NJ Department of Environmental Protection
Water Monitoring and Standards

Techniques and Practices for *Vibrio* Reduction – Use of Shading and Rapid Cooling (ice slurry) to Control *Vibrio* Growth

November 2015

State of New Jersey
Chris Christie, Governor
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NJ Department of Environmental Protection
Bob Martin, Commissioner

Techniques and Practices for *Vibrio* Reduction – Use of Shading and Rapid Cooling (ice slurry) to Control *Vibrio* Growth

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Cover Photo – Delaware Bay Oyster Boats – Jon C. Peterson and the Howard W. Sockwell, Circa 2015

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EXECUTIVE SUMMARY

The New Jersey Department of Environmental Protection's (NJDEP's), Division of Water Monitoring and Standards (DWM&S), Bureau of Marine Water Monitoring (BMWM) wishes to thank the Interstate Shellfish Sanitation Conference (ISSC) for their provision of grant funding, which enabled the process of this *Vibrio* Reduction/Shading/Rapid Cooling project to take place. This year's ISSC Grant for *Vibrio* monitoring in relation to the utilization of rapid cooling (ice slurry) was intended to provide valuable monitoring information and shellfish harvest management practicum for a given state and associated industries from the perspective of public health and safety.

It is well known and documented that certain *Vibrio* species and strains are potentially harmful estuarine living bacteria, which can be found within the tissue of raw shellfish (particularly oysters). As a genus, *Vibrio* and its variant species and strains are found throughout the world. Research and monitoring projects that provide an understanding of *Vibrio* growth limiting factors, as is the nature of this project and grant, are intended to provide industry and management practices that minimize public illness related to the consumption of raw shellfish.

Past studies by this Bureau and other monitoring or research groups suggest the faster harvested shellfish product is cooled, the greater the reduction in *Vibrio* growth. This grant incorporated the use of rapid cooling between the months of May – August, utilizing ice slurry over a variety of post-harvest time frames. Sampling of Oysters from subtidal sites in Delaware Bay started the first week of May, 2015 and continued through August 31st, 2015. An examination of *Vibrio* levels in relation to varied post-harvest handling time frames and practices was undertaken.

Evaluations were made between temperature and the prevalence of pathogenic strains of *Vibrio parahaemolyticus* (*Vp*) and *Vibrio vulnificus* (*Vv*). Various oyster handling techniques and suggested Best Management Practices (BMP's) were also examined with the intent to further understand potential oyster industry practice in relation to *Vibrio* levels resulting from harvested oysters.

DWM&S/BMWM's advanced microbiology lab analyzed oyster tissue for *Vibrio* using two procedures for the enumeration of genes, specific to total and pathogenic *Vibrio*. The first of the two methods used for identification and characterization or the Direct Plating Technique, provided final results presented in colony forming units (CFU's). With this method, a DNA gene probe was used for identification after bacterial colony growth, using an agar nutrient for plating. Bacterial colonies that grew on the plate were subsequently lifted, then placed on a filter for probe detection. Secondarily, bacterial isolates, derived from a three-tube, three dilution process, followed by a plating, screening, and a confirmation process, were verified with the use of a PCR (Polymerase Chain Reaction), DNA gene detection probe. This methodology provided a most probable number (MPN) through the use of a 3-tube-MPN table. All samples were tested for total *Vp* (*tlh*+), pathogenic *Vp* (*tdh*+ and *trh*+), and *V. vulnificus* (*vvh*).

EXECUTIVE SUMMARY CONTINUED

In brief summary, continuous temperature loggers indicated the use of ice slurry was an effective means of rapidly cooling oyster meat temperatures to 10^{o} C (50^{o}F) in 10 minutes or less.

There were no significant differences in *Vibrio* levels at initial harvest, 1 hour shading, and 3 hours shading associated with the use of ice slurry. At 5 hours shading, followed by ice slurry, a marked increase in *Vibrio* levels occurred.

Vibrio growth continued during mechanical refrigeration preceded by five hours of shading. Rapid cooling using ice slurry following five hours of shading was more effective at slowing *Vibrio* growth than going to mechanical refrigeration following shading.

The data for this project and former projects further suggests *Vibrio* levels are not always highest during months with the highest water and air temperatures. The highest levels of *trh* and *tdh* genes (virulent strains) occurred during late June through early July.

While elevated levels of *Vibrio* vulnificus (*Vv*) were found in oyster meats, New Jersey has never had a reported illness, associated with this genus and species.

Current New Jersey subtidal oyster harvest locations, in particular Bennies Sand (Station ID G) and Shell Rock (Station ID H), where sampling for this project took place are shown on the next page (Figure 1). General field and lab descriptions for data base fields are presented in Table 1.

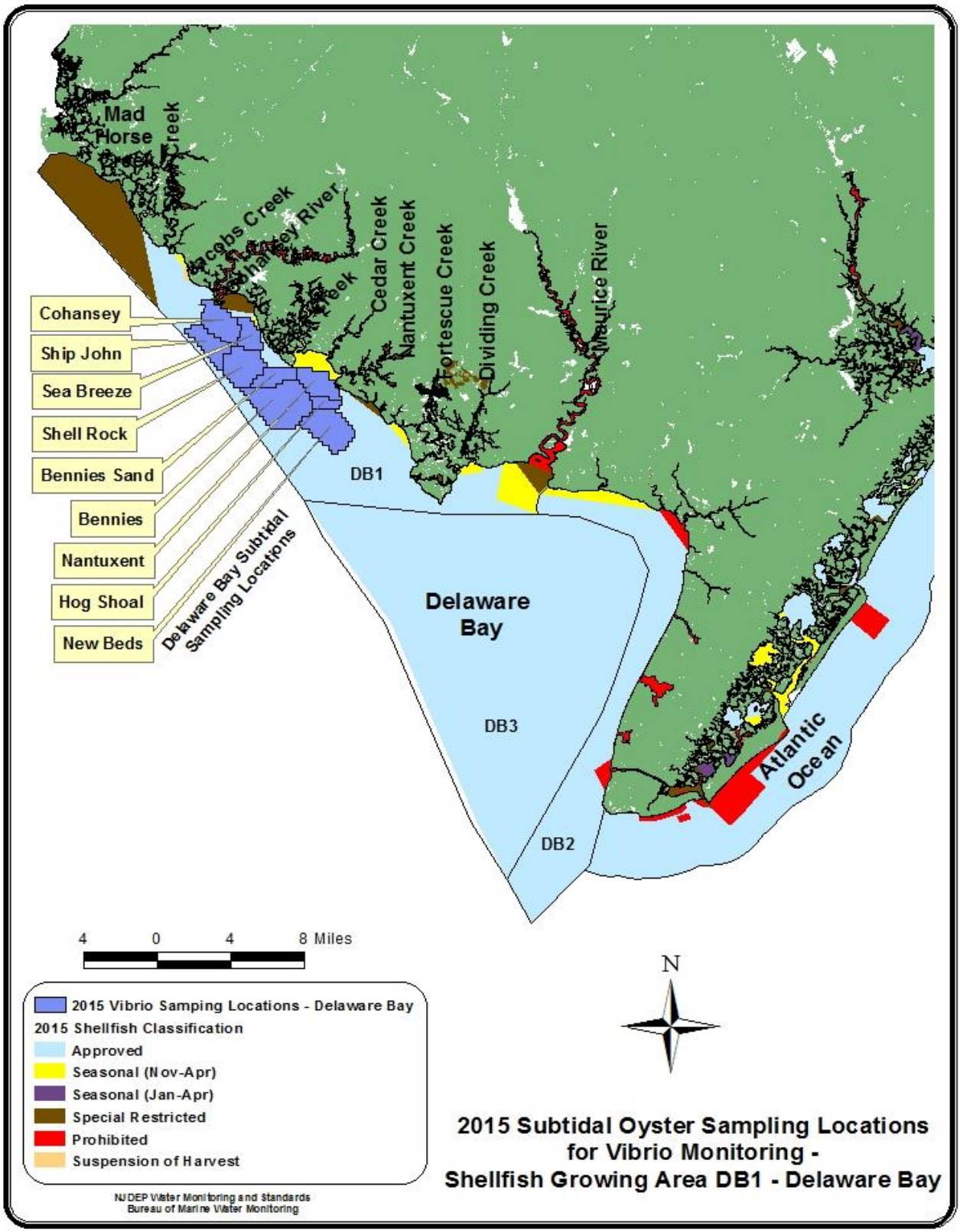

Figure 1: NJ Oyster Harvest Locations - 2015 ISSC *Vibrio* Monitoring Project and Grant

Table 1: General Field and Lab Data Base Descriptions for 2015 *Vibrio* Sampling and Analysis

