Department of Biological Sciences

P.O. Box 2021
Thibodaux, LA 70310
985.448.4700 or 448.4872
Fax: 493.2496

NICHOLLS
STATE UNIVERSITY
A MEMBER OF THE UNIVERSITY OF LOUISIANA SYSTEM

May 15, 2012

Ken Moore, Director
Interstate Shellfish Sanitation Conference
209-2 Dawson Road
Columbia, SC 29223

Dear Mr. Moore,

Attached is the final report for our project "Two Log Reduction of *Vibrio parahaemolyticus* in Gold Band Oyster Products Using Hydrostatic High Pressure Post Harvest Processing."

Thank you for your support and for this opportunity to test the "two log reduction method for *V. p*. It is an extremely expensive and time consuming protocol, but we were successfully able to validate Motivatit Seafoods, Inc.'s State of LA, State of CA and FDA approved Gold Band oyster protocol using hydrostatic high pressure (HHP) post harvest treatment with their Plant 2's Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor to effect a two (2) log reduction of naturally occurring *Vibrio parahaemolyticus* in commercially harvested and processed Louisiana oysters.

I apologize for the delay, but we had several other research projects that required work at the same time and limited resources of trained students to be able to do this work. We also were required to purchase a number of required certifications of our current instruments and buy several FDA approved thermometers. Additionally, we were running the FDA Proficiency tests at the same time and had to have the *V. parahaemolyticus* TLH -AP gene probe replaced by DNA Technology after we tried it a couple of times because as they emailed me eventually, "*It does not have a high affinity for coupling to the AP enzyme.*" They sent us another probe, but it had to be constructed and is shipped all the way from Denmark.

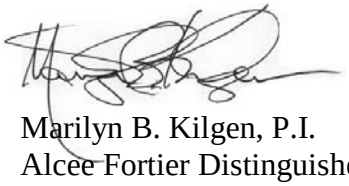
The good news is that after the certified Gold Band Oyster HHP -PHT at Motivatit Seafoods, Inc. we did not have a single positive *V. parahaemolyticus* tube in all of the MPN tubes required for all three Lots of samples.

Thank you and the ISSC for your patience. Universities are not designed to do full-time commercial laboratory work. We mainly are engaged in research for our graduate students theses, and this type of lab work is difficult and time consuming, but it cannot be used for thesis research since it does not involve a new discovery.

I hope this work will help the ISSC and the oyster industry.

Please do not hesitate to call me if you need further information.

Sincerely,



Marilyn B. Kilgen, P.I.
Alcee Fortier Distinguished Service Professor and Director of the Institute for Seafood Studies
Department of Biological Sciences
Nicholls State University
Thibodaux, LA 70310

Office Phone: 985-448-4701
Cell Phone: 985-803-6866
FAX: 985-493-2496
Email: marilyn.kilgen@nicholls.edu

cc: Mike Voisin, Industry Co-PI, CEO, Motivatit Seafoods, Inc.
412 Palm Ave.
P.O. Box 3916
Houma, LA, 70361-3916

Institute for Seafood Studies

NICHOLLS STATE UNIVERSITY

Department of Biological Sciences
P.O. Box 2021
Phone: 985-448-4701
Fax: 985-493-2496

FINAL REPORT TO THE

ISSC

Interstate Shellfish Sanitation Conference

Two Log Reduction of *Vibrio parahaemolyticus* in Gold Band ® Oyster Products Using Hydrostatic High Pressure Post Harvest Processing

Marilyn B. Kilgen, Ph.D.
Alcee Fortier Distinguished Service Professor
Department of Biological Sciences
Nicholls State University

Angela L. Corbin, M.S.
Assistant Professor of Biological Sciences
Department of Biological Sciences
Nicholls State University

Stacy Martinez
M.S. Candidate
Department of Biological Sciences
Nicholls State University

Mike Voisin, CEO
Motivatit Seafoods, Inc.
PO Box 3916
Houma, LA 70361

May 15, 2012

1. EXECUTIVE SUMMARY

This study was proposed in response to the FDA *Vibrio parahaemolyticus* Control Plan Guidance in the NSSP 2009a Section IV Chapter IV. Naturally Occurring Pathogens, Section .03 *Vibrio parahaemolyticus* Interim Control Plan .03 *Vibrio parahaemolyticus* Control Plan Guidance B. Control Measures 1. Post Harvest Processing (PHP) (NSSP 2009a). In part II.B. (Control Measures) of this plan the list of control measures include 1. post harvest processing (PHP) as one of (7) seven control measures listed. Some other measures include closing the area to oyster harvest; restricting oyster harvest to product labeled "For Cooking Only"; limit time from harvest to refrigeration to no more than five (5) hours or other times based on modeling and sampling in consultation with FDA; limit time from harvest to refrigeration such that levels of total *Vp* after completion of cooling to 60 °F do not increase more than 0.75 log from levels at harvest; or other control measures based on appropriate scientific studies. Of these (7) seven control measures, PHP is the only measure that if validated will provide a certified oyster product for interstate commerce with approval of States, ISSC and FDA. However, there is the problem of having no FDA BAM media that is selective and differential for *V. parahaemolyticus* as there is mCPC for *V. vulnificus* (Oliver et al. 1992). The use of a multiplex qPCR does not require the isolation of the organisms on a selective or differential media. They can be identified in single MPN tubes or single oyster tissues. In our laboratory we have identified both *V. parahaemolyticus* and *V. vulnificus* in MPN alkaline peptone water (APW) cultures inoculated with a full gram of oyster tissue using the American Biosystems, Inc. 7500 Fast qPCR with its Vibrio multiplex kit for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*. We have not isolated *V. cholerae* with this extremely rapid and sensitive method. Unfortunately, however the Applied Biosystems Fast 7500 qPCR instrument and Vibrio Multiplex Kit which can identify these three important *Vibrio* species simultaneously in mixed samples in about 30 minutes is not yet approved by FDA or the ISSC.

