FINAL PROGRESS REPORT

Project Title	Development of more efficient methods of *Vibrio sp.* detection and identification of *Vibrio sp.* abundance in cultured oysters from Northeast U.S. farms and from retail sites post-harvest.
Reporting Period	September 1, 2014 – August 31, 2015
Author (Project Coordinator)	Roxanna Smolowitz
Key Word	
Funding Level	Total funds allocated for this project to date. *Year One:* $96,844 *Year Two:* $90,180
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Project Objectives	A. Develop a multiplex quantitative real time PCR (mqPCR) method for the detection of Vv and Vp using an oyster DNase inhibitor (activated carbon coated with bentonite;ACCB) and compare sensitivity and specificity with the FDA MPN/PCR method. Develop two additional multiplex methods for evaluation of samples for both Vp and Vv pathogenic genes using previously published methods with the oyster DNase inhibitor. B. Intensively monitor cultured oysters, water and sediment from two locations (one in RI and one in MA) over a year using the MPN/mqPCR and the ACCB/mqPCR method side by side in order to understand the Vv and Vp cycle in the northeast environment and compare sensitivity of both tests. C. Identify the occurrence of pathogenic and non-pathogenic strains of *Vibrio sp.* in oysters (1). at two time periods after collection of oysters from culturists who reside in 5 northeastern states (2). from ten retail stores/restaurants in July/August. The source of oysters (culturists from which animals were collected in 2.1.A. and 2.1.B.), and post-harvest handling (time between harvest and chilling and length of time chilled before sampling) will be identified as selection criteria. D. Provide findings (via presentations, websites and brochure/white paper) to culturists and extension agents and diagnostic laboratories at regional and national meetings, at local meetings for culturists and extension agents and to representatives of the Food and Drug Administration.
Anticipated Benefits	State briefly how the project will benefit the aquaculture industry – directly or indirectly. Our lab (Aquatic Diagnostic Laboratory, ADL) has developed a less labor intense and yet specific multiplex quantitative real time PCR (mqPCR) method of identification of Vp and Vv organisms in composites of oyster tissue. We are currently using the method in other studies to determine Vp and Vv levels in various situations in order to understand how those situations affect abundance of the bacteria in oysters. We also developed a multiplex mqPCR test for the pathogenic genes of Vp. We have found that the MPN method is not accurate or specific and in fact can be very incorrect (many other bacteria grow in the media and cause falsely high results) and that only by using the mqPCR methods can accurate and specific quantification of Vp and Vv be determined in a homogenate of oysters. The other major finding was that in almost all samples we examined, only a low level of Vp and Vv were identified in our MA and RI samples. This finding is very different from information concerning levels of Vp and

	Vv in southern U.S. areas where Vp and Vv abundances are high in the water column and freshly harvested animals, especially at certain times of the year. The information provides more incentive to increase management for northeast oysters by developing and using post-harvest oyster handling methods for northeast culturists, and provides the information showing that these methods may be more important in controlling Vp and Vv abundance in the northeast, than the levels of Vp and Vv in the water and the oysters upon harvesting. Such information can help regulators, aquaculturists and extension agents to further examine handling methods and consider their effect as a cause for high Vp and Vv in northeast oysters, rather than consider concentration of Vp and Vv in the oysters/water when oysters are harvested. Examination of oysters shipped from other locations in the northeast strongly supported the need for using appropriate handling methods when shipping; specifically the need for using a better insulated boxes for shipping and the need for precooling the oyster and the box before shipment. Our restaurant work showed that when oysters are handled properly, the levels of Vp and Vv remained low. When oysters were mistreated bacterial levels elevated in the oysters. Although the bacteria that proliferates may not be Vp or Vv. Interestingly, the information suggests there may be a component of "stress" involved in the abundance of Vp and Vv in oysters as well as a temperature relationship.
Project Progress	Summarize concisely for each objective the progress toward accomplishment to date. This has an 8,000 character limit. A. Develop a multiplex quantitative real time PCR (mqPCR) method for the detection of Vv and Vp using an oyster DNase inhibitor (activated carbon coated with bentonite;ACCB) and compare sensitivity and specificity with the FDA MPN/PCR method. Develop two additional multiplex methods for evaluation of samples for both Vp and Vv pathogenic genes using previously published methods with the oyster DNase inhibitor. We have developed a speedy and cost-effective multiplex quantitative real time PCR (mqPCR) method of identification of Vp and Vv organisms in composites of oyster tissues that is more accurate and sensitive than the FDA approved MPN method. We also developed an mqPCR test for the detection and quantification of pathogenic genes of Vp. This test results in percentages of pathogenic genes vs. the total number of Vp in the sample. In this study we compared the method of quantification of Vp and Vv to our test. We have found that the MPN method is not accurate or specific and that only by using the mqPCR methods can accurate and specific quantification of Vp and Vv be determined.

For Reference: The FDA BAM-MPN method used in our lab. Ten oysters were homogenized and a serial dilution of 10g, 1g and 1:10-1:10000 were enriched overnight in Alkaline Peptone Water (APW). All tubes were run in triplicate. After overnight enrichment the tubes were evaluated for growth. Any tube with growth was called a positive and was used to calculate the number of bacteria/gram of oyster tissue by using the BAM-MPN calculation spreadsheet (provided by M. Gomez-Chiarri).

mqPCR methods: A mqPCR assay was designed using primers and probes from Nordstrom et al. 2007 for Vp (tlh gene) and Takahashi et al. (2005) for Vv (toxR gene) (Figure 1). Modification were made to thermal cycling parameters, mastermix set up and primer and probe ratios to fit the Bio-Rad qPCR thermal cycler used in the Aquatic Diagnostic Lab (ADL). The initial assay was developed for purified gDNA from pure cultures of Vp and Vv. The primers and probe for Vp described by Nordstrom et al (2007) utilize detection of the thermolabile hemolysin *tlh* gene; Forward primer (*tlh*): ACTCAACACAAGAAGAGATCGACAA; Reverse primer (*tlh*): GATGAGCGGTTGATGTCCAA; TaqMan probe (*tlh*): Texas Red flourophore CGCTCGCGTTCACGAAACCGT. The primers and probe for Vv are described by Takahashi et al (2005) and detect the *toxR* gene; Forward primer- Tox-130: TGTTCGGTTGAGCGCATTAA; Reverse primer- Tox-200: GCTTCAGAAGCTGCGTCATTC; TaqMan probe FAM flourophore; Tox-152: CGCTCCTGTCAGATTCAACCAACAACG. The method uses 300nM concentrations of both Vp and Vv primers, and 200nM concentrations of both Vp and Vv probes using 10µ of Bio-Rad iQ Multiplex Powermix (cat# 1725849). One microliter of each standard curve plasmids DNA (see below) and 2µlof unknown DNA are used in the mqPCR. Plasmids DNA is diluted 10 fold over eight orders of magnitude starting at 10^8 to 10 copies. The cut off for this mqPCR is 10 copies. The thermal cycler protocol is as follows: one initial 95°C for 3min cycle, followed by 95°C for 15 seconds, 60°C for 60seconds, repeating for 45 cycles in total, and a hold at 4°C forever. Each assay for Vp and Vv was run independently, and as a duplex, to ensure the same results once duplexed. All parameters for an optimized assay were achieved (Efficiency of 90-110% and R^2 values close to 0.990).