Station Name	Station ID	Tidal Zone	Report Area	Location	Latitude	Longitude	Species Type	Species	Matrix	Sampling Method
Cohansey	C	Subtidal	DB1	Delaware Bay – 2.5 miles WSW of Cohansey Cove	Lat. N 39˚19'20.42"	Long. W -75˚21'38.28"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
Ship John	D	Subtidal	DB1	Delaware Bay – 3 ¼ miles SW of Cohansey Cove	Lat. N 39˚18'28.71"	Long. W -75˚22'11.39"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
New Beds	E	Subtidal	DB1	Delaware Bay – 3 miles SE of Nantuxent Cove	Lat. N 39˚14'49.0"	Long. W -75˚15'9.0"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
Sea Breeze	F	Subtidal	DB1	Delaware Bay – 2 ¾ miles SE of Cohansey Cove	Lat. N 39˚18'50.10"	Long. W -75˚19'54.16"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
Bennies Sand	G	Subtidal	DB1	Delaware Bay – 3 miles WSW of Nantuxent Cove	Lat. N 39˚16'47.0"	Long. W -75˚19'9.0"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
Shell Rock	H	Subtidal	DB1	Delaware Bay – 3.5 miles SE of Cohansey Cove	Lat. N 39˚17'27.44"	Long. W -75˚20'11.92"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
Bennies	I	Subtidal	DB1	Delaware Bay – 3 miles SW of Nantuxent Cove	Lat. N 39˚15'41.63"	Long. W -75˚17'48.26"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
Hog Shoal	J	Subtidal	DB1	Delaware Bay – 3 miles SE of Nantuxent Cove	Lat. N 39˚15'44.57"	Long. W -75˚15'17.15"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling
Nantuxent	K	Subtidal	DB1	Delaware Bay – mouth of Nantuxent Cove	Lat. N 39˚16'33.89"	Long. W -75˚15'42.65"	Eastern Oyster	Crassostrea virginica	Tissue	Dredge then hand culling

ABSTRACT

V. parahaemolyticus and *V. vulnificus* were isolated weekly for 95 oyster samples, with six different handling methods. Samples were collected from two of nine commercial harvest locations in Delaware Bay, New Jersey from May - August 2015. *V. parahaemolyticus* isolates were tested for the total *tlh* gene, pathogenic *tdh* and *trh* genes, and *V. vulnificus* was tested for the *vvh* gene using a multiplex real-time PCR assay.

Average out of water shell temperature was 19.2^{o} C; 22.98^{o} C; 25.1^{o} C and 25.6^{o} C for May, June, July, and August respectively. Although the highest average monthly out of water temperature was in August, the highest incidence of *tlh*, *tdh*, *trh* and *vvh* was recorded in June. Early July showed a similar gene pattern although *tdh* levels were low. The effects of ice slurry on the rapid cooling of oysters for four handling methods showed that it took maximum of six minutes to cool the oyster shell/meat temperature to below 50^{o} F (10^{o} C), whereas it took over 2.5 hours to achieve the same temperature for five hour shaded oysters followed by refrigeration.

Serotyping for K and O antigens was carried out on 225 strains of *V. parahaemolyticus*; 53.78% (121 isolates) of the 225 environmental isolates of *V. parahaemolyticus* were typed. 46.22% (104) were untypeable for O or K antigen. The dominant serotype was O5, with KUT at 8.00% followed by O2, with K28 at 7.56%.

INTRODUCTION

Vibrio parahaemolyticus and V. vulnificus are natural inhabitants of marine and estuarine environments worldwide (Baker-Austin *et al*., 2010). *V. parahaemolyticus* is the leading cause of gastroenteritis from seafood in the United States, with most infections resulting from the consumption of raw or mishandled seafood (Altekruse *et al*., 2000; Mead et al., 1999). Strains carrying the *tdh* gene, encoding the thermostable direct hemolysin (TDH), or the *trh* gene, encoding the TDH-related hemolysin (TRH), or both genes are considered virulent strains (Nishibuchi and Kaper, 1995).

V. vulnificus produces a rapidly fatal septicemia associated with the consumption of raw oysters (Blake *et al*., 1979; Linkos and Oliver, 1999). Wound contact with seawater or shellfish can also lead to infections that can progress to septicemia. The most frequent target of the species-specific *V. vulnificus* gene is the hemolysin/cytolysin gene, *vvhA* (Yamamoto *et al*., 1990).

Water temperature influences the presence of *V. parahaemolyticus*. Higher densities of *V. parahaemolyticus* in U.S. oysters have been observed in samples collected in spring and summer and are significantly correlated with higher water temperature (DePaola *et al.,* 2003). Specifically, *V. parahaemolyticus* densities decrease in oysters in harvest water less than 14 to 15° C (Johnson *et al*., 2010; Parven *et al*., 2008). Densities of *V. parahaemolyticus* are also influenced by temperature during postharvest transport and processing, resulting in multiplication of the bacterium to potentially hazardous levels if oysters are not promptly

refrigerated (Gooch *et al*., 2002). A recent market survey of oyster microbiological quality performed in the United States found that 15% of tested lots exceeded the 10,000 most probable number (MPN)/g *V. parahaemolyticus* criterion established by the U.S. Food and Drug Administration (FDA) (FDA, 2007; DePaola *et al*., 2010). This emphasizes the need to improve risk management practices for this food-borne bacterium.

MATERIALS AND METHODS

OYSTER SAMPLES

Oysters (*Crassostrea virginica*) used in this study were harvested on a weekly basis from May through August 2015 by two assigned DWM&S/BMWM boat captains at two Delaware subtidal commercial harvest locations [Bennies Sand (Station ID G) and Shell Rock (Station ID H)]. Each visit required the collection of 72 to 90 oysters depending on size, which were used for six different harvest/post-harvest handling method comparisons. Twelve to 15 oysters were used for each handling method. The handling method identifiers and methods of handling are presented below (Table 2).

Table 2: Handling Methods – Identifiers and Descriptions

Handling Method ID	Handling Method Description
A	Collect 15 -20 oysters, immediately place on ice, transport, shuck, and analyze. Do during weeks of (05/18/15, 06/15/15, 06/22/15, 07/13/15, and 08/17/15). Lab processes on arrival during those weeks!
N	Baseline/Zero Hour: Immediate rapid cooling of 15 – 20 harvested oysters for 10 minutes to meat temperature of 10°C or 50° F or less using ice slurry! Take shell & meat temps after slurry. Place on ice after slurry. Lab processes on arrival.
O	1 hour from harvest of 15 – 20 oysters to meat temperature of 10°C or 50° F or less using ice slurry (50 min on deck in shade then into slurry for 10 minutes rapid cooling)! Take shell and meat temps. after slurry. Place on ice after slurry. Lab processes on arrival.
P	3 hours from harvest of 15 – 20 oysters to meat temperature of 10°C or 50° F or less using ice slurry (2 hours 50 min on deck in shade then into slurry for 10 minutes rapid cooling)! Field crew/lab take shell and meat temps after slurry on P, as slurry might be done at lab! Collect and keep shaded for 2 hours and 50 minutes only; then place in slurry, and if needed, follow w/ ice. Lab processes on arrival or after slurry.
Q	5 hours from harvest of 15 – 20 oysters to meat temperature of 10°C or 50° F or less using ice slurry (4 hours 50 min on deck in shade then into slurry for 10 minutes rapid cooling)! Field crew/lab take shell and meat temps after slurry on Q, as slurry likely done at lab. Collect and keep shaded for 4 hours and 50 minutes only, then place in slurry, and if needed, follow w/ ice. Lab processes on arrival or after slurry process at lab.
R	NSSP standard VPCP: Shade 15 – 20 oysters for 5 hours from harvest then into traditional mechanical temperature control for 10 hours to an internal temperature of 10° C or 50° F. Lab takes shaded product, places in refrigerator for 10 hours of cooling and then lab takes pre-process shell and meat temperature and processes next day!

Out of water shell and meat temperature, post slurry shell and meat temperature, and pre-process shell and meat temperature were measured using a VWR Wide Range Infrared Thermometer. Air and water temperature, Dissolved Oxygen (DO), pH, and salinity were measured using YSI Professional Plus, YSI Incorporated, Yellow Springs, Ohio, USA. Continuous temperature recordings of shell and meat temperature as well as pre-process shell and meat temperature were measured using the ACR SmartButton, ACR Systems Inc., Surrey, Canada.

VIBRIO ISOLATION

As mentioned in the Oyster Samples section, 72 to 90 oysters were harvested to accommodate each of 6 handling methods. This was done on a weekly basis for 18 weeks. Oysters were cleaned prior to analysis. Isolation of *Vibrio parahaemolyticus* and *V.vulnificus* were performed according to FDA BAM (*Vibrio*) using the MPN method. A 3-mm loopful from the top 1 cm of alkaline peptone water (APW) tubes containing the three highest dilutions of sample showing growth were streaked onto *Vibrio* selective, Thiosulfate Citrate Bile Sucrose Agar (TCBS Agar) and modified cellobiose-polymyxin B-colistin (mCPC Agar) for *V. vulnificus* isolation. They were incubated at 35 ±2° overnight. *V. parahaemolyticus* appeared as round, opaque, green or bluish colonies, 2 to 3 mm in diameter on TCBS. *V.vulnificus* colonies appeared as round, flat, opaque, yellow, and 1 to 2 mm in diameter on mCPC. Screening of suspected *V. parahaemolyticus* and *V. vulnificus* was performed using API 20E test strips (Biomerieux, France).

DNA EXTRACTION

Crude cell lysates were prepared from 1 mL of bacterial culture from positive MPN tubes by boiling for 10 minutes in 1.5 mL microcentrifuge tubes (Blackstone *et al*., 2003) and centrifuging for 2 minutes at 12,000 rpm. The lysates were stored at -20º C in a lab freezer until ready for testing. The genomic DNA (2µl) contained in the boiled lysates was used as a template for development of the real-time PCR assay.