For this reason, the recently improved DNA ***tlh*** (thermolabile hemolysin) alkaline phosphatase (AP) gene probe (5'Xaa agc gga tta tgc aga agc act g 3') where X is the AP label (McCarthy et al. 1999) was used for this project. This NSSP approved method for confirmation of *V. parahaemolyticus* following enrichment in MPN/g alkaline peptone water (APW) medium per the Bacteriological Analytical Manual (BAM) protocol for sample preparation of shellstock for *Vibrio* species analysis (FDA 2007) was used to confirm the validation of 2 log reduction of *V. parahaemolyticus* in Gulf of Mexico oysters (NSSP 2009b) post harvest treated with hydrostatic high pressure (HHP) using the Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor at Motivatit Seafoods, Inc., Houma, LA. Using this FDA protocol and the approved Motivatit Seafoods, Inc., post harvest treatment of hydrostatic high pressure for post harvest treatment of shellstock Gold Band® oysters, the 2 log reduction was achieved with "0" positive MPN tubes of oyster tissue in all of the HHP treated samples in three separate Lots of commercially harvested and processed oysters.

2. SCOPE, APPROACH AND METHODOLOGY

The *Vibrio parahaemolyticus* NSSP Model Ordinance newly approved 2 log reduction method (NSSP 2009b) was used to confirm the validation of reduction of *V. parahaemolyticus* to NSSP acceptable levels in oysters post harvest treated with hydrostatic high pressure (HHP) using the

Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor at Motivatit Seafoods, Inc., Houma, LA. Specifically, this assay was used for confirmation of MPN/g *V. parahaemolyticus* following enrichment in alkaline peptone water (APW) as per the Bacteriological Analytical Manual (BAM) protocol for sample preparation of shellstock for *Vibrio* species analysis (FDA 2007).

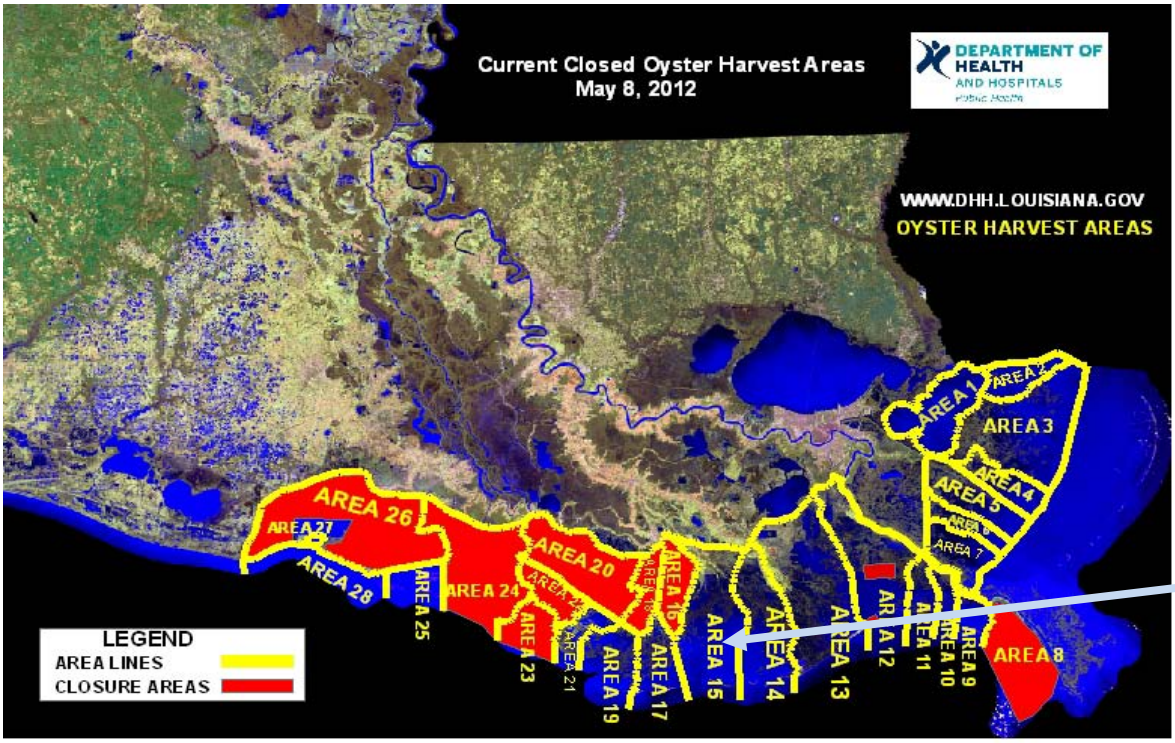
This proposed post harvest treatment (PHT) valildation was in collaboration with Motivatit Seafoods, Inc., Houma, LA using their Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor to reduce *Vibrio parahaemolyticus* in Gold Band oyster products by 2 logs according to the National Shellfish Sanitation Program's Sec II. Model Ordinance Chapter II Risk Assessment and Risk Management, @.05 *Vibrio parahaemolyticus* Control Plan (4) (b) (i) Post harvest processing using a process that has been validated to achieve a 2 log reduction in the levels of total *Vibrio parahaemolyticus* for Gulf and Atlantic Coast oysters, National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2009. (NSSP 2009b).