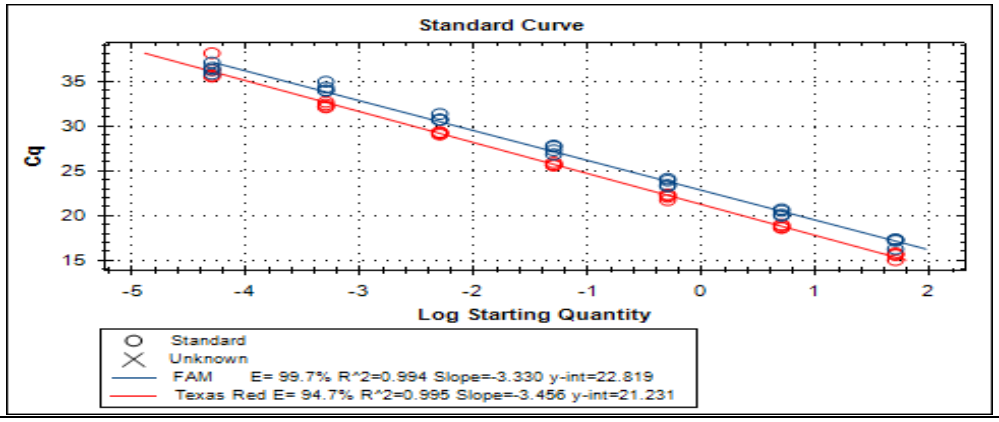

Figure 1. Optimized standard curve of *Vibrio paraheamolyticus* (Texas Red) and *Vibrio vulnificus* (FAM).

We determined that the standard method of boiled cell lysate extraction of oyster tissues for PCR work is inefficient and too variable. By comparing the BCL method with commercial kit extraction methods available (data not shown), we determined that use of a bench top extraction method using a MoBio PowerFood® Microbial DNA Isolation kit, is the best tissue extraction method to use. It appears more sensitive than other methods and can be used to detect Vv and Vp in oyster tissues without the need for pretreatment to eliminate "inhibition" or for pretest enrichment. It also appears to be more accurate and sensitive than the boiled cell lysate extraction method used in other protocols (Cox and Gomez-Chiarri, 2013; Nordstrom et al., 2007).

We found that using cultured bacteria as controls produced high levels of variability in the standard curve. So, we developed plasmids for the two genes (tlh and toxR) to use as controls in the mqPCR method. Plasmids were created using the TOPO-TA cloning kit from Invitrogen. Plasmids made with the genomic DNA (from cultured bacteria) are now routinely used for the standard curves in our test. The refined assay is now quantifiable down to 10 copies of plasmid DNA which is equated to 10 bacterial cells. Below this number, we determine the sample to be negative. Unknown samples were run and compared to the standard curve using plasmid copy numbers. Results were equated to the standards in order to determine how many copies of the gene are present. Standard curves using plasmids also were also run independently and as a duplex to ensure the same results once duplexed. All parameters for an optimized assay were achieved (Efficiency of 90-110% and R^2 values close to 0.990).

We have also optimized a multiplex for tlh, trh and tdh for Vp, have created plasmids for the trh and tdh genes and are using plasmids in the mqPCR assay. (Figure 2 and Figure 3). The primers and probe for Vp and pathogenic genes was described by Nordstrom et al (2007) and utilize detection of *tlh* gene; forward primer (*tlh*): ACTCAACACAAGAAGAGATCGACAA; reverse primer (*tlh*): GATGAGCGGTTGATGTCCAA; TaqMan probe (*tlh*): Texas Red flourophore CGCTCGCGTTCACGAAACCGT. The primers and probe for tdh; forward primer (tdh): TCCCTTTTCCTGCCCCC; Reverse primer (tdh): CGCTBCCATTGTTTTATC; TaqMan probe (tdh): FAM flourophore TGACATCCTACATGACTGTG. The primers and probe for trh; forward primer (trh): TTGCTTTCAGTTTGCTATTGGCT; Reverse primer (trh): TGTTTACCGTCATATAGGCGCTT; TaqMan probe (trh): TET fluorophore AGAAATACAACAATCAAAACTGA. The same thermal cycler protocol was used as describe above.

Evaluation of the **Activated Carbon Coated with Bentonite (ACCB)** method, as described in Wang and Levin, (2011) and Luan and Levin (2008), was used to treat oyster homogenate in order to remove potential inhibitors from oyster tissue before use in the mqPCR method to detect Vv and Vp. Activated carbon was coated with bentonite as previously described by Luan and Levin, (2008). In this process, 4.2g activated carbon (1-2mm particles) (Calgon Carbon) was washed with deionized water in a 400mL beaker until the drained water was clear. Then, 0.4g of bentonite (Fisher Scientific Cat# B235-500) was mixed with 200mL of deionized water and suspended by an Osterizer blender at high speed for 1 minute. The suspension was transferred to 250 mL bottles and centrifuged at 80 x g for 1 minute at room temperature. The supernatants were then transferred into a beaker along with the previously washed activated carbon, and placed onto a rotary shaker at 150 rpm at 37°C overnight. The next day, the beaker was placed at 55°C until the ACCB was dry. Drying time varied from one to three days.

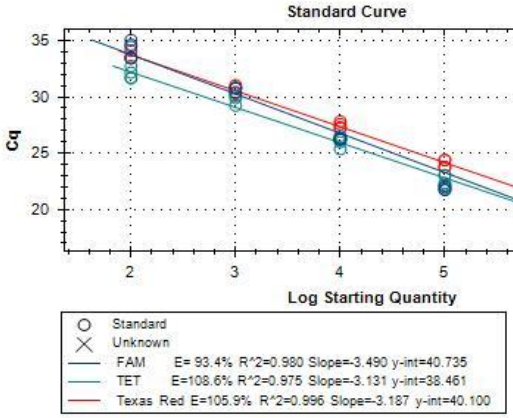

Fig. 2.