REAL-TIME PCR AMPLIFICATION

The real-time PCR amplification was optimized for the multiplex detection and quantification of the *tlh, tdh, trh* (*V. parahaemolyticus*) and *vvh* (*V.vulnificus*) genes and the IAC according to Nordstrom *et al*., 2007. PCR was run using 25µl volume of the reaction mixture components in final concentrations: 1x PCR buffer (Invitrogen, Carlsbad, CA), 5.0 mM $MgCl_2$ (Invitrogen), 300 nM of each of the dNTPs (Roche, Indianapolis), 300 nM each of the *trh* and *vvh* forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 200 nM *tlh* forward and reverse primers (Integrated DNA Technologies), 100 nM of *tdh* forward and reverse primers (Integrated DNA Technologies), 75 nM for *tlh* and *vvh* IAC forward and reverse primers (Integrated DNA Technologies), 25 nM for *tdh*/*trh* IAC forward and reverse primers (Integrated DNA Technologies), 150 nM probe for *tlh* and IAC (Integrated DNA Technologies), 75 nM probe for *tdh* and *trh* (Life Technologies), 200 nM probe for *vvh* (Integrated DNA Technologies), and 1.50 U, 1.12 U 2.25 U platinum *Taq* polymerase (Invitrogen) for *tlh, vvh,* and *tdh/trh*, respectively.

The rest of the reaction mixture volume consisted of a previously quantified IAC DNA (2µl), nuclease-free water and a DNA template (2µl of boiled cell lysates). The Real-time primer and probe sequences used in assay are shown below (Table 3):

Table 3: Real-time Primer and Probe Sequences Used in Assays

***tlh* forward**	5'-ACTCAACACAAGAAGAGATCGACAA-3'
***tlh* reverse**	5'-GATGAGCGGTTGATGTCCAAA-3'
***tlh* probe**	JOE-5'- CGCTCGCGTTCACGAAACCGT -3'-BHQ2
***tdh* forward**	5'-TCCCTTTTCCTGCCCCC-3'
***tdh* reverse**	5'-CGCTGCCATTGTATAGTCTTTATC-3'
***tdh* probe**	6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ
***trh* forward**	5'-TTGCTTTCAGTTTGCTATTGGCT-3'
***trh* reverse**	5'-TGTTTACCGTCATATAGGCGCTT-3'
***trh* probe**	VIC-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ
***vvh* forward**	5'-TGTTTATGGTGAGAACGGTGACA-3'
***vvh* reverse**	5'-TTCTTTATCTAGGCCCCAAACTTG-3'
***vvh* probe**	6FAM-5'-CCGTTAACCGAACCACCCGCAA-BHQ2-3'
IAC forward	5'-GACATCGATATGGGTGCCG-3'
IAC reverse	5'-CGAGACGATGCAGCCATTC-3'
IAC probe	CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG -3'- BHQ2

Real-time PCR thermal cycling was performed using a LightCycler 480 instrument from Roche (Indianapolis, IN). The cycling parameters for *tlh* and *tdh*/*trh* consisted of a 95º C hold for 60 s for the initial denaturation and activation of the hot-start *Taq* polymerase , followed by 45 cycles of amplification, with each cycle consisting of denaturation at 95º C for 5 s and a combined annealing /extension step at 59º C for 45 s. For *vvh*, the cycling parameters consisted of a 95º C hold for 60 s for the initial denaturation and activation of the hot-start *Taq* polymerase, followed by 45 cycles of amplification, with each cycle consisting of denaturation at 95º C for 15 s, annealing at 57º C for 15 s and an extension step at 72º C for 25 s . Positive controls, consisting of the *V. parahaemolyticus* strain, possessing all three target genes (*tlh, tdh* and *trh*) as well as the *V. vulnificus* strain, possessing the *vvh* gene, and a negative control (nuclease-free water added as a template) were prepared for each PCR master mix.

SEROTYPING OF *V. parahaemolyticus* PATHOGENIC STRAINS

Pathogenic strains (positive *tdh*/*trh* or both – Table 7) of *V. parahaemolyticus* isolated from these studies were serotyped for K and O antigens using *Vibrio parahaemolyticus* Antisera by Denka-Seiken (Tokyo).

DWMS/BMWM was able to serotype 53.78% of the 225 environmental isolates of *V. parahaemolyticus.* 46.22% were untypeable for O or K antigen. The dominant serotype was O5: KUT at 8% followed by O2:K28 at 7.56% (Table 4).

Table 4: Serotype of *V. parahaemolyticus* Isolated

Serotype	No. of Isolate	% of total typeable strains
O1:K20	1	0.44
O1:K25	1	0.44
O1:K35	2	0.89
O1:K38	1	0.44
O1:K41	1	0.44
O1:K5	1	0.44
O1:K64	6	2.67
O1:K69	2	0.89
O1:KUT	4	1.78
O2:K28	17	7.56
O2:K3	1	0.44
O2:KUT	8	3.56
O3:K28	4	1.78
O3:K31	2	0.89
O3:K33	2	0.89
O3:K37	5	2.22
O3:K48	2	0.89
O3:KUT	5	2.22
O4:K10	1	0.44
O4:K13	1	0.44
O4:K20	1	0.44
O4:K42	2	0.89
O4:K49	1	0.44
O4:K9	1	0.44
O4:KUT	12	5.33
O5:K15	1	0.44
O5:K30	3	1.33
O5:KUT	18	8
O6:K18	1	0.44
O8:K12	1	0.44
O8:K39	1	0.44
O8:KUT	5	2.22
09:K23	1	0.44
O9:KUT	1	0.44
O10:KUT	2	0.89
O11:KUT	3	1.33
Untypeable	104	46.22

RESULTS

TEMPERATURE RESULTS

Oyster samples were collected and handled with six different methods, as described in the Materials and Methods section, sub-section, Oyster Samples. Shell and internal meat temperatures of oysters were taken when the product was first harvested and removed from the water to evaluate temperature differentiation between both out of water shell and meat temperature data (Tables 5 – 7). The 2015 seasonal averages for the shell and meat temperature

data shows nearly similar temperatures for the shell and meat at given months during the time frame of this project (Figure 2). Figure 2 also shows the lowest out of water shell and meat temperatures were recorded in May while the highest temperatures for both out of water oyster shell and meat occurred in July and August.

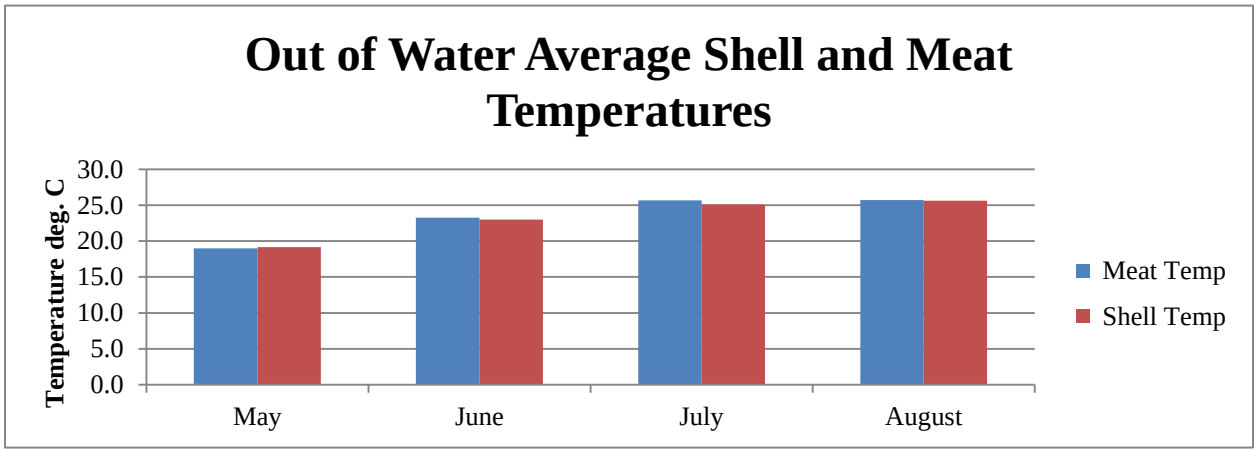

Figure 2: Comparison of Initial Out of Water Shell and Internal Meat Temperature

Temperature of both the shell and internal meat temperatures were taken post-slurry cooling for initial out of water oysters, oysters after 10 minutes in slurry following shading for 50 minutes (1 hour), 2 hours and 50 minutes shading (3 hours), and 4 hours and 50 minutes shading (5 hours) on the boat deck. Average results for the 2015 season by month for each handling method shows that 10 minutes in the ice slurry was adequate to cool the product externally and internally to 10^{o} C (50^{o} F) or less. The data also shows a significant difference between the post slurry shell and internal meat temperatures, as the shell was more rapidly cooled than the internal meat temperature. In addition, the highest post-slurry monthly average internal meat temperature for the 3 and 5 hour handling methods occurred during the month of June (Figures 7 - 8).

The effects of ice slurry on handling methods N to Q are presented in Figures 3, 6, 7, and 8. For handling method N (initial sample to slurry) shown in Figure 3, shell and meat temperature steadily increased for each month tested, with the lowest temperatures recorded in May and the highest occurring in August.

