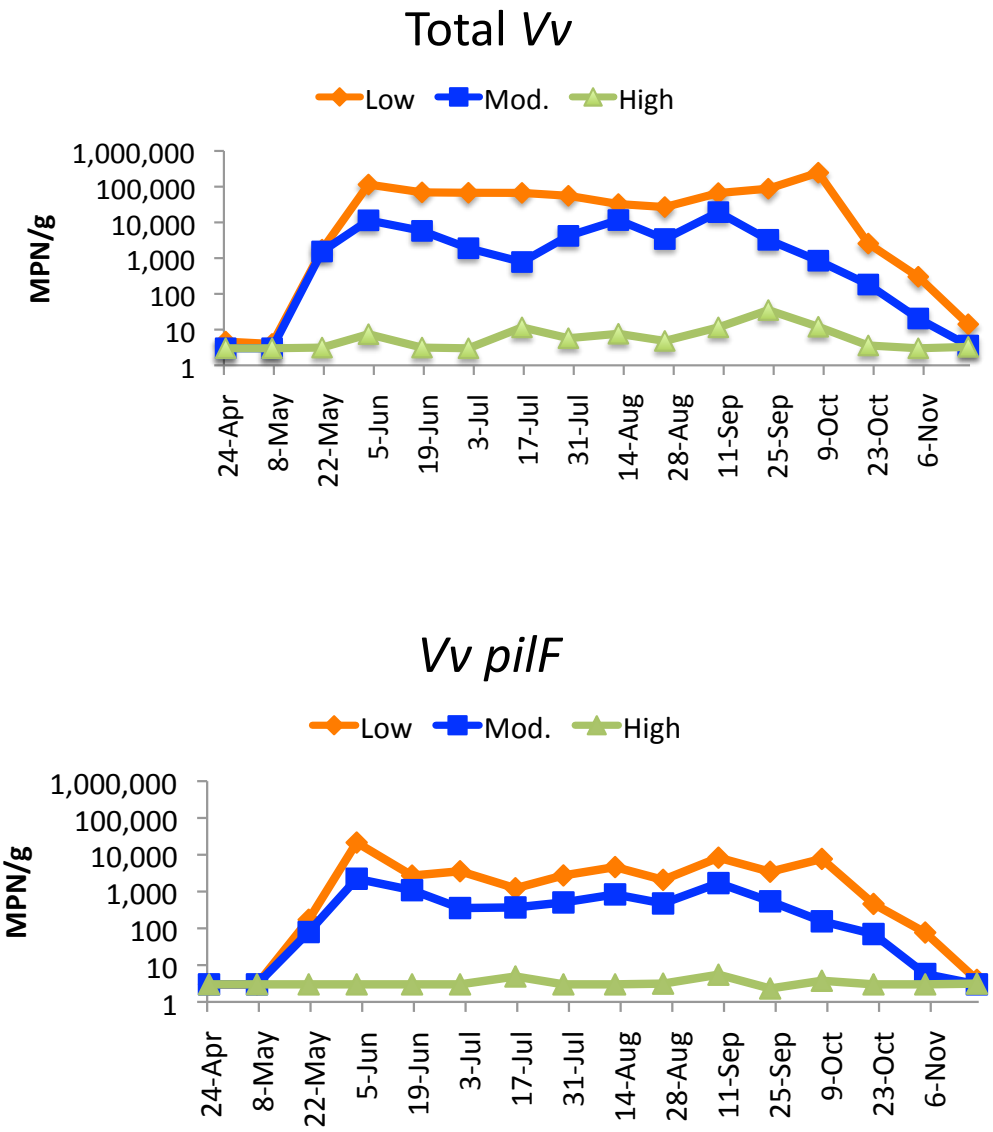

Fig. 2: Concentrations in oysters of total *V. vulnificus* (A) and clinical *V. vulnificus* possessing the *pilF* gene (B) measured at the 3 sites.