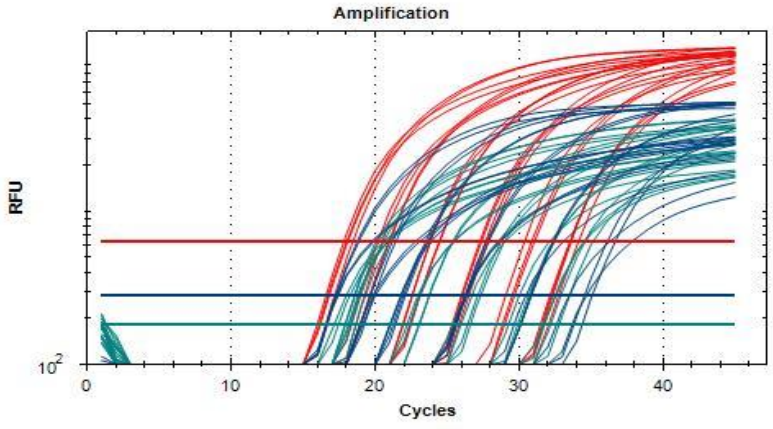

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Fig. 3

Figure 2 and Figure 3: Figures show the multiplex for the detection of total

	and pathogenic Vp. Based on the Nordstrom 2007 paper and optimized in the RWU ADL. Treatment of oyster homogenate was adapted from the methods previously described in Wang and Levin, (2011). Thirty grams of oyster homogenate was mixed with 300mL 1.5% saline solution and homogenized in an Osterizer blender for 3 minutes. A 100mL subsample of the homogenate and 1.5% saline solution was then transferred into sterile 250mL centrifuge bottles and centrifuged at 1000rpm (160 x g) for 10 minutes. The supernatants were collected and centrifuged at 10,000g for 10 minutes. The supernatants were discarded and the pellets re-suspended to a volume of 4.5mL with a 1.5% saline solution. This suspension was combined with the washed ACCB in 20.5mL of PBS (pH 7.5), and agitated at 160rpm for 15 minutes at room temperature. After agitation, the suspension of PBS, oyster pellet and saline solution was harvested from the activated carbon by decanting the liquid layer directly into a 50mL falcon tube. The bottom layer of activated carbon was discarded. Samples were stored at -80°C until downstream DNA extraction. After treatment with ACCB, the 30mL eludates were extracted as described previously for APW cultured (enriched) samples using the MO-BIO PowerFood® Microbial DNA Isolation Kit. A two sample t-test assuming equal variances was performed to compare the levels of detection of Vv and Vp between ACCB treated vs untreated oysters, 0.1g vs 0.25g direct extractions, 0.1g v ACCB treated oysters, and 0.25g v ACCB treated oysters. Problems: Detection of Vv and Vp increased when ACCB treatment of spiked oyster homogenate was compared to control (same method without ACCB absorption). Additionally, ACCB treated homogenate extracted using a MO-BIO Powerfood protocol, as compared to the TZ lysis method described by Luan and Levin (2008), also resulted in greater retrieval of Vp (10^8 as compared to 10^5 plasmid copies). Wang and Levin (2010) did demonstrates that there is oyster DNase activity occurring in oyster tissues, but our results show that although this DNase is present, it does not appear to interfere with our mqPCR. When spiked oyster homogenate is extracted directly using the MO-BIO PowerFood protocol. And, importantly, ACCB treated oysters homogenate did not have a higher sensitivity than untreated homogenate when both were extracted using the MO-BIO PowerFood Protocol. Coating the activated carbon with bentonite can take several days, when drying time is added to the procedure. Additionally, because we noted no difference in sensitivity between oyster homogenate treated with ACCB vs oyster homogenate not treated, when both were extracted with the MO BIO PowerFood protocol, **the use of ACCB is not is not a feasible diagnostic tool.**

Further evaluation of the mqPCR Vv and Vp detection method:

We determined that unenriched (not incubated in APW) samples do not contain sufficient Vp and Vv to detect reliably in the mqPCR methods since real levels of these bacteria are commonly very low in homogenates. We also determined a 5 hour incubation of oyster homogenate in APW media produced a sufficient growth to easily detect Vv and Vp in the mqPCR method. We saw viable detection of both Vp and Vv in the samples for 1g dilution samples (diluted in APW as described in the MPN method) in the 5 hour incubated tube. Unfortunately, while the 5 hour incubation is sufficient for evaluation of oyster homogenates, it does not provide a useable laboratory method due to workday schedules. So, we used a 20 hour enrichment of oyster homogenate in APW followed by extraction and compared those results to the MPN method in our work (Figure 4). Also, after evaluation of all dilation of several MPN evaluations, comparison of the dilution tubes results using our mqPCR showed that higher dilutions undergo percentage changes in the amount of one bacteria vs. the other and that this probably represents competition between these two bacteria (and any others in the tubes). This results in false positives as well as inaccurate results in the MPN and, if selecting the higher dilutions for extraction, identification of Vp and Vv in the mqPCR test method. We decided to use the 1g dilution (x3) as our standard enriched samples from which extractions for Vp and Vv and pathogenic gene determination would be made.

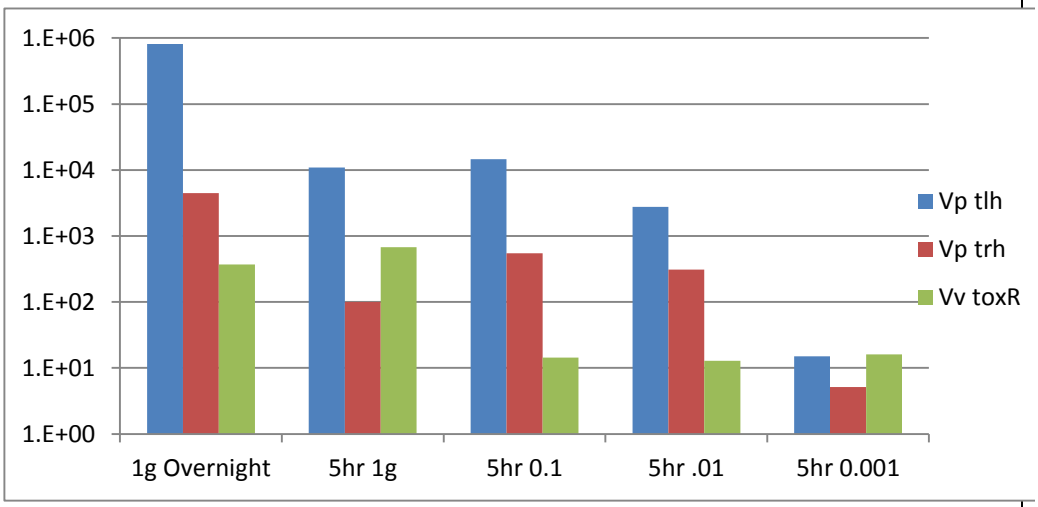

Figure 4. After 5 hours of enriching in APW media, viable detection of total Vp, pathogenic Vp (trh gene) and total Vv levels is easily accomplished using the qPCR.