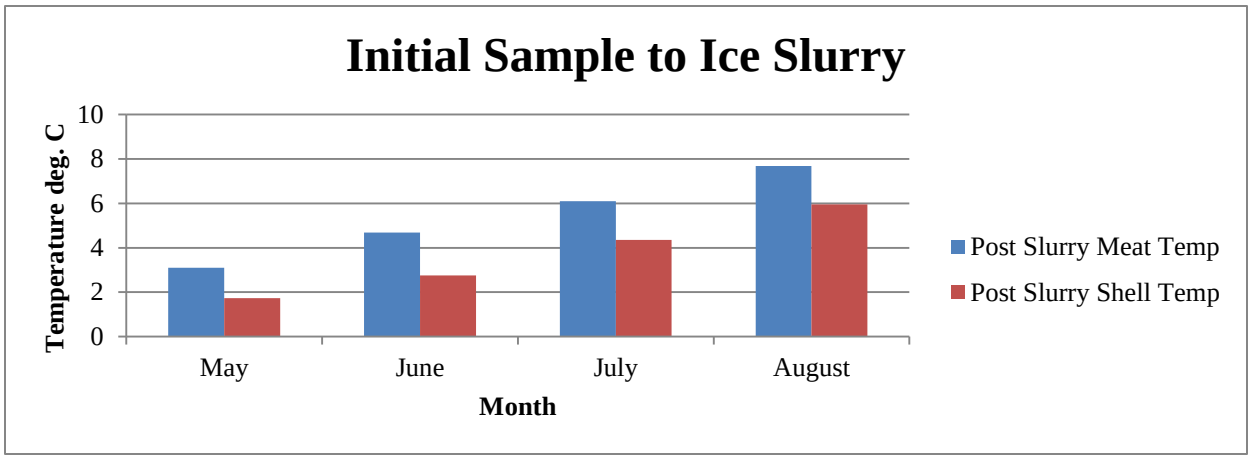

Figure 3: Post 10 Minute Ice Slurry, Shell and Meat Temperature (Initial Out of Water Process – Handling Method N)

The initial sample to slurry was additionally utilized to capture and analyze *Vibrio* presence in oyster meat in relation to water temperature at the time of harvest. Total *Vp* in oyster meat reached its peak (approx. 10,000 MPN/g) during the 1st week of July with water temperatures at 25 ^{o} C. *Vv* was observed to be most prevalent (11,000 – 15,000 MPN/g) from mid-June through the first week of July at 23.5^{o} - 25^{o} C (Figure 4).

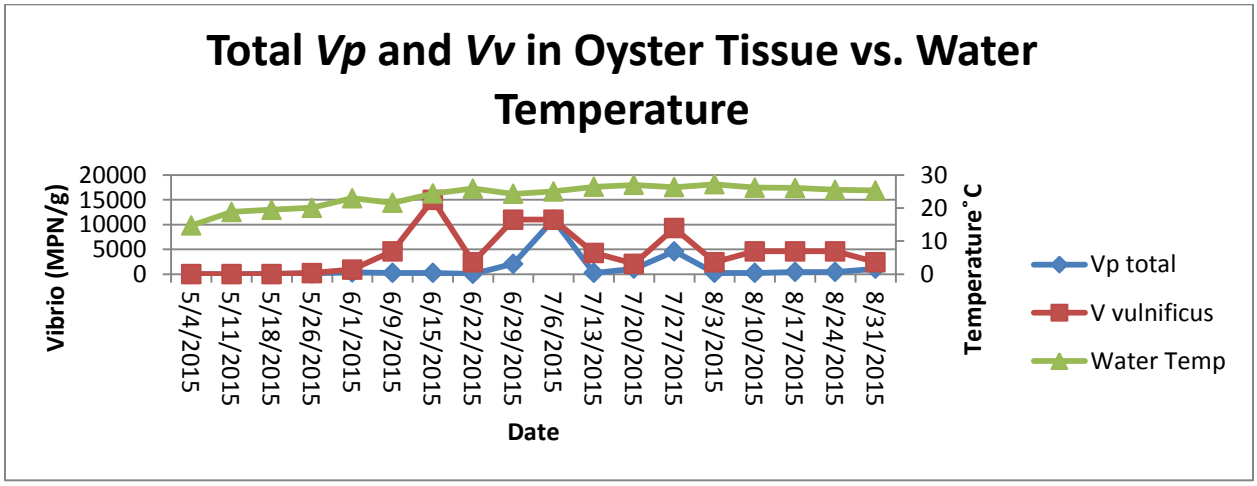

Figure 4: Total *Vp* and *Vv* in Oyster Tissue vs. Water Temperature

Results for initial sample to slurry in relation to *Vp* virulent strains (*tdh* and *trh* genes) present in oyster meat versus water temperature at the time of harvest showed *tdh* in oyster meat results were higher for New Jersey waters as compared to *trh.* Results for either gene were relatively low, particularly *trh* which had nearly negligible results. The *tdh* gene had two higher peaks of greater presence shown in the results with the first presented at the start of June (approx. 90

MPN/g) at water temperatures of 22.5° C and that gene (*tdh*) showed highest results toward the end of June (approx. 110 MPN/g) with water temperatures at 23.5° C (Figure 5).

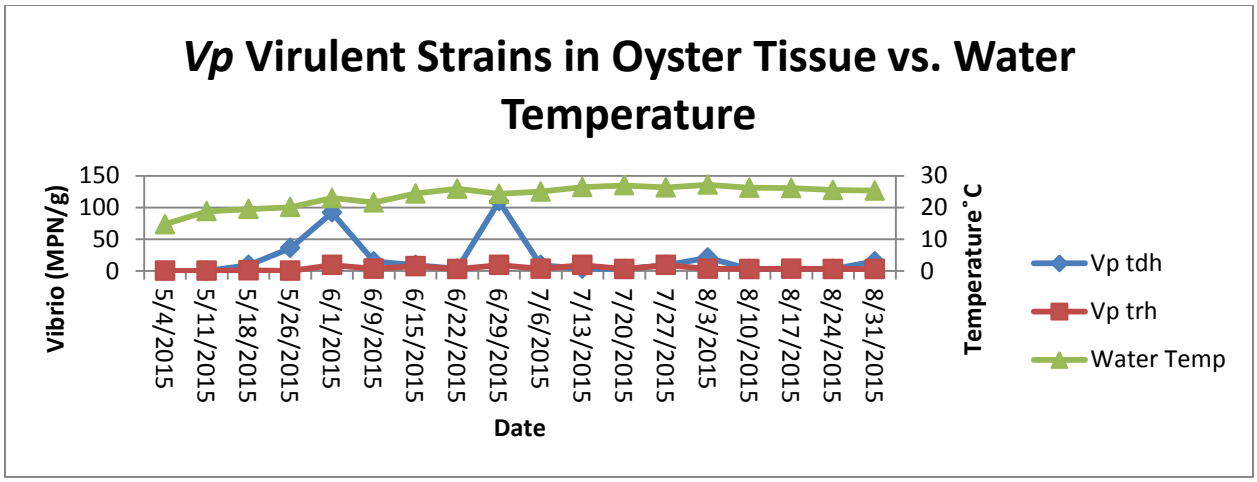

Figure 5: *Vp* Virulent Strains (*tdh* - *trh* genes) in Oyster Tissue vs. Water Temperature

Handling method O (50 minutes on board shading, 10 minutes in ice slurry – one hour process) had the highest post slurry shell temperature compared to all other handling methods. With the exception of the month of May shown in Figure 8, for handling method Q (4 hours 50 minutes on board shading, 10 minutes in ice slurry – five hour process), the results of post slurry internal meat temperature by month and handling methods revealed that months of June, July and August presented higher temperatures.

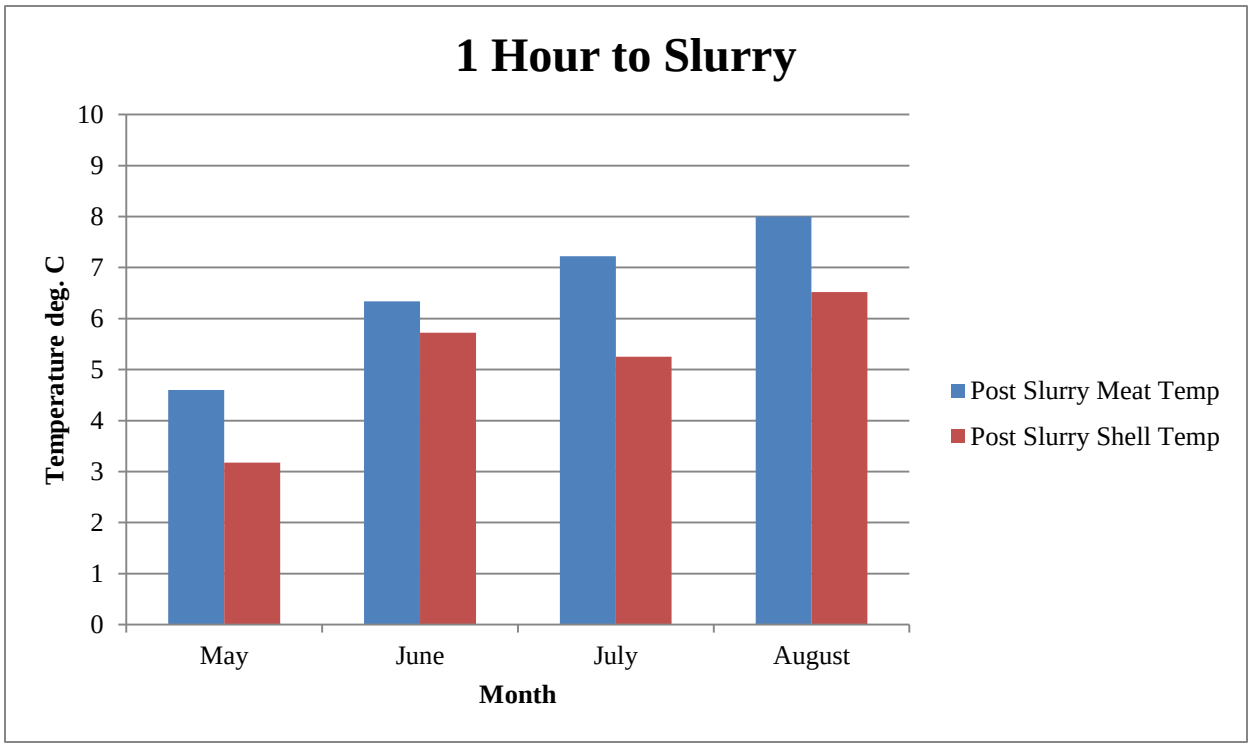

Figure 6: Post 10 Minute Ice Slurry, Shell and Meat Temperature for the 50 Minute Shaded Sample (1 Hour Process – Handling Method O)