METHODOLOGY

Three separate commercially harvested Lots of shellstock oysters for this project were all obtained from a saltier area of the Terrebonne National Estuary in Louisiana's oyster harvesting Area 15 south of Montegut, LA. in LA Area 15. See Figure 1 from the LA Department of Health and Hospitals, Office of Public Health, Oyster Monitoring Program. http://new.dhh.louisiana.gov/assets/images/NewsReleases/NOROVIRUS582012.jpg

This is the most recent map of the LA oyster harvesting areas showing oyster harvest closures after a recent Norovirus outbreak in oyster harvesting areas to the west and northwest of Area 15.

Figure 1. Area 15 South of Montegut, LA



Saltier oyster harvest waters were specifically requested in the winter harvest to have a minimal number of *Vibrio vulnificus* and to be able to isolate mainly *Vibrio parahaemolyticus*, which survives cold water of the Gulf in the winter and also can live in the saltier waters of the estuaries.

The oysters were commercially harvested as three separate "Lots" on three separate weeks in January and transported in a commercial refrigerated truck and delivered to Motivatit Seafoods, Inc., the oysters were unloaded and one sack of oysters was held in a 10^{O}C cold room at Motivatit overnight for preservation of the *V. parahaemolyticus*.

OYSTER PREPARATION METHODS

Sample Collection, Preservation and Storage Requirements:

Sample collection, preservation, preparation, storage, and clean up followed procedures currently described by NSSP, as cited in the BAM (FDA 2007), for validation of oyster PHP using MPN analysis of *V. parahaemolyticus* in control and HHP-PHT sample oysters for each of the 3 Lots as directed in the NSSP, 2009b, Sec II. Model Ordinance Chapter II Risk Assessment and Risk Management, @.05 *Vibrio parahaemolyticus* Control Plan (4) (b) (i) Post harvest processing using a process that has been validated to achieve a 2 log reduction in the levels of total *Vibrio parahaemolyticus* for Gulf and Atlantic Coast oysters. National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2009.
http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/UCM046413

The oysters were commercially harvested as three separate "Lots" on three separate weeks in January, 2012 and transported in a commercial refrigerated truck. At Motivatit Seafoods, Inc., the oysters were unloaded and one sack of oysters was held in a cold room at Motivatit at 10^{O}C (50^{O}F) overnight to preserve the *V. parahaemolyticus* which is more cold tolerant than *V. vulnificus.*

A. Two (2) Log Reduction Process Validation

The newly approved protocol for the NSSP Model Ordinance 2 Log reduction in the levels of total *Vibrio parahaemolyticus* for Gulf and Atlantic Coast oysters according to the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2009b was used for validation of Vp 2 log reduction by HHP-PHT.

B. Equipment Validation

Motivatit Seafood's Inc. has two hydrostatic high pressure post harvest treatment process instruments that are validated by the State of Louisiana's DHH-OPH, Oyster Monitoring Program, the State of California's Health Department and the FDA for reducing *V. vulnificus*

from 10^5 MPN/g levels to <3 MPN/g. They have been maintaining quarterly verification of their HHP equipment to <3MPN/g for *V. vulnificus* for many years. Their new Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor in their Plant 2 was used for this Vp 2 log reduction PHT verification project.

C. **Initial Load Testing (from FDA 2007 and NSSP 2009b)**

Procedures and methods recommended by the FDA Bacteriological Analytical Manual (BAM) for the examination of shellfish (FDA 2007) and specifically for the validation of a PHT (HHP) 2 log reduction of MPN/g *V. parahaemolyticus* by the NSSP (2009b) were used.

Table 1 below shows the dates for the Initial Load Testing for each of the 3 Lots of oysters over a period of 3 weeks in January, 2012. For each initial load testing, 12 oysters were randomly selected from the sack of commercially harvested oysters from Area 15 that was held overnight at 10^OC (50^OF) in a cold room. Ten samples of 12 oysters each were also Gold Band® processed by HHP-PHT and then stored in a commercial cooler at 1.7^OC (10^OF) at Motivatit until the initial number of *V. parahaemolyticus* in the Control sample was determined. This determination of initial loads of *V. parahaemolyticus* in the oysters took about 1 week of time. The 12 Control oysters for the initial load testing for each Lot were shucked commercially at Motivatit into a sanitized plastic 12 oz container for shucked oysters according to the BAM (FDA 2007) and delivered the same day to Nicholls State University's Environmental and Public Health Microbiology Laboratory in 208 Gouaux Hall, Thibodaux, LA. Each of the 3 Lots of Control shucked oysters were analyzed for *V. parahaemolyticus* according to the methods and protocols found in the BAM (FDA 2007) and the ISSC 2009b protocols at Nicholls State University

The required 10 HHP-PHT samples of 12 oysters each from each of the three Lots were also processed at Motivatit Seafoods, Inc. the same day as their Control sample was processed at Nicholls State University. They were post harvest treated with HHP in Motivatit's Plant 2 with their Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor. See Appendix 1 for the Results of both the Control sample of 12 oysters for each Lot and the HHP-PHT 10 samples of 12 oysters each from each Lot. The 10 samples of HHP-PHT oysters were stored in Motivatit's commercial cooler at 1.7^OC (35^OF) for approximately 1 week before being transported on frozen gel packs to the Nicholls State University's Environmental and Public Health Microbiology Laboratory in 208 Gouaux Hall, Thibodaux, LA. See Table 1 below for the exact Dates of harvest and processing of each of the three Lots' Control oyster sample and their corresponding 10 HHP-PHT oyster samples.