B. Intensively monitor cultured oysters, water and sediment from two locations (one in RI and one in MA) over a year using the MPN/mqPCR and the ACCB/mqPCR method side by side in order to understand the Vv and Vp cycle in the northeast environment and compare sensitivity of both tests.

Because the ACCB method provided no advantage to the mqPCR test method, it was not used. Instead we compared the MPN method results to the mqPCR results extracted using the MO-BIO PowerFood® Microbial DNA Isolation Kit. Using the MPN methodology, cultured oysters from four locations in both RI and MA were tested in August of 2012 for assumed *Vibrio* sp. content (Figures 5 and 6). From this testing, one location in MA and one location in RI were chosen for monitoring starting in Aug/Sept of 2013, Monthly/bimonthly sampling began in the spring of 2014 and has continued through Fall of 2014 in the specific aquaculture farms in RI and MA. We conducted MPN evaluations of samples of composites of 10 oysters at each sample period (we also added control test tubes to the traditional MPN method to exclude any lab contamination error in findings). We began using the 20 hour enrichment of oyster homogenate in APW followed by extraction to eliminate sources of incubation error (not corrected for in the published MPN method).

Day	MPN	Vp	Vv	tlh	trh	tdh	% trh/ tlh	% tdh/ tlh
3/18	15.662	**0**	**1**	0	0	0	0.00	0.00
5/8*#	TMTD	**0**	**4**	0	0	0	0.00	0.00
6/11	73.993	**3016217**	**1**	8814583	259070	0	2.94	0.00
7/9	424.172	**75457**	**26**	205921	**927**	0	0.45	0.00
8/4***	919.07	**12027**	**12593**	53697	1067	0	1.99	0.00
9/2	230.343	**78472**	**382**	253289	844	0	0.33	0.00
10/1	424.172	**196489**	**307**	196489	14477	0	7.37	0.00

Fig. 5. RI sample results

Day	MPN	Vp	Vv	tlh	trh	tdh	% trh/ tlh	% tdh/ tlh
4/28	499.952	**1**	**0**	0	0	0	0.00	0.00
5/1*#	TMTD	**2**	**4**	0	0	0	0.00	0.00
6/11	42.392	**314460**	**1140**	740124	70249	0	9.49	0.00
6/29	91.783	**20184**	**48**	77307	1561	0	2.02	0.00
7/16	21454.97	**138681**	**5937**	99950	2161	0	2.16	0.00
7/31**	424.172	**45480**	**3935**	147904	1076	0	0.73	0.00
7/31	424.172	**43004**	**13974**	95858	858	0	0.90	0.00
8/24	91.783	**84635**	**7368**	289526	6033	20	2.08	0.01
9/8	1468.898	**225519**	**970**	615181	3293	88	0.54	0.01
9/23	230.343	**88009**	**4770**	463497	2732	53	0.59	0.01
10/7	91.783	**20950505**	**0**	missing	missing	missing		

Fig. 6. MA sample results.
TMTD = greater than 106850.57 cells/gram enriched oyster tissue
* High numbers of colonies cultured on CHROMagar and SWT culture plates with subsequent sequencing (URI Genomics Lab) identified as *Alcaligenes* sp. Other *Vibrio* sp. also identified, but no Vp or Vv.
# No Vp identified when evaluated on a PCR with Vp primers.
**Higher MPN dilutions (10^{-2} and 10^{-3}) showed high levels of *V. alginolyticus* dominating the cultures (as compared to lower MPN dilutions)

Finding show some general trends that need to be investigated further. First, when comparing the spring and fall MPN data to the mqPCR data, there are large discrepancies in results between MPN and mqPCR data (RI, 3/18 and 5/8; MA, 4/2 and 5/1). We examined these samples using additional evaluation methods (culturing on Chromagar and SWT media before and after enrichment) and by conduction PCR using Vp, Vv and Va primers on both post-enrichment extractions and on cultured colonies.

In several samples other bacteria were identified, sometimes in seemingly high proportion on the culture plates and in the dilutions of the MPN test. These findings indicate the nonspecific and poor quantifiability of the MPN method. Specifically as an example, after plating some of our diluted enriched oyster homogenates we found that there was a change in the predominate bacteria growing in the tubes. We found that the primary bacteria in the 5/1 MA and 5/8 RI samples causing the high MPN values (growth at moderate to high dilutions), was not Vp and Vv, but rather other bacterial species, esp. *Alcaligenes* sp. Additionally samples labeled 7/31 MA and 8/4 RI both showed high levels of *V. alginolyticus* dominating the higher dilutions as compared to undiluted and low dilutions. These findings reinforced early data identified in the development of the mqPCR which showed spiked homogenate samples developed higher Vv levels at the higher

dilutions as compared to lower dilutions where Vp predominated. The problem with the MPN method is an unequal growth of bacteria in the MPN tubes resulting from competition of the species in the tubes. The MPN method assumes that growth of bacterial in the media is selective for Vibrio and especially for Vp and Vv and that growth is not competitive between species, and other species do not proliferate well in the APW media. But that is not correct, thus resulting in an unreliable ability to predict levels of Vp and Vv in oysters using the MPN test.

Water samples were taken at each RI and MA sample time in triplicate. Each of the water samples were cleanly and sequentially filtered through three sizes of Millipore Nitrocellulose filters (0.8, 0.44 and 0.22 µm). Nucleic acids were later extracted from the filters using the MO-Bio Power Water™ DNA Isolation Kit following manufacturer protocols. The DNA was then quantified with the NanoDrop 2000c and was run in a duplex qPCR to detect Vp and Vv. Only a few water samples were positive for Vp and Vv in the mqPCR detection method (Figure 7). The inability to detect Vp and Vv in most of the water sampled probably reflects the low level of Vp/Vv in the water column or interference with detection by unknown factors. No filter pore size was more likely to retain Vp than any other which may be a function of particle size in the water column since Vp and Vv which are surfacing colonizing bacteria may attach to various sized particles.