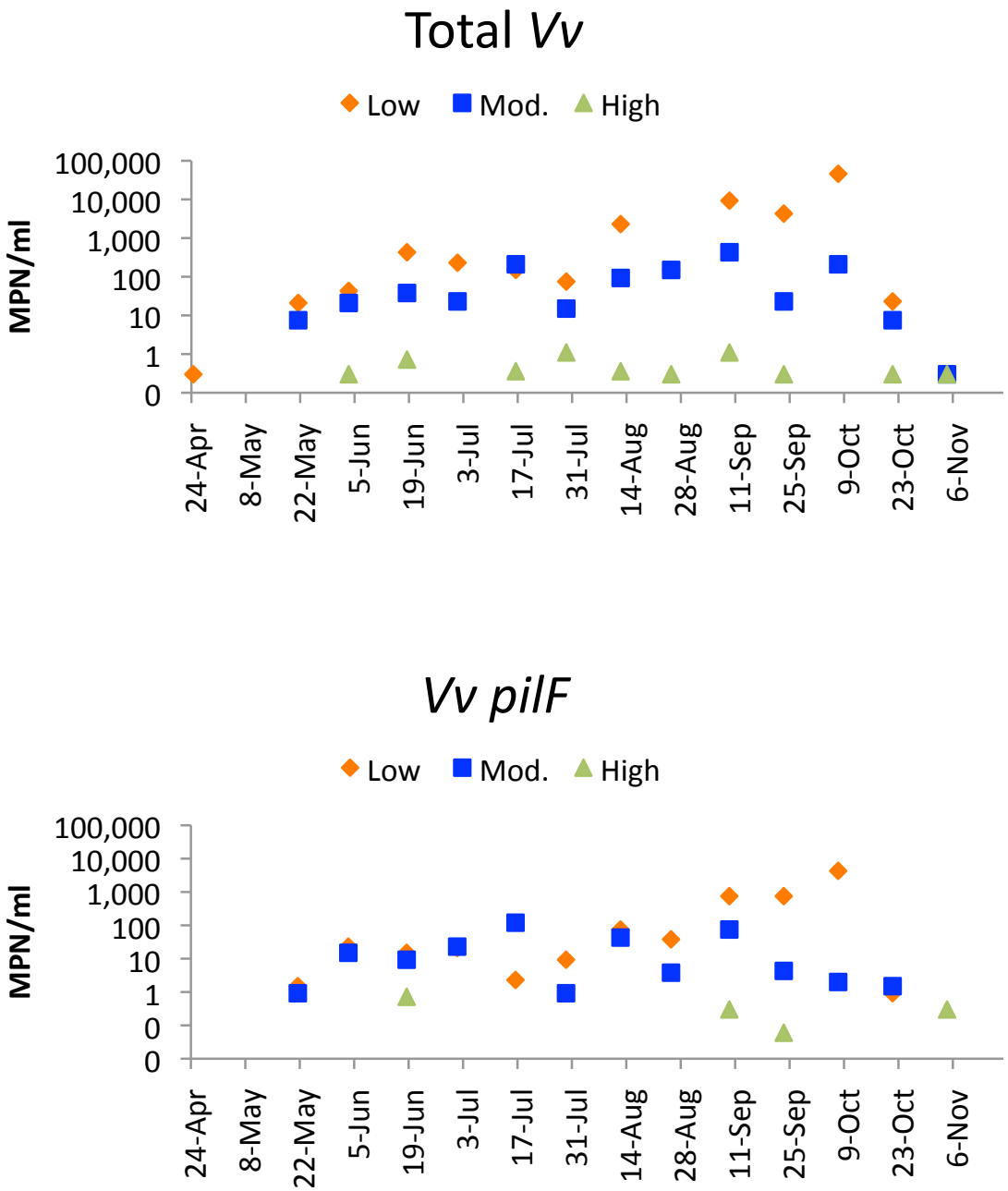

Fig. 3: Concentrations in water samples of total *V. vulnificus* (A) and clinical *V. vulnificus* possessing the *pilF* gene (B) measured at the 3 sites.

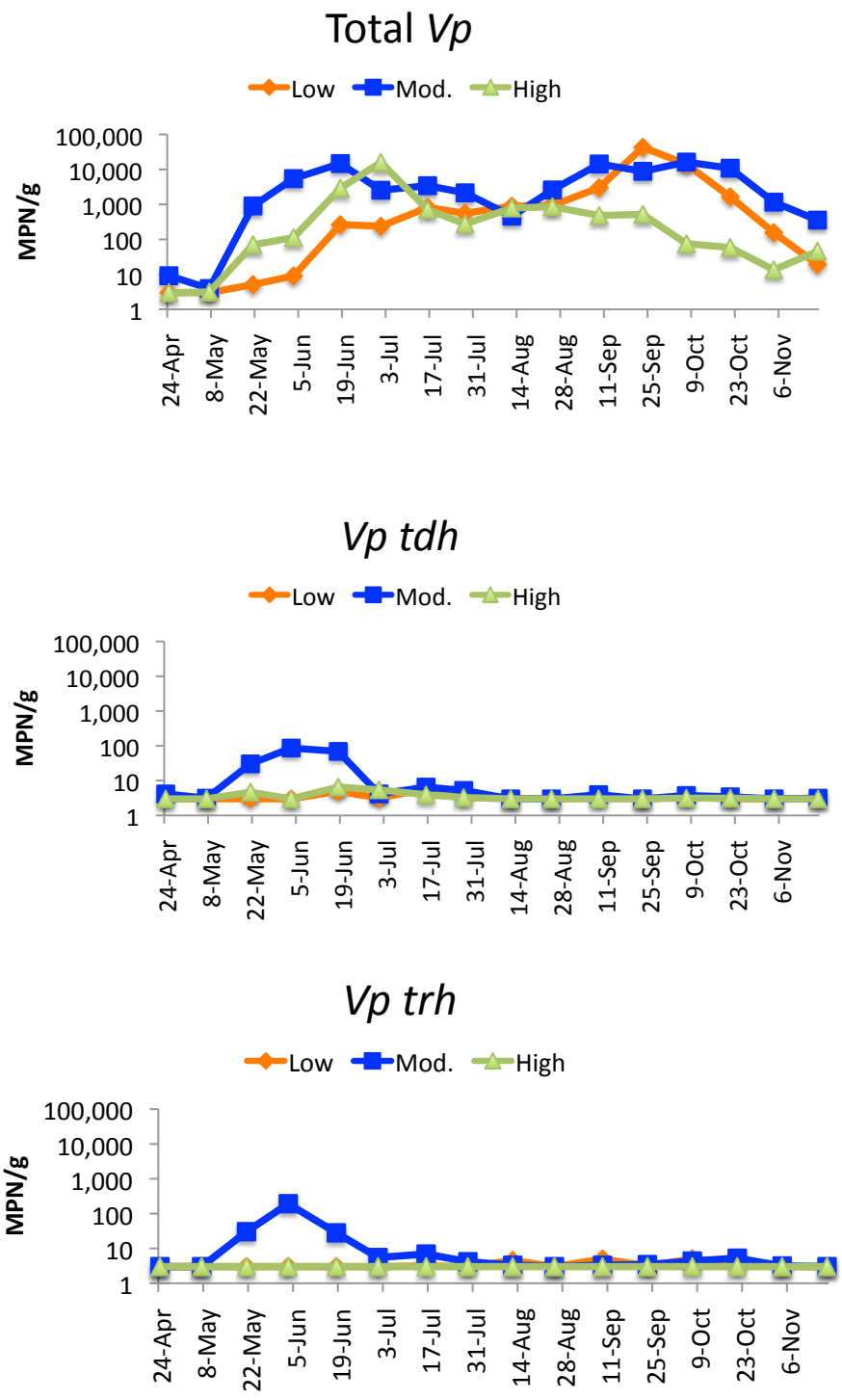

Fig. 4: Concentrations in oysters of total *V. parahaemolyticus* (A), pathogenic V. *parahaemolyticus* possessing the *tdh* gene (B) and pathogenic V. *parahaemolyticus* possessing the *trh* gene (C).

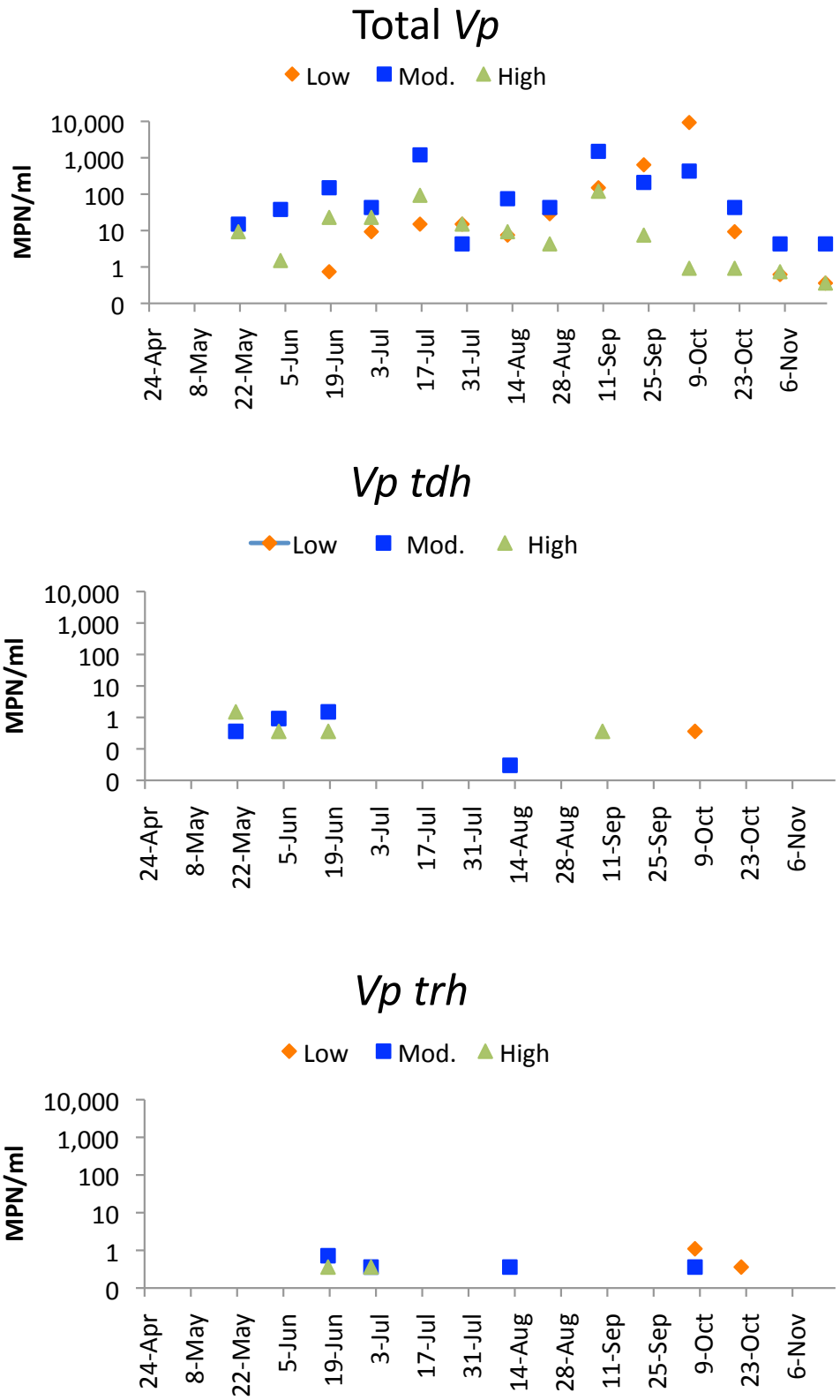

Fig. 5: Concentrations in water samples of total *V. parahaemolyticus* (A), pathogenic V. *parahaemolyticus* possessing the *tdh* gene (B) and pathogenic V. *parahaemolyticus* possessing the *trh* gene (C).