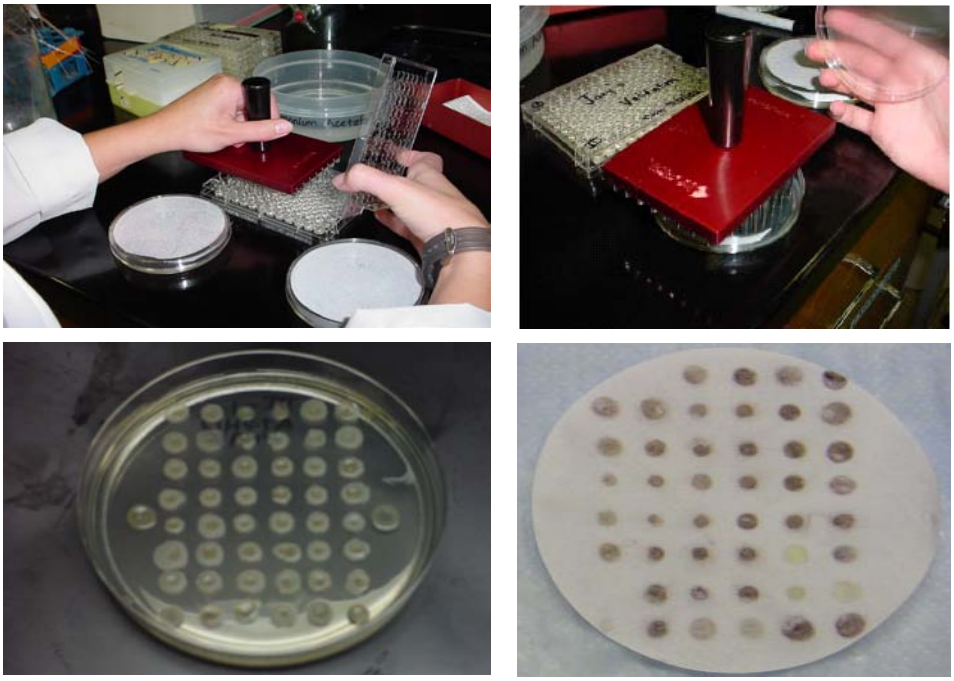
Table 1. Dates of Harvest and Processing of Control and HHP-PHT Oyster Samples

Oyster Control and HHP-PHT Lot Numbers	Date Harvested	Date Processed at Motivatit	Date Processed at Nicholls State
Lot # 1 Control Sample	1/15/12	1/17/12	1/17/12
Lot # 1 HHP-PHT Samples	1/15/12	1/17/12	1/24/12 - 1/26/12
Lot # 2 Control Sample	1/22/12	1/24/12	1/24/12
Lot # 2 HHP-PHT Samples	1/22/12	1/24/12	2/02/12 - 2/6/12
Lot # 3 Control Sample	1/30/12	1/31/12	1/31/12
Lot # 3 HHP-PHT Samples	1/30/12	1/31/12	2/8/12 - 2/13/12

Samples were all run according to the protocols and methods of the FDA BAM (2007) and the protocols of the ISSC 2009b Vp 2 log reduction PHT Validation.

Samples were enriched in alkaline peptone water (APW) at 35^{O}C overnight and then streaked for colony isolation on Thiosulfate-Citrate-Bile-Sucrose (TCBS) *Vibrio* agar and incubated overnight at 37^{O}C to isolate presumptive *Vibrio parahaemolyticus* colonies. The green colony isolates from TCBS were picked with sterile applicator sticks or toothpicks and isolated into alkaline peptone water (APW) in 96 well plates at 35^{O}C overnight. The 96 well plates were then replicated to T1N3 (BAM, FDA 2007) Agar using a 48 prong replicator. The colonies can be blotted for the alkaline phosphatase (AP) gene probe hybridization identification using DNA Technology's *V. parahaemolyticus tlh* gene probe - 5'Xaa agc gga tta tgc aga agc act g 3' where X is the AP-label (McCarthy et al. 1999). This alkaline phosphatase gene probe hybridization method is also found in the FDA BAM (FDA 2007). See Figure 2 below for the procedure to replicate green presumptive *Vibrio parahaemolyticus* isolates from 96 well plates onto T_1N_3 agar (has 3% NaCl) with a 48 prong replicator. After the isolates have grown at 35^{O}C overnight they are blotted onto Whatman 541 filter paper and processed for the *tlh* AP gene probe according to protocols found in the FDA BAM 2007 for the confirmation of *V. parahaemolyticus*. Positive V.p. *tlh* colony blots are a reddish brown color. Negative blots are pale yellow. See Figure 2 below.

Figure 2. Replicating Presumptive *Vibrio parahaemolyticus* isolates from APW in 96 well plates to T1N3 Agar Plates and Final Vp *tlh*-AP Blot Results*



***** *These pictures are from a previous validation, but the same procedure was used for this project.*

3. PROJECT DELIVERABLES (RESULTS - TABLE 1 IN APPENDIX)

Motivatit Seafoods, Inc.'s Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor validated the required 2 log reduction of *Vibrio parahaemolyticus* in their Gold Band® oyster products according to the approved Sec. II. Model Ordinance Chapter II Risk Assessment and Risk Management @.05 *Vibrio parahaemolyticus* Control Plan (4) (b) (i) Post harvest processing using a process that has been validated to achieve a 2 log reduction in the levels of total *Vibrio parahaemolyticus* for Gulf and Atlantic Coast oysters (NSSP 2009b).

See Appendix 1 for the results of the Lots # 1, 2 and 3 Samples of Control non-processed oysters for the calculation of the initial load of *V. parahaemolyticus* in the oysters and the 2 log Vp reduction results of the HHP-PHT oysters for each Lot.

4. PROJECT MANAGEMENT APPROACH

Marilyn B. Kilgen, Ph.D., Principle Investigator
Alcee Fortier Distinguished Service Professor of Biological Sciences and Project Director of the Institute for Seafood Studies
Kilgen will be responsible for overall management of the project and for the commercial hydrostatic high pressure experimental research design in collaboration with the Industry partner, Mike Voisin, CEO of Motivatit Seafoods, Inc.