MA Vp			**MA Vv**		
Filter size	Date	# of cells	Filter Size	Date	# of cells
0.8	6/11	0	0.22	4/28	1
0.8	6/11	0	0.22	4/28	14
0.8	6/11	32	0.22	4/28	0
0.22	9/8	11	0.22	6/11	30
0.22	9/8	17	0.22	6/11	30
0.22	9/8	186	0.22	6/11	0
0.45	9/8	21	0.45	8/24	0
0.45	9/8	11	0.45	8/24	30
0.45	9/8	30	0.45	8/24	0
0.22	9/23	13	0.22	9/8	11
0.22	9/23	36	0.22	9/8	17
0.22	9/23	15	0.22	9/8	186
0.8	9/23	0	0.45	9/8	21
0.8	9/23	0	0.45	9/8	11
0.8	9/23	179	0.45	9/8	30
0.22	10/7	13	0.22	9/23	13

0.22	10/7	5	0.22	9/23	36
0.22	10/7	5	0.22	9/23	15
0.45	10/7	14	0.45	9/23	5
0.45	10/7	10	0.45	9/23	6
0.45	10/7	4	0.45	9/23	6
			0.8	9/23	5
			0.8	9/23	6
			0.8	9/23	30
			0.45	10/7	1
			0.45	10/7	0
			0.45	10/7	13

RI Vp			**RI Vv**		
Filter Size	Date	# of cells	Filter Size	Date	# of cells
0.80	5/8	283	0.22	5/8	24
0.80	5/8	0	0.22	5/8	11
0.80	5/8	0	0.22	5/8	16
0.80	7/9	0	0.45	5/8	94
0.80	7/9	0	0.45	5/8	0
0.80	7/9	1748	0.45	5/8	3
0.80	9/2	1	0.80	5/8	113
0.80	9/2	0	0.80	5/8	1
0.80	9/2	31	0.80	5/8	3
			0.22	10/1	16
			0.22	10/1	7
			0.22	10/1	0
			0.45	10/1	15
			0.45	10/1	21
			0.45	10/1	7
			0.80	10/1	1
			0.80	10/1	18
			0.80	10/1	3

Figure 7. Positive water samples from MA and RI sample sites. Negative results were not included.

Sediment samples were taken at each RI and MA sample time in triplicate. Sediment samples were taken by using a 50 ml sterile conical tube and either removing a “plug” of sediment, then freezing and removing only the top 1/8 layer for extraction or by using the sterile conical and lightly and superficially

scooping sediment from the surface of undisturbed sediment. Sediments extracted with the MO-Bio Power Soil™ DNA Isolation Kit after sediment collection using 1.0g of sediment. DNA quantification was determined using the NanoDrop 2000c and was run in a duplex qPCR to detect Vp and Vv. All sediment samples were negative for Vp and Vv in the mqPCR method. It is probable that levels of Vp and Vv in the sediment were too low to detect in the mqPCR method or there was interference (we believe this is the less likely reason because spiking of sediments resulted in reliable detection).

Chlorophyll

Three one liter samples of the collected seawater were filtered through two 47mm, 0.45um synthetic fiber filters (Wilkem Scientific) in 500 mL increments. Three drops of 1% $MgCO_3$ was added to the seawater for each filter. For each sample, the filters were stored together in a desiccator at -20°C until processed at which time they were transferred to a 15 mL centrifuge tube where 15 mL of 90% acetone was added. After storing for 24 hours in the dark at 4°C the tube was centrifuged and decanted into a 1-cm path length spectrophotometer cuvette and measured at the following wavelengths: 750, 664, 647, and 630. The wavelengths were then corrected and the chlorophyll levels were formulated according to Parsons et al., (1984) (Fig. 8).

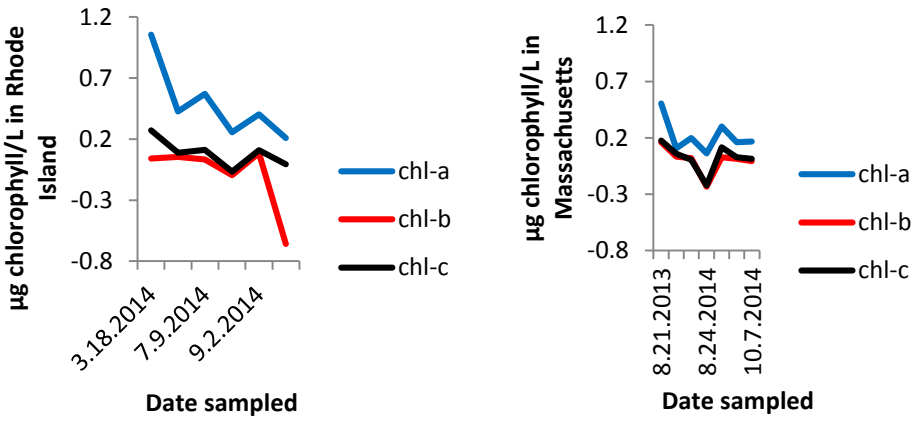

Fig. 8 Average µg chlorophyll a, b, and c levels per liter for sample sites in Rhode Island and MA.

Temperature and salinity at the two sample sites:

Temperature data was recorded using a StowAway TidbiT Temperature Data Logger (Fig. 9). The highest sea water temperatures exhibited in both Massachusetts and Rhode Island was during the months of July and August, 2014. The average temperature during these two months was 23.5°C and 24.4°C for Rhode Island and Massachusetts respectfully. A sharp decline in temperature occurred in both states at the end of August then the temperature

steadily decreases into the winter months with the next increase exhibited in late March or early April.

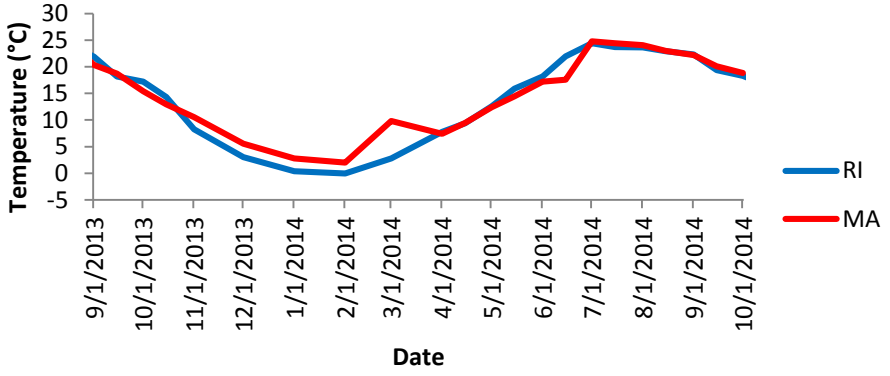

Figure 9. Average daily water temperature, in degrees Celsius, of collection sites in Rhode Island and Massachusetts.

Salinity

Salinities stayed relatively stable in the MA location, but varied someone in the RI location.

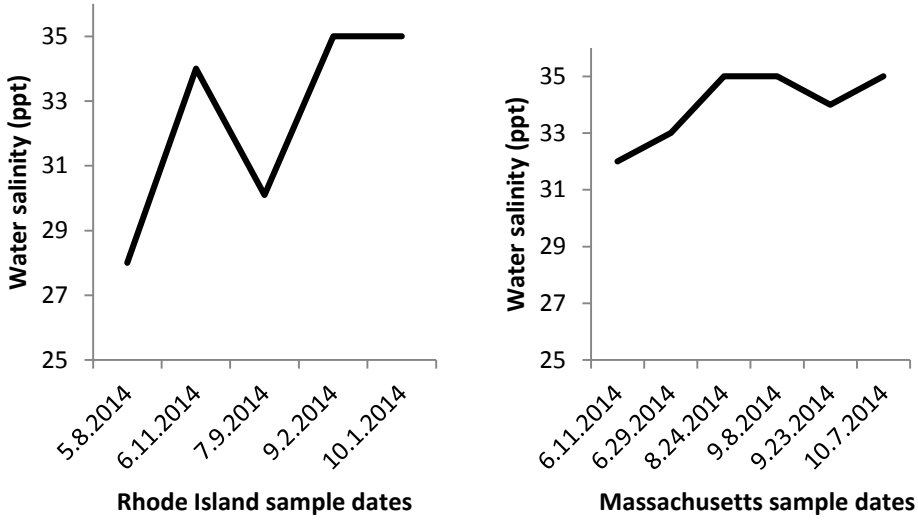

Figure 10. Water salinity, in ppt, of collection sites in Rhode Island and Massachusetts.

C. Identify the occurrence of pathogenic and non-pathogenic strains of *Vibrio sp*. in oysters

(1). at two time periods after collection of oysters from culturists who reside in five northeastern states

(2). from ten retail stores/restaurants in July/August. The source of

oysters (culturists from which animals were collected in 2.1.A. and 2.1.B.), and post-harvest handling (time between harvest and chilling and length of time chilled before sampling) will be identified as selection criteria.

(1). During July/August and again in Sept of 2014, we collected and processed oyster samples from our unfunded participates in NJ, NY, MD, and ME (Fig. 11). Additionally we collected a sample from a location in RI. As we found in previous site work, the MPN did not show consistent equivalency to the mqPCR method.

State	Date	MPN	Vp	Vv	tlh	trh	tdh	% trh	% tdh
RI	5/29	2	**25374**	**0**	106329	255	0	0.2	0.0
NY	6/4	92	**152968**	**0**	513529	0	0	0.0	0.0
NJ	6/10	21455	**472676**	**17584**	1416455	16664	17413	1.2	1.2
NJ	6/11	4408	**67154**	**6501**	230201	3258	4976	1.4	2.2
ME	6/25	42	**5446943**	**6**	16379822	761880	1088	4.7	0.0
NJ	8/26	424	**80723**	**29189**	337288	3779	2578	1.1	0.8
ME	9/3	230	**432030**	**896**	1999557	71734	565	3.6	0.0
MD	9/10	9322	**5**	**24032913**	566345	2005	166	0.4	0.0

Figure 11. Result of MPN and mqPCR evaluation of samples provided by other extension agents.

Temperature recorders (Onset Tidbits) were included in shipping boxes. All boxes were shipped overnight to the ADL. Animals were processed when received. The temperature of all samples exceeded 50° F for at least two hours in each of the shipments. In some shipments the temperature in the containers never went below 60° F. Two types of shipping containers were used. It appears that shipping samples in coolers is not as effective at maintaining temperatures as shipping in Styrofoam boxes within a cardboard box. The temperature data is not shown in this report. These findings point out the need for using a better insulated box for shipping and the need for precooling the oyster and the box before shipment. Results from this work did show a higher Vp and Vv in most of these samples as compared to findings in RI and MA.

(2). During the summer of 2014, we collected samples from restaurant settings. After consultation with extension agents, we changed the original methods somewhat to include multiple samples from each restaurant instead of one to two samples per restaurant. As a result we decreased the total number of restaurants sampled. The number and type of samples varied somewhat between each of the three locations, but the method was designed to help us to understand how the handling of oysters in different ways effects the Vp/Vv levels in the samples as they move from the harvest into the restaurant. We collected from four to 14 samples from each of the 3

scenarios/restaurants. Each sample was run in triplicates. Shown are averages of the triplicate data.

Restaurant one was a vertically integrated business (Fig. 12). Animals were cultured in an estuary close to the restaurant and were served in the restaurant. Treatments are describe in the chart (the 2nd day on the raw bar reflects an overnight storage in the walk-in refrigerator). Temperatures in the kitchen were cool on the day of the experiment.

Treatment	Date	MPN	Vp	Vv	tlh	trh	tdh	% trh/ tlh	% tdh/ tlh
Immediately place on ice after collection	7/15	919.07	**14031**	**6220**	**49211**	**1693**	44	3.44	0.09
1.5 hour delay before placed on ice	7/15	2310.77	**4323**	**3330**	**17063**	**1888**	41	11.07	0.24
Placed in walk-in overnight	7/16	424.17	**15438**	**14616**	54175	8471	28	15.64	0.05
After 3 hours on the raw bar	7/16	204.37	**777**	**41357**	7110	1588	8	22.33	0.11
After 3 hours in the kitchen	7/16	919.07	**226**	**2902**	1654	102	33	6.14	2.00
2nd day on raw bar	7/17	424.17	**8598**	**26837**	28868	3776	0	13.08	0.00

Figure 12. Results of MPN and mqPCR evaluation of samples for restaurant one.

Restaurant 2

This scenario represented the collection of animals from a lease that was delivery on Ice to an unrelated restaurant. All samples were place in a walk-in refrigerator on arrival. Then, approximately 3 hours later, one sample was placed on ice in a cooler by the raw bar, one sample was on the raw bar, one sample was place on the kitchen table for 4 hours and one sample remained in the refrigerator for the 4 hour duration. After 4 hours, all samples were placed back in the walk-in refrigerator then transferred to the lab on ice and processed the next day (Fig. 13). Temperatures in the kitchen were very hot during the time animals were on the table.

Treatment	M/D	MPN	Vp	Vv	tlh	trh	tdh	% trh/ tlh	% tdh/ tlh

placed in walk in frig upon arrival	8/7	73.99	285171	448	603	712	714	118	118.44
place in cooler, on ice, beside the raw bar for 4 hours	8/7	2310.77	**3.54**	**1.20**	**0.00**	**0.00**	**0.00**	0.00	**0.00**
on raw bar for 4 hours	8/7	424.17	**50.50**	**0.00**	**0.00**	**0.00**	**0.00**	0.00	**0.00**
placed on table surface in kitchen	8/7	106850	**4.28**	**4.95**	**0.00**	**0.00**	**0.00**	0.00	**0.00**

Figure 13. Results of MPN and mqPCR evaluation of samples for restaurant 2.

Interesting highlights are first, that the animals held in the kitchen showed a high MPN. However, the Vp and Vv abundance as low when run in the mqPCR. Later investigations showed this high MPN was not caused by Vp or Vv. Instead abundant *Vibrio alginolyticus* was identified as the probable cause of the high MPN using PCR identification. This finding shows ambient temperature is very important.

Restaurant 3

This scenario actually encompassed 5 treatments using animals from the same initial aquaculture farm and included the following sample locations: farm, wholesaler, transport, restaurant and cleaning (Fig. 14). Descriptions for each treatment are as listed in Fig 15.

Treatment	M/D	MPN	Vp	Vv	tlh	trh	tdh	% trh/ tlh	% tdh/ tlh
At Farm "A"	8/14	741	**2803**	**74**	12552	1061	1573	8.46	0.07
At Farm "B"	8/15	424	**34208**	**957**	198299	3424	1958	1.73	0.00
At Farm "C"	8/15	919	**6881**	**40**	40364	465	1	1.15	0.00
Wholesale "A"	8/20	92	**8371**	**27**	40229	54	0	0.13	0.00
Wholesale "B"	8/20	92	**95616**	**0**	479491	5409	0	1.13	0.00
Wholesale "C"	8/20	42	**10578**	**747**	60820	349	38	0.57	0.00
Wholesale "D"	8/20	147	**47758**	**815**	280932	19945	0	7.10	0.00
Transport "A"	8/21	230	**2144**	**8**	11617	61	0	0.53	0.00
Transport "B"	8/21	4269	**27144**	**304**	140742	8812	0	6.26	0.00
At Restaurant "A"	8/26	92	**50202**	**4477**	213041	5369	0	2.52	0.00
At Restaurant "B"	8/26	92	**6754**	**283**	40368	876	0	2.17	0.01
At Restaurant "C"	8/26	919	**65730**	**2674**	313334	2194	678	0.70	0.00
At Restaurant "D"	8/27	42	**88824**	**1838**	422635	4753	0	1.12	0.00
Cleaning group A (immed. on ice)	9/8	42	**17780**	**1026**	84817	720	0	0.85	0.00
Cleaning group B (cleaned)	9/8	23926	**8033**	**69**	46538	3347	0	7.19	0.02

Figure 14. Results of MPN and mqPCR evaluation of samples for restaurant 3.

Treatment	
At Farm "A"	immediately iced upon collection from the water
At Farm "B"	held overnight on ice after collected
At Farm "C"	held overnight on ice then 2 hours in sun un-iced before processing
Wholesale "A"	held on ice and in fridge immediately after collected till processed
Wholesale "B"	held on ice during grading of other oysters, beside animals being graded
Wholesale "C"	not placed on ice, set beside grading table, beside animals being graded
Wholesale "D"	part of group of oysters being graded
Transport "A"	on ice during trip to restaurant
Transport "B"	not iced during trip to restaurant, kept at 60-70°F, left in at room temp overnight before processing
At Restaurant "A"	kept in cooler while "B" & "C" were undergoing treatments
At Restaurant "B"	placed beside raw bar for 3 hours then on ice
At Restaurant "C"	on kitchen counter for 3 hours then on ice
At Restaurant "D"	placed beside rare bar for 3hrs then in fridge for 12-15 hrs and then back on raw bar for 3 hrs before collected
Cleaning group A	removed from group to be cleaned and put back on ice in fridge
Cleaning group B	after cleaning, first cleaned and then returned to ice.

Fig. 15. Explanations of treatment for the restaurant 3 scenario.

Interesting highlights are that the Vp and Vv levels do not correlate well with the MPN data. Combined with previous work described above, it appears that the MPN data may not be accurate in the assessment of Vp and Vv in a sample of animals.

At the wholesale facility: The MPN cell/g abundance increased during handling. This was reflected in the Vp and Vv qPCR values. Interestingly there was a marked increase in the trh gene in samples handled, but kept on ice. None of these samples were evaluated for occurrence of other organisms in the cultures, but it is possible, based on other findings, that the Vp/Vv may have been outcompeted by other organisms in the C sample thus resulting in

	low Vp/Vv for that sample or that handling followed by icing may result in increased numbers of bacteria carrying pathogenic genes. The transport scenario showed at least a log increase in the MPN level and in the VP and Vv levels. Additionally the abundance of the trh gene increased in animals exposed to room temperatures (Transport B). The restaurant scenario MPN data did not provide marked differences in results. The Cleaning treatment showed a higher MPN value for the cleaned group but did not show an increase in Vp/Vv organisms in this group indicating other organisms may have caused the increase in MPN. However, there was a marked increase in occurrence of the trh gene in the Vp organisms present in the cleaned animals. References: Cox, A. M., and Gomez-Chiarri, M. 2012. *Vibrio parahaemolyticus* in Rhode Island Coastal Ponds and the Estuarine Environment of Narragansett Bay. Applied and Environmental Microbiology 78(8): 2996-2999. Luan, C., R.E. Levin. 2008. Use of activated carbon coated with bentonite for increasing the sensitivity of pcr detection of *Escherichia coli* O157:H7 in Canadian oyster (*Crassostrea gigas*) tissue. J. Microbio Methods 72:67-72. Nordstrom, J. L., Vickery, M. C. L., Blackstone, G. M., Murray, S. L., and DePaola, A. 2007. Development of a Multiplex Real-Time PCR Assay with an Internal Amplification Control for the Detection of Total and Pathogenic *Vibrio parahaemolyticus* Bacteria in Oysters. Applied and Environmental Microbiology 73(18): 5840-5847. Wang, S. and R.E. Levin. 2011. Interference of Real-time PCR Quantification of *Vibrio vulnificus* by a Novel DNase from the Eastern Oyster (*Crassostrea virginica*). Food Biotech. 24: 121-134

Accomplishments:

Outreach Overview	Describe in general how your results have been extended to the intended users. OR, if they haven't yet, explain when & how this will occur. We have provided MPN data to all aquaculturists involved thus helping them to understand how their handling is affecting the animals and what the MPN levels are in their animals both out of the water and during different handling scenarios and thus allowing for development of better handling methods within their situations. We are currently working with Barnstable Extension and the Dept. of

	Marine Fisheries in MA to identify levels of Vp and Vv in samples in MA waters using both the MPN and qPCR methods.
Targeted Audiences	Provide information on the **target audience** for efforts designed to **cause a change in knowledge, actions, or conditions**. We have presented methods developed and information comparing the MPN method to our mqPCR information at several meeting where lab personnel who are interested in diagnostic method development are present and were both regulators and aquaculturists who will use the information to develop better handling methods are in attendance. Additionally, we have had several conversations with people who work in these different capacities promoting the use of the qPCR methods and the inexactness of the MPN method.
Outputs:	Outputs are tangible, measurable products (website, events, workshops, products [AV, curricula, models, software, technology, methods, websites, patents, etc.], trainees, etc.). Do NOT include publications as they're listed separately. Our laboratory now provides the mqPCR diagnostic test for a fee and we will provide a summary of findings on our website after publication has occurred. We will provide methods to other laboratories and share data/findings for use in developing post-harvest handling methods to regulators and extension agents.
Outcomes /Impacts:	Describe how findings, results, techniques, or other products that were developed or extended from the project generated or contributed to an outcome/impact. **Outcomes/impacts are defined as changes in Knowledge, Action, or Condition.** These study shows that the MPN method of Vp and Vv evaluation is neither specific nor sensitive and is not helpful in determining the status of oysters in a bay. Rather, we need to examine the abundance of these organisms with standardized mqPCR test. Our work began that process both in identifying the level of Vp and Vv in oyster homogenates and in developing an mqPCR test for 2 important pathogenic genes. Continued refinement, and development of controls are needed and we will continue to work on the method in the next few years. Further, these findings show the abundance of Vp and Vv in the environment in the northeast U.S. is very low most of the year (unlike the southern states).
Impacts Summary	Provide short statements (2-3 sentences) about each of the following: (pre-established fields for Researchers to complete short statement answers) 1. **Relevance:** Issue – what was the problem? The levels of Vp and Vv in oyster samples is not accurately determined using the currently approved MPN method and the levels of Vp and Vv in the northeast are different on an annual basis from levels in the southeast. 2. **Response:** What was done? We designed two mqPCR methods; one to detect Vp and Vv and one to detect two known pathogenic genes in oyster homogenates. We compared finding to MPN levels in the same homogenates. We determined the level of Vp and Vv in oysters from two location over the

	course of a season and compared results to samples from other regions in the northeast. We looked at the effects of handling on oysters destined to be a raw food product. **Results:** How did your work make a difference **(change in knowledge, actions, or conditions)** to the target audiences? We have shown the MPN method is neither sensitive nor specific and actually may be a very poor predictor of Vp and Vv abundance in an oyster sample. We have developed mqPCR methods for both Vp and Vv, and pathogenic genes for Vp, that can be used in laboratories for determination of levels in the same oyster homogenates as would be used for the MPN method, but that would provide accurate data vs the MPN method. We have shown that the natural levels of Vp and Vv in oysters, sediment and water column is very low during most of the year in the northeast and that handling and shipping has significant effects on the levels of Vp and Vv and pathogenic genes in oysters post-harvest. 3. **Recap:** One- sentence summary Multiplex quantitative PCR methods are significantly more accurate in determining Vibrio parahaemolyticus and Vibrio vulnificus and pathogenic gene abundance in oysters than the Most Probably Number Methods, that levels of Vp and Vv in northeast oysters is lower than southeast oysters and that handling methods significantly affect the proliferation of Vp and Vv in oysters.
Publications	Follow the format to list publications in the following categories: **Oral Presentation:** Smolowitz, R. *Vibrio* vs. the eastern oyster. Am Vet. Med. Assoc. San Diego, CA, August, 2012. Smolowitz, R. Identification of *Vibrio* sp. abundance in cultured oyster from northeast U.S. farms. 34Th Milford Aquaculture Seminar, Feb. 24-26, 2014 Markey, K. and R. Smolowitz, Variation in data: the importance of sample preparation and processing protocol of oyster homogenates for the detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Rhode Island and Massachusetts sample. 34Th Milford Aquaculture Seminar, Feb. 24-26, 2014 Smolowitz, R., D. Murphy, J. Reitsma and K. Markey. Identification of *Vibrio* sp. abundance in cultured oyster from northeast U.S. farms. Presented to Massachusetts Aquaculture Association Annual meeting. March, 2014. Markey, K. and R. Smolowitz. Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Rhode Island and Massachusetts oyster homogenates pre- and post-enrichment in alkaline peptone water: variation in data generation based on sample processing protocol. 106th Annual Meeting, National Shellfisheries Association, Jacksonville, FL, April 3, 2014. **Poster Presentations:** Jaillet, W., K. Markey and R. Smolowitz. Development of a new bentonite based quantitative PCR test method to detect *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oyster tissues. 106th Annual Meeting, National Shellfisheries

	Association, Jacksonville, FL, April 3, 2014 Brown, A., W. Jaillet, K. Markey and R. Smolowitz. Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oyster tissue, sediment, and water samples, and their correlation to chlorophyll levels, throughout Rhode Island and Massachusetts, 107th Annual meeting, NSA, Monterey Bay, CA, March 22, 2015.
Students/ Participants:	Provide the following information for **every** student that worked with you during the reporting period: • Name: • Whether Degree was completed during the reporting period (name, yes/no): • New or Continuing Student: • Capstone/Thesis Title (actual or anticipated): • Date of Graduation: • Provide link to thesis/dissertation document: **Undergraduate Students at RWU** 1. Whitney Jaillet, BS, continuing student, Undergraduate Senior Thesis: Development of a new bentonite based quantitative PCR test method to detect *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oyster tissues; May,2014; no link available. 2. Alexandra Brown, BS, continuing student, Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oyster tissue, sediment, and water samples, and their correlation to chlorophyll levels, throughout Rhode Island and Massachusetts, May, 2014, no link available 3. Chris Materna, no degree currently, continuing student, participating in collecting and processing of samples in the lab, will graduate in May, 2016. 4. Mary Agnew, no degree currently, continuing student, participating in collecting and processing of samples in the lab, will graduate in 2017. 5. Ashley Powell, BS, continuing student, participating in collecting and processing of samples in the lab, May 2015. 6. Catherine Grimm, BS, continuing student, participating in collecting and processing of samples in the lab, graduated in May 2014. 7. Jill Hamlin, BS, new student, participating in collecting and processing of samples in the lab, graduated in May 2015.
Partnerships	List any partners that you worked with on your project. Provide the following information for each Partner:

Partner	**Specific Type**	**Level**	**Nature of Partnership**
1. Aquacultures from one location in RI and one in MA (these names and specific locations will not be provided for reasons	Participant in	Active	

	describe in the grant). 2. Restaurants/aquaculturists (3 different groups/companies; one in RI and two in MA). These partners provided their time and energy to help us develop appropriate restaurant scenarios for their locations/types of growing, harvesting and serving activities in both RI and MA. They then provided access to their facilities and provided oyster samples when conducting the exposure and sampling work. 3. Barnstable County Extension Service (D. Murphy and J. Reitsma)	restaurant and field studies Provided design and sample collection help	Active	Provided samples and helped develop restaurant plans Helped develop restaurant plans and helped in collection of animals