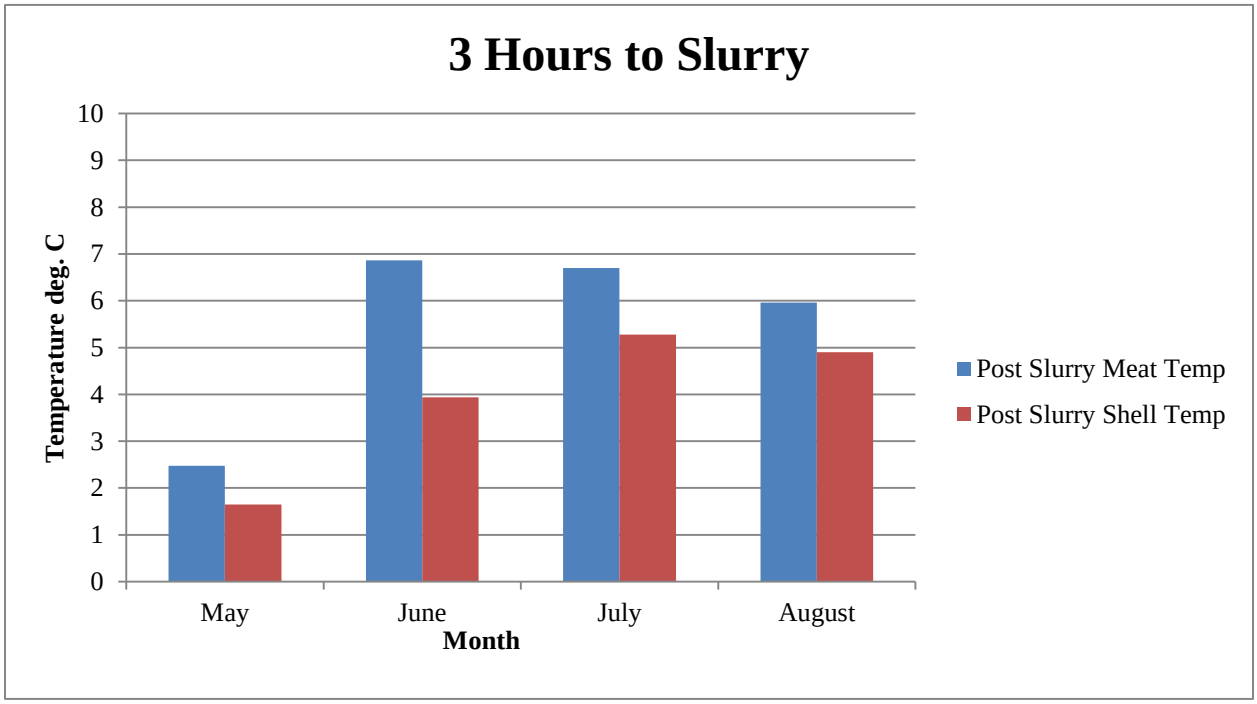

Figure 7: Post 10 Minute Ice Slurry, Shell and Meat Temperature for the 2 hour 50 Minute Shaded Sample (3 Hour Process – Handling Method P)

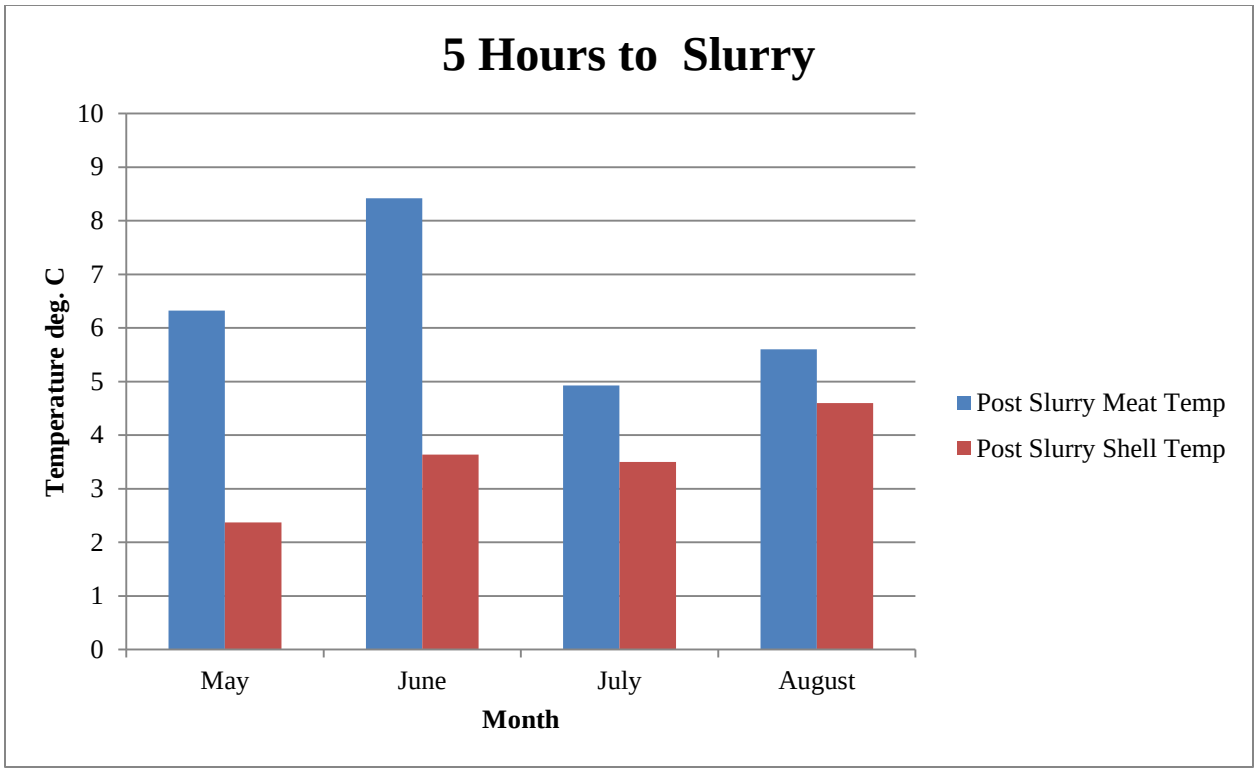

Figure 8: Post 10 Minute Ice Slurry, Shell and Meat Temperature for the 4 hour 50 minute Shaded Sample (5 Hour Process – Handling Method Q)

In mid-June 2015, ACR SmartButtons were employed for both external and internal shellfish temperatures from harvest time through handling and lab processing. These buttons recorded temperature data at one minute intervals. This provided DWM&S/BMWM an evaluative tool to assess the amount of time it took internal meat temperatures to reach 10^{o} C (50^{o} F) or less for all handling methods, and presented a picture of temperature response for those methods.

Figure 9 shows the time required for internal meat temperature to cool down to 10^{o} C (50^{o} F) or less and the initial temperature before slurry or refrigeration. For handling methods N to Q, it took 5 to 6 minutes on average to cool the internal meat temperature to 10 ^{o} C/50^{o} F or less. However, it took over 2 and half hours to cool the temperature down to below 10^{o} C (50^{o}F) for handling method R [5 hours shading, then into mechanical temperature control (refrigerator) for 10 hours].

When the product is shaded for 1 and 3 hours, the data additionally showed seasonal average pre-slurry temperatures dropped when compared to the initial out of water pre-slurry temperature. This could be due to cooling from evaporation or the consideration that air temperatures are cooler during morning harvest, or a combination of the two factors.

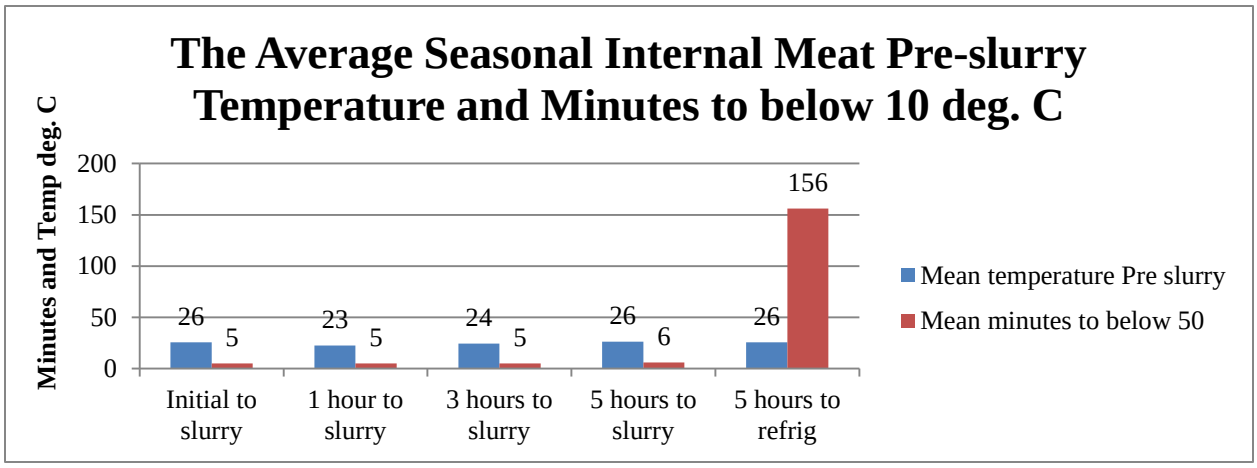

Figure 9: Seasonal Average Pre- cooling Internal Meat Temperatures and Minutes to below 50^{o} F for Each Handling Method (N - Q) and R (Refrigeration)

VIBRIO RESULTS

The forms of vibrios analyzed and isolated from positive APW tubes and confirmed using API 20E strips included total *Vibrio parahaemolyticus* (*Vp*) - (tlh gene isolate), pathogenic *Vp* (*trh* and *tdh* gene isolates), and *Vibrio vulnificus* (*Vv*) - (*vvh* gene isolates). *Vibrio* and project parameter data are shown in Tables 5 – 7.

The average results for all handling methods during each month show total *Vp* and *Vv* are highest during July, but that the pathogenic genes of *Vp*, or *trh* and *tdh* are higher in the month of June with levels for all vibrios presented as low during May (Figure 10). In addition, the *tdh* gene for each month showed the highest counts occurred in handling method R (refrigerated product) during the study period (Figure 11).

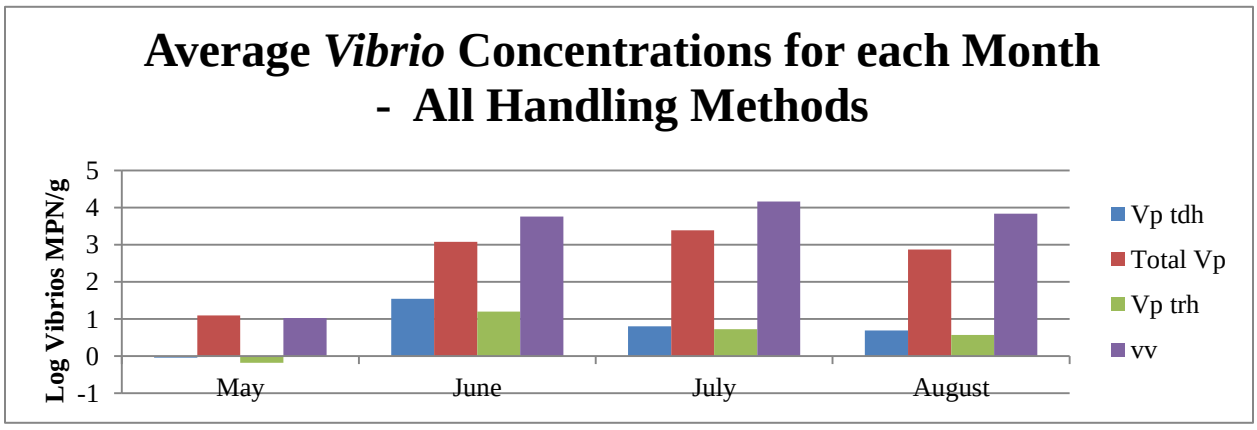

Figure 10: 2015 Overall Average *Vibrio* Levels by Month

Average Monthly data for *Vp tdh* was evaluated for each handling method. The data show that from the initial sample out of water up to 3 hours shaded, there is no significant increase in

Vibrio tdh levels and for some months the levels at 1 and 3 hours shaded then to ice slurry showed lower average levels. In May, July, and August there is no significant increase in *tdh* levels from initial out of water samples through all handling methods including overnight mechanical refrigeration.

The month of June shows increasing *tdh* levels with 4 hour, 50 minute shaded samples followed by ice slurry, and a greater increase with the 5 hours shaded samples followed by overnight refrigeration. This suggests that the month of June and the 5 hour methodology is the significant time frame for vibrios and *Vibrio* growth post-harvest in New Jersey, and that once *Vibrio* growth starts, mechanical refrigeration does not cool quickly enough to keep levels low (Figure 11).

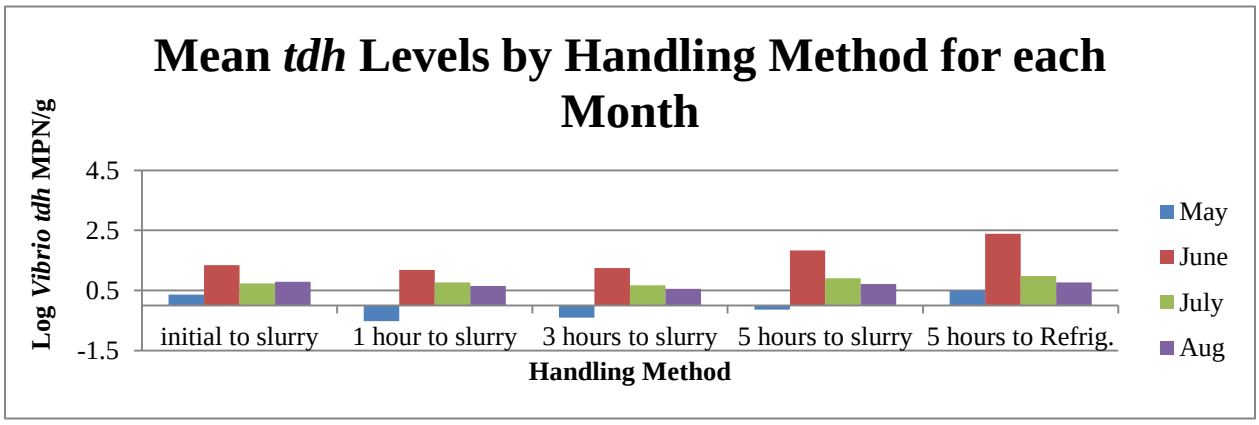

Figure 11: Average *Vp tdh* Levels by Month for each Handling Method

Table 5: Detection of *V. parahaemolyticus* and *V. vulnificus* in Oyster Samples

Date	Station ID	Handling method	Air Temp ℃	Water Temp ℃	Out of Water Shell Temp ℃	Out of Water Meat Temp ℃	Post Slurry Meat Temp ℃	Post Slurry Shell Temp ℃	pH s.u.	Dissolved Oxygen (mg/l)	Salinity	*Vp tdh* (MPN/g)	*Vp* tlh (MPN/g)	*Vp trh* (MPN/g)	*Vv* (MPN/g)
5/4/15	G	N	13.2	14.7	15.1	15.1	0.9	-0.5	8	14.07	14.57	0.3	0.92	0.3	15
5/4/15	G	O					2.2	0.2				0.3	0.3	0.3	1.5
5/4/15	G	P					-4.5	-1.8				0.3	0.3	0.3	9.2
5/4/15	G	Q					3.3	0.9				0.3	0.92	3.6	15
5/4/15	G	R										0.92	0.92	0.3	3.6
5/11/15	G	N	19.5	18.8	18.1	17.9	5.4	2.5	8	11.29	15.16	0.3	2.9	0.3	0.3
5/11/15	G	O					6.7	5.4				0.3	0.36	0.3	0.62
5/11/15	G	P					6.4	3.2				0.3	4.3	0.3	1.2
5/11/15	G	Q					4.9	0.9				0.3	15	0.3	0.3
5/11/15	G	R										0.3	15	0.3	0.3
5/18/15	G	A										1.1	24	0.3	0.3
5/18/15	G	N	20.4	19.5	22.9	22.4	4	3.5	8	7.43	15.31	9.2	15	0.92	15
5/18/15	G	O					6.1	4.9				0.3	9.3	0.3	0.3
5/18/15	G	P					4.5	2.6				0.3	4.3	0.3	2100
5/18/15	G	Q					10.1	2.1				2.1	24	15	200
5/18/15	G	R										9.3	46	9.3	93
5/26/15	G	N	20.3	20.1	20.5	20.6	2.1	1.4	8	8.25	14.91	36	150	0.3	210
5/26/15	G	O					3.4	2.2				0.3	150	0.3	15
5/26/15	G	P					3.5	2.6				0.92	1100	0.36	460
5/26/15	G	Q					7	5.6				1.5	460	1.5	460
5/26/15	G	R										35	7500	15	460
6/1/15	G	N	23.3	23	23.1	22.9	6.6	1.6	8	8.15	14.69	92	350	9.2	930
6/1/15	G	O					7.4	5.5				62	930	9.2	750
6/1/15	G	P					6.2	4.1				72	2100	93	1500
6/1/15	G	Q					3.9	4.8				230	7500	21	9300
6/1/15	G	R										200	2100	93	11000
6/9/15	G	N	20.8	21.6	20.8	21	3.7	2.6	8	7.13	14.69	15	240	3.6	4600
6/9/15	G	O					7.9	6.6				20	460	15	9300
6/9/15	G	P					10.5	4.7				93	1500	23	9300
6/9/15	G	Q					3.6	-4.1				64	1500	150	2400
6/9/15	G	R										430	2100	210	4600
6/15/15	G	A										15	240	3.6	4300
6/15/15	G	N	28.3	24.4	25.1	25.2	3.4	2.1	8	6.34	14.61	9.2	240	7.4	15000
6/15/15	G	O					5.9	3.9				21	2400	9.2	4300
6/15/15	G	P					6.6	2.6				23	2400	43	9300
6/15/15	G	Q					10.1	6.1				93	2400	43	3600
6/15/15	G	R										1500	15000	92	2400