Marilyn Kilgen has 11 years of experience in validation of the post harvest processing using 1) hydrostatic high pressure (HHP) for live shellstock oysters banded shut to prevent oysters from falling out of the shell after treatment, 2) HHP shucking of oysters for packing in commercial plastic containers, 3) combination HHP and cryogenic freezing in a N2 tunnel for HHP shucked oysters on a half shell, 4) cryogenic freezing with liquid CO2 of manually shucked oysters placed on a half shell and stored for 6 weeks, and 5) commercial ionizing irradiation in live shellstock, and in shucked oysters in commercial plastic containers (8 oz and 12 oz).

The initial proposal for this project had a detailed list of all of the oyster products that have been validated by the PI.

Angela L. Corbin, M.S., Co-PI and QA Manager for the Project
Assistant Professor of Biological Sciences and QA Manager for the Environmental and Public Health Microbiology at Nicholls State University.

Angela Corbin has 25 years of QA Director management experience at the Thibodaux Regional Medical Center as the Microbiologist in charge of FDA lab and blood bank inspections and certifications. She also wrote the SOP's and the QAPP for this project.

She is now an Assistant Professor in the Department of Biological sciences where she is greatly involved in research. She has a lot of clinical experience with Vibrio species in human infections from her tenure at the Thibodaux Regional Medical Center. She has taught Pathogenic Microbiology for many years at Nicholls and has been an extremely valued colleague in the Environmental and Public Health Lab since she returned to Nicholls as an M.S. student to earn a

Marine and Environmental Biology M.S. degree. She has since worked with MRSA clinical and community associated MRSA, microbial source tracking with male specific phage and human polyoma virus BK, water quality, *Vibrios* in seafoods as both pathogens of aquaculture shrimp and in immunocompromised humans, and post harvest processing.

Professional Support for the Management Team:

Mike Voisin, CEO, Motivatit Seafoods, Inc., Houma, LA.
Mike Voisin was the industry collaborator for this project and he worked with the P.I. for the research design for the Vp 2 log PHP reduction validation. The P.I. has worked with this progressive company for more than 30 years in oyster safety and technology issues.

Melissa "Lizzie" Evans, FDA, served as a point of contact for regulatory policy advice and interpretations.

Overall, the P.I. and the Co-PI and QA Manager were in contact with Motivatit Seafoods throughout the process to assure the success of this project and were responsible for training the M.S. Graduate Assistant and the undergraduate students assisting in the research.

Cited References:

FDA Bacteriological Analytical Manual (BAM) Online. 2007. Chapter 9. Vibrio. Updated 05/09/2009. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/UCM070830

McCarthy, S. A., A. DePaola, D. W. Cook, C. A. Kaysner, and W. E. Hill. 1999. Evaluation of alkaline phosphates- and digoxigenin-labeled probes for detection of the thermolabile hemolysin (*tlh*) gene of *Vibrio parahaemolyticus*. Lett. Appl. Microbiol. 28:66-70.

National Shellfish Sanitation Program (NSSP). 2009a. Section IV. Guidance Documents Chapter IV. Naturally Occurring Pathogens .03 *Vibrio parahaemolyticus* Control Plan Guidance B. Control Measures 1. Post Harvest Processing (PHP). National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2009. **http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/UCM061639**

National Shellfish Sanitation Program (NSSP). 2009b. Sec II. Model Ordinance Chapter II Risk Assessment and Risk Management, @.05 *Vibrio parahaemolyticus* Control Plan (4) (b) (i) Post harvest processing using a process that has been validated to achieve a 2 log reduction in the levels of total *Vibrio parahaemolyticus* for Gulf and Atlantic Coast oysters. National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2009.

http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FederalStatePrograms/NationalShellfishSanitationProgram/UCM046413

Oliver, J.D., K. Guthrie, J. Preyer, A. Wright, L.Simpson, R. Seibling, J.G. Morris, Jr. 1992. Use of Colistin-Polymyxin B-Cellobiose Agar for Isolation of Vibrio vulnificus from the Environment. Appl Environ Micro. 58(2): 737-739.

5. BUDGET REQESTED AND INDUSRY MATCH

TITLE: Two Log Reduction of *Vibrio parahaemolyticus* in Gold Band ® Oyster Products Using Hydrostatic High Pressure Post Harvest Processing					
Marilyn B. Kilgen, Ph.D., P.I. Angela L. Corbin, M.S., Co-P.I. and Q.A. Manager Department of Biological Sciences Nicholls State University, Thibodaux, LA 70310			Mike Voisin, CEO and Co-PI Motivatit Seafoods, Inc. PO Box 3916 Houma, LA 70361		
REVISED BUDGET AND BUDGET JUSTIFICATION: July 1 2011 - March 31, 2011					
	REQUESTED	**BALANCE**		**INDUSTRY MATCH**	**BALANCE**
PERSONNEL TOTAL	12,000	0	**PERSONNEL TOTAL**	9,375	0
Research Assistant (per 4 month grant period)	8,000	0	Chief Executive Officer (75 hrs.@ $75)	5,625	0
Undergraduate students (2) 200 hrs ea at $10/hr	4,000	0	HACCP & QC (75 hrs.@ $25)	1,875	0
BENEFITS 7.65% FICA N. Broussard	118.22	0	Plant Manager and Processing personnel (75 hrs @ $25)	1,875	0
SUPPLIES	11,858.78	0	**SUPPLIES**	500	0
Gene probe expendable supplies; microbial media and chemicals for buffers, pH reference buffers, disposable plastic ware		0	Oysters and Boxes	500	0
EQUIPMENT	0	0	**EQUIPMENT (use)**	1,500	0
OPERATING SERVICES	2,400	0		0	0

TRAVEL	123	0	**TRAVEL**	123	0
About five 50 mi round trips to Motivatit Seafoods in Houma, LA to set up sampling design and to observe HHP processing and collect samples. ($.49/mi)		0	About five 50 mi round trips to Nicholls State University to discuss sampling design and to observe laboratory efforts ($.49/mi)		
TOTAL DIRECT COSTS	26,500	0	**TOTAL DIRECT COSTS**	11,498	0
INDIRECT COSTS (none allowed)		0	**INDIRECT COSTS (none allowed)**	0	0
TOTAL COSTS	**$26,500**	0	**TOTAL MATCH COSTS**	**$11,498**	**0**

Budget Justification Nicholls State University	**Budget Justification Motivatit Seafoods, Inc., Houma LA**
PERSONNEL TOTAL: $12,000	**PERSONNEL TOTAL: $9,375**
Research Assistant/ Graduate Student ($8,000/grant period)	Chief Executive Officer (75 hrs at $75/hr = $5,625)
Nicole Broussard (Res., Assist. $1,545.41 for July 1 - July 25, 2011; Stacy Martinez (Graduate Stud) $6,454.59 for Aug 11, 2011 -Oct 31, 2011	HACCP & QC (75 hrs @ $25 = $1,875)
BENEFITS: $ 118.22 FICA (7.65%) paid for Nicole Broussard	**BENEFITS: $0.00**

OPERATING SERVICES: $2,400	
Calibrations and certifications for our Traceable RTD Platinum Thermometer ($120); our Fluke-63 Infrared Thermometer ($180); our Mettler Toledo analytical balance ($300); and repair, replacement of parts, and certification and claibration after repair and portal to portal travel for our BF4 Bioflow chambe ($1,800). This is to meet all FDA QC requirements.	
SUPPLIES: $11,858.78	**SUPPLIES: $500**
Molecular expendable supplies; microbial media, chemicals for buffers and media, pH reference buffers, disposable plastic ware , and glassware.	Oysters and Boxes - $500
TRAVEL: $123	**TRAVEL: $123**
About five approximately 50 mi round trips to Motivatit Seafoods in Houma, LA to set up sampling design and to observe HHP processing and collect samples. ($.49/mi)	About five 50 mi round trips to Nicholls State University to discuss sampling design and to observe laboratory efforts. ($.49/mi)
TOTAL DIRECT COSTS: $26,500	**TOTAL DIRECT COSTS: $11,498**
INDIRECT COSTS (none allowed by ISSC)	**INDIRECT COSTS (none allowed by ISSC)**
TOTAL REQUESTED COSTS: $26,500	**TOTAL MATCH COSTS: $11,498**
BALANCE: $0.00	**BALANCE: $0.00**

The funds in the requested Nicholls State Budget have been completely expended for the project.

The matching funds from Motivatit Seafoods, Inc. have also been provided.

6. Appendix:

References

The P.I. has conducted successful State, ISSC and FDA approved validations for post harvest processes to reduce *Vibrio vulnificus* to FDA defined acceptable levels using hydrostatic high pressure, HHP shucking and cryogenic freezing with liquid N2 on the half shell (combination PHP), and cryogenic freezing with liquid CO2 on the half shell with these companies:

1) Mike Voisin, CEO, Motivatit Seafoods, Inc., Houma, LA - HHP validation for banded shellstock and combination HHP shucking and cryogenic freezing (N2 tunnel) validation for frozen half shell oyster products

2) Frank Bellavia, President, Joey Oysters, Inc., Amite, LA – HHP validation for shucking oysters for fresh oysters packaged in different size plastic containers.

3) Rodney Fox, President, R&A Seafood, Inc., Bayou Labatre, AL – cryogenic liquid CO2 freezing and 6 week storage validation of shucked half-shell frozen oysters.

NOTE: Although all of these validations were done using the FDA BAM and NSSP standard required methods for oyster validations for *V. vulnificus*, gene probes were used for confirmation of *V. vulnificus.*

7. Project Team Staffing

Project Principal Investigator: Marilyn B. Kilgen, Ph.D., Alcee Fortier Distinguished Service Professor and Project Director of the Institute for Seafood Studies, Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310

Project Co-Principal Investigator: Angela L. Corbin, M.S., Assistant Professor of Biological Sciences and Q.A. Manager for the Marine and Environmental Microbiology Laboratory
More than 25 experience in Clinical Microbiology with expertise in clinical and environmental *Vibrios* and MRSA. Also 25 years of experience in adjunct teaching in Pathogenic Microbiology and Anatomy and Physiology at Nicholls State University and 11 years as an NSU faculty member with a lot of research experience in the Environmental and Public Health Microbiology Lab at Nicholls.

Industry Collaborator: Mike Voisin, CEO, Motivatit Seafoods, Inc., Houma, LA.
His company developed hydrostatic high pressure post harvest processing in collaboration with Marilyn Kilgen, P.I. He is a member of the ISSC and is a leader for the national oyster and entire seafood industry.

Research Associate: Nicole Broussard, M.S.
Has extensive microbiology experience and worked on a *V. vulnificus* validation for R&A Oysters cryogenically frozen half-shell oyster product.

Research Associate: Stacy Martinez, M.S. Candidate
Extensive undergraduate and now graduate experience with microbiology. Will take over for Nicole Broussard on August 10, 2011 to complete project.

Undergraduate Research Assistants: **Ross Adams, Drew Ledet and Andrew Morris**
These 3 undergraduate student research assistants were selected to work on this project They had microbiological experience with *Vibrios.*

APPENDIX 1

RESULTS

Two Log Reduction of *Vibrio parahaemolyticus* in Gold Band ® Oyster Products Using Hydrostatic High Pressure Post Harvest Processing

Nicholls State University Environmental and Public Health Microbiology Laboratory Room 208 Gouaux Hall Marilyn B. Kilgen PhD

Motivatit Seafood, Inc. Two Log Reduction *Vibrio parahemolyticus* Validation

Lot # 1 Control

Lot Number: Lot # 1	Oyster weight: 261 g
Date Harvested: 1/15/12	Initial V.P. Measurement
Site: LA Area 15	Homogenate Mass: 1, 0.1, 0.01 g
Date processed at Motivatit: 1/17/12	Tube code: 10, 5 , 3
Notes:	

Sample	Process-NSU	APW	TCBS	TLH-AP Probe	Results
	Initial	Turbidity (+)	Green (+)	Brown - rust (+)	
Tech:	S Martinez M Kilgen	S Martinez M Kilgen	S Martinez, M Kilgen, A Corbin	A. Corbin S Martinez	
Date processed at NSU:	1/17/2012	1/18/2012	1/19/2012	1/23/2012	
Controls:					MBK
V. parahaemolyticus (17802)		+	+	+	
V. vulnificus (27562)		+	+	-	
V. harveyi (14126)		+	YELLOW	-	
V. alginolyticus (33840)		+	YELLOW	-	
Homogenate Mass:	10 MPN Tubes/Dilut.				
1 g	A	+	+	+	
	B	+	+	+	
	C	+	+	+	
	D	+	+	+	
	E	+	+	+	Total positives:
	F	+	+	+	10
	G	+	+	+	
	H	+	+	+	
	I	+	+	+	
	J	+	+	+	
0.1 g	A	+	+	+	
	B	+	-		
	C	+	-		
	D	+	+	+	
	E	+	+	+	Total positives:
	F	+	-		5
	G	+	+	+	
	H	+	+	+	
	I	+	-		
	J	+	-		
0.01 g	A	+	-		
	B	+	+	+	
	C	+	-		
	D	+	-		
	E	+	-		Total positives:
	F	+	-		3
	G	+	+	+	
	H	+	+	-	
	I	+	+	-	
	J	+	+	+	
0.001 g	A	-			
	B	-			
	C	-			
	D	-			
	E	-			Total positives:
	F	-			
	G	-			
	H	-			
	I	-			
	J	-			
0.0001 g	A	-			
	B	-			
	C	-			
	D	-			
	E	-			Total positives:
	F	-			
	G	-			
	H	-			
	I	-			
	J	-			

Lot # 1 Hydrostatic High Pressure PHT Samples

Control Number Lot 1	Initial V.P. Measurement (from control)	Sample homogenate mass (from SOP chart): 0.1 g
Date Harvested : 1/15/2012	Control Tube code : 10, 5 , 3	Number of tubes per sample required: 5
Site : LA Area 15	Control Homogenate mass: 1, 0.1 , 0.01 g	Positive tubes allowed: 0

Date processed at Motivatit : 1/17/2012

Nicholls State University Environmental and Public Health Microiology Laboarotory Room 208 Gouaux Hall Marilyn B. Kilgen PhD

Motivatit Seafood, Inc. Two Log Reduction *Vibrio parahemolyticus* Validation

Sample	Process-NSU Initial	APW Turbidity (+)	TCBS Green (+)	Probe Brown - rust (+)	Pass / Fail	Sample	Process-NSU Initial	APW Turbidity (+)	TCBS Green (+)	Probe Brown - rust (+)	Pass/ Fail
Tech:	S Martinez	S Martinez	A Corbin		MBK	Tech:	S Martinez	S Martinez	A Corbin		MBK
Date:	1/24/2012	1/25/2012	1/26/2012	NA	NA	Date:	1/24/2012	1/25/2012	1/26/2012	NA	NA
***V. parahaemolyticus* (17802**		+	+			Sample 6	A	+	-		Pass
***V. vulnificus* (27562)**		+	+			Weight:	B	+	-		
***V. harveyi* (14126)**		+	yellow			204.5 g	C	+	-		
***V. alginolyticus* (33840)**		+	yellow				D	+	-		
Sample 1	A	+	-		Pass	HHP	E	+	-		
Weight:	B	+	-			average	F				
163 g	C	+	-			38241 -	G				
	D	+	-			38002	H				
HHP	E	+	-			psi	I				
average	F						J				
38064 -	G					Sample 7	A	+	-		Pass
38337	H					Weight:	B	+	-		
psi	I					191.5 g	C	+	-		
	J						D	+	-		
Sample 2	A	+	-		Pass	HHP	E	+	-		
Weight:	B	+	-			average	F				
204.8	C	+	-			38219 -	G				
	D	+	-			37995	H				
HHP	E	+	-			psi	I				
average	F						J				
38107 -	G					Sample 8	A	+	-		Pass
37886	H					Weight:	B	+	-		
	I					198.2 g	C	+	-		
	J						D	+	-		
Sample 3	A	+	-		Pass	HHP	E	+	-		
Weight:	B	+	-			average	F				
214.2 g	C	+	-			38265 -	G				
	D	+	-			38018	H				
HHP	E	+	-			psi	I				
average	F						J				
38127 -	G					Sample 9	A	+	-		Pass
37910	H					Weight:	B	+	-		
psi	I					133.9 g	C	+	-		
	J						D	+	-		
Sample 4	A	+	-		Pass	HHP	E	+	-		
Weight:	B	+	-			average	F				
180.2 g	C	+	-			38095 -	G				
	D	+	-			37879	H				
HHP	E	+	-			psi	I				
average	F						J				
38346 -	G					Sample 10	A	+	-		Pass
38072	H					Weight:	B	+	-		
psi	I					148.2 g	C	+	-		
	J						D	+	-		
Sample 5	A	+	-		Pass	HHP	E	+	-		
Weight:	B	+	-			average	F				
218.3 g	C	+	-			38072 -	G				
	D	+	-			37881	H				
HHP	E	+	-			psi	I				
average	F						J				
38219 -	G										
37995	H										
psi	I										
	J										

Comments: There were "0" positive Vp tubes in this set of 10 HHP-PHT samples of 0.1g/tube 5 tube MPN tubes, as required.

Nicholls State University Environmental and Public Health Microiology Laboratory Room 208 Gouaux Hall Marilyn B. Kilgen PhD

Motivatit Seafood, Inc. Two Log Reduction *Vibrio parahemolyticus* Validation

Lot # 2 Control

Lot Number: Lot # 2

Date Harvested: 1/22/2012

Site: Area 15

Date processed at Motivatit: 1/24/2012

Oyster weight: 140.4 g

Initial V.P. Measurement

Homogenate Mass: 0.1, 0.01, 0.001

Tube code: 8, 5, 0

Notes:

Sample	Process-NSU	APW	TCBS	TLH-AP Probe	Results
	Initial	Turbidity (+)	Green (+)	Brown - rust (+)	
Tech:	S Martinez	S Martinez	A Corbin S Martinez	A Corbin S Martinez	
Date processed at NSU:	01/24/12	01/25/12	01/26/12	01/30/12	
Controls:					MBK
V. parahaemolyticus		+	+	+	
V. vulnificus (27562)		+	+	-	
V. harveyi (14126)		+	yellow	-	
V. alginolyticus (33840)		+	yellow	-	
Homogenate Mass:	10 MPN Tubes/Dilution				
1 g	A	+	+	+	
	B	+	+	+	
	C	+	-		
	D	+	+	+	
	E	+	+	+	Total positives: 9
	F	+	+	+	
	G	+	+	+	
	H	+	+	+	
	I	+	+	+	
	J	+	+	+	
0.1 g	A	+	+	-	
	B	+	+	+	
	C	+	+	+	
	D	+	+	+	
	E	+	+	+	Total positives: 8
	F	+	+	+	
	G	+	+	+	
	H	+	+	+	
	I	+	-		
	J	+	+	+	
0.01 g	A	+	+	+	
	B	+	-		
	C	+	-		
	D	+	+	+	
	E	+	+	+	Total positives: 5
	F	+	-		
	G	+	-		
	H	+	-		
	I	+	+	+	
	J	+	+	+	
0.001 g	A	+	-		
	B	+	-		
	C	+	-		
	D	+	-		
	E	+	-		Total positives: 0
	F	+	-		
	G	+	-		
	H	+	+	-	
	I	+	-		
	J	+	-		
0.0001 g	A	+	-		
	B	-			
	C	-			
	D	-			
	E	-			Total positives:
	F	-			
	G	+	-		
	H	+	-		
	I	+	-		
	J	-			

Nicholls State University Environmental and Public Health Microiology Laboarotory Room 208 Gouaux Hall Marilyn B. Kilgen PhD

Motivatit Seafood, Inc. Two Log Reduction *Vibrio parahemolyticus* Validation

Lot # 2 Hydrostatic High Pressure PHT Samples

Control Number : Lot #2
Date Harvested : 1/22/2012
Site : LA area 15

Initial V.P. Measurement (from control)
Control Tube code : 8,5,0
Control Homogenate mass: 0.1,0.01, 0.001 g

Sample homogenate mass (from SOP chart): 1 g
Number of tubes per sample required: 10
Positive tubes allowed: 4

Date processed at Motivatit : 1/24/2012

	Process-NSU	APW	TCBS	Probe	Results
Sample	Initial	Turbidity (+)	Green (+)	Brown - rust (+)	Pass / Fail
Tech:	S Martinez	S Martinez	A Corbin	A Corbin S Martinez	MBK
Date:	2/2/2012	2/3/2012	2/4/2012	2/6/2012	
***V. parahaemolyticus* (17802)**		+	+	+	
***V. vulnificus* (27562)**		+	+	-	
***V. harveyi* (14126)**		+	yellow	-	
***V. alginolyticus* (33840)**		+	yellow	-	
Sample 1 Weight: 234 g HHP average 38119 - 37894 psi	A	+	-		Pass
	B	+	-		
	C	+	-		
	D	+	-		
	E	+	-		
	F	+	-		
	G	+	-		
	H	+	-		
	I	+	-		
	J	+	-		
Sample 2 Weight: 206.3 g HHP average 38295 - 38041 psi	A	+	-		Pass
	B	+	-		
	C	+	-		
	D	+	+	-	
	E	+	-		
	F	+	+	-	
	G	+	-		
	H	+	-		
	I	+	-		
	J	+	-		
Sample 3 Weight: 174.6 g HHP average 38195 - 37979 psi	A	+	+	-	Pass
	B	+	-		
	C	+	+	-	
	D	+	-		
	E	+	-		
	F	+	-		
	G	+	-		
	H	+	+	-	
	I	+	-		
	J	+	-		
Sample 4 Weight: 197.2 g HHP average 38207 - 37987 psi	A	+	-		Pass
	B	+	-		
	C	+	-		
	D	+	-		
	E	+	-		
	F	+	+	-	
	G	+	-		
	H	+	-		
	I	+	-		
	J	+	-		
Sample 5 Weight: 218.3 g HHP average 38288 - 38033 psi	A	+	-		Pass
	B	+	-		
	C	+	-		
	D	+	-		
	E	+	-		
	F	+	-		
	G	+	-		
	H	+	-		
	I	+	-		
	J	+	-		

	Process-NSU	APW	TCBS	Probe	Results
Sample	Initial	Turbidity (+)	Green (+)	Brown - rust (+)	Pass / Fail
Tech:	S Martinez	S Martinez	A Corbin	A Corbin S Martinez	MