Massachusetts Division of Marine Fisheries

Techniques and Practices for *Vibrio parahaemolyticus* Reduction in Massachusetts

Final Report to the Interstate Shellfish Sanitation Conference

Submitted August 31, 2017

TECHNIQUES AND PRACTICES FOR VIBRIO *PARAHAEMOLYTICUS* REDUCTION IN MASSACHUSETTS

Final Report Submitted to the Interstate Shellfish Sanitation Conference (ISSC)
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In 2016, the Interstate Shellfish Sanitation Conference (ISSC) published a Request for Proposals to identify and evaluate factors, that contribute to increased risk of Vibrio *parahaemolyticus* (Vp) illnesses, and evaluate the effectiveness of techniques and practices that could potentially reduce the risk of Vp illnesses. The Massachusetts Division of Marine Fisheries (DMF) in partnership with *Mass*DPH and the University of New Hampshire Center for Vibrio Disease and Ecology (UNH), submitted a proposal to conduct work that would complement on-going environmental surveillance and Vp control validation efforts in the state, by bolstering sampling frequency and adding a quantitative ST36/ST631 strain-specific assay to the ongoing analyses for total and hemolysin producing Vp. The proposed work included a detailed analysis of illness history in relation to localized environmental conditions in an attempt to understand potential drivers of the observed increase in Vp illness occurrence in Massachusetts and their management implications. Finally, strain identification of environmental isolates by PFGE and /or end point PCR was proposed in an attempt to match confirmed clinical strain types with environmental isolates obtained from shellstock samples collected from harvest areas following confirmed Vp illness.

EXECUTIVE SUMMARY

In the last two decades, *Vibrio parahaemolyticus* (Vp) has become the leading cause of seafood-borne poisoning from bacteria in the US and world-wide (Daniels *et al*., 2000; Scallan *et al*. 2011). Especially concerning is the increased incidence of Vp. cases in Northeastern US, where illnesses linked to locally harvested product were previously rare (DePaola *et al*; 2000; Jones, 2011; Xu *et al*., 2015; Urquhart *et al*. 2016). Since 2012, however, Vp has rapidly emerged as a significant public health threat in the region, resulting in harvest area closures, recalls, and the implementation of costly control measures for the harvesting and handling of oysters during summer months. The historic rarity of outbreaks in this region was presumed the result of a generally lower abundance of Vp bacteria compared to regions with higher illness prevalence, and the absence of pathogenic strains. Changing climate, leading to warmer summer waters, has however created increasingly favorable conditions for the growth of these bacteria and may be contributing to the observed increased infection rates (CDC, 2005). Concurrent with changes in climate, previously undocumented virulent strains have emerged and spread into the region and increased infections (Martinez-Urtaza *et al*.,2013; Xu *et al*., 2015; Xu et al., 2017). Other contributing factors include that while illnesses have increased, reporting of illnesses has improved and we suspect under-reporting is less frequent, as well as both the demand for raw oyster consumption during summer and the production of oysters in Massachusetts and the Northeast have dramatically increased in recent years.

Most recently ST36 (O4:K12), which caused infections in 13 US states in 2013 (CDC, 2013), and the majority of Massachusetts infections between 2013 and 2016, is believed to have invaded the Atlantic from the Pacific, and has established populations in several harvest areas where it now causes recurrent seasonal infections (Newton et al., 2014; Haendeges *et al*., 2015; Xu *et al*., 2015; Unpublished data). Additionally a resident strain (ST631) has also caused infections from Massachusetts sources and remains the second most prevalent strain from clinical sources in the state (Xu *et al*., 2015, Xu et al., 2017a). Together, these two strains cause approximately 85% of local source gastric infections, most of which occurred after consumption of oysters.

Due to the relatively rapid onset of Vp. as a major public health concern in the region a concerted effort to assemble background information on the variability and abundance of total and pathogenic Vp. bacteria in shellfish growing areas, and the environmental factors or harvest

practices that may be driving the observed increase in Vp. illness, have only recently begun in earnest. These efforts have primarily relied on analysis of total Vp. abundance and temperature as an indicator of risk (USFDA, 2005). Two hemolysin genes (*tdh* and *trh*), often present in strains recovered from infected patients, are also currently used as markers to discriminate relative abundance of potentially pathogenic strains in surveillance efforts (Nordstrom *et al*., 2007; Honda and Iida, 1993; Hiyoshi *et al*., 2010; Panicker *et al*.; 2004; Shirai *et al*., 1990). However, the increased abundance of total Vp. and/or the two hemolysin genes, more often than not, do not correlate strongly with disease incidence beyond a seasonal level (Turner *et al*., 2013; Zimmerman *et al*., 2007; unpublished). Although we do not discount the utility of total Vp and hemolysin abundance detection as surrogates for risk, with background knowledge of the clinical strains causing the majority of illnesses in a region, a concerted surveillance effort to monitor for the specific identified human pathogenic strains that result in the majority of document illnesses would likely provide the best information when developing a monitoring based risk management strategy. Additionally developing a greater understanding of how common culture practices and proposed Vp. risk mitigation strategies impact the abundance of these strains in oysters would further the current state of research and assist in directing Vp. management efforts on the local level.

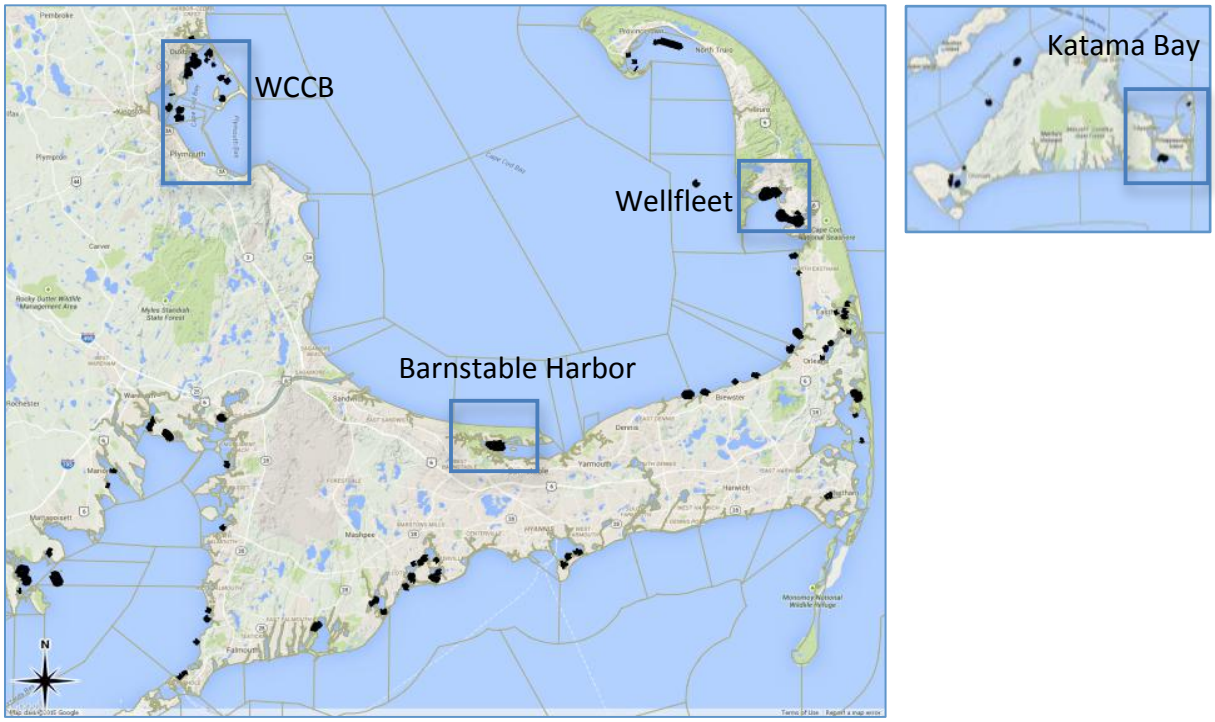
We complemented on-going environmental surveillance and Vp control validation efforts in Massachusetts by adding a quantitative ST36/ST631 strain-specific assay to the ongoing total and hemolysin producing Vp. analysis in select growing areas to determine if it is a feasible and potentially better indicator of risk than total or hemolysin producing Vp. We also conducted an analysis of illness occurrence between 2014 and 2016 to determine if a relationship exists between environmental conditions and illness occurrence, and strain identification of environmental isolates by PFGE and /or end point PCR was proposed in an attempt to match confirmed clinical strain types with environmental isolates obtained from shellstock samples collected from harvest areas.

ENVIRONMENTAL MONITORING FOR VP

Routine environmental monitoring of oyster shellstock samples for total, *tdh*+, and *trh*+ Vp has been ongoing in Massachusetts since 2014. Samples are generally collected from June to October from high production harvest areas. Collection of oysters and quantification of Vp. follow standard protocols (Ellis et al, 2012, Jones *et al*., 2011; Nordstrom *et al*., 2007). Total and hemolysin producing Vp. concentration in oysters are determined by a combination of MPN RT-PCR using fluorescently labeled probes and culture based MPN, which allows the isolation of bacteria to augment strain collections used in genomics analysis, comparative genomics, and validation of strain detection protocols against collections of environmental isolates.

Statewide Vp levels presented in this report are from oyster shellstock samples collected from five growing areas in Massachusetts (Barnstable Harbor, Dennis East Coastal, Duxbury Bay, Katama Bay and Wellfleet- See Map 1). Site-specific data are presented for Barnstable Harbor, Duxbury Bay and Katama. To account for impacts of tidal exposure, oysters from intertidal harvest sites were collected within 1-hour of tidal exposure. Following collection, all oysters were stored immediately on ice. Oysters were either transported directly on ice or overnight shipped on ice packs to laboratory facilities in MA and NH. . Oysters were cleaned, shucked and homogenized according to FDA BAM protocols. The homogenate was diluted according to the FDA BAM protocols using a 3-tube, five dilution or more series and incubated at 35-37°C. For culture based enumeration and isolate collection, turbid tubes were quadrant streaked on agar, where colonies were tested by PFGE or end-point PCR using published primers (Paniker et al., 2004) with slight modifications to allow the identification of ST36 (Whistler et al., 2015) or ST631 (unpublished). For RT-PCR enumeration, a 1 mL sample from turbid tubes was boiled to lyse cells, the debris cleared by centrifuging, and 2 ul of the lysate analyzed. The tiered analysis first employs the FDA/NSSP MPN RT-PCR procedure (Nordstrom et al., 2007) to quantify total Vp using the Vp species specific marker tlh and an internal amplification control (IAC). Positive tlh samples were then examined in duplex for the presence of hemolysin containing strains using primers and probes specific to tdh/trh. Samples were collected weekly unless sampling was not possible due to unforeseeable issues or scheduling limitations. Some samples were not processed or included in the results if time temperature abuse during transport was identified. To adjust for variability in the detection threshold across labs, sample results for tlh, trh and tdh that were <3 MPN/g were not considered positive in the following analysis.

Map 1 Environmental sample collection sites. Black polygons denote licensed aquaculture sites.



Detection of Vp

Between 2014 and 2016 a total of 323-oyster shellstock samples were analyzed for total Vp. (*tlh*), 314 samples were analyzed for the hemolysin gene *tdh*, and 286 samples were analyzed for the hemolysin gene *trh*. Gene occurrence and descriptive statistics for all samples are presented in Table 1. For the purposes of data normality, all MPN/g values for Vp concentrations were $\log_{10}$-transformed prior to analysis.

Figure 1 Log total and hemolysin-positive Vp MPN/g from 2014-2016 for all sample sites

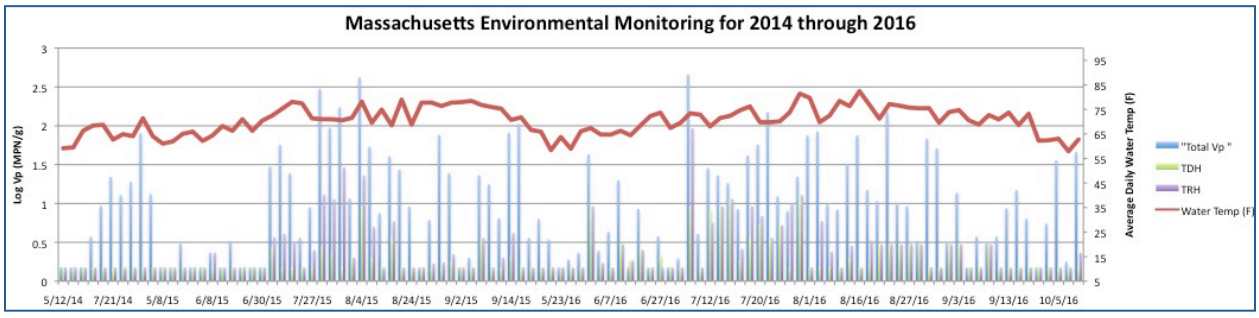


Table 1 descriptive statistics for Total Vp and hemolysin-positive frequency of detection and MPN concentrations

Total Vp	
tlh+	253/323
Mean	25.6
Standard Deviation	9.5
Minimum	3.0
Maximum	110,000.0
tdh	
tdh+	76/313
Mean	8.7
Standard Deviation	11.5
Minimum	3.0
Maximum	110,000.0
trh	
trh+	104/285
Mean	9.1
Standard Deviation	8.4
Minimum	3.0
Maximum	110,000.0

Monitoring of Environmental Conditions in Harvest Areas

Environmental parameters (water temperature, salinity, pH, turbidity (NTU), depth (m), and chlorophyll *a* (ug/l) were measured at 15-minute intervals from the Duxbury Bay and Barnstable Harbor sites. Data was collected using YSI EXO2 multi-parameter data sondes (YSI, Inc. Yellow Springs, OH), owned and maintained by The Barnstable County Cooperative Program. In Katama Bay, water temperature, salinity (PPT) and depth (m) were measured via Onset Computer data sondes (Onset MA) at 15-minute intervals. Due to cost limitations, chlorophyll *a* (ug/l), pH and turbidity (NTU) data were acquired from an EXO2 multi-parameter data sonde located in Cotuit Bay and these data are surrogate data for Katama Bay. Discrete sampling via a YSI ProDSS for pH and turbidity (NTU), and a bench top Thermo Fisher Scientific filter fluorometer for chlorophyll *a* (ug/l), was conducted to evaluate the suitability of Coutit Bay data as a surrogate for Katama Bay.

Linear Regression Statistical Analysis

The measure of linear association between log-transformed Vp concentrations and paired environmental parameter data were calculated by Pearson's product-moment correlation with

significant relationships determined by the degrees of freedom at an alpha level of 0.05. For the purposes of data normality, all MPN/g values for Vp concentrations were $\log_{10}$ transformed prior to statistical analysis. For the purposes of observation of data trends within the normal range of Vp concentrations, outlying, relatively high Vp concentration data points (determined by the absolute value of 2 times the standard deviation) were excluded from the correlation analysis. Additional data points in which concurrent environmental data were not available were also excluded from the correlation analysis.

Table 2 Results of Pearson's correlation analysis for total and hemolysin-positive Vp from all sites (2014 – 2016). Bold values are significant at an alpha level of .05.

		Total Vp	***tdh***	***trh***
Average of Temperature	**R**	**0.28143**	**0.13047**	**0.17019**
	R Standard Error	**0.00295**	**0.00331**	**0.00354**
	t	**5.18047**	**2.2679**	**2.8589**
	p-value	**4.68946E-7**	**0.02422**	**0.00462**
	H0 (5%)	***rejected***	***rejected***	***rejected***
Average of Salinity	**R**	0.0562	0.05456	0.09872
	R Standard Error	0.00371	0.00393	0.00429
	t	0.92326	0.87089	1.50784
	p-value	0.3568	0.38469	0.13291
	H0 (5%)	*accepted*	*accepted*	*accepted*
Min of Depth (m)	**R**	**-0.2093**	-0.00394	**-0.19052**
	R Standard Error	**0.00327**	0.00361	**0.00379**
	t	**-3.65747**	-0.06554	**-3.09301**
	p-value	**0.00031**	0.9478	**0.00222**
	H0 (5%)	***rejected***	*accepted*	***rejected***
Average of pH	**R**	**-0.1987**	-0.06386	-0.08681
	R Standard Error	**0.00358**	0.00385	0.00419
	t	**-3.319**	-1.02979	-1.34145
	p-value	**0.00104**	0.30414	0.18104
	H0 (5%)	***rejected***	*accepted*	*accepted*
Average of Turbidity (NTU)	**R**	0.10356	-0.06374	0.01725
	R Standard Error	0.00404	0.00431	0.00465
	t	1.62973	-0.97076	0.25301
	p-value	0.10447	0.33265	0.80048
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of Chlorophyll (ug/l)	**R**	**0.23834**	0.05518	**0.19533**
	R Standard Error	**0.00361**	0.00405	**0.00431**
	t	**3.96469**	0.86682	**2.97421**
	p-value	**0.0001**	0.38691	**0.00324**
	H0 (5%)	***rejected***	*accepted*	***rejected***

Correlation coefficients for environmental parameters and total and hemolysin producing (*trh and tdh)* Vp from pooled statewide data are presented in table 2. Significantly correlated parameters are identified in bolded text. Average Temperature was positively correlated with total, *tdh*+ and *trh*+ Vp. No other parameters were significantly correlated with the occurrence of *tdh*. Depth was negatively correlated with total and *trh*+ Vp. Chlorophyll was positively correlated with both total and *trh*+ Vp and pH was negatively correlated with total Vp.

Table 3 descriptive statistics for environmental variables when total Vp was detected from all sites for 2014 – 2016.

Average of Temperature		**Average of pH**	
Mean	70.8	*Mean*	7.84
Standard Deviation	5.8	*Standard Deviation*	0.14
Minimum	55.9	*Minimum*	7.44
Maximum	82.4	*Maximum*	8.29
Average of Salinity (PPT)		**Average of Turbidity (NTU)**	
Mean	29.7	*Mean*	3.08
Standard Deviation	1.7	*Standard Deviation*	2.26
Minimum	23.1	*Minimum*	0.32
Maximum	32.1	*Maximum*	12.90
Average of Depth (m)		**Average of Chlorophyll (ug/l)**	
Mean	*1.3*	*Mean*	7.58
Standard Deviation	*0.9*	*Standard Deviation*	3.63
Minimum	*-0.09*	*Minimum*	1.73
Maximum	*3.2*	*Maximum*	18.54

Duxbury Bay Detection of Vp

Between 2014 and 2016 a total of 114-oyster shellstock samples were collected from Duxbury Bay and analyzed for total Vp. (*tlh*), 113 samples were analyzed for the hemolysin gene *tdh*, and 113 samples were analyzed for the hemolysin gene *trh*. Gene occurrence and descriptive statistics for all Duxbury Bay samples are presented in Table 4.

Figure 2 log total, tdh and trh Vp MPN/g for Duxbury Bay 2014-2016

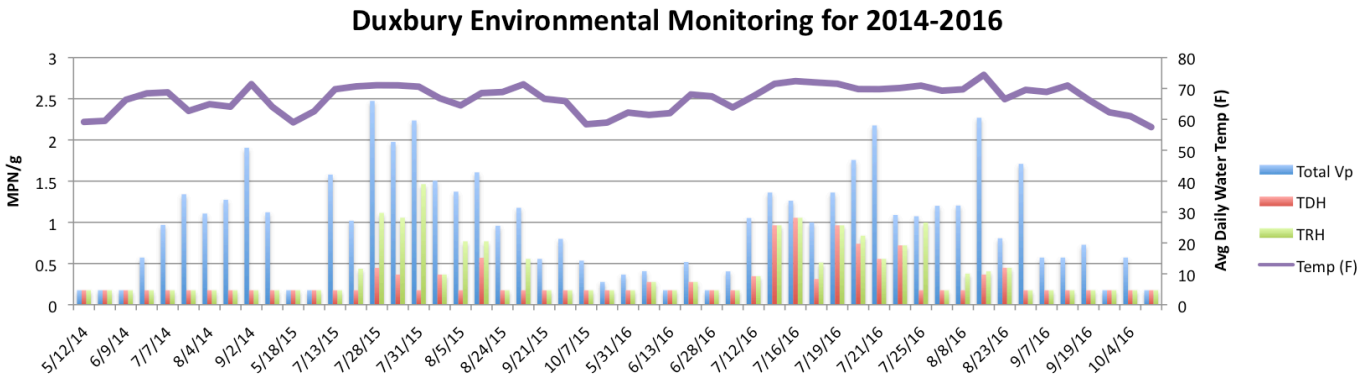


Table 4 descriptive statistics for Total Vp and hemolysin-positive frequency of detection and MPN concentrations for Duxbury

Duxbury	
Total Vp	
tlh+ samples	85/114
Mean	21.5
Standard Deviation	5.1
Minimum	3.0
Maximum	15,000.0
tdh	
tdh+ samples	26/113
Mean	5.0
Standard Deviation	1.9
Minimum	3.0
Maximum	43.0
trh	
trh+ Samples	40/113
Mean	7.8
Standard Deviation	2.9
Minimum	3.0
Maximum	240.0

Table 5 results of Pearson's correlation analysis for total and hemolysin-positive Vp from Duxbury Bay 2014 – 2016. Bold values are significant at an alpha level of .05

		Total Vp Log	*tdh* log	*trh* Log
Average of Temperature	**R**	**0.54965**	**0.31586**	**0.41805**
	R Standard Error	**0.00623**	**0.00811**	**0.00743**
	t	**6.96307**	**3.50737**	**4.84845**
	p-value	**4.8402E-10**	**0.0007**	**5.03741E-6**
	H0 (5%)	***rejected***	***rejected***	***rejected***
Average of Salinity (PPT)	**R**	**0.54511**	0.10952	0.1767
	R Standard Error	**0.01019**	0.01453	0.01425
	t	**5.40098**	0.90856	1.48038
	p-value	**5.1585E-7**	0.36596	0.14219
	H0 (5%)	***rejected***	*accepted*	*accepted*
Average of Depth (m)	**R**	**-0.23548**	**0.02497**	-0.19919
	R Standard Error	**0.01027**	0.01098	0.01055
	t	**-2.32395**	0.23829	-1.93904
	p-value	**0.02233**	0.81219	0.05556
	H0 (5%)	***rejected***	*accepted*	*accepted*
Average of pH	**R**	0.07171	-0.19253	-0.17767
	R Standard Error	0.0117	0.01146	0.01153
	t	0.6628	-1.7982	-1.65465
	p-value	0.50911	0.07543	0.1014
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of Turbidity (NTU)	**R**	-0.12732	0.04059	-0.05848
	R Standard Error	0.01069	0.01097	0.01095
	t	-1.23121	0.38749	-0.55886
	p-value	0.22138	0.69929	0.57762
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of Chlorophyll (ug/l)	**R**	**0.39259**	**0.40325**	**0.38984**
	R Standard Error	**0.00919**	**0.0092**	**0.00932**

	t	**4.09431**	**4.20369**	**4.03838**
	p-value	**0.00009**	**0.00006**	**0.00011**
	H0 (5%)	***rejected***	***rejected***	***rejected***

Correlation coefficients for environmental parameters and total and hemolysin producing (*trh+ and tdh+)* Vp from pooled Duxbury Bay data are presented in table 5. Significantly correlated parameters are identified in bolded text. Average temperature and chlorophyll were both positively correlated with Total, *tdh+* and *trh+* Vp. No other parameters were significantly correlated with the occurrence of *tdh+ or trh+* Vp. Depth was negatively correlated with total Vp and salinity was positively correlated with Vp.

Table 6 descriptive statistics for environmental variables when total Vp was detected in Duxbury Bay 2014 – 2016.

Average of Temperature		**Average of pH**	
Mean	67.14	*Mean*	7.98
Standard Deviation	4.21	*Standard Deviation*	0.08
Minimum	57.49	*Minimum*	7.87
Maximum	74.40	*Maximum*	8.29
Average of Salinity		**Average of Turbidity (NTU)**	
Mean	29.15	*Mean*	3.57
Standard Deviation	1.88	*Standard Deviation*	1.38
Minimum	25.19	*Minimum*	0.97
Maximum	32.02	*Maximum*	7.69
Minimum of Depth (m)		**Average of Chlorophyll (ug/l)**	
Mean	0.70	*Mean*	7.54
Standard Deviation	0.31	*Standard Deviation*	2.75
Minimum	-0.09	*Minimum*	2.83
Maximum	1.31	*Maximum*	12.58

KATAMA BAY DETECTION OF VP

Between 2014 and 2016 a total of 104-oyster shellstock samples were collected from Katama Bay and analyzed for total Vp. (*tlh*), 100 samples were analyzed for *tdh,* and 98 samples were analyzed for the *trh.* Gene occurrence and descriptive statistics for all Katama Bay samples are presented in table 7.

Figure 3 log total and hemolysin-positive Vp MPN/g for Katama Bay

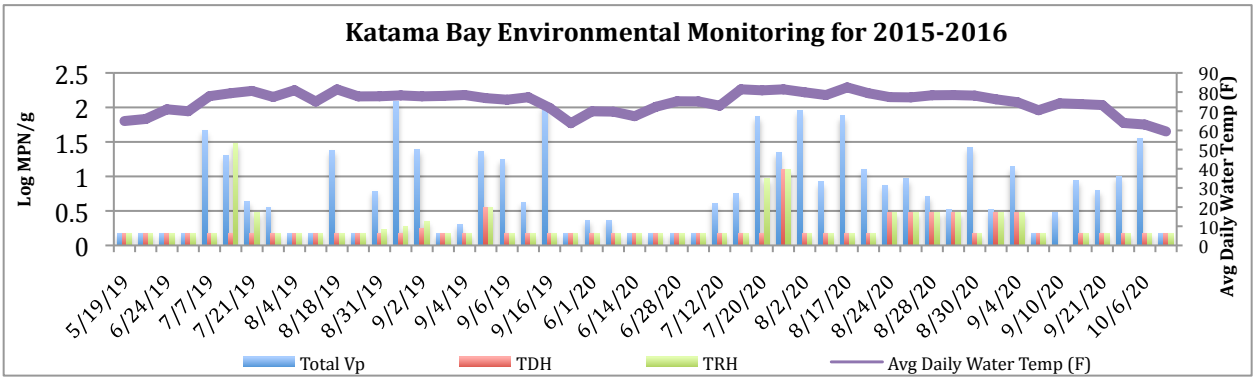


Table 7 Descriptive statistics for Total Vp and hemolysin-positive frequency of detection and MPN concentrations for Katama Bay

Total Vp	
Count	75/104
Mean	23.6
Standard Deviation	12.6
Minimum	3.0
Maximum	110,000
tdh	
Count	19/100
Mean	28.2
Standard Deviation	51.2
Minimum	3.0
Maximum	110,000
trh	
Count	25/98
Mean	20.2
Standard Deviation	37.7
Minimum	3.0
Maximum	110,000

Table 8 Pearson's correlation analysis for total and hemolysin-positive Vp from Katama Bay 2015 – 2016. Bold values are significant at an alpha level of .05

		Total Vp Log	***tdh* Log**	***trh* Log**
Average of Temperature	**R**	**0.31427**	0.16588	**0.23099**
	R Standard Error	**0.0092**	0.01035	**0.01029**
	t	**3.27714**	1.63087	**2.27711**
	p-value	**0.00166**	0.10761	**0.02598**
	H0 (5%)	***rejected***	*accepted*	***rejected***
Average of Salinity	**R**	**0.06814**	**0.18796**	**0.14409**
	R Standard Error	0.01016	0.01026	0.01064
	t	0.67615	1.85546	1.39668
	p-value	0.50127	0.06793	0.16712
	H0 (5%)	*accepted*	*accepted*	*accepted*
Min of Depth (m)	**R**	**0.00447**	**0.12507**	**0.03403**
	R Standard Error	0.0102	0.01047	0.01086
	t	0.04427	1.22219	0.32664

	p-value	0.96482	0.22592	0.74496
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of pH	**R**	**-0.179**	**-0.07913**	**-0.08075**
	R Standard Error	0.00998	0.01069	0.0108
	t	-1.79184	-0.76546	-0.77704
	p-value	0.07767	0.44669	0.43987
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of Turbidity (NTU)	**R**	**-0.10348**	**-0.10778**	**-0.08882**
	R Standard Error	0.01393	0.01475	0.01526
	t	-0.87667	-0.88742	-0.71896
	p-value	0.3838	0.37803	0.47466
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of Chlorophyll (ug/l)	**R**	**0.15425**	**-0.07182**	**-0.03837**
	R Standard Error	0.01436	0.01554	0.01611
	t	1.2874	-0.57604	-0.30234
	p-value	0.20238	0.56652	0.76333
	H0 (5%)	*accepted*	*accepted*	*accepted*

Correlation coefficients for environmental parameters and total and hemolysin producing Vp from pooled Katama Bay data are presented in table 9. Significantly correlated parameters are identified in bolded text. Average temperature was positively correlated with total and *trh*+ Vp. No parameters were significantly correlated with *tdh*+ Vp.

Table 9 descriptive statistics for environmental variables when total Vp was detected in Katama Bay 2014 – 2016.

Average of Temperature		Average of pH	
Mean	76.55	*Mean*	7.73
Standard Deviation	4.14	*Standard Deviation*	0.10
Minimum	63.07	*Minimum*	7.47
Maximum	82.44	*Maximum*	8.09
Average of Salinity		Average of Turbidity (NTU)	
Mean	30.53	*Mean*	1.32
Standard Deviation	0.66	*Standard Deviation*	0.31
Minimum	28.98	*Minimum*	0.73
Maximum	31.46	*Maximum*	2.78
Avg of Depth (m)		Average of Chlorophyll (ug/l)	
Mean	2.39	*Mean*	5.89
Standard Deviation	0.08	*Standard Deviation*	1.82
Minimum	2.28	*Minimum*	2.59
Maximum	2.63	*Maximum*	10.33

Barnstable Harbor Detection of Vp

In 2014 and 2016 a total of 29-oyster shellstock samples were collected from Barnstable Harbor and analyzed for total Vp. 29 samples were analyzed for the hemolysin gene *tdh*, and 21 oyster

shellstock samples were analyzed for the hemolysin gene *trh*. Gene occurrence and descriptive statistics for all Barnstable Harbor samples are presented in table 10.

Figure 4 Log total and hemolysin-positive Vp MPN/g for Barnstable Harbor

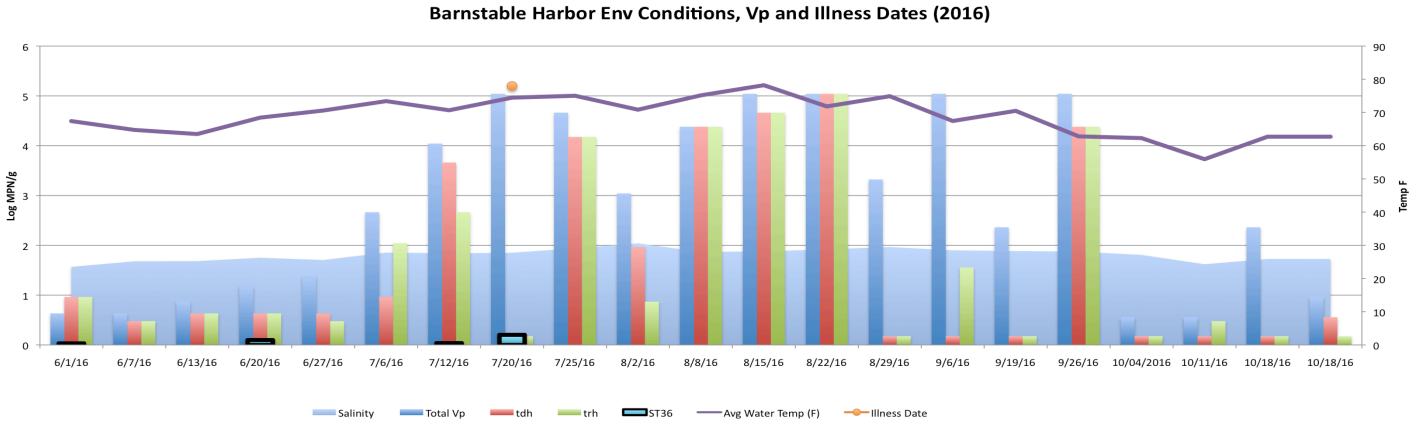


Table 10 Descriptive statistics for Total Vp and hemolysin-positive frequency of detection and MPN concentrations Barnstable Harbor. Note samples were not collected in 2015 from Barnstable Harbor due to staffing limitations.

Total Vp	
Count	20/29
Mean	100.0
Standard Deviation	11.0
Minimum	3.6
Maximum	12000
tdh	
Count	5/29
Mean	23.6
Standard Deviation	19.5
Minimum	4.3
Maximum	4600
trh	
Count	6/21
Mean	28.8
Standard Deviation	7.3
Minimum	3.6
Maximum	460

Table 11 results of Pearson's correlation analysis for total and hemolysin-positive Vp from Barnstable Harbor for 2014 and 2016.

Barnstable Harbor		Total Vp log	*tdh* Log	*trh* Log
Average of Temperature	**R**	**0.73129**	**0.22396**	**0.43737**
	p-value	0.0002	0.31938	0.11143
	H0 (5%)	*rejected*	*accepted*	*accepted*
Average of Salinity	**R**	**0.67506**	**0.10993**	**0.3804**
	p-value	0.00085	0.62758	0.17341

	H0 (5%)	*rejected*	*accepted*	*accepted*
Min of Depth (m)	**R**	**-0.06863**	**0.33444**	**0.09311**
	p-value	0.76233	0.13207	0.75018
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of pH	**R**	**-0.12114**	**0.01603**	**-0.23662**
	p-value	0.59276	0.94375	0.41131
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of Turbidity (NTU)	**R**	**-0.13643**	**-0.11976**	**-0.36121**
	p-value	0.57803	0.6257	0.19838
	H0 (5%)	*accepted*	*accepted*	*accepted*
Average of Chlorophyll (ug/l)	**R**	**-0.12969**	**0.17321**	**-0.09581**
	p-value	0.57651	0.45449	0.75367
	H0 (5%)	*accepted*	*accepted*	*accepted*

Correlation coefficients for environmental parameters and total and hemolysin producing (*trh+ and tdh+)* Vp from pooled Barnstable Harbor data are presented in table 11. Significantly correlated parameters are identified in bolded text. Average temperature and salinity were both positively correlated with Total Vp. No other parameters were significantly correlated with the occurrence of total, *tdh+ or trh+* Vp.

Table 12 descriptive statistics for environmental variables when total Vp was detected in Barnstable Harbor for 2014 and 2016.

Average of Temperature		**Average of pH**	
Mean	67.35	*Mean*	7.67
Standard Deviation	4.65	*Standard Deviation*	0.10
Minimum	55.94	*Minimum*	7.46
Maximum	74.91	*Maximum*	7.85
Average of Salinity (PPT)		**Average of Turbidity (NTU)**	
Mean	26.20	*Mean*	4.05
Standard Deviation	1.89	*Standard Deviation*	1.62
Minimum	23.16	*Minimum*	1.91
Maximum	30.60	*Maximum*	7.80
Min of Depth (m)		**Average of Chlorophyll (ug/l)**	
Mean	0.59	*Mean*	4.76
Standard Deviation	0.24	*Standard Deviation*	1.70
Minimum	0.27	*Minimum*	2.68
Maximum	1.11	*Maximum*	9.98

Discussion of Environmental Monitoring for Vp

Significant correlations between environmental parameters and total and hemolysin Vp vary considerably between sites, as do the environmental conditions when Vp is first detected and most prevalent. Consistent with other studies from the region total Vp correlated strongly with water temperature and season. Water temperature when Vp was first detected (~55 °F) and when Vp was most prevalent (~67 °F) was similar between both Cape Cod Bay sample sites (Duxbury Bay and Barnstable). Water temperatures when Vp. was first detected in Katama Bay (~63°F), and when it was most prevalent (~76°F) were significantly higher than the two Cape Cod Bay sites. This highlights the high variability and thermal tolerance of Vp in oysters in Massachusetts. Beyond temperature there was significant variability in what environmental conditions correlated with total and hemolysin producing Vp. Trends in correlation for *trh*+ Vp were more consistent with total Vp than those observed for *tdh*+ Vp, which showed very little significant correlation with observed environmental conditions. The exception would be the Duxbury site where total and both *trh*+ and *tdh*+ Vp was significantly correlated with chlorophyll levels at the time of sampling.

Observed mean total Vp levels were similar between Duxbury Bay and Katama Bay (20-23 MPN/g) whereas Barnstable harbor had a higher mean total Vp (100 MPN/g). The converse relation was observed for mean *trh*+ and *tdh*+ Vp level with values in Katama Bay and Barnstable Harbor between 23-28 MPN/g for *tdh* and 20- 28 MPN/g *trh*, whereas Duxbury Bay had a mean *tdh*+ level of 5.0 *tdh*+ and a mean *trh*+ value of 7.8.

Quantitative ST36 Analysis

Quantitative ST36 (*prp*+/*flp*+) analysis of *tdh*+ and *trh*+ enrichments from 30 shellstock samples from Barnstable Harbor and Katama Bay, and 87 *tdh*+ and *trh*+ lysates from shellstock samples collected from Duxbury and Katama Bay in 2016 were conducted. ST36 was confirmed in 19 individual shellstock samples as determined by the co-occurrence of *prp* and *flp*. Results are presented in Table 13.

Table 13 MPN-RT-PCR *prp* or *flp positive* environmental samples. ND denotes a sample below the detection threshold

Sample Type	Area	Treatment	Log MPN/g FLP	Log MPN/g PRP
Lysate	Duxbury	Environmental	0.96	0.96
Lysate	Duxbury	48hr Abused Sample*	2.38	1.63
Lysate	Duxbury	48hr Abused Sample*	2.38	1.63
Lysate	Duxbury	2 Days Re-Submerged*	1.32	0.56
Lysate	Duxbury	2 Days Re-Submerged	1.63	0.96
Lysate	Duxbury	4 Days Re-Submerged	0.96	0.56
Lysate	Duxbury	Environmental	0.96	0.56
Lysate	Duxbury	Environmental	1.18	0.56
Lysate	Duxbury	Environmental	0.96	0.56
Lysate	Duxbury	Environmental	ND	0.56
Lysate	Duxbury	Environmental	1.63	ND
Lysate	Duxbury	Environmental	1.58	ND
Lysate	Duxbury	Environmental	0.56	ND
Lysate	Duxbury	Environmental	0.96	ND
Lysate	Duxbury	Environmental	0.87	ND
Lysate	Duxbury	Environmental	0.96	ND
Lysate	Duxbury	Environmental	0.56	ND
Lysate	Duxbury	Environmental	0.56	ND
Lysate	Katama Bay	2 Days Re-Submerged	0.87	ND
Lysate	Katama Bay	2 Days Re-Submerged	0.48	ND
Lysate	Katama Bay	Environmental	0.48	ND
Enrichment	Katama Bay	Environmental	0.48	ND
Enrichment	Katama Bay	Environmental	0.48	0.48
Enrichment	Katama Bay	Environmental	0.48	ND
Enrichment	Katama Bay	Environmental	2.08	2.08
Enrichment	Katama Bay	Environmental	2.38	3.04
Enrichment	Katama Bay	Environmental	0.48	ND
Enrichment	Katama Bay	Environmental	0.48	ND
Enrichment	Katama Bay	Environmental	0.48	ND
Enrichment	Katama Bay	Environmental	ND	ND
Enrichment	Barnstable	Environmental	0.41	0.56
Enrichment	Barnstable	Environmental	0.56	0.56
Enrichment	Barnstable	Environmental	1.48	1.48
Enrichment	Barnstable	Environmental	0.56	0.56
Enrichment	Barnstable	Environmental	2.56	2.56
Enrichment	Barnstable	Environmental	2.38	3.04

Discussion of ST36 Analysis

Whistler et al. (2015) demonstrated analysis of clinical and environmental strains using multiplex PCR for the genes *prp* and *flp* accurately identified confirmed ST36 strains 100% of

the time. However, the *prp* and *flp* genes are not entirely unique to ST36 and can be present individually in other strain types. This presents a challenge for the identification of ST36 in lysates from environmental shellstock samples, as it is unknown if a single strain is responsible for their co-occurrence or if they are being detected in different strains within a single lysate. Confirmation of ST36 can be conducted on isolates by an additional marker (*cps*) or by other typing methods including PFGE and serotyping by MLST or whole genome sequencing. These methods are likely too cost prohibitive for state shellfish control authorities to conduct on a routine basis and they require isolation of bacteria from fresh enrichments and cannot be conducted on lysates. Confirmation of both *prp* and *flp* genes via MPN-RT-PCR of *tdh*+ and *trh*+ lysates is likely a strong indicator of the presence of ST36, and may provide a useful quantitative method of detection of ST36 abundance, but they do not 100% positively confirm the presence of ST36 in an individual enrichment or lysate and more analysis of samples is needed to determine the range of specificity and accuracy of these assays.

As stated above, fresh enrichments can be streaked and individual colonies processed via end-point PCR, PFGE or WGS to confirm the presence of ST36, however due to the relatively low abundance of these pathogenic strains in the environment it is time consuming and rare for a confirmed clinical isolate to be cultured and identified in an environmental sample. Other genetic markers or enrichment strategies may improve assay specificity and sensitivity, and are the subject of ongoing work.

GENETIC ANALYSIS OF ENVIRONMENTAL ISOLATES

Despite the challenges associated with streaking turbid tubes from enrichments of environmental samples and identification of individual isolates , *MassDPH* was able to identify a single ST36 isolate from an environmental sample collected in 2015 via PFGE. The isolate was subsequently confirmed via whole genome sequencing by UNH. Between 2015 and 2016, turbid tubes from 57 environmental samples were quadrant streaked on Vibrio CHROMAgar (CHROMagar, Paris, France) by UNH. There were 462 Vp+ environmental isolates identified from the 57 samples. Vp+ samples were analyzed via end-point PCR for *tdh* and *trh*. 54 of 462 cultured isolates from environmental sample were both *trh*+ and *tdh*+, and the whole genomes of 41 isolates were sequenced via Illumina technology. Sequences of seven housekeeping loci were isolated from the resulting sequence data for each isolate to compare with allele combinations in previously

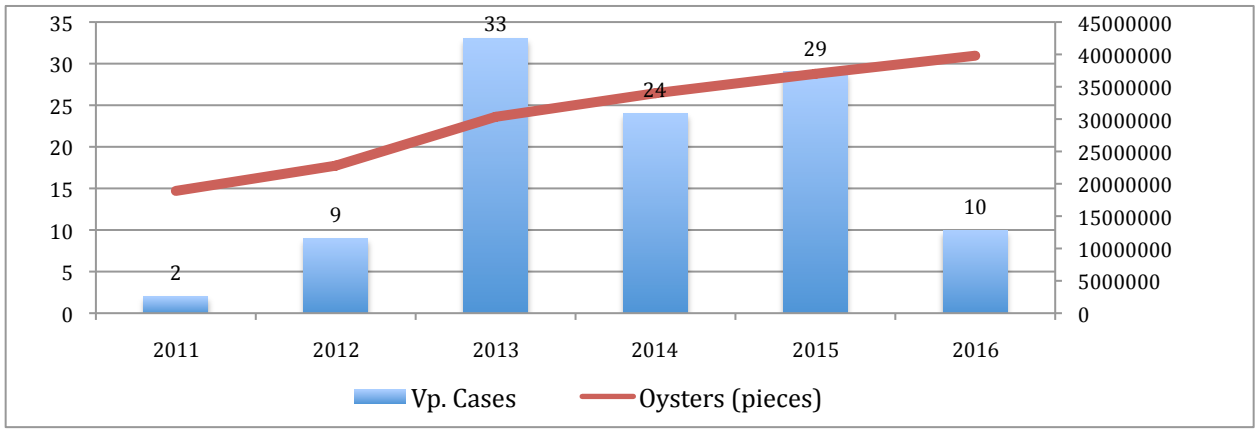
reported strains in the MLST database. ST 631 was the only known pathogenic strain to be isolated, and this was a member of the less clinically prevalent clade I ST631 for which only one infection has been reported. Most environmental isolates harboring hemolysin genes have either only been reported from environmental sources and never an infection (e.g. ST1185), or they have never been previously reported from any source (e.g. NF= not found in database representing a unique ST).

Table 14 results of genetic analysis of cultured environmental isolates.

Strain Type	# of Isolates
631	1
771	1
1185	6
1156	2
1187	1
1399	1
NF	29

Illness Occurrence in Massachusetts

Figure 5 epi-curve and landings for statewide illness occurrence (2011-2016)



Between 2011 and 2016, 107 cases of Vp involving oyster consumption have been epidemiologically linked to single Massachusetts harvest areas. This rapid emergence of Vp in Massachusetts in recent years has resulted in a significant burden on public health managers and the state's rapidly growing oyster aquaculture industry; and highlighted the ability for pathogenic

strains to rapidly change regional risk profiles. ST36 (O4:K12), which caused infections in 13 US states in 2013 (CDC, 2013), and the majority of Massachusetts infections between 2013 and 2016 (UNH-Unpublished), is believed to have invaded the Atlantic from the Pacific, and has established populations in several harvest areas where it now causes recurrent seasonal infections (Newton et al., 2014; Haendeges *et al*., 2015; Xu *et al*., 2015; Unpublished data). Additionally a resident strain (ST631) has also caused infections from Massachusetts sources and remains the second most prevalent strain from clinical sources in the state (Xu *et al*., 2015, Xu et al., 2017a; Xu et al., 2017b). Together, these two strains caused approximately 85% of local source gastric infections from 2011- 2015, most of which occurred after consumption of oysters attributed by food borne illness investigations to two harvest areas, Katama Bay and Duxbury Bay; however, sporadic illnesses have also been attributed to harvest areas in Eastern Cape Cod Bay (ECCB), Buzzards Bay and the Outer Cape.

Between 2014 and 2015 illnesses attributed to Katama Bay have exclusively been ST36. In 2016 ST 199, a broadly distributed Atlantic strain, was attributed to a Katama Bay illness marking the first non-ST36 strain associated with illness in Katama Bay. The majority of illnesses attributed to Western Cape Cod Bay (Duxbury, Plymouth and Kingston Bays) are ST36, with ST631 as the second leading cause of infection in the region (8/6). The majority of infections attributed to ECCB (Barnstable Harbor to Provincetown) harvest areas are considered endemic strain types with Atlantic lineages, but ST36 has been associated in a number of illnesses from the region. Table 15 provides an overview of strain types associated with sole source illness by location from 2014- 2016.

Table 15 Results of whole genome sequencing and MLST of clinical isolates (2014-2016)

Area	ST36	631	199	670	1127	1156	1728
Katama Bay	16		1				
WCCB	8	6			1		
ECCB	4		1	1	1	1	1

CORRELATION OF ILLNESS OCCURRENCE AND ENVIRONMENTAL CONDITIONS

Trends in confirmed sole source illness occurrence from 2014-2016 were analyzed in relation to environmental conditions observed in the implicated harvest areas on the most likely harvest date

identified in the illness trackback. Illness reported between 2014 and 2016 with corresponding environmental data (n=44) occurred between June and October, with peak occurrence between July 1 and September 15 (figure 7). Differences in the timing of occurrence between growing areas and across years were observed.

Figure 7 Epi-curve of sole source illnesses from all areas between 2014 and 2016

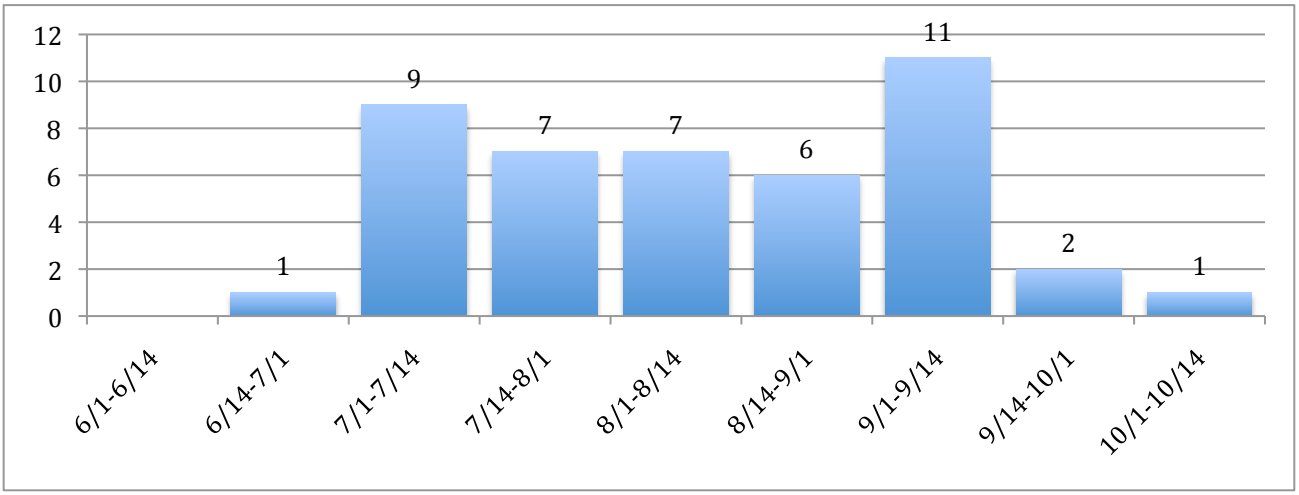


Table 16 Frequency Distribution of Environmental Conditions on Harvest Dates Associated with Sole Source Illnesses Statewide 2014- 2016

Statewide Frequency Distribution of Environmental Conditions on Harvest Dates Associated With Sole Source Illnesses 2014- 2016			
Max Air Temp	*Count*	*Percent*	*Cumulative Percent*
75 To 80	14.	0.31818	0.47727
80 To 85	13.	0.29545	0.77273
85 To 90	9.	0.20455	0.97727
70 To 75	6.	0.13636	0.15909
65 To 70	1.	0.02273	0.02273
90 To 95	1.	0.02273	1.
Average Water Temp	*Count*	*Percent*	*Cumulative Percent*
74 To 76	20.	0.45455	0.63636
76 To 78	10.	0.22727	0.86364
72 To 74	6.	0.13636	0.18182
78 To 80	4.	0.09091	0.95455
80 To 82	2.	0.04545	1.
66 To 68	1.	0.02273	0.02273
70 To 72	1.	0.02273	0.04545
Average of Salinity	*Count*	*Percent*	*Cumulative Percent*
29 To 30	18.	0.40909	0.61364
30 To 31	11.	0.25	0.86364
28 To 29	7.	0.15909	0.20455

31 To 32	6.	0.13636	1.
26 To 27	1.	0.02273	0.02273
27 To 28	1.	0.02273	0.04545
Min of Depth	*Count*	*Percent*	*Cumulative Percent*
2 To 2.5	17.	0.38636	1.
0.5 To 1	14.	0.31818	0.56818
0 To 0.5	11.	0.25	0.25
1 To 1.5	2.	0.04545	0.61364
Average of pH	*Count*	*Percent*	*Cumulative Percent*
7.7 To 7.75	14.	0.31818	0.54545
7.65 To 7.7	8.	0.18182	0.22727
7.8 To 7.85	7.	0.15909	0.77273
7.85 To 7.9	4.	0.09091	0.86364
7.75 To 7.8	3.	0.06818	0.61364
7.9 To 7.95	3.	0.06818	0.93182
7.6 To 7.65	2.	0.04545	0.04545
7.95 To 8	2.	0.04545	0.97727
8 To 8.05	1.	0.02273	1.
Average of Turbidity+	*Count*	*Percent*	*Cumulative Percent*
0 To 5	36.	0.81818	0.81818
5 To 10	4.	0.09091	0.90909
10 To 15	3.	0.06818	0.97727
15 To 20	1.	0.02273	1.
Average of Chlorophyll	*Count*	*Percent*	*Cumulative Percent*
5 To 10	23.	0.52273	0.75
0 To 5	10.	0.22727	0.22727
10 To 15	9.	0.20455	0.95455
15 To 20	2.	0.04545	1.

KATAMA BAY ILLNESS TRENDS

17 sole source cases were reported from Katama Bay between 2014 and 2016 with occurrence between July 15 and October 1 (figure 8) and peak occurrence between July 15 and September 1.

Figure 8 Katama Bay Epi Curve (2014-2016)

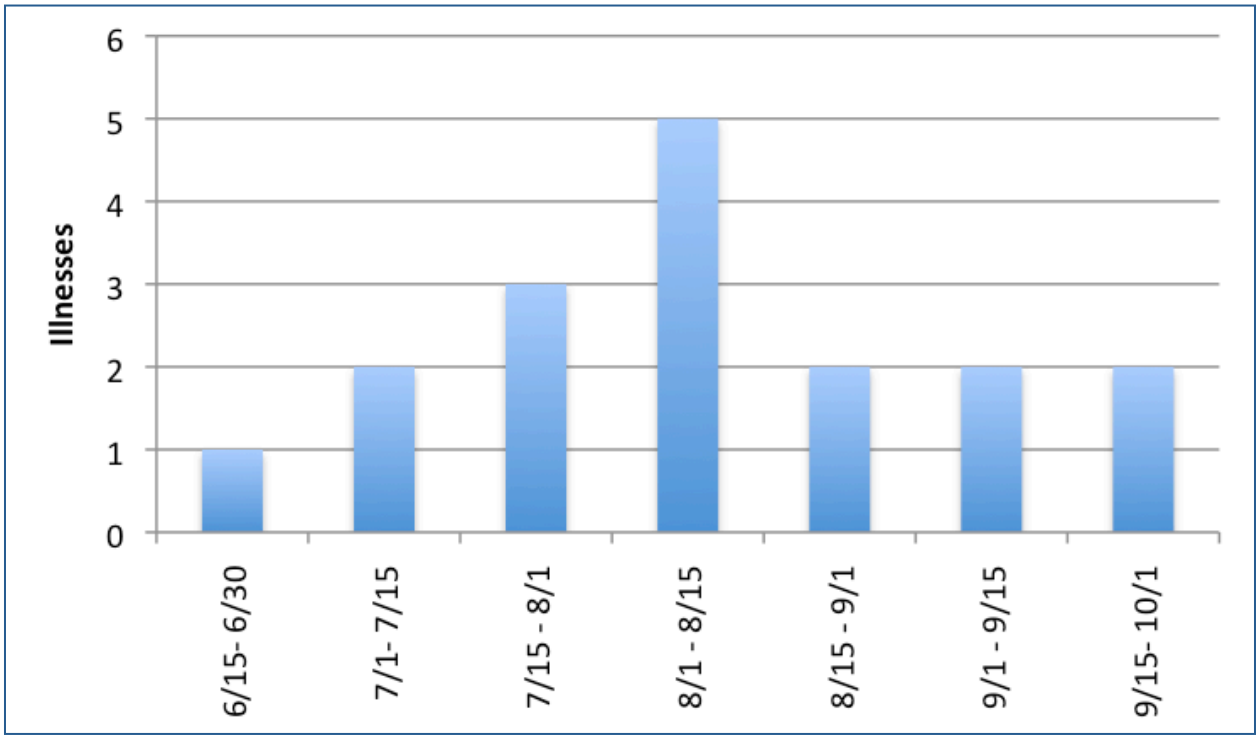


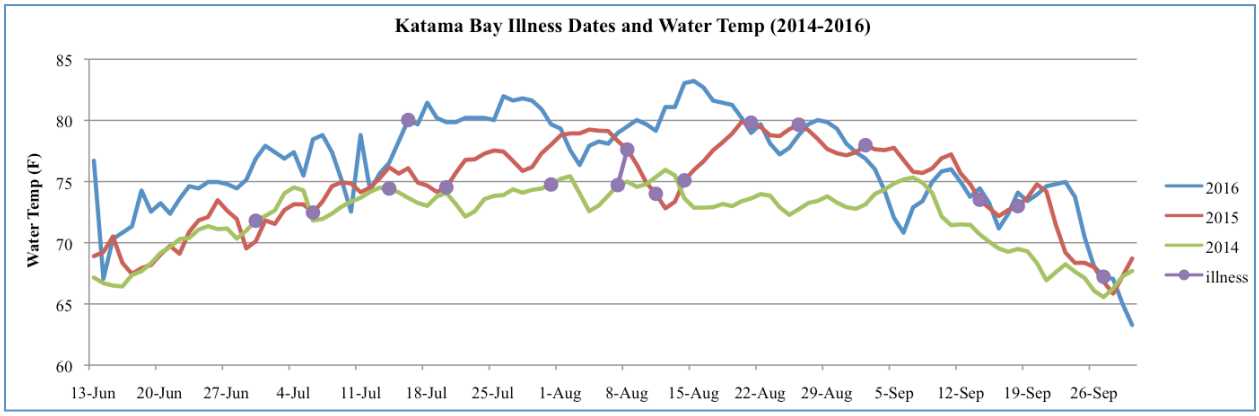
Table 17 Katama Bay Frequency Distribution of Environmental Conditions on Harvest Dates Associated With Sole Source Illnesses 2014- 2016

Katama Bay Frequency Distribution of Environmental Conditions on Harvest Dates Associated With Sole Source Illnesses 2014- 2016

Max Air Temp	*Count*	*Percent*	*Cumulative Percent*
80 To 85	6.	0.35294	0.88235
75 To 80	5.	0.29412	0.52941
70 To 75	3.	0.17647	0.23529
85 To 90	2.	0.11765	1.
65 To 70	1.	0.05882	0.05882
Average Water Temp	*Count*	*Percent*	*Cumulative Percent*
74 To 76	7.	0.41176	0.70588
72 To 74	3.	0.17647	0.29412
76 To 78	2.	0.11765	0.82353
78 To 80	2.	0.11765	0.94118
66 To 68	1.	0.05882	0.05882
70 To 72	1.	0.05882	0.11765
80 To 82	1.	0.05882	1.
Average of Salinity	*Count*	*Percent*	*Cumulative Percent*
29.5 To 30	7.	0.41176	0.70588
28.5 To 29	2.	0.11765	0.17647
29 To 29.5	2.	0.11765	0.29412

30 To 30.5	2.	0.11765	0.82353
30.5 To 31	2.	0.11765	0.94118
28 To 28.5	1.	0.05882	0.05882
31 To 31.5	1.	0.05882	1.
Min of Depth	*Count*	*Percent*	*Cumulative Percent*
2.3 To 2.35	9.	0.52941	0.70588
2.35 To 2.4	4.	0.23529	0.94118
2.25 To 2.3	3.	0.17647	0.17647
2.4 To 2.45	1.	0.05882	1.
Average of pH	*Count*	*Percent*	*Cumulative Percent*
7.7 To 7.75	6.	0.35294	0.58824
7.65 To 7.7	3.	0.17647	0.23529
7.75 To 7.8	2.	0.11765	0.70588
7.8 To 7.85	2.	0.11765	0.82353
7.85 To 7.9	2.	0.11765	0.94118
7.6 To 7.65	1.	0.05882	0.05882
7.95 To 8	1.	0.05882	1.
Average of Turbidity	*Count*	*Percent*	*Cumulative Percent*
0.5 To 1	5.	0.29412	0.52941
0 To 0.5	4.	0.23529	0.23529
1 To 1.5	3.	0.17647	0.70588
1.5 To 2	3.	0.17647	0.88235
2 To 2.5	1.	0.05882	0.94118
2.5 To 3	1.	0.05882	1.
Average of Chlorophyll	*Count*	*Percent*	*Cumulative Percent*
6 To 8	8.	0.47059	0.88235
0 To 2	5.	0.29412	0.29412
8 To 10	2.	0.11765	1.
2 To 4	1.	0.05882	0.35294
4 To 6	1.	0.05882	0.41176

Figure 9 Illness Occurrence and Water Temperature 2014-2016



KATAMA BAY TRENDS IN VP LEVELS AND ENVIRONMENTAL CONDITIONS PROXIMATE TO ILLNESS OCCURRENCE

Figure 10 Katama Bay Water and Air Temp and Total Vp proximate to Illness dates 2015

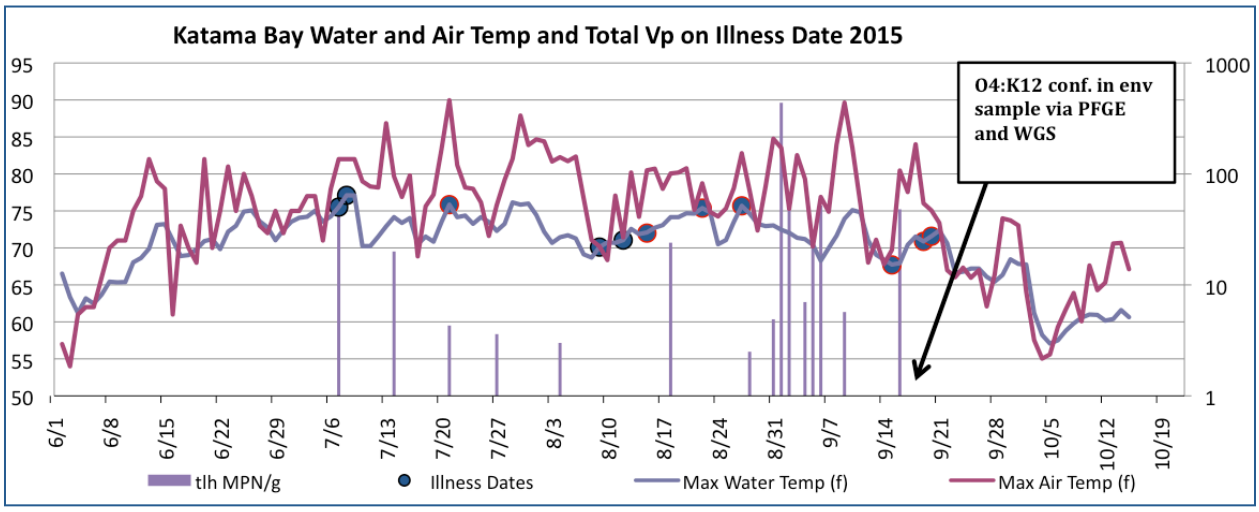
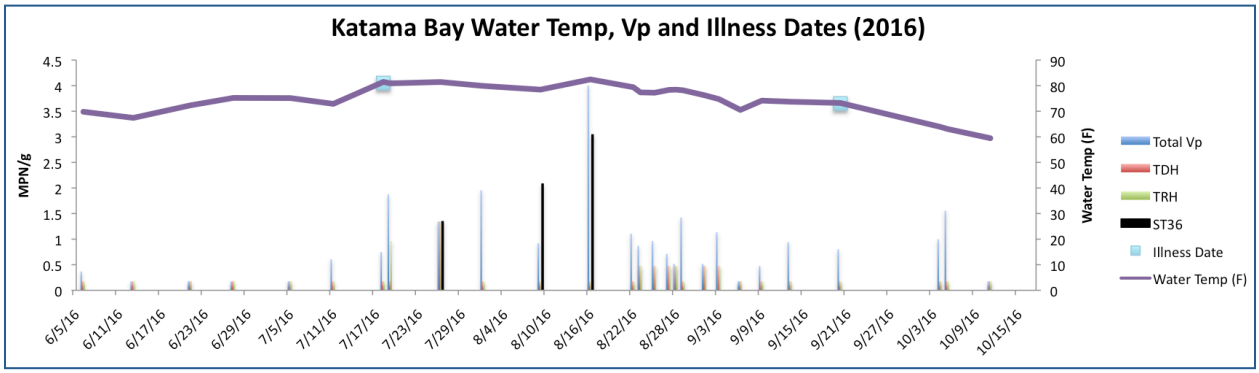


Figure 11 Katama Bay Water Temp, Vp and illness Dates 2016



WESTERN CAPE COD BAY

16 sole source cases were reported from Katama Bay between 2014 and 2016 with peak occurrence between September 1 and September 15 (figure 11).

Figure 11 epi curve for WCCB (2014- 2016)

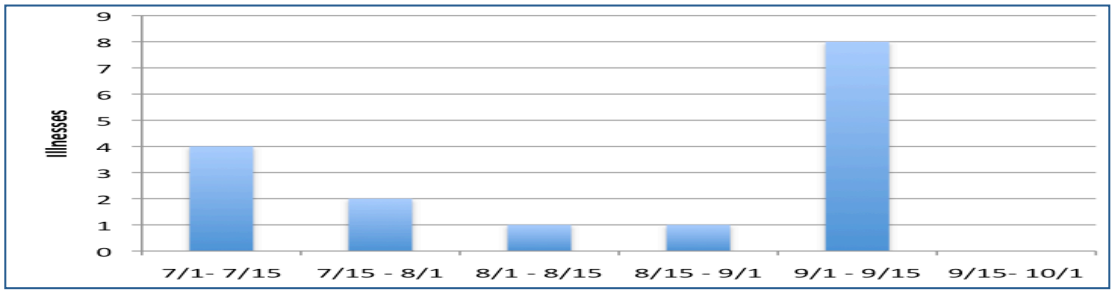


Table 18 Western Cape Cod Bay (Duxbury Bay and Plymouth Harbor) Frequency Distribution of Environmental Conditions on Harvest Dates Associated With Sole Source Illnesses 2014- 2016

Western Cape Cod Bay (Duxbury Bay and Plymouth Harbor) ***Frequency Distribution of Environmental Conditions on Harvest Dates Associated With Sole Source Illnesses 2014- 2016***			
Max Air Temp	*Count*	*Percent*	*Cumulative Percent*
85 To 90	6.	0.375	0.9375
75 To 80	4.	0.25	0.375
80 To 85	3.	0.1875	0.5625
70 To 75	2.	0.125	0.125
90 To 95	1.	0.0625	1.
Average Water Temp	*Count*	*Percent*	*Cumulative Percent*
74 To 76	9.	0.5625	0.6875
76 To 78	3.	0.1875	0.875
72 To 74	2.	0.125	0.125
78 To 80	2.	0.125	1.
Average of Salinity	*Count*	*Percent*	*Cumulative Percent*
29.5 To 30	4.	0.25	0.5
30.5 To 31	4.	0.25	0.8125
31 To 31.5	3.	0.1875	1.
29 To 29.5	2.	0.125	0.25
28 To 28.5	1.	0.0625	0.0625
28.5 To 29	1.	0.0625	0.125
30 To 30.5	1.	0.0625	0.5625
Min of Depth	*Count*	*Percent*	*Cumulative Percent*
0.2 To 0.4	7.	0.4375	0.4375
0.6 To 0.8	4.	0.25	0.8125
0.4 To 0.6	2.	0.125	0.5625
0.8 To 1	2.	0.125	0.9375
1 To 1.2	1.	0.0625	1.
Average of pH	*Count*	*Percent*	*Cumulative Percent*
7.65 To 7.7	4.	0.25	0.25
7.7 To 7.75	4.	0.25	0.5
7.8 To 7.85	3.	0.1875	0.6875
7.85 To 7.9	2.	0.125	0.8125
7.9 To 7.95	1.	0.0625	0.875
7.95 To 8	1.	0.0625	0.9375
8 To 8.05	1.	0.0625	1.
Average of Turbidity+	*Count*	*Percent*	*Cumulative Percent*
0 To 2	7.	0.4375	0.4375
2 To 4	3.	0.1875	0.625
8 To 10	2.	0.125	0.875
10 To 12	1.	0.0625	0.9375
12 To 14	1.	0.0625	1.
4 To 6	1.	0.0625	0.6875
6 To 8	1.	0.0625	0.75

Average of Chlorophyll	Count	Percent	Cumulative Percent
10 To 12	5.	0.3125	0.8125
6 To 8	4.	0.25	0.3125
8 To 10	3.	0.1875	0.5
12 To 14	2.	0.125	0.9375
14 To 16	1.	0.0625	1.
4 To 6	1.	0.0625	0.0625

Figure 12 Duxbury Bay Illness Data and Water Temperature 2014-2016

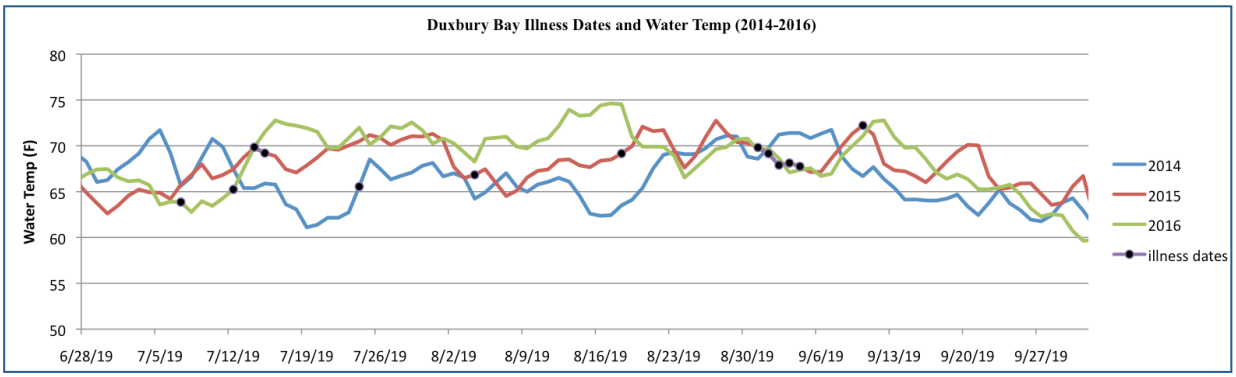
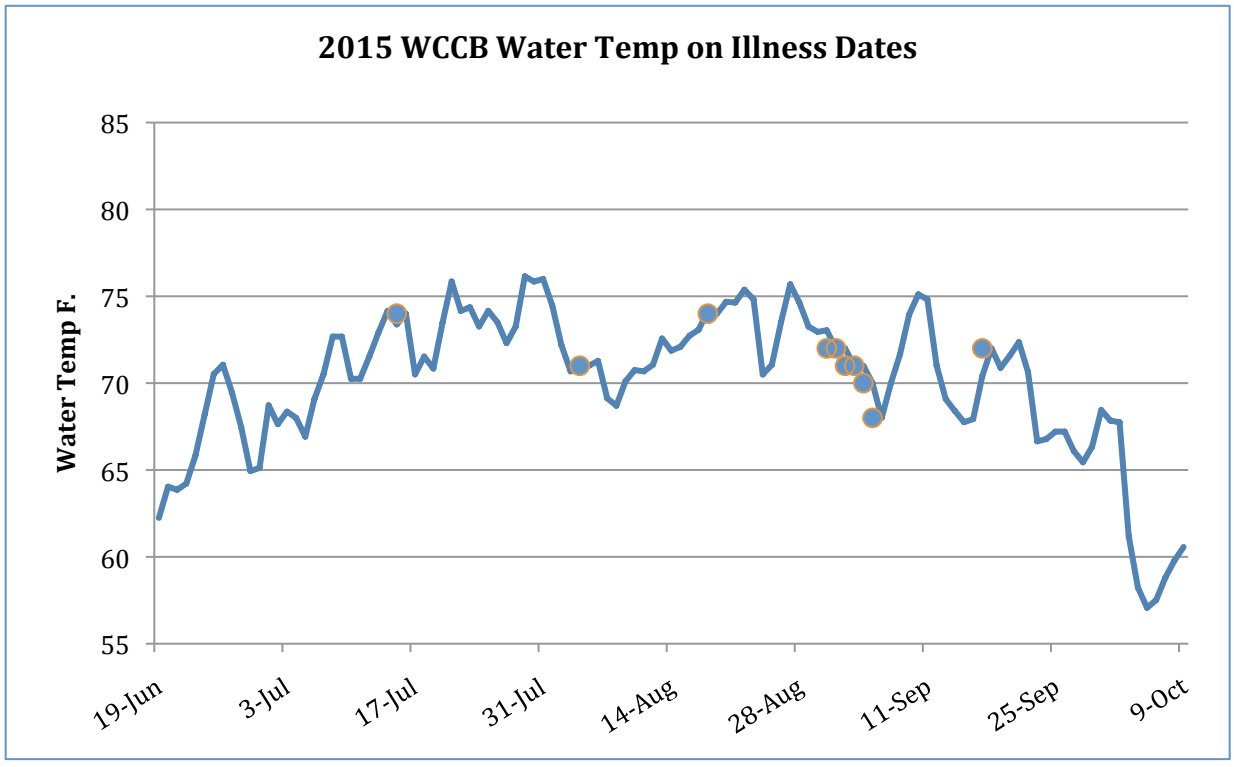
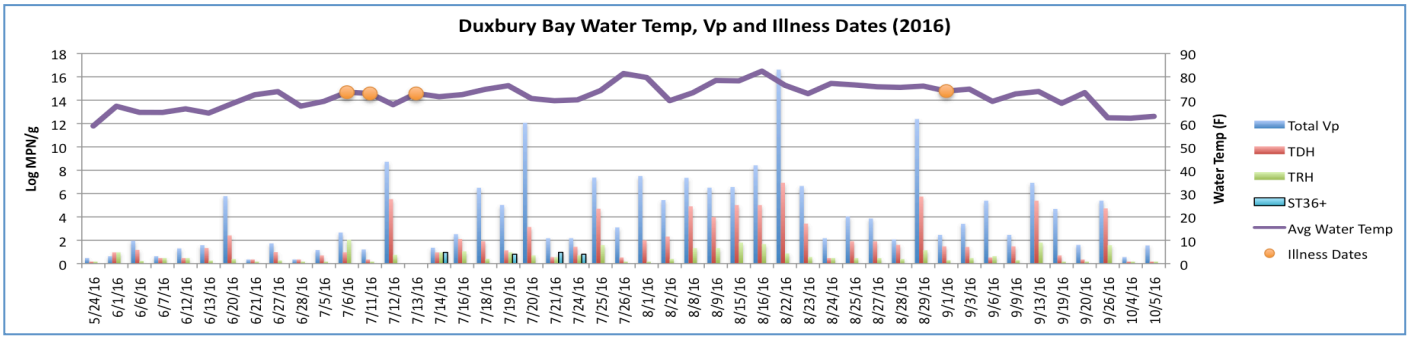


Figure 13 WCCB Water Temperature on Illness Dates.



DUXBURY BAY TRENDS IN VP LEVELS PROXIMATE TO ILLNESS OCCURRENCE

Figure 14



EASTERN CAPE COD BAY

11 sole source cases were reported from ECCB between 2014 and 2016 with peak occurrence between July 15 and September 15 (figure 15)

Figure 15 epi curve ECCB (2014-2016)

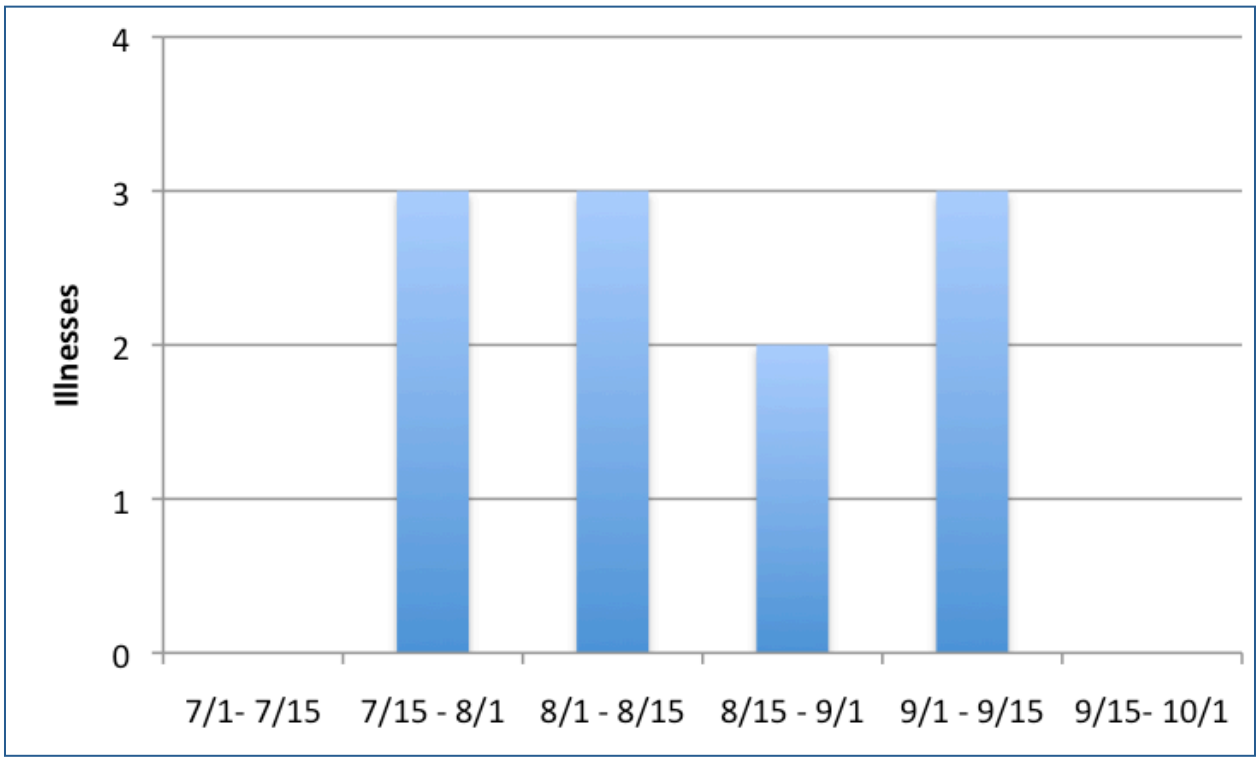


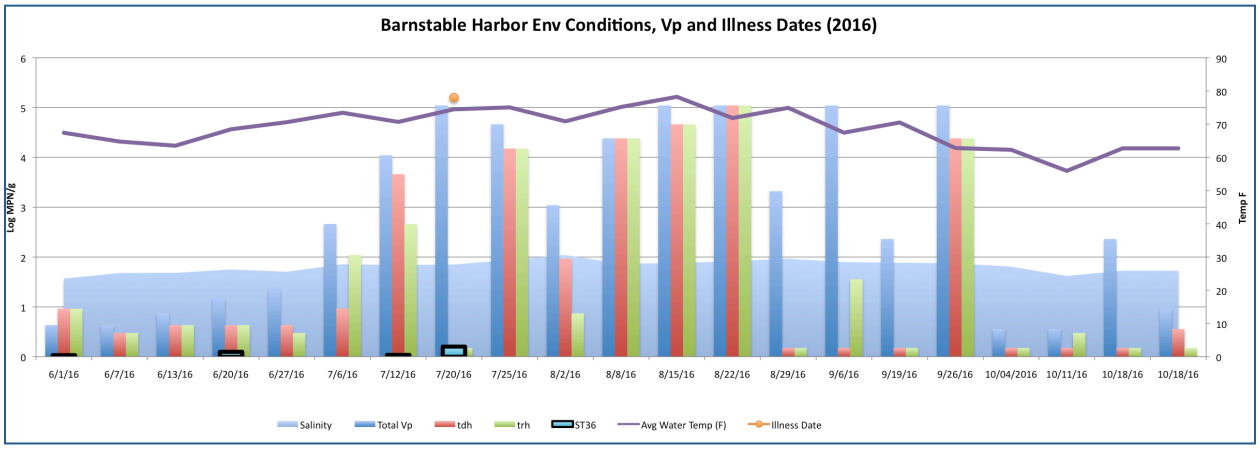
Table 19 Eastern Cape Cod Bay (Barnstable to Wellfleet) Frequency Distribution of Environmental Conditions on Harvest Dates Associated With Sole Source Illnesses 2014- 2016

Eastern Cape Cod Bay (Barnstable to Wellfleet) ***Frequency Distribution of Environmental Conditions on Harvest Dates Associated With Sole Source Illnesses 2014- 2016***			
Max Air Temp	*Count*	*Percent*	*Cumulative Percent*
75 To 80	5.	0.45455	0.54545
80 To 85	4.	0.36364	0.90909
70 To 75	1.	0.09091	0.09091
85 To 90	1.	0.09091	1.
Average Water Temp	*Count*	*Percent*	*Cumulative Percent*
76 To 78	5.	0.45455	0.90909
74 To 76	4.	0.36364	0.45455
72 To 74	1.	0.09091	0.09091
80 To 82	1.	0.09091	1.
Average of Salinity	*Count*	*Percent*	*Cumulative Percent*
29 To 30	3.	0.27273	0.63636
28 To 29	2.	0.18182	0.36364
30 To 31	2.	0.18182	0.81818
31 To 32	2.	0.18182	1.
26 To 27	1.	0.09091	0.09091
27 To 28	1.	0.09091	0.18182
Min of Depth	*Count*	*Percent*	*Cumulative Percent*
0.6 To 0.8	6.	0.54545	0.90909
0.2 To 0.4	3.	0.27273	0.27273
0.4 To 0.6	1.	0.09091	0.36364
1 To 1.2	1.	0.09091	1.
Average of pH	*Count*	*Percent*	*Cumulative Percent*
7.7 To 7.75	4.	0.36364	0.54545
7.8 To 7.85	2.	0.18182	0.81818
7.9 To 7.95	2.	0.18182	1.
7.6 To 7.65	1.	0.09091	0.09091
7.65 To 7.7	1.	0.09091	0.18182
7.75 To 7.8	1.	0.09091	0.63636
Average of Turbidity+	*Count*	*Percent*	*Cumulative Percent*
0 To 5	8.	0.72727	0.72727
10 To 15	1.	0.09091	0.90909
15 To 20	1.	0.09091	1.
5 To 10	1.	0.09091	0.81818

Average of Chlorophyll	*Count*	*Percent*	*Cumulative Percent*
0 To 5	4.	0.36364	0.36364
5 To 10	4.	0.36364	0.72727
15 To 20	2.	0.18182	1.
10 To 15	1.	0.09091	0.81818

BARNSTABLE HARBOR TRENDS IN VP LEVELS PROXIMATE TO ILLNESS OCCURRENCE (2016)

Figure 16 Barnstable Harbor Environmental Conditions, Vp and Illness Dates.



DISCUSSION OF ILLNESS TRENDS

The majority of Vp risk assessment and mitigation strategies are based on the basic relationship between increasing temperature and increasing bacterial abundance at harvest, and evaluate risk broadly by region. As such, in the US the predicted Vp illness risk is highest for harvest areas in the Gulf Coast region and lowest for those in the North Atlantic and Pacific Northwest regions, yet the increase in illness occurrence in recent years in the North Atlantic and Pacific Northwest regions suggests other trends are driving risk and illness. Often risk is considered on a regional basis, but occurrence in Massachusetts suggests Vp community composition and localized conditions in growing areas can also create significant variation on a small geographic scale.

Under current risk assessment models for the Pacific Northwest and Atlantic regions, the estimated risk is higher for intertidal harvest areas than sub-tidal harvest areas where shellfish are

always submerged. Again the relationship of risk and tidal exposure is based on a predicted increase in Vp abundance as a result of exposure to elevated ambient air temperatures at low tide. Although total and hemolysin-producer Vp abundance, and the environmental factors such as temperature that correlate with abundance, may be useful for broadly managing risk, in many cases illness patterns do not correlate with these basic risk models and may be resulting in inadequate controls, as was the case leading up to the 2012 and 2013 Vp outbreaks in the US Northeast. Additionally, the majority of implicated harvest areas during the 2013 outbreak were not those subject to the greatest amount of tidal exposure or the highest average water temperatures.

What can be determined from the provided data is that seasonality is likely the strongest driving factor associated with Vp illness occurrence in Massachusetts, with 42 of 44 of the cases analysis between 2014 and 2016 occurring between July 1 and September 15. Approximately two thirds (30/44) of illnesses occurred when average water temperatures were between 74°F and 78 °F, and over two thirds (33/44) of reported cases implicate only two growing areas in the state. These basic observations have driven the Massachusetts Vp control strategy which focuses elevated controls during the time of year and in the locations when most illness occur.

IMPACT OF RESUBMERGENCE AND TRANSPLANT ON OF TOTAL AND HEMOLYSIN-PRODUCER VP. IN OYSTERS

RESUBMERGENCE

Oyster production is decreased by the presence of bio-fouling organisms on oysters and oyster culture equipment. These organisms compete with oysters for resources and can negatively affect growing conditions and marketability. The least expensive and most common means for anti-fouling is by exposing oysters and equipment to air to dry and kill off attached organisms. Various management production strategies where this practice is included are floating cages and bag that are flipped to expose oysters and cages to air, and removal of submerged cages containing oyster to air. These practices are efficient at removing fouling organisms with limited oyster mortality. During warm summer conditions, however, these practices are highly conducive to stimulating the growth of Vp, including potentially pathogenic strains.

The length of time required for Vp to be reduced or to return to background levels can be a production restraint as it may prohibit producers from being able to market their oysters before bio-fouling organisms begin to re-colonize the oysters again. Current time frames are based on limited-scope, state specific, studies. It is currently unclear what effects these practices have on pathogenic strains of Vp, and not just the total Vp populations.

We conducted total and hemolysin-producer Vp on oysters exposed to extended handling and specific re-submergence intervals to determine the time needed for Vp levels to return to background following such activities. Work was conducted in 2015 and 2016 in Katama Bay and Duxbury Bay. Results identified as controls were placed on ice immediately upon harvest. Results identified as 48Hr abused were exposed to 48hrs of ambient air to simulate common air drying practices. Results identified as 2 day, 4 day, 7 day, and 14day resub, indicate the number of days since time temperature abused oyster have been returned to the water. Duplicate treatment and control samples were collected at each interval. Data from the 4 study periods were pooled for the purposes of statistical analysis.

Statistical Analysis of Resubmerged Oysters

Differences between the means of the treatment groups were evaluated using One-Way Analysis of Variance (ANOVA) of the log transformed total, tdh+ and trh+ *Vp*.

Table 20 Descriptive statistics report for Vp resubmergence samples.

Treatment Name	N	Mean Vp MPN/g	Std Dev	SEM
Control *tlh*	46	15.2	6.2	1.3
Control *tdh*	46	2.6	2.2	1.1
Control *trh*	46	3.8	2.9	1.2
48 hr abused *tlh*	8	1978.0	6.1	1.9
48 hr abused *tdh*	8	28.9	6.3	1.9
48 hr abused *trh*	8	69.4	12.2	2.4
2 day re-sub *tlh*	8	136.2	11.5	2.4
2 day re-sub *tdh*	8	4.8	3.8	1.6
2 day resub *trh*	8	17.0	5.8	1.9
4 day re-sub *tlh*	8	66.0	3.7	1.6
4 day re-sub *tdh*	8	3.3	2.0	1.3
4 day resub *trh*	8	4.2	2.6	1.4
7 day re-sub *tlh*	8	7.8	3.0	1.5
7 day re-sub *tdh*	8	1.4	2.4	1.4

7 day resub *trh*	8	1.7	2.7	1.4
14 day re-sub *tlh*	8	15.1	3.4	1.5
14 day re-sub *tdh*	8	2.7	1.8	1.2
14 day resub *trh*	8	4.5	2.1	1.3

Table 21 One Way Analysis of Variance report for total Vp resubmergence samples

Source of Variation	*SS*	*df*	*MS*	*F*	*p-value*
Between Groups	38.23342	5	7.64668	13.18569	2.28564E-9
Within Groups	46.39383	80	0.57992		
Total	84.62725	85			

Table 22 One Way Analysis of Variance report for tdh+ Vp resubmergence sample

Source of Variation	*SS*	*df*	*MS*	*F*	*p-value*
Between Groups	9.11647	5	1.82329	10.1649	1.45997E-7
Within Groups	14.34974	80	0.17937		
Total	23.46621	85			

Table 23 One Way Analysis of Variance report for trh+ Vp resubmergence samples.

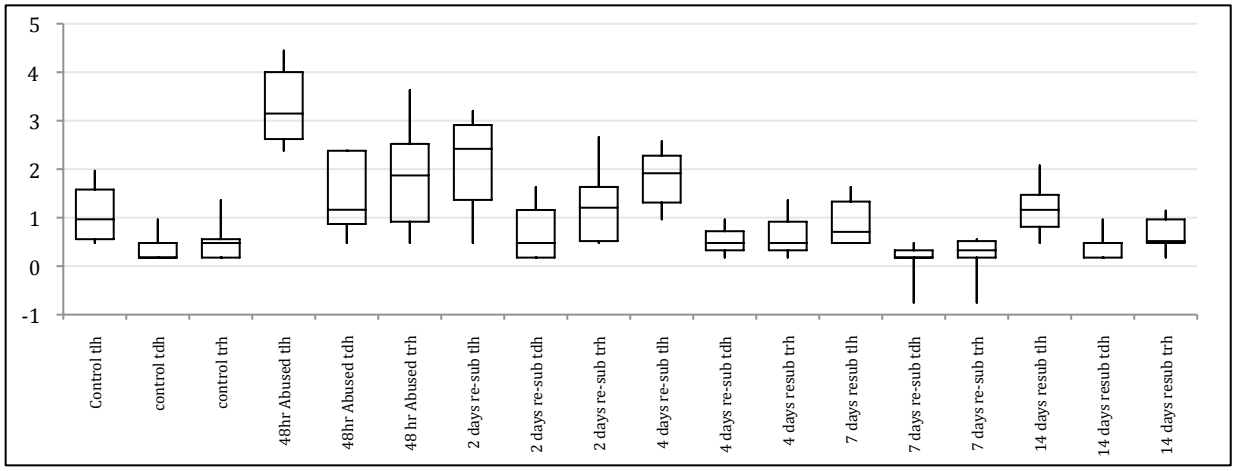
Source of Variation	*SS*	*df*	*MS*	*F*	*p-value*
Between Groups	15.06914	5	3.01383	9.45617	4.11171E-7
Within Groups	25.49724	80	0.31872		
Total	40.56638	85			

To determine significance between treatment groups pairwise comparison was conducted using Student's T-Test assuming unequal variance between groups.

Table 24 Pairwisecomparisons between control and resubmergence interval samples.

Resubmergence Interval	p-value	STD DEV
Control *tlh*/ 48 hr Abused *tlh*	0.00004	2.22814
Control *tlh*/ 2 day resub *tlh*	0.04178	2.306
Control *tlh*/ 4 day resub *tlh*	0.01854	2.17881
Control *tlh*/ 7 day resub *tlh*	0.17549	2.13145
Control *tlh*/ 14 day resub *tlh*	0.99062	2.16037
Control *tdh*/ 48 hr Abused *tdh*	0.007944313	2.364624252
Control *tdh*/ 2 day resub *tdh*	0.23617733	2.306004135
Control *tdh*/ 4 day resub *tdh*	0.348444196	2.228138852
Control *tdh*/ 7 day resub *tdh*	0.093319811	2.262157163
Control *tdh*/ 14 day resub *tdh*	0.869514148	2.17881283
Control *trh*/ 48 hr Abused *trh*	0.014421821	2.364624252
Control *trh*/ 2 day resub *trh*	0.047400073	2.306004135
Control *trh*/ 4 day resub *trh*	0.793972062	2.228138852
Control *trh*/ 7 day resub *trh*	0.064440584	2.228138852
Control *trh*/ 14 day resub *trh*	0.588019056	2.17881283

Figure 7 Box plots of total, trh+ and tdh+ Vp log MPN/g concentration in oysters from all sites prior to and following air drying, as well as following 4 re-submergence intervals. The band inside each box indicates the median value. Lower and upper lines of the box represent the 25th and 75th percentiles, respectively. Lower and upper limits of the whiskers represent the 10th and 90th percentiles, respectively.



Discussion of Resubmergence Study

Following 48-hours of exposure to ambient air temperatures significant level of total and hemolysin-producer Vp growth was observed as compared to un-abused control samples. In two of eight abused samples the indicator genes for ST36 (*prp* and *flp)* were observed. Following two days of resubmergence total and trh+ Vp remained significantly higher than control samples and in four of eight samples the indicator genes for ST36 (*prp* and *flp)* were observed. Following four days of resubmergence total and trh+ Vp remained significantly higher than control samples and in one of eight samples the indicator genes for ST36 (*prp* and *flp)* were observed. Following seven days of resubmergence there was no significant differences between abused re-submerged oysters and control samples was observed and the indicator genes for ST36 (*prp* and *flp)* we below the detection threshold in all samples.

Katama Bay to Eel Pond Off-Shore Transplant

The transplant of oysters from a growing area with environmental conditions deemed more conducive to Vp production (lower salinity, higher temperature), or an area with a history of Vp illness, to an area with environmental conditions considered less conducive to Vp abundance (higher salinity/lower temperature) prior to harvest has been considered as a Vp risk management strategy. We conducted total and hemolysin-producer Vp analysis on oysters

relocated from Katama Bay (Edgartown, MA) to Eel Pond Off-Shore (V13), to examine whether this could be a viable proactive aquaculture practice for Vp risk reduction. To show a reduction in background levels oysters from Katama Bay were exposed to ambient air temperatures for 48 hours prior to transplant and sampled at specific re-submergence intervals to determine the time needed for Vp levels to return to background following transplant from areas with elevated levels. Results identified as controls were placed on ice immediately upon harvest. Results identified as 48Hr abused were exposed to 48hrs of ambient air to simulate common culture practices. Results identified as 2 day, 4 day, 7 day, and 14day resub, indicate the number of days since time temperature abused oyster have been moved to the transplant area. Duplicate treatment and control samples were collected at each resubmergence interval.

Map 2 Katama Bay and Eel Pond Transplant Area

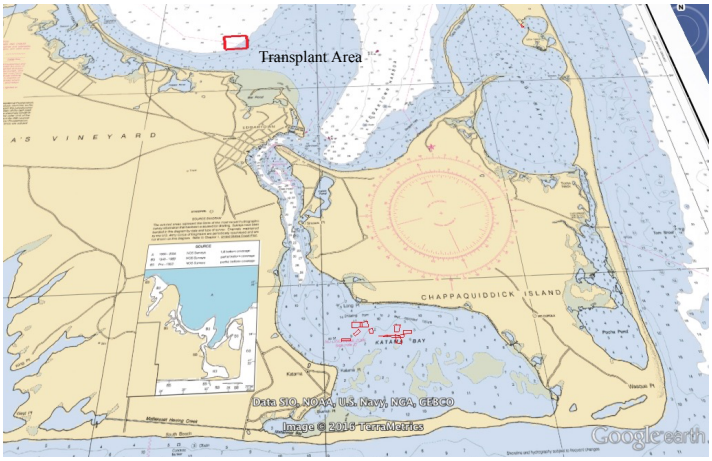


Figure 8 Temperature Graph of Katama Bay (V20) and the Transplant Area (V13) shows lower temperatures deems less conducive to Vp growth in the off-shore transplant area.

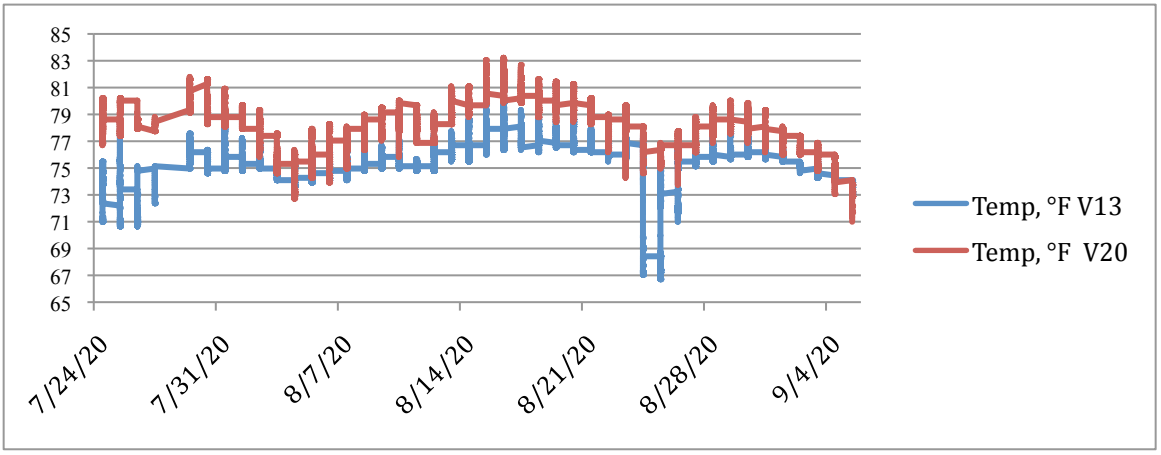
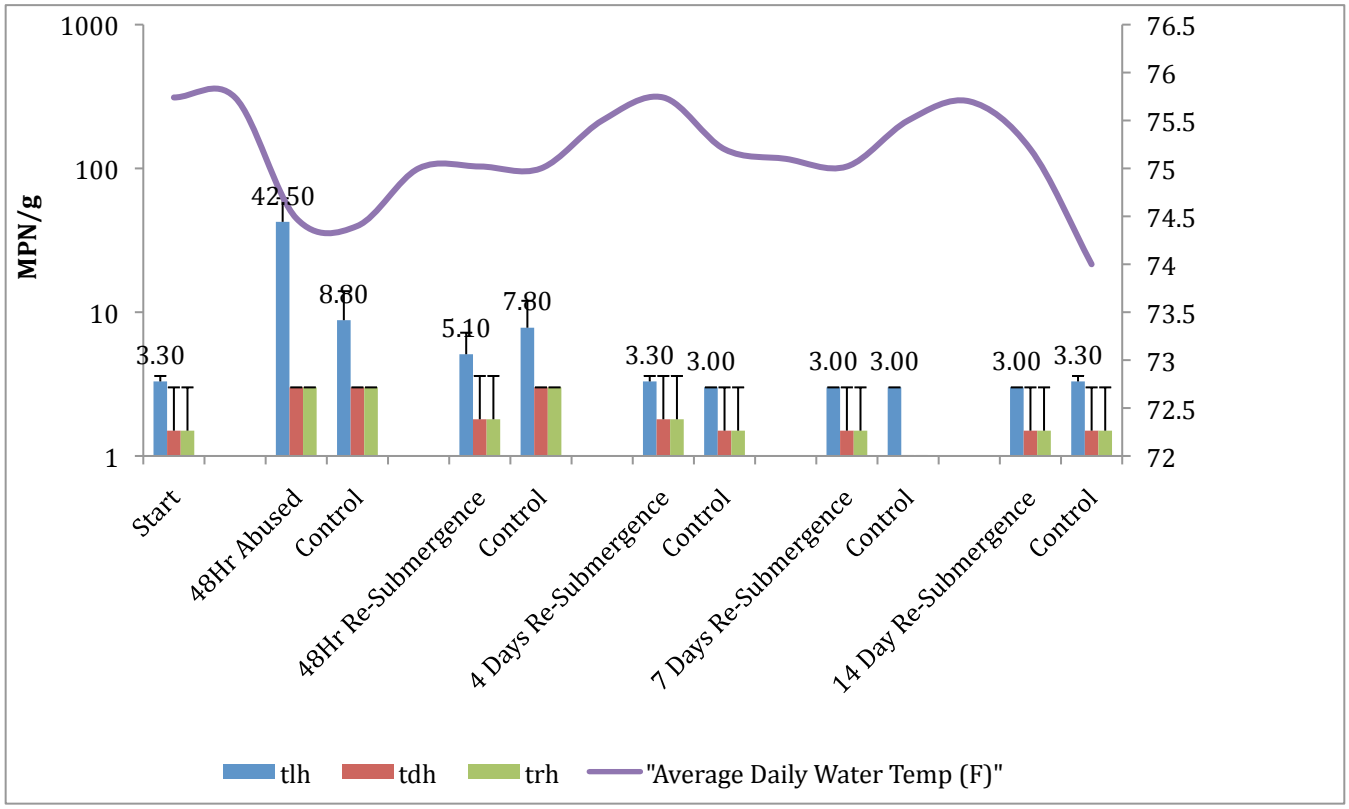


Figure 9 Graph of Total and Hemolysin-Producer Vp Following 48 hours of Abuse and Resubmergence



Discussion of Transplant

Background levels of total Vp at the start of the sample period were at the detection threshold. Background levels of hemolysin producing Vp duplicate samples were at and below the detection threshold. Following 48 hours of abuse no significant difference was observed between abused and control samples for total and hemolysin-producing Vp, and no significant difference was observed in the subsequent re-submergence samples. Additional transplant work is ongoing.

While data did not show significant difference between Katama Bay and V13, During the 2016 Vp season approximately 35% Katama Bay production was diverted from Katama Bay to V13 for two week resubmergence prior to harvest. No illnesses were reported from oysters harvested from V13 and a sustainable reduction in reported Vp illnesses occurred between 2015 and 2016 (11 in 2015 vs. 2 in 2016).

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