Table 6: Detection of *V. parahaemolyticus* and *V. vulnificus* in Oyster Samples Continued

Date	Station ID	Handling method	Air Temp ℃	Water Temp ℃	Out of Water Shell Temp ℃	Out of Water Meat Temp ℃	Post Slurry Meat Temp ℃	Post Slurry Shell Temp ℃	pH s.u.	Dissolved Oxygen (mg/l)	Salinity	*Vp tdh* (MPN/g)	*Vp* tlh (MPN/g)	*Vp trh* (MPN/g)	*Vv* (MPN/g)
6/22/15	H	A										3.6	460	3.6	930
6/22/15	H	N	24.3	25.9	24.2	26.2	6.1	5.4	8	6.15	11.55	3.6	43	3	2400
6/22/15	H	O					6.4	5				3	460	3.6	11000
6/22/15	H	P					7.6	5.6				3	240	3	46000
6/22/15	H	Q					7	4.9				15	240	7.4	24000
6/22/15	H	R										15	4600	20	9300
6/29/15	H	N	18.1	24.3	21.7	21	3.6	2.1	8	6.18	14.06	110	2100	9.2	11000
6/29/15	H	O					4.1	7.6				11	9300	3.6	11000
6/29/15	H	P					3.4	2.7				3.6	2400	15	1500
6/29/15	H	Q					17.5	6.5				75	7500	15	24000
6/29/15	H	R										460	2400	93	24000
7/6/15	H	N	24	25	24.3	24.5	3.9	3.4	8	5.78	11.68	9.2	11000	3.6	11000
7/6/15	H	O					8.5	6.6				3	1100	3	460
7/6/15	H	P					5.9	5.5				3.6	2900	3.6	24000
7/6/15	H	Q					5.5	6				20	11000	11	11000
7/6/15	H	R										43	46000	9.2	15000
7/13/15	H	A										3.6	240	3.6	9300
7/13/15	H	N	22.7	26.4	24.1	25.5	4.5	3.7	8	6.63	11.69	3.6	240	9.2	4300
7/13/15	H	O					7.2	5.9				43	460	43	9300
7/13/15	H	P					8.9	7.1				15	2400	15	24000
7/13/15	H	Q					4.5	0.5				9.2	4600	9.2	240000
7/13/15	H	R										3	4600	3	93000
7/20/15	H	N	25.9	27	27.3	27.6	10.6	5.7	8	6.5	11.55	3	1100	3	2100
7/20/15	H	O					8.8	5.6				3	1100	3	4600
7/20/15	H	P					7.9	5.9				3	24000	3	4600
7/20/15	H	Q					4.7	3.5				3	2400	3	46000
7/20/15	H	R										9.2	46000	3.6	24000
7/27/15	H	N	22.5	26.3	24.7	25	5.4	4.6	8	6.24	14.2	9.2	4600	9.2	9300
7/27/15	H	O					4.4	2.9				3	150	7.2	24000
7/27/15	H	P					4.1	2.6				3	240	3	21000
7/27/15	H	Q					5	4				7.4	4600	3.6	120000
7/27/15	H	R										7.4	2400	3	24000
8/3/15	H	N	23.8	27.2	25.7	25.5	6.5	4.6	7	6.21	12.51	21	240	3.6	2400
8/3/15	H	O					8.6	6.7				3	240	3	2400
8/3/15	H	P					8.6	6.7				3	240	3	4300
8/3/15	H	Q					5.5	5.5				32	2800	3.6	46000
8/3/15	H	R										3	3800	3	9300

Table 7: Detection of *V. parahaemolyticus* and *V. vulnificus* in Oyster Samples Continued

Date	Station ID	Handling method	Air Temp ℃	Water Temp ℃	Out of Water Shell Temp ℃	Out of Water Meat Temp ℃	Post Slurry Meat Temp ℃	Post Slurry Shell Temp ℃	pH s.u.	Dissolved Oxygen (mg/l)	Salinity	*Vp tdh* (MPN/g)	*Vp* tlh (MPN/g)	*Vp trh* (MPN/g)	*Vv* (MPN/g)
8/10/15	H	N	21.2	26.2	25.9	25.1	5.5	5.4	8	5.89	17.51	3	240	3	4600
8/10/15	H	O					7.1	6.6				3.6	1100	3.6	2400
8/10/15	H	P					6.2	4.9				3	460	3	4600
8/10/15	H	Q					4	3				3.6	1100	3.6	4600
8/10/15	H	R										23	1100	23	46000
8/17/15	H	A										3.6	240	3	9300
8/17/15	H	N	23.2	26.1	26.3	26.4	7.6	6.4	8	6.34	13.74	3	460	3.6	4600
8/17/15	H	O					6.4	3.6				3	240	3	4300
8/17/15	H	P					4.5	1.5				3	2400	3	4300
8/17/15	H	Q					6	3.5				3.6	2400	3.6	9300
8/17/15	H	R										3.6	240	3.6	4300
8/24/15	H	N	21.1	25.5	25.3	25.8	8.2	7.2	8	7.01	14.26	3	460	3	4600
8/24/15	H	O					7.2	7.8				7.4	1100	3.6	24000
8/24/15	H	P					5.4	4.9				3.6	1100	3.6	15000
8/24/15	H	Q					6.5	4.5				3	460	3	24000
8/24/15	H	R										9.2	1100	9.2	24000
8/31/15	H	N	24.1	25.3	24.8		10.6	6.2	8	6.44	15.28	15	1100	3	2400
8/31/15	H	O					10.7	7.9				7.4	240	3	2400
8/31/15	H	P					5.1	6.5				6.1	2400	6.1	2400
8/31/15	H	Q					6	6.5				3	1100	3	11000
8/31/15	H	R										3	2400	3	11000

DISCUSSION

Overall, the month of May produced low *Vibrio* levels regardless of the post-harvest handling method. Shading product for up to 2 hours, 50 minutes post-harvest, followed by rapid ice slurry cooling, then storing oysters in an ice filled cooler produced results similar to samples collected directly from the water with an immediate ice slurry implementation. This was true for all months of this study.

In July and August, the post-harvest shaded pre-slurry time frame can be extended up to 5 hours and still produce *Vibrio* level values similar to the initial levels recorded from the out of water to immediate slurry.

Consistent with previous New Jersey *Vibrio* studies and historical illnesses for the State, the month of June had the overall highest levels of the pathogenic *Vp* genes (*tdh* and *trh*), and *tdh* also increased substantially more with post-harvest handling times. Temperature data also

DISCUSSION CONTINUED

showed that June exhibited higher average post-slurry internal meat temperatures than other months, suggesting that product heat retention is greater post-harvest in June although the ice slurry was still able to bring the product to 10^{o} C (50^{o} F) or less during that month. In addition, ice slurry was able to bring meat temperature below 10^{o} C (50^{o} F) in six minutes or less for all post-harvest handling practices.

Mechanical refrigeration (handling method R) following 5 hours of shading showed an increase in *tdh* levels accompanied by a slower cooling process. It took about two and half hours to achieve temperatures of 10^{o} C (50^{o} F) or less in mechanical refrigeration as compared to 6 minutes or less in ice slurry.

In summarization, Ice slurry is an effective means for rapid cooling and is more effective than mechanical refrigeration alone. Illnesses and highest *trh* and *tdh* levels do not always occur during months with highest air and water temperatures. The data supports 3 hours, as the critical time for rapid cooling to prevent growth of *trh* and *tdh*.

Throughout a broad range of state's and countries, species have been found that are similar to those reported on in this grant study. Further research on strains may prove beneficial, though. The occurrence of *Vibrio* illness in relation to species presence and prevalence of apparent strains or gene types seems varied by region. For example, Gulf waters of the US have *Vv*, and this report supports the presence of *Vv* in New Jersey waters. However, illnesses have not been reported for *Vv* in New Jersey. It may be that more virulent strains of *Vv* exist in some regions as opposed to others.

Regionally, differences also result from climate and harvest/distribution practice. In closing, *Vibrio* management might be most effective if created at the State level in concert with a process of continued enabling promulgation provided by the ISSC and National Shellfish Sanitation Program (NSSP) with additional oversight provided by the US Food and Drug Administration (USFDA).

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Date	Station ID	Handling method	Air Temp ℃	Water Temp ℃	Out of Water Shell Temp ℃	Out of Water Meat Temp ℃	Post Slurry Meat Temp ℃	Post Slurry Shell Temp ℃	pH s.u.	Dissolved Oxygen (mg/l)	Salinity	Vp tdh (MPN/g)	Vp tlh (MPN/g)	Vp trh (MPN/g)	Vv (MPN/g)
5/4/15	G	N	13.2	14.7	15.1	15.1	0.9	-0.5	8.3	14.07	14.57	0.3	0.92	0.3	15
5/4/15	G	O					2.2	0.2				0.3	0.3	0.3	1.5
5/4/15	G	P					-4.5	-1.8				0.3	0.3	0.3	9.2
5/4/15	G	Q					3.3	0.9				0.3	0.92	3.6	15
5/4/15	G	R										0.92	0.92	0.3	3.6
5/11/15	G	N	19.5	18.8	18.1	17.9	5.4	2.5	8.15	11.29	15.16	0.3	2.9	0.3	0.3
5/11/15	G	O					6.7	5.4				0.3	0.36	0.3	0.62
5/11/15	G	P					6.4	3.2				0.3	4.3	0.3	1.2
5/11/15	G	Q					4.9	0.9				0.3	15	0.3	0.3
5/11/15	G	R										0.3	15	0.3	0.3
5/18/15	G	A										1.1	24	0.3	0.3
5/18/15	G	N	20.4	19.5	22.9	22.4	4	3.5	7.76	7.43	15.31	9.2	15	0.92	15
5/18/15	G	O					6.1	4.9				0.3	9.3	0.3	0.3
5/18/15	G	P					4.5	2.6				0.3	4.3	0.3	2100
5/18/15	G	Q					10.1	2.1				2.1	24	15	200
5/18/15	G	R										9.3	46	9.3	93
5/26/15	G	N	20.3	20.1	20.5	20.6	2.1	1.4	7.94	8.25	14.91	36	150	0.3	210
5/26/15	G	O					3.4	2.2				0.3	150	0.3	15
5/26/15	G	P					3.5	2.6				0.92	1100	0.36	460
5/26/15	G	Q					7	5.6				1.5	460	1.5	460
5/26/15	G	R										35	7500	15	460
6/1/15	G	N	23.3	23	23.1	22.9	6.6	1.6	8.14	8.15	14.69	92	350	9.2	930
6/1/15	G	O					7.4	5.5				62	930	9.2	750
6/1/15	G	P					6.2	4.1				72	2100	93	1500
6/1/15	G	Q					3.9	4.8				230	7500	21	9300
6/1/15	G	R										200	2100	93	11000
6/9/15	G	N	20.8	21.6	20.8	21	3.7	2.6	7.83	7.13	14.69	15	240	3.6	4600
6/9/15	G	O					7.9	6.6				20	460	15	9300
6/9/15	G	P					10.5	4.7				93	1500	23	9300
6/9/15	G	Q					3.6	-4.1				64	1500	150	2400
6/9/15	G	R										430	2100	210	4600
6/15/15	G	A										15	240	3.6	4300
6/15/15	G	N	28.3	24.4	25.1	25.2	3.4	2.1	7.67	6.34	14.61	9.2	240	7.4	15000
6/15/15	G	O					5.9	3.9				21	2400	9.2	4300
6/15/15	G	P					6.6	2.6				23	2400	43	9300
6/15/15	G	Q					10.1	6.1				93	2400	43	3600
6/15/15	G	R										1500	15000	92	2400
6/22/15	H	A										3.6	460	3.6	930
6/22/15	H	N	24.3	25.9	24.2	26.2	6.1	5.4	7.66	6.15	11.55	3.6	43	3	2400
6/22/15	H	O					6.4	5				3	460	3.6	11000
6/22/15	H	P					7.6	5.6				3	240	3	46000

6/22/15	H	Q					7	4.9				15	240	7.4	24000
6/22/15	H	R										15	4600	20	9300
6/29/15	H	N	18.1	24.3	21.7	21	3.6	2.1	7.75	6.18	14.06	110	2100	9.2	11000
6/29/15	H	O					4.1	7.6				11	9300	3.6	11000
6/29/15	H	P					3.4	2.7				3.6	2400	15	1500
6/29/15	H	Q					17.5	6.5				75	7500	15	24000
6/29/15	H	R										460	2400	93	24000
7/6/15	H	N	24	25	24.3	24.5	3.9	3.4	7.57	5.78	11.68	9.2	11000	3.6	11000
7/6/15	H	O					8.5	6.6				3	1100	3	460
7/6/15	H	P					5.9	5.5				3.6	2900	3.6	24000
7/6/15	H	Q					5.5	6				20	11000	11	11000
7/6/15	H	R										43	46000	9.2	15000
7/13/15	H	A										3.6	240	3.6	9300
7/13/15	H	N	22.7	26.4	24.1	25.5	4.5	3.7	7.81	6.63	11.69	3.6	240	9.2	4300
7/13/15	H	O					7.2	5.9				43	460	43	9300
7/13/15	H	P					8.9	7.1				15	2400	15	24000
7/13/15	H	Q					4.5	0.5				9.2	4600	9.2	240000
7/13/15	H	R										3	4600	3	93000
7/20/15	H	N	25.9	27	27.3	27.6	10.6	5.7	7.71	6.5	11.55	3	1100	3	2100
7/20/15	H	O					8.8	5.6				3	1100	3	4600
7/20/15	H	P					7.9	5.9				3	24000	3	4600
7/20/15	H	Q					4.7	3.5				3	2400	3	46000
7/20/15	H	R										9.2	46000	3.6	24000
7/27/15	H	N	22.5	26.3	24.7	25	5.4	4.6	7.99	6.24	14.2	9.2	4600	9.2	9300
7/27/15	H	O					4.4	2.9				3	150	7.2	24000
7/27/15	H	P					4.1	2.6				3	240	3	21000
7/27/15	H	Q					5	4				7.4	4600	3.6	120000
7/27/15	H	R										7.4	2400	3	24000
8/3/15	H	N	23.8	27.2	25.7	25.5	6.5	4.6	7.48	6.21	12.51	21	240	3.6	2400
8/3/15	H	O					8.6	6.7				3	240	3	2400
8/3/15	H	P					8.6	6.7				3	240	3	4300
8/3/15	H	Q					5.5	5.5				32	2800	3.6	46000
8/3/15	H	R										3	3800	3	9300
8/10/15	H	N	21.2	26.2	25.9	25.1	5.5	5.4	7.95	5.89	17.51	3	240	3	4600
8/10/15	H	O					7.1	6.6				3.6	1100	3.6	2400
8/10/15	H	P					6.2	4.9				3	460	3	4600
8/10/15	H	Q					4	3				3.6	1100	3.6	4600
8/10/15	H	R										23	1100	23	46000
8/17/15	H	A										3.6	240	3	9300
8/17/15	H	N	23.2	26.1	26.3	26.4	7.6	6.4	7.81	6.34	13.74	3	460	3.6	4600
8/17/15	H	O					6.4	3.6				3	240	3	4300
8/17/15	H	P					4.5	1.5				3	2400	3	4300
8/17/15	H	Q					6	3.5				3.6	2400	3.6	9300
8/17/15	H	R										3.6	240	3.6	4300
8/24/15	H	N	21.1	25.5	25.3	25.8	8.2	7.2	7.97	7.01	14.26	3	460	3	4600
8/24/15	H	O					7.2	7.8				7.4	1100	3.6	24000
8/24/15	H	P					5.4	4.9				3.6	1100	3.6	15000

8/24/15	H	Q				6.5	4.5				3	460	3	24000
8/24/15	H	R									9.2	1100	9.2	24000
8/31/15	H	N	24.1	25.3	24.8	10.6	6.2	7.75	6.44	15.28	15	1100	3	2400
8/31/15	H	O				10.7	7.9				7.4	240	3	2400
8/31/15	H	P				5.1	6.5				6.1	2400	6.1	2400
8/31/15	H	Q				6	6.5				3	1100	3	11000
8/31/15	H	R									3	2400	3	11000

Table 2: Serotype of V. parahaemolyticus isolated

Serotype	No. of Isolate	% of total typeable strains
O1:K20	1	0.44
O1:K25	1	0.44
O1:K35	2	0.89
O1:K38	1	0.44
O1:K41	1	0.44
O1:K5	1	0.44
O1:K64	6	2.67
O1:K69	2	0.89
O1:KUT	4	1.78
O2:K28	17	7.56
O2:K3	1	0.44
O2:KUT	8	3.56
O3:K28	4	1.78
O3:K31	2	0.89
O3:K33	2	0.89
O3:K37	5	2.22
O3:K48	2	0.89
O3:KUT	5	2.22
O4:K10	1	0.44
O4:K13	1	0.44
O4:K20	1	0.44
O4:K42	2	0.89
O4:K49	1	0.44
O4:K9	1	0.44
O4:KUT	12	5.33
O5:K15	1	0.44
O5:K30	3	1.33
O5:KUT	18	8
O6:K18	1	0.44
O8:K12	1	0.44
O8:K39	1	0.44
O8:KUT	5	2.22
09:K23	1	0.44
O9:KUT	1	0.44
O10:KUT	2	0.89
O11:KUT	3	1.33
Untypeable	104	46.22
	225	99.93