CONNECTICUT DEPARTMENT OF AGRICULTURE BUREAU OF AQUACULTURE
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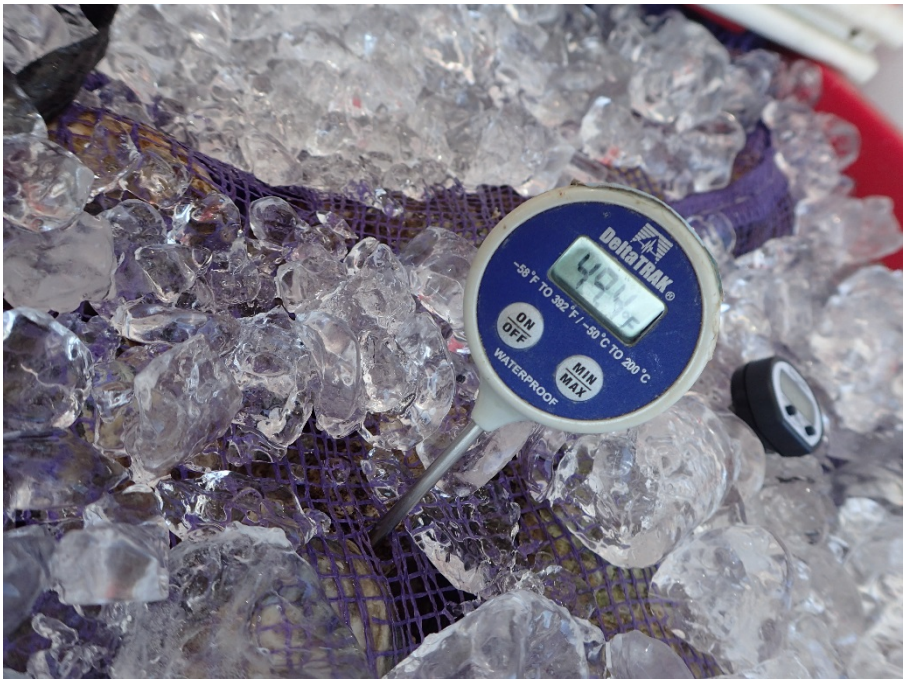
Techniques and Practices for Vibrio Reduction: Connecticut

Final Report to the Interstate Shellfish Sanitation Conference Submitted March 21, 2016



Submitted by Kristin DeRosia-Banick, David H. Carey, Joseph DeCrescenzo

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Submitted by:
Kristin DeRosia-Banick,[a] David H. Carey,[a] Joseph DeCrescenzo[a]
State of Connecticut
Department of Agriculture Bureau of Aquaculture[a]
Milford, Connecticut, USA

Project Partners:
Michael Whitney[b], Evan Ward[b]
University of Connecticut Department of Marine Sciences
Groton, Connecticut, USA[b]

In 2014, the Interstate Shellfish Sanitation Conference (ISSC) published a Request for Proposals for Techniques and Practices for Vibrio Reduction. The purpose of the Request for Proposals, was to invite qualified entities to propose studies that could offer viable process options for the shellfish industry that would reduce risk of Vibrio illnesses. The Connecticut Department of Agriculture, Bureau of Aquaculture and Laboratory Services (CT DA/BA) program managers sought and were successful in having their proposal selected; the result of the proposal was to accomplish several complementary objectives through the ISSC funding opportunity, described below in detail.

One objective of this study was to compare the levels of total, *tdh+, and trh+ V. parahaemolyticus* in oysters subjected to one (1) of five (5) post-harvest cooling treatments at specified intervals from the time of harvest. Treatments included the use of ice slurry to reduce the internal temperature of oysters to less than 10°C (50°F) immediately post-harvest, and to an internal temperature of 50°F (10°C) at one (1) hour, three (3) hours, and five (5) hours from harvest, respectively. The National

Shellfish Sanitation Program (NSSP) standard *Vibrio parahaemolyticus* Control Plan (VPCP) option of placing oysters under mechanical refrigeration at or below 45°F (7.2°C) within five (5) hours of harvest and reducing the internal temperature of oysters to 50°F (10°C) within ten (10) hours of being placed under refrigeration was also evaluated. Most probable number (MPN) real-time polymerase chain reaction (MPN-Rti-PCR) methods were used for enumeration of total *V. parahaemolyticus* and pathogenic (*tdh*+ and/or *trh*+) *V. parahaemolyticus*. *V. parahaemolyticus* was detected in all process study samples, with a median result of 1.88 log MPN/g for all oyster samples tested.

Differences in total *V. parahaemolyticus* levels between the VPCP traditional mechanical refrigeration method and treatments using ice slurry to cool to an internal temperature of 50°F (10 °C) immediately following harvest, and within one (1) hour and three (3) hours of harvest were statistically significant ($P < 0.005$). The difference in the means of total *V. parahaemolyticus* levels between the five (5) hour to 50°F (10°C) ice slurry treatment and both the zero (0) hour (0Hr) and one (1) hour treatments were also statistically significant. These data indicate that post-harvest growth of *V. parahaemolyticus* is more effectively controlled by rapidly cooling oysters to an internal temperature of 50°F (10°C) within three (3) hours of harvest as compared to the traditional process of placing oysters under mechanical refrigeration within five (5) hours of harvest and reducing internal temperatures of oysters to 50°F (10°C) with ten (10) hours of being placed under refrigeration. The results can be used to evaluate and refine Vibrio control plan cooling strategies employed by risk managers and State Shellfish Control Authorities (SSCAs).

Complementary to the post-harvest controls study, the study provided a mechanism to gain a better understanding of *V. parahaemolyticus* levels in the environment and their relevance to implementing

meaningful Vibrio controls in Connecticut growing waters. The 2014/2015 monitoring plan included the collection of environmental parameters, e.g. water temperature, air temperature, salinity, and depth in order to assess their relationship to levels of *V. parahaemolyticus* bacteria in shellfish. Vibrio monitoring and continuous environmental observations have been used to inform the understanding of the temporal variability and spatial distribution of *V. parahaemolyticus* in Long Island Sound (LIS) oyster production areas. These data allowed the state to proactively manage *V. parahaemolyticus* during 2015 by requiring more stringent controls under those specific environmental conditions that have historically correlated to a higher risk of illness, rather than relying on a trigger based on the specific dates associated with illness alone.

Executive Summary

Vibrio parahaemolyticus is a Gram-negative, curve-shaped rod found in estuarine and marine environments worldwide (Lampel, 2012). The incidence of vibriosis in the United States increased between 1996 through 2010, driven primarily by an increase in *V. parahaemolyticus* which increased from 0.01 to 0.13 per 100,000 population via Cholera and Other Vibrio Illness Surveillance (COVIS) and from 0.06 to 0.23 via the Foodborne Diseases Active Surveillance Network (Food-Net) (Newton, 2012). *V. parahaemolyticus* typically manifests as mild to moderate gastroenteritis, however wound infection and septicemia may also occur (Lampel, 2012). There are many pathogenic and non-pathogenic strains of this bacterium, which are typically identified at higher concentrations in shellfish in the northeast region from April through October when coastal waters are warm. Consumers may be exposed to these pathogenic bacteria by eating raw or undercooked molluscan shellfish and crustaceans.

During the summers of 2012 and 2013, *V. parahaemolyticus* infections of a strain previously traced

only to the Pacific Northwest were associated with consumption of oysters and other shellfish from several Atlantic Coast harvest areas (Martinez-Urtaza, et al., 2013). Connecticut growing waters were the source of at least 23 confirmed cases of *V. parahaemolyticus* during the summer of 2013, with an additional 15 multi-source cases potentially linked to Connecticut waters. Connecticut shellfish growing areas had not been the confirmed source of an outbreak in the years prior to 2013, however the *V. parahaemolyticus* risk evaluation conducted by the SSCA for Connecticut had determined the need for a VPCP beginning in 2012. For Connecticut, the high risk season was determined to be between June 1 and September 30, based on seasonal air and water temperatures and salinity levels in the optimal range for *V. parahaemolyticus* proliferation. The VPCP that was in place at the time of the 2013 outbreak included the NSSP standard time to temperature control measure of limiting time from harvest to refrigeration to no more than five (5) hours, and required the original dealer to cool oysters to an internal temperature of 50°F (10°C) or below within ten (10) hours after placement into refrigeration. Unfortunately, the national standard *V. parahaemolyticus* controls that were in place at the time of the 2013 outbreak were inadequate to prevent illnesses from occurring, and on-board rapid-cooling was selected by the SSCA for the 2014 and 2015 *V. parahaemolyticus* seasons in order to reduce the risk of illness associated with oysters harvested from the outbreak area.

In 2014, the Connecticut Department of Agriculture Bureau of Aquaculture (DA/BA) acquired real-time PCR technology (Life Technologies 7500 Fast Real Time PCR System) which has allowed the Bureau in their role as the SSCA to conduct both environmental monitoring as well as post-harvest process studies for total, *tdh+* and *trh+ V. parahaemolyticus* bacteria.

Evaluation of Post-Harvest *V. parahaemolyticus* Controls

The primary objective of the Connecticut Techniques and Practices for Vibrio Reduction study was to evaluate the effectiveness of post-harvest controls that could potentially reduce the risk of Vibrio illnesses. The use of ice slurry for rapidly cooling the internal temperatures of oysters to 50°F (10°C) was compared to the NSSP standard VPCP controls requiring placement under temperature control [in this case, mechanical refrigeration at or below 45°F (7.2°F)] within five (5) hours of harvest and cooling to an internal temperature of 50°F (10°C) within ten (10) hours of being placed under temperature control. The effectiveness of several post-harvest time and temperature strategies were evaluated using continuous temperature data loggers (ACR Smart Button) to record the length of time each sample took to reach 50°F (10°C) and via enumeration of total, *tdh+*, and *trh+ V. parahaemolyticus* associated with each treatment sample.

The project industry partner's (Norm Bloom & Son Norwalk, CT) on-vessel ice slurry equipment was used for rapidly cooling shellfish to an internal temperature of 50°F (10°C). The ice slurry process used by Norm Bloom & Son was first evaluated in 2014 and approved for use under Connecticut's rapid cooling VPCP during the 2014 and 2015 Vibrio seasons. A rapid cooling control plan was required during 2014 and 2015 for the harvest of oysters from the municipalities of Westport, Norwalk, and Darien growing areas confirmed and implicated in the 2013 outbreak. Studies completed by the DA/BA during 2014 conclusively proved that via the use of on-vessel ice slurry, harvesters were able to rapidly cool oysters to an internal temperature of 50°F (10°C) in less than thirty (30) minutes throughout the Vibrio season.

The post-harvest controls study period was July 14 through September 23, 2015, inclusive. The CT DA/BA collected eight (8) shellstock samples approximately every two weeks for a total of 51

samples; occasional runs were rescheduled or sample runs missed due to scheduling limitations. Each of the eight (8) samples collected had been subjected to one (1) of five (5) post-harvest treatments, plus replicates. Shellfish samples were analyzed for total *V. parahaemolyticus* using (MPN-Rti-PCR) as previously described by Kinsey et al (Kinsey, Lydon, Bowers, & Jones, 2015). treatments, plus replicates. Shellfish samples were analyzed for total *V. parahaemolyticus* using a MPN-Rti-PCR method as previously described by Kinsey et al (Kinsey, Lydon, Bowers, & Jones, 2015). A second multiplex Rti-PCR method targeting the *tdh* and *trh* hemolysin genes was used for identification and MPN enumeration of pathogenic *V. parahaemolyticus*. Norm Bloom & Son's on-vessel global positioning system (GPS) was used to verify that oyster samples were collected from the same shellfish lease location each sample run.

Processes investigated include:

1) Zero (0) Hour (Baseline): Immediate post-harvest rapid cooling to internal temperature of 50°F (10°C) or less using ice slurry, and
2) One (1) hour from harvest to internal temperature of 50°F (10°C) or less using ice slurry (45 minutes on deck then into slurry for 15 minutes rapid cooling), and
3) Three (3) hours from harvest to internal temperature of 50°F (10°C) or less using ice slurry (two (2) hours 45 minutes on deck prior to slurry for 15 minutes), and
4) Five (5) hours from harvest to internal temperature of 50°F (10°C) or less using ice slurry (four (4) hours 45 minutes on deck prior to slurry for 15 minutes), and
5) NSSP standard VPCP: Five (5) hours from harvest into mechanical refrigeration at or below 45°F (7.2°C) and maximum of ten (10) hours to an internal temperature of 50°F (10°C).

Environmental Monitoring for *V. parahaemolyticus*:

Complementary to the post-harvest controls study, the SSCA sought to gain a better understanding of *V. parahaemolyticus* levels in the environment and their relevance to implementing meaningful Vibrio controls in Connecticut growing waters. The 2014/2015 monitoring plan included the collection of environmental parameters, e.g. water temperature, air temperature, salinity, and depth that may correlate to levels of Vibrio bacteria in shellfish. The SSCA uses Vibrio monitoring and continuous environmental observations to understand the temporal variability and spatial distribution of *V. parahaemolyticus* in Long Island Sound (LIS) oyster production areas. These data allowed the state to proactively manage *V. parahaemolyticus* during 2015 by requiring more stringent controls under those specific environmental conditions that have historically correlated to a higher risk of illness, rather than relying on a trigger based on the specific dates associated with illness alone.

The environmental monitoring study period was June 15 to October 31, 2014, and June 1 through October 31, 2015, inclusive. The CT DA/BA collected eight (8) shellstock samples every two weeks for a total of 101 samples (n = 101). Shellfish samples were analyzed for total *V. parahaemolyticus* using (MPN-Rti-PCR). A second multiplex Rti-PCR method targeting the *tdh* and *trh* hemolysin genes was used for identification and MPN enumeration of pathogenic *V. parahaemolyticus* as described above. On-vessel global positioning system (GPS) was used to verify the location from which each sample was collected. The primary oyster production areas in Connecticut waters were targeted, with more intensive sampling focused on the Westport/Norwalk/Darien inner island growing area associated with the 2013 outbreak.

Materials and Methods

Post-Harvest Controls Study

The study tested the hypothesis that post-harvest control of oyster temperatures utilizing ice slurry for on-board rapid cooling of oysters to 50°F (10°C) within one (1) hour of harvest effectively limits the proliferation of Vibrio bacteria (total, *tdh*+ and *trh*+ *V. parahaemolyticus*) and is more effective than the NSSP standard VPCP cooling allowing five (5) hours from harvest to temperature control at or below 45°F (7.2°C) and ten (10) hours to an internal temperature of 50°F (10°C). Treatments investigated during the 2015 study period include:

1) Zero (0) Hour (Baseline): Immediate post-harvest rapid cooling to internal temperature of 50°F (10°C) or less using ice slurry, and
2) One (1) hour from harvest to internal temperature of 50°F (10°C) or less using ice slurry (45 minutes on deck then into slurry for 15 minutes rapid cooling), and
3) Three (3) hours from harvest to internal temperature of 50°F (10°C) or less using ice slurry (two (2) hours 45 minutes on deck prior to slurry for 15 minutes), and
4) Five (5) hours from harvest to internal temperature of 50°F (10°C) or less using ice slurry (four (4) hours 45 minutes on deck prior to slurry for 15 minutes), and
5) NSSP standard 5/10 Hour VPCP: Five (5) hours from harvest into mechanical refrigeration at or below 45°F (7.2°C)and maximum of ten (10) hours to an internal temperature of 50°F (10°C).
6) Replicate sample Zero (0) Hour (Baseline)
7) Replicate sample One (1) hour
8) Replicate sample NSSP 5/10 Hour VPCP

On each sample collection run, the industry vessel captain harvested 160 oysters from oyster lease 103-L-43, located in Conditionally Approved Area #1 in the municipality of Norwalk, CT. All samples were collected and processed by the state Vibrio manager with the assistance of one staff analyst. Internal shellfish temperatures were recorded at the time of collection by using a gloved hand and partially shucking the oyster then inserting a calibrated probe thermometer into the deepest part of the tissue. Eight (8) oysters were partially shucked keeping the adductor muscle intact, a Smart Button data logger (ACR Systems Inc. Surrey, British Columbia) was inserted, and the oyster zip-tied closed. These oysters were placed with remaining oysters and held in a bushel basket on the deck of the vessel. The basket was located in a shaded area on the deck of the vessel to represent conditions that a typical commercial harvest would be exposed to during the VPCP control months when shading is required. Consecutive samples were pulled from the basket at identified intervals. Ambient post-harvest air temperatures were recorded during each sample collection run using a Smart Button data logger attached to the basket.

Each sample consisted of twenty (20) oysters: three (3) oysters were used to take internal shellfish tissue temperatures using a calibrated probe thermometer (DeltaTRAK® 08C1), one (1) oyster was used for the Smart Button temperature logger, and sixteen (16) oysters were brought to the lab, of which twelve (12) were used in the analysis.

For samples 1 and 6, oysters were placed into a mesh bag and placed immediately into ice slurry, allowing for 15 minutes in the ice slurry to reach an internal temperature of 10°C (50°F) or less prior to collection. Following rapid cooling via on-board ice slurry, samples were placed into a plastic bag, labeled with the sample identification, and placed on ice in an insulated cooler for transport to the laboratory.

Samples 2, 3, 4, and 7 were held on deck for 45 minutes, 165 minutes, 285 minutes, and 45 minutes, respectively, prior to being placed in a mesh shellstock bag and placement into the ice slurry, allowing for 15 minutes in the ice slurry to reach an internal temperature of 10°C (50°F) or less prior to sample collection. Following rapid cooling, samples were placed into a plastic bag, and placed on ice in an insulated cooler for transport to the laboratory.

Samples 5 and 8 were held on either the deck of the boat or on shore for five (5) hours prior to placement in the shellfish dealer's mechanical refrigeration unit at or below 45°F, five (5) hours from the time of harvest. In order to achieve the ten (10) hour cool-down rate, samples were placed in plastic bags and wrapped in bubble wrap, then placed inside an insulated cooler in order to slow the cooling process to meet the ten (10) hour time window as recorded by the temperature loggers. Based on previous VPCP verification studies conducted at Norm Bloom & Son's facility, the expected time for oyster internal temperatures to reach 10°C (50°F) in the mechanical refrigeration unit was known to be three (3) hours or less, however in this study the attempt was to achieve the ten (10) hours to 50°F (10°C) cool down currently required by NSSP *V. parahaemolyticus* Control Plan options (NSSP Model Ordinance 2013 Revision) in the absence of a more restrictive state VPCP. Samples were cooled to an internal temperature of 10°C (50°F) within ten (10) hours after placement under temperature control at or below 45°F and held overnight. Samples were collected by DA/BA staff the following morning from the dealer's mechanical refrigeration unit and placed into an insulated cooler on ice for transport to the laboratory.

Environmental Monitoring

In June of 2014, DA/BA environmental analysts deployed 16 HOBO® Water Temp Pro v2 (U22-001) (Onset Corp. Bourne, MA) temperature data loggers at near-bottom depth and six (6) DST

conductivity, temperature, and depth (CTD) data loggers (Star-Oddi, Iceland) were deployed at near-surface and near-bottom depth at three (3) shellfish cage locations in the municipalities of Westport and Milford (Figure 1). One Vantage Pro 2 remote weather station (Davis Instruments, Vernon Hills, IL) was deployed in the municipality of Milford to monitor meteorological conditions, including rainfall and air temperature. During the June 2015 deployment, Star-Oddi DST loggers were replaced with Hobo® conductivity and temperature (U24-002-C) loggers to record the near-bottom temperature and salinity; Hobo® temperature loggers (U22-001) were used to record near-surface temperatures.

Stations were located to provide spatial coverage throughout Connecticut growing waters that are actively in use for oyster cultivation. A higher intensity of data collection focused on the waters of the municipalities of Norwalk and Westport, CT, where the majority of oysters associated with the 2013 *V. parahaemolyticus* outbreak were harvested.

From July 1 to September 30, 2014, and June 1 through September 30, 2015, eight (8) shellstock samples were collected on a bi-weekly basis for environmental monitoring and analyzed for total, *tdh*+, and *trh*+ *V. parahaemolyticus* levels (Figure 2). Temperature and salinity (near-bottom and near-surface) were measured at the time of collection using an YSI Model 30 or Pro30 (YSI, Inc. Yellow Springs, OH). Latitude, longitude, and water depth at the time of collection were recorded from the on-vessel GPS and depth finder. Internal shellfish temperatures were recorded at the time of collection by partially shucking and inserting a calibrated probe thermometer into the deepest part of the tissue.

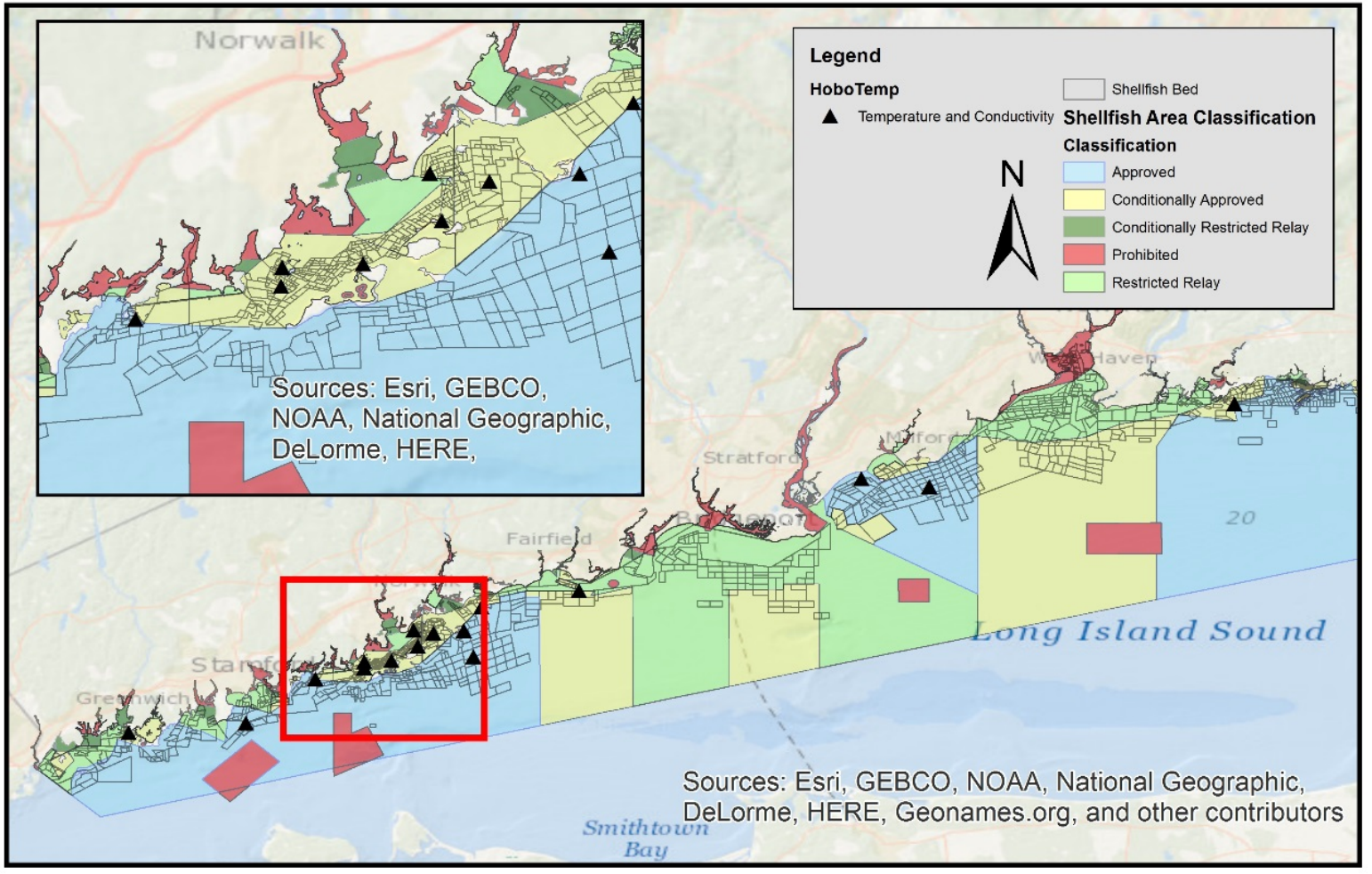

Figure 1. 2014/2015 Vibrio parahaemolyticus environmental data monitoring locations.

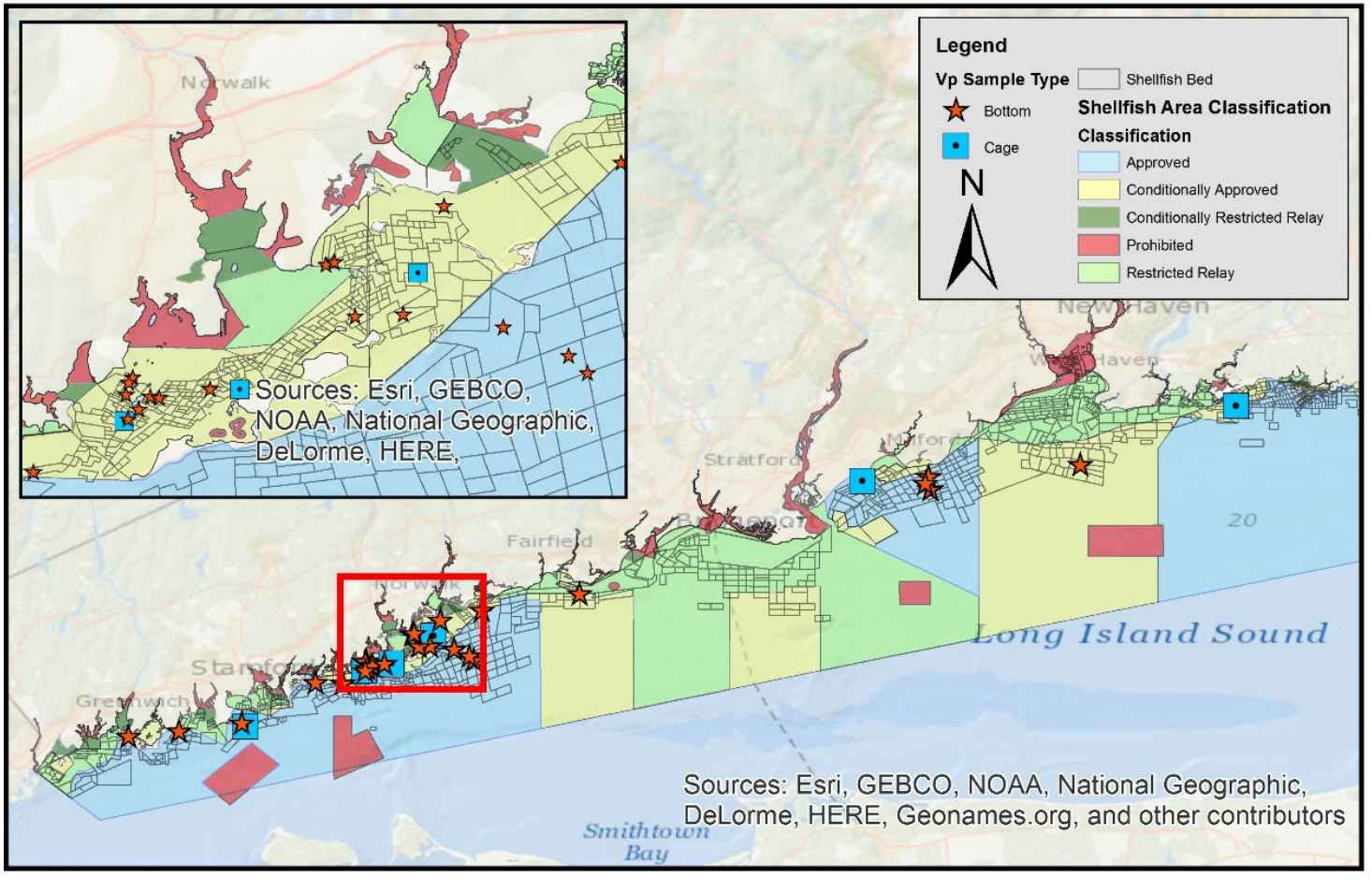

Figure 2. 2014/2015 Vibrio parahaemolyticus sample collection locations. Samples analyzed for total, tdh+, and trh+ V. parahaemolyticus levels.

Sample Analysis

Sample analysis was initiated within 24 hours of sample collection. Shellfish samples were analyzed for *Vibrio* spp. using most probable number (MPN) real-time (Rti) PCR. For each sample, the entire shell contents of 12 animals were aseptically removed and homogenized. The homogenate was used to prepare a three-tube, multiple-dilution MPN series in alkaline peptone water (APW) and incubated overnight (18-24 hours) at 35°C. A second multiplex Rti-PCR method targeting the *tdh, tlh* and *trh* genes, with an internal amplification control (IAC), was used for identification of both total and pathogenic *V. parahaemolyticus* as per Kinsey et al, 2015. All primers and nuclease style probes were purchased from Integrated DNA Technologies (IDT) (Coralville, IA) or Life Technologies. Cycling was conducted on an Applied Biosystems 7500 Real Time PCR System with an initial denaturation/polymerase activation at 95°C for 60 seconds, followed by 45 cycles of 95°C for 5 seconds and 59°C for 45 seconds with instrument optics turned to the on position. Default instrument analysis parameters were used, except that the threshold was set at 0.02 and the background end cycle set at 10.

Statistical Analysis: Post-Harvest Controls

All analyses were conducted using SigmaPlot 3.5 (Systat Software, Inc., San Jose, CA). Plots and graphs were created in Excel or Sigma Plot 3.5. Data tables were created in Excel.

One-Way Analysis of Variance

Differences between the means of the treatment groups were evaluated using One-Way Analysis of Variance (ANOVA) of the log transformed total *V. parahaemolyticus* observations. Pairwise comparisons between treatment groups were conducted following Fisher's protected LSD

procedure with an overall significance level of 0.05.

Results

A total of 51 shellfish samples were collected for the post-harvest controls study during 2015 (n = 51). *V. parahaemolyticus* was detected in 51 of the 51 samples collected during the study period. Total *V. parahaemolyticus* data was log transformed for analysis. The mean level of total *V. parahaemolyticus* in the zero (0) hour treatment (0Hr) was 1.908, in the one (1) hour treatment (1Hr) 1.980, in the three (3) hour treatment (3Hr) 2.201, in the five (5) hour treatment (5Hr) 2.581, and in the five (5) hours to 50°F(10°C) treatment (5/10) 2.918. The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant between group difference (P = 0.002).

Table 1. Descriptive statistics report for V. parahaemolyticus post-harvest controls treatments.

Group Name	N	Mean	Std Dev	SEM
0Hr	13	1.908	0.51	0.141
1Hr	7	1.98	0.585	0.221
3Hr	7	2.201	0.308	0.117
5Hr	13	2.581	0.582	0.162
5/10	11	2.918	0.924	0.279

Table 2. One Way Analysis of Variance report for V. parahaemolyticus post-harvest controls treatments.

Source of Variation	DF	SS	MS	F	P
Between Groups	4	7.898	1.975	4.947	0.002
Residual	46	18.36	0.399		
Total	50	26.258			

Given a significant difference between treatment groups overall, Fisher's protected LSD procedure was applied to evaluate all pairwise comparisons between treatment groups. All comparisons were

conducted at a significance (alpha) level of 0.05, unadjusted for the total number of pairwise comparisons and implying a type I error rate of 0.05 per comparison (Table 3). Comparison results indicate that the difference of the means between the five (5) hours to mechanical refrigeration and ten (10) hours to 50°F (10°C) treatment (5/10) and the zero hour (0Hr), one (1Hr) and three hour (3Hr) ice slurry treatments are statistically significant. Also, the difference of the means between the five (5) hour to 50°F (10°C) ice slurry treatment (5Hr) and both the zero hour (0Hr) and one hour (1Hr) are statistically significant. There was no statistically significant difference identified between any of the other pairs of treatments.

Table 3. All Pairwise Multiple Comparison Procedures means of post-harvest control treatments.

All Pairwise Multiple Comparison Procedures (Fisher LSD Method):				
Comparisons for factor: Process Study Code				
Comparison	Diff of Means	LSD(alpha=0.050)	P	Diff >= LSD
5/10 vs. 0Hr	1.01	0.521	<0.001	Yes
5/10 vs. 1Hr	0.938	0.615	0.004	Yes
5/10 vs. 3Hr	0.717	0.615	0.023	Yes
5/10 vs. 5Hr	0.337	0.521	0.199	No
5Hr vs. 0Hr	0.673	0.499	0.009	Yes
5Hr vs. 1Hr	0.601	0.596	0.048	Yes
5Hr vs. 3Hr	0.38	0.596	0.206	No
3Hr vs. 0Hr	0.294	0.596	0.327	No
3Hr vs. 1Hr	0.222	0.68	0.515	Do Not Test
1Hr vs. 0Hr	0.072	0.596	0.809	Do Not Test

Shown are box plots of total *V. parahaemolyticus* log MPN/g concentration in oysters following each of five post-harvest treatments (Figure 3). Results identified as 0Hr were placed into ice slurry immediately upon harvest. Results identified as 1Hr, 3Hr and 5Hr were cooled using ice slurry and indicate the time interval from harvest to an internal temperature of 10°C (50°F). The results

identified as 5/10 is the National Shellfish Sanitation Program *Vibrio parahaemolyticus* Control Plan (VPCP) treatment of 5 hours from harvest to refrigeration and 10 hours to an internal temperature of 10°C (50°F). The band inside each box indicates the median value. Lower and upper lines of the box represent the 25^{th} and 75^{th} percentiles, respectively. Lower and upper limits of the whiskers represent the 10th and 90th percentiles, respectively.

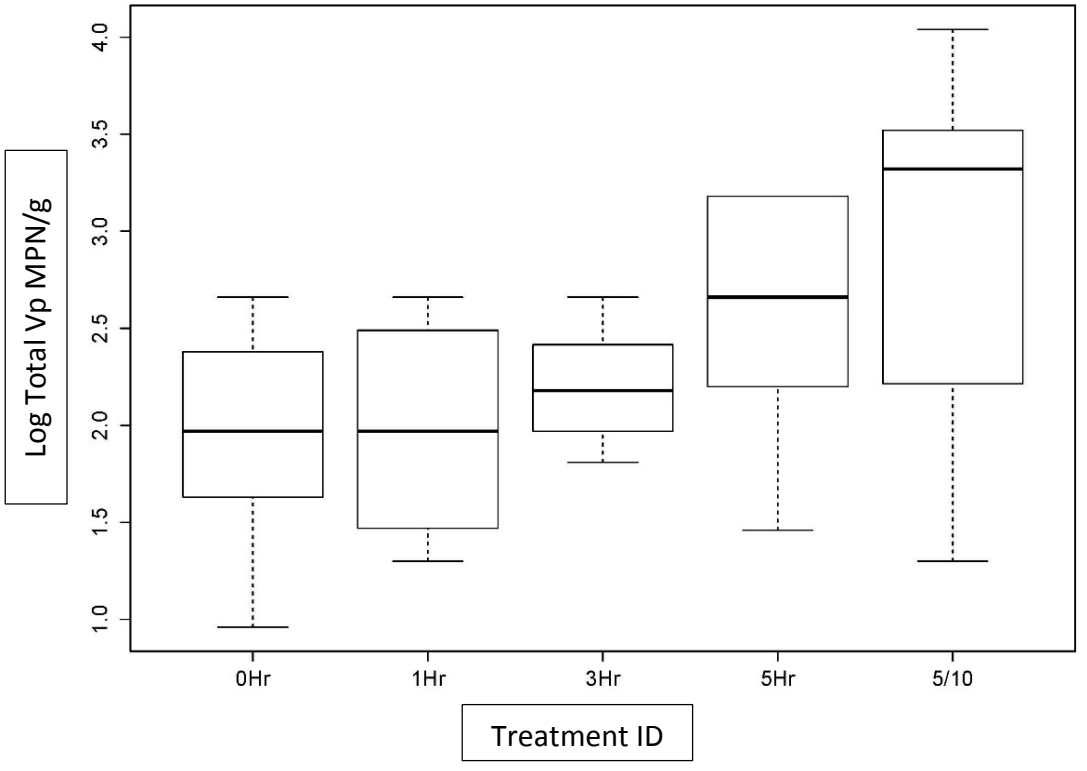

Figure 3. V. parahaemolyticus levels in shellfish harvested from Long Island Sound. Shown are box plots of total V. parahaemolyticus log MPN/g concentration in oysters following each of five post-harvest treatments. Results identified as 0Hr were placed into ice slurry immediately upon harvest. Results identified as 1Hr, 3Hr and 5Hr were cooled using ice slurry and indicate the time interval from harvest to an internal temperature of 10°C. The results identified as 5/10 is the National Shellfish Sanitation Program Vibrio parahaemolyticus Control Plan (VPCP) treatment of 5 hours from harvest to refrigeration and 10 hours to an internal temperature of 10°C. 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.

Environmental Monitoring for *V. parahaemolyticus*

Environmental monitoring of shellfish for total, *tdh+*, and *trh+ V. parahaemolyticus* was conducted July 1 through September 30, 2014 and June 1 through September 30, 2015. Results are presented as log Vp in MPN/g and plotted against the bottom water temperature in °C at the time of collection (Figure 4). A variety of descriptive statistics, exploratory data analyses, and linear regression were performed on the data, with the most significant predictive variable for total *V. parahaemolyticus* being bottom seawater temperature at the time of collection. In general, findings suggest that environmental total *V. parahaemolyticus* is identified at low levels (<2.0 MPN/g) early in the Vibrio season when near-bottom and near-surface water temperatures are less than 20°C, and levels climb steadily through the summer as water temperatures increase. Total *V. parahaemolyticus* in the environment peaks when water temperatures are at their highest; during 2014 and 2015 near-bottom seawater temperatures reached 24°C to 25°C by the end of August and into early September. During 2014, levels remained relatively elevated even as water temperatures dropped off through September. In 2015, total *V. parahaemolyticus* dropped off rapidly as water temperatures dropped through September.

Association of *Vibrio parahaemolyticus* with Environmental Parameters

A total of 101 shellfish samples were collected during 2014 and 2015 (n = 101). *V. parahaemolyticus* was detected in 100 of the 101 samples collected during the study period. Median *V. parahaemolyticus* levels were 1.380 log MPN/g and ranged from the limit of detection (LOD = -0.523 log MPN/g) to 4.362. *V. parahaemolyticus* tdh^+ was identified in 19 of 101 samples analyzed with median *tdh+* levels of -0.444 log MPN/g, ranging from the LOD to 0.362 log MPN/g. *V. parahaemolyticus trh+* was identified in 18 of 101 samples with median *trh+* levels

of -0.444 log MPN/g, ranging from the LOD to 0.362 log MPN/g.

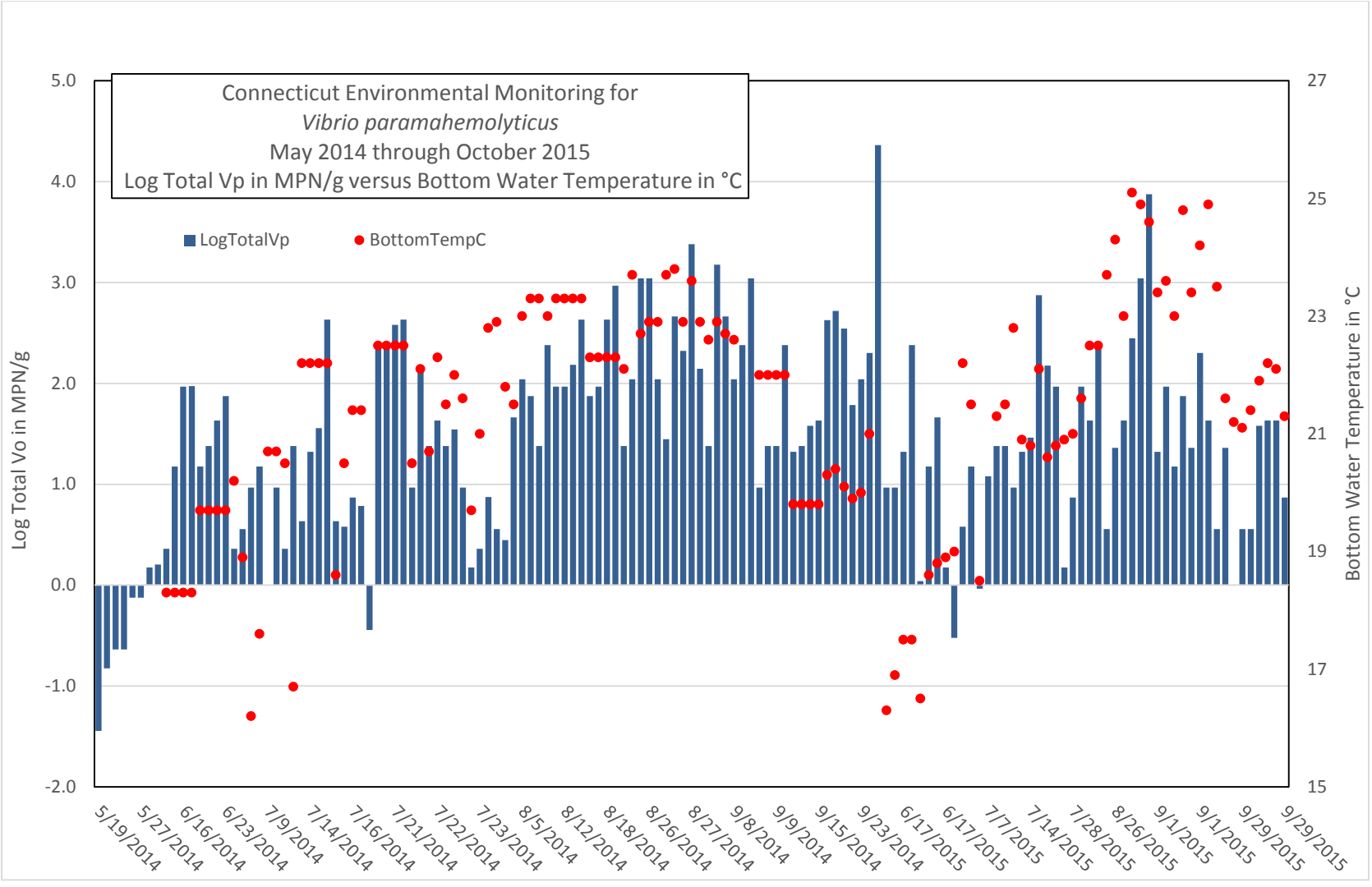

Figure 4. Log total Vp in MPN/g plotted versus near-bottom water temperature in °C recorded at the time of collection. Background environmental monitoring for Vp conducted between May 2014 and September 2015.

The majority of monitoring samples collected during the study period were oysters of the species *Crassostrea virginica* (n = 98 of 101 total samples), which is the species of concern in terms of *V. parahaemolyticus* illnesses associated with Connecticut waters. Three hard clam samples of the species *Mercenaria mercenaria* were analyzed during the study period (n = 3 of 101 total samples).

Near-surface salinity ranged from 22.2 to 27.8 parts per thousand (ppt) with a median salinity of 25.0 ppt during the study period. Near-bottom salinity ranged from 22.2 to 27.9 with a median salinity of 25.1 ppt.

The near-bottom seawater temperatures recorded at the time of sample collection (n = 97) ranged from 16.2 to 25.1°C, with a median temperature of 21.6°C. Near-surface seawater temperatures ranged from 17.2 to 25.6°C with a median temperature of 22.6°C. Minimum, maximum and average of near-bottom temperatures collected during each sample run are plotted in Figure 5.

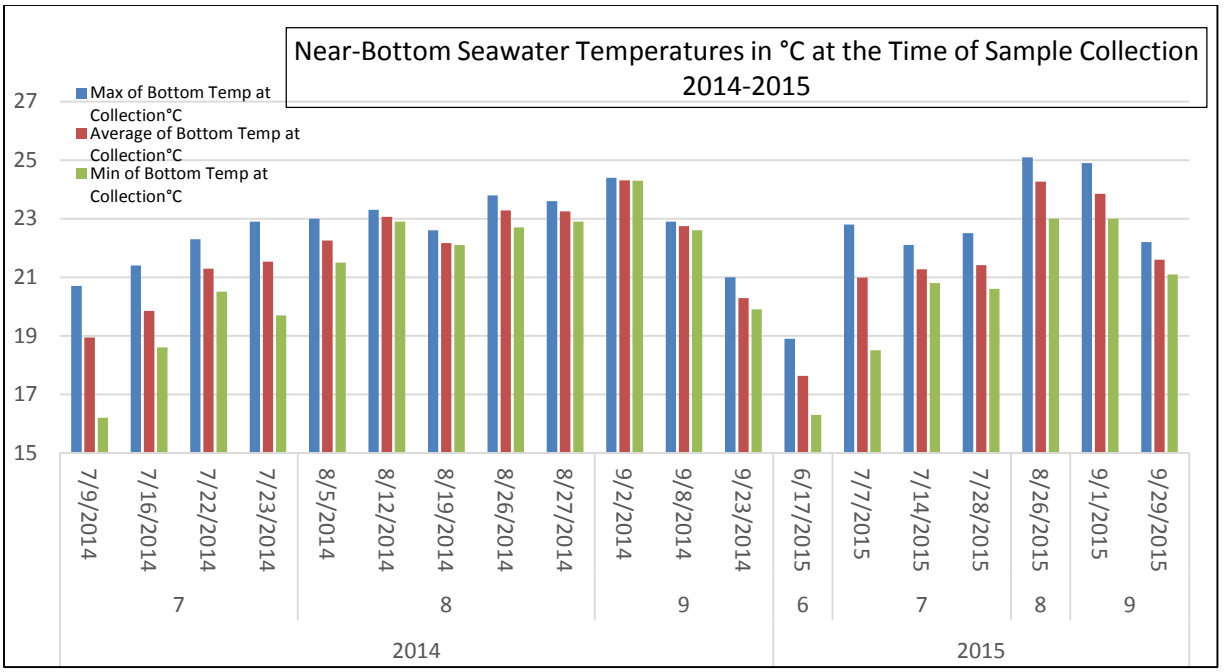

Figure 5. Near-bottom seawater temperatures plotted by collection date. Maximum, average, and minimum temperature in °C for each collection date during the 2014-2015 study period.

Near-bottom water temperatures and internal shellfish temperature in °C at the time of harvest are plotted in Figure 6. Temperatures were averaged for each month during 2014 and 2015. Internal tissue temperatures were significantly higher than near-bottom water temperatures based on a Mann-Whitney Rank Sum Test (P=0.003), and the use of bottom temperature appears to be the better predictor of *V. parahaemolyticus* of the two parameters.

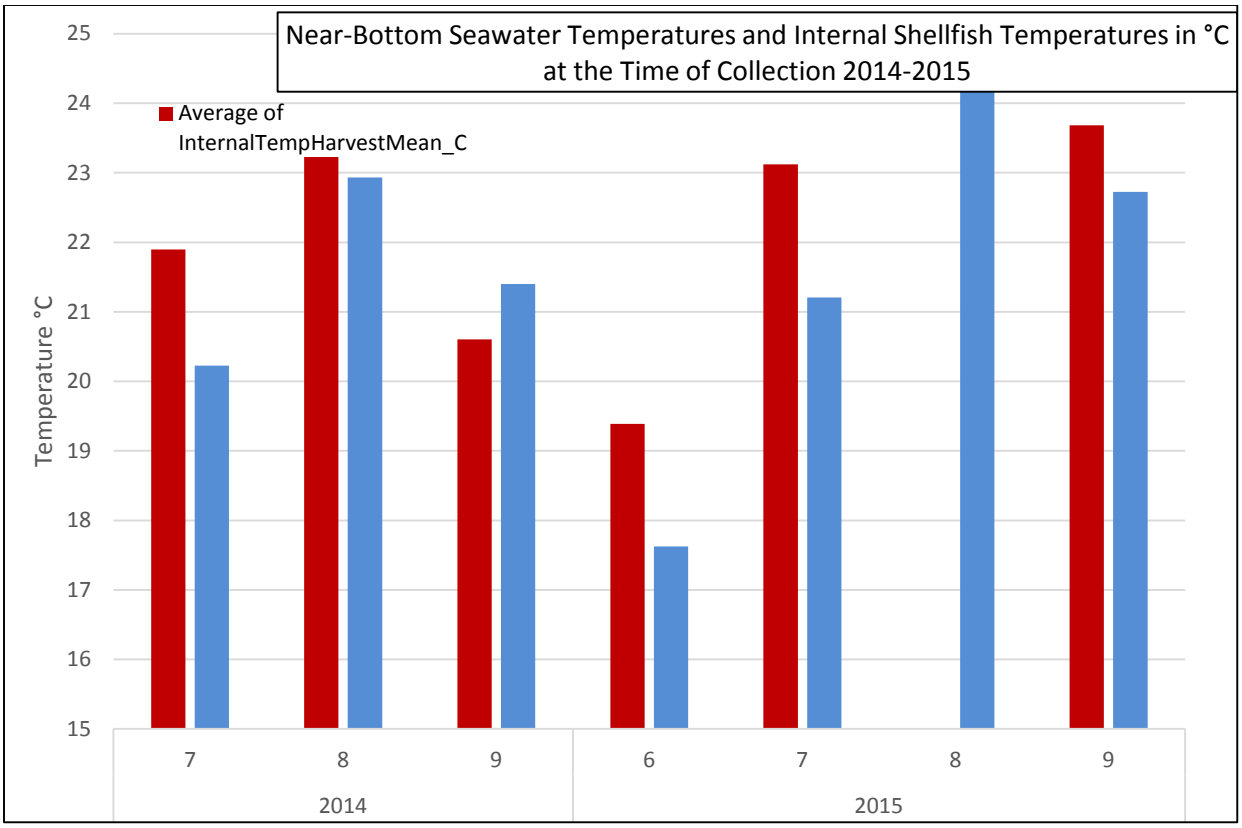

Figure 6. Average monthly near-bottom temperatures and internal shellfish temperatures in °C at the time of collection.

Forward stepwise regression for total *V. parahaemolyticus* including environmental parameters of water depth at the time of collection, surface salinity, bottom salinity, surface temperature, near-bottom temperature, and internal temperature was performed.

Backward stepwise regression for total *V. parahaemolyticus* including environmental parameters of water depth at the time of collection, surface salinity, bottom salinity, surface temperature, near-bottom temperature, and internal temperature was performed.

The most significant predictor of total *V. parahaemolyticus* in any of the models explored for this dataset was near-bottom temperature, and a simple linear regression model was chosen for predicting

total *V. parahaemolyticus,* which included only bottom temperature. A significant positive correlation was identified between total *V. parahaemolyticus* and bottom temperature (R = 0.432, P = <0.001).

Near-bottom temperature accounts for 18.7% of the variation in total *V. parahaemolyticus* when the simple linear regression model is applied. The linear regression results [including raw data, confidence interval of the regression, and confidence interval of the population] are plotted in Figure 7 and presented in Table 4. These initial regression model findings should be considered preliminary, as additional parameters and models will be continue to be explored in future modeling efforts.

Linear regression models were also tested for the *V. parahaemolyticus tdh*+ and *trh*+ data, however none of the variables tested appear able to predict the levels of *tdh*+ or *trh*+. The majority of *tdh* and *trh* results were below the level of detection in this dataset (*tdh*+ N = 83; *trh*+ N = 85 less than the LOD).

Additional environmental parameters will be added to the environmental monitoring program during 2016, in order to build a more robust predictive model for Connecticut *V. parahaemolyticus* data. In addition to the parameters collected in this study, variables to be explored in 2016 will include turbidity, chlorophyll a, and dissolved oxygen.

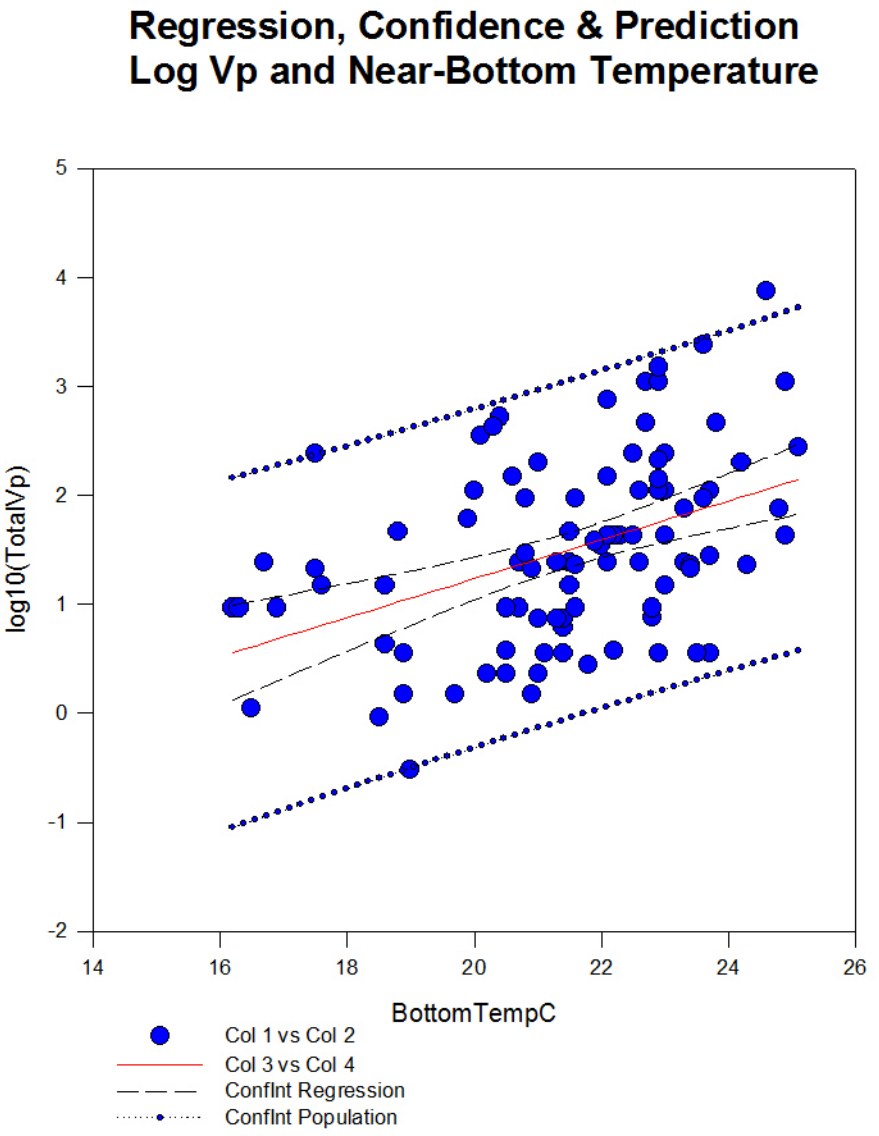

Figure 7. Linear regression, confidence interval of regression, and confidence interval of population.

Table 4. Linear Regression Results Table Log Total V. parahaemolyticus vs. Near-Bottom Temperature at Time of Collection.

log10(TotalVp) = -2.334 + (0.179 * BottomTempC)					
N = 94	Missing Observations = 7				
R = 0.432	Rsqr = 0.187	Adj Rsqr = 0.178			
Standard Error of Estimate = 0.776					
	Coefficient	Std. Error	t	P	
Constant	-2.334	0.837	-2.788	0.006	
BottomTempC	**0.179**	**0.0388**	**4.6**	**<0.001**	
Analysis of Variance:					
	DF	SS	MS	F	P
Regression	1	12.735	12.735	21.159	<0.001
Residual	92	55.37	0.602		
Total	93	68.105	0.732		
Normality Test:	Passed	(P = 0.585)			
Constant Variance Test:	Passed	(P = 0.445)			
Power of performed test with alpha = 0.050: 0.993					

Connecticut's 2015 *Vibrio parahaemolyticus* Risk Assessment

Based on Connecticut's 2015 *V. parahaemolyticus* Risk Assessment, environmental conditions present at the time of illnesses were assessed for correlation to risk of illness. Water temperature was identified as the most important parameter for Connecticut's shellfish growing area in terms of triggering the need for *V. parahaemolyticus* Controls. In-situ water temperature data for the growing areas associated with the 2013 illness outbreak were not available due to a lack of sensors recording continuous data at that time. To overcome that challenge, water temperatures were hind-cast for the 2013 outbreak, using the Long Island Sound Vp Prediction System (Whitney, Ward, & DeRosia-Banick, 2016). Examples of how the hind cast data were developed may be seen in the figures below, but to summarize, satellite sea-surface temperatures were acquired (Figure 8) and incorporated into an existing Long Island Sound hydrodynamic model (Figure 9) to predict and estimate the bottom temperatures in the growing area. Several different model-predicted and in-situ water temperature parameters were associated with each illness in the database (n = 82 including multi-state cases) going back to 2010 to 2014 and confirmed Connecticut cases (n = 34) were plotted in order to assess the water temperature associated with the highest risk of illness over the period between 2010 and 2014 (Figure 10).

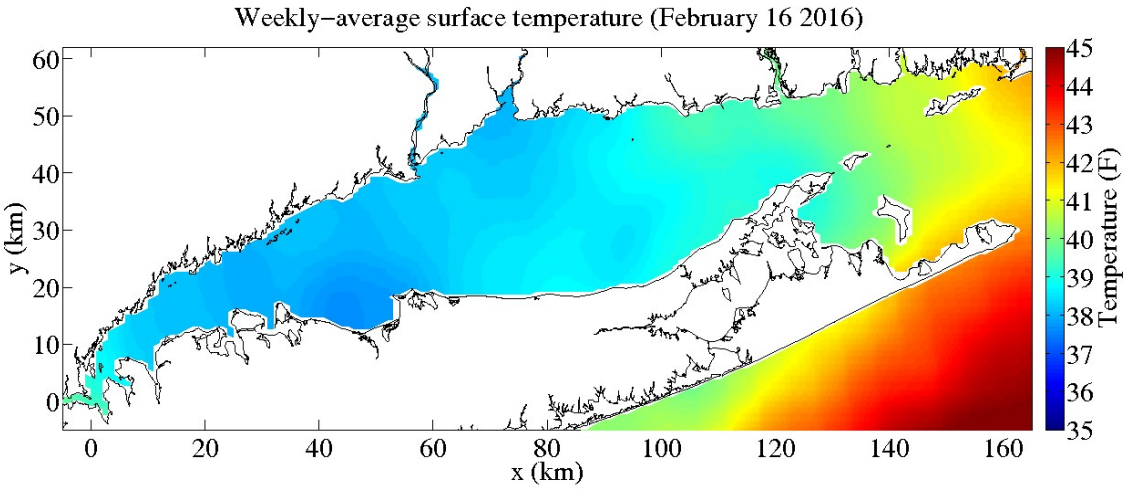

Figure 8. Daily sea-surface temperature (SST) data are acquired from the G1SST product (from the NASA Jet Propulsion Laboratory) that includes observations from satellites. The prior week (7 days) of SST are averaged together to construct the weekly-averaged surface temperature field throughout LIS.

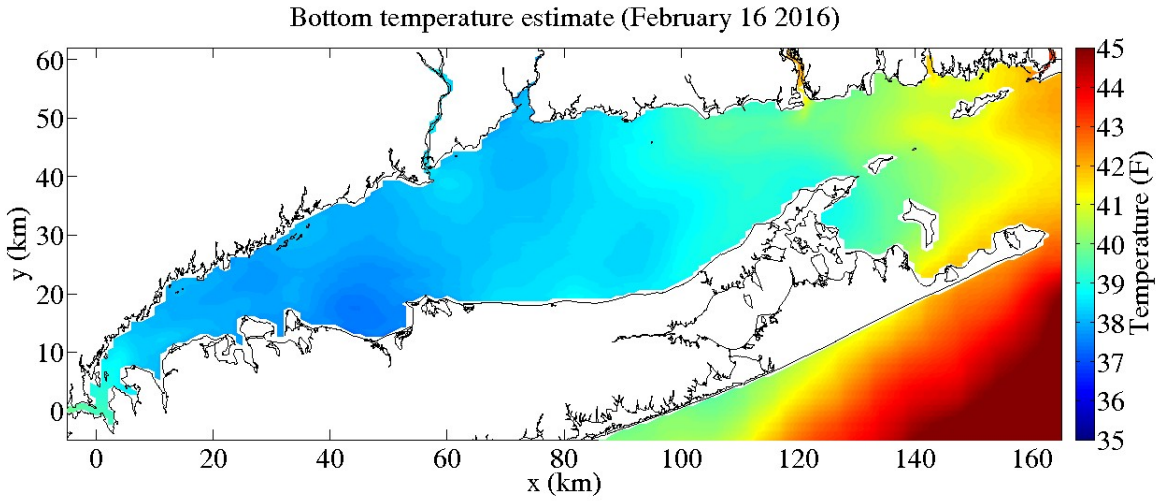

Figure 9. Previous results from a hydrodynamic model of LIS and adjacent coastal waters (run by Mike Whitney's research group) were analyzed to determine the top-to-bottom temperature differences (ΔT) at locations throughout LIS. Four years of model results (2009-2012) were averaged together to determine the average annual cycle of ΔT at each location. Temperature differences are smallest during the winter and largest during the summer; the differences typically are larger for deeper areas. The ΔT estimate from the model-based average annual cycle then is subtracted from the weekly-averaged surface temperatures to produce an estimate of the bottom temperature field.

All confirmed illnesses associated with a Connecticut growing area (n =34 of 82) have occurred when surface seawater temperatures exceeded 19.9°C (67.8°F). Illnesses coded 1 were traced back to a single Connecticut growing area. Illnesses coded 2 were traced back to one of several Connecticut growing areas. Sea surface temperatures for traceback code 1 or 2 as measured by the NASA G1SST temperature estimate at each harvest area on each harvest date ranged from 20.5 to 26.4°C. Sea surface temperatures as measured at the nearest NOAA coastal buoy (BRHC3-Bridgeport, CT) ranged from 19.9 to 26.5°C.

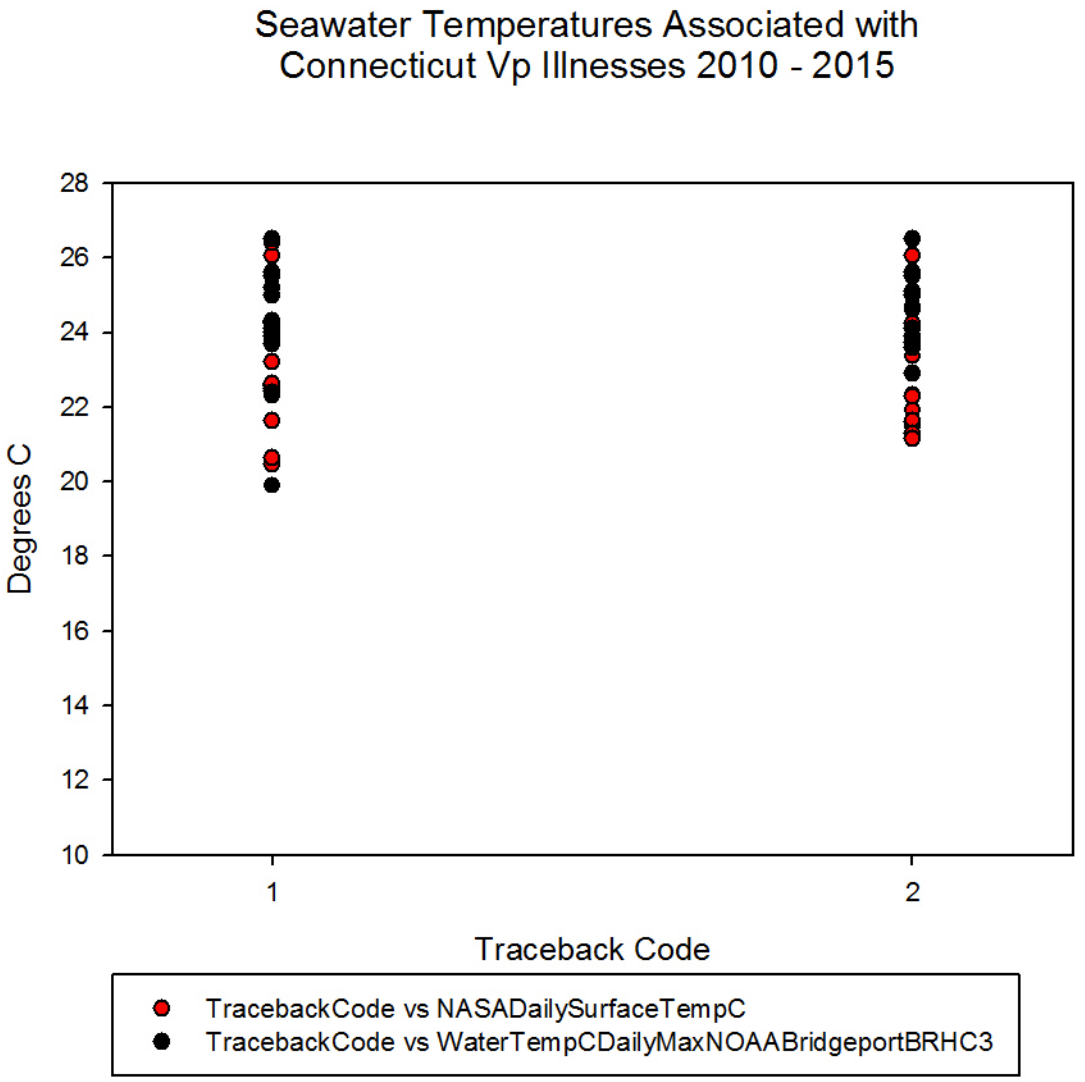

Figure 10. NASA G1SST Daily Sea Surface Temperature in C and Maximum NOAA BRHC3 daily Seawater Surface Temperature in C associated with Vp illnesses 2010 to 2015, plotted by Traceback Code. Code 1 cases are single CT source harvest location/date, code 2 cases were confirmed CT source, multiple potential CT harvest location/date.

Table 5. Confirmed V. parahaemolyticus cases linked to Connecticut shellfish, 2010 through 2015.

Year	Confirmed Cases Linked to CT Shellfish	Multi-State Shellfish Cases Including CT Source
2010	1	2
2011	1	2
2012	1	3
2013	23 (23 outbreak area)	11
2014	1 (1 outbreak area)	2
2015	2 (1 outbreak area)	8

DISCUSSION

The primary objective of the Connecticut Techniques and Practices for Vibrio Reduction study was to evaluate the effectiveness of post-harvest controls that could potentially reduce the risk of Vibrio illnesses. The use of ice slurry for rapidly cooling the internal temperatures of oysters to 50°F (10°C) was compared to the NSSP standard VPCP controls requiring placement under temperature control [in this case mechanical refrigeration at or below 45°F (7.2°F)] within five (5) hours of harvest and cooling to an internal temperature of 50°F (10°C) within ten (10) hours of being placed under temperature control. The effectiveness of several post-harvest time and temperature strategies were evaluated using continuous temperature data loggers (ACR Smart Button) to record the length of time each sample took to reach 50°F (10°C) and enumerating the total *V. parahaemolyticus*, *tdh*+ and *trh*+ associated with each treatment sample.

In order apply the findings of this study to the process of choosing appropriate *V. parahaemolyticus* controls, a SSCA might consider the rate at which different cooling methods can bring the internal temperature of oysters down to a target temperature where the risk of post-harvest *V.*

parahaemolyticus growth is effectively minimized, or to an internal temperature of 50°F (10°C) or less as per NSSP guidance for *V. parahaemolyticus* growth. Based on the findings of this study, the greatest benefit in terms of limiting post-harvest *V. parahaemolyticus* growth can be achieved by meeting this target temperature within three (3) hours of harvest. By five (5) hours post-harvest, the mean *V. parahaemolyticus* level increased from 1.908 log MPN/g to 2.581 log MPN/g.

The use of traditional controls based only on time from harvest to mechanical refrigeration should be applied with caution in growing areas that have been associated with *V. parahaemolyticus* outbreaks, as the time to an internal temperature of 50°F (10°C) can vary greatly and is much less consistent in terms of controlling the rate of cooling when compared to the use of ice slurry results. An exposure time of five (5) hours to ambient air temperatures followed by placement under mechanical refrigeration at 45°F (7.2°C) or less during the *V. parahaemolyticus* season allows for significantly more growth than rapid cooling controls utilizing ice slurry which drop the internal temperatures of the oysters to 50°F (10°C) within three (3) hours of harvest or tidal exposure. While this study focused on the effectiveness of the traditional controls on limiting *V. parahaemolyticus* growth, our agency has several seasons of data evaluating the effectiveness of a wide range of mechanical refrigeration units in reducing the internal temperature of oysters to 50°F (10°C). We have observed that the times to 50°F (10°C) can range from less than one (1) hour to greater than 24 hours and the rate of cool down depends on a number of factors, including size of the cooler, condenser maintenance, stocking density and placement, air circulation, temperature of incoming product, etc. Refrigeration units may be intended for holding cold product, rather than for cooling down warm product, and any cool down process utilizing mechanical refrigeration must be carefully designed, controlled and validated.

A rapid cooling process utilizing ice slurry is much faster and more consistent than a cooling process using only mechanical refrigeration, and has been observed to cool product to an internal temperature of 50°F (10°C) within 30 minutes; in many instances cool down was achieved in a little as 15 minutes or fewer.

In addition to evaluating the effectiveness of these control strategies on limiting post-harvest *V. parahaemolyticus* growth, Connecticut also has had two seasons of *V. parahaemolyticus* illness data to evaluate in order to assess the practical effectiveness of the application of rapid cooling in reducing the number of illnesses associated with Connecticut shellfish growing areas.

As previously mentioned, Connecticut growing waters were the source of 23 confirmed cases of *V. parahaemolyticus* during the summer of 2013, with an additional 15 multi-source cases potentially linked to Connecticut waters (Table 5). The VPCP that was in place at the time of the 2013 outbreak included the National Shellfish Sanitation Program standard time to temperature control measure of limiting time from harvest to refrigeration to no more than five (5) hours, and required the original dealer to cool oysters to an internal temperature of 50°F (10°C) or below within ten (10) hours after placement into refrigeration. Unfortunately the standard *V. parahaemolyticus* controls that were in place at the time of the 2013 outbreak were inadequate to prevent illnesses from occurring, and on-board rapid-cooling was selected by the Authority for the 2014 and 2015 *V. parahaemolyticus* seasons in hopes of reducing the risk of illness associated with oysters harvested from the outbreak area.

The number of illnesses associated with shellfish growing areas within the municipalities of Westport, Norwalk and Darien was reduced from 23 during 2013 to one (1) case in 2014 and one (1) in 2015, achieving an illness reduction of 95.6% in each of those years as compared to the 2013

season. Clinical isolates associated with confirmed cases linked to Connecticut growing areas in 2014 and 2015 provide evidence that the O4:K12 virulent strain is still present in Connecticut growing areas. Based on the results of the post-harvest controls study and on recent illness data, we believe that the use of the standard five (5) hour harvest to temperature controls and mechanical refrigeration for cool down were contributing factors to the 2013 outbreak and provide convincing evidence that the use of ice slurry for rapid cooling has prevented similar outbreak events in 2014 and 2015.

Complementary to the post-harvest controls study, this ISSC project also provided a mechanism for the SSCA to gain a better understanding of *V. parahaemolyticus* levels in the environment and their relevance to implementing meaningful Vibrio controls in Connecticut growing waters. These data have been incorporated into a Long Island Sound *V. parahaemolyticus* Prediction System (Whitney, Ward, & DeRosia-Banick, 2016). Vibrio monitoring and continuous environmental observations have been used to inform the understanding of the temporal variability and spatial distribution of *V. parahaemolyticus* in Long Island Sound (LIS) oyster production areas. This data has also been used to look at environmental conditions leading up to illness events and has allowed the state to proactively manage *V. parahaemolyticus* during 2015 by requiring more stringent controls under those specific environmental conditions that have historically been correlated to a higher risk of illness, rather than relying on a trigger based on a specific date alone.

Water temperature was identified as the most important parameter for Connecticut's shellfish growing area in terms of triggering the need for *V. parahaemolyticus* controls. All illnesses confirmed to be associated with Connecticut shellfish harvest areas have occurred when surface water temperatures are greater than 68°F (20°C). Several different model-predicted and in-situ water

temperature parameters were associated with each illness in the database (n = 82 including multi-state cases) going back to 2010 to 2015 and confirmed Connecticut cases (n = 34) were plotted in order to assess the water temperature associated with the highest risk of illness over the period between 2010 and 2015.

In 2015, Connecticut's *V. parahaemolyticus* Control Plan (VPCP) for the 2013 outbreak area was triggered when surface seawater temperatures reached 68°F (20°C) as measured using the NASA G1SST product [incorporated into the Long Island Sound hydrodynamic model] and the NOAA BRHC3 coastal buoy located in Bridgeport, CT. The use of a trigger based on environmental conditions rather than a pre-determined start date proved to be effective during 2015, as no illnesses were confirmed prior to the June 19, 2015 start date of the rapid cooling VPCP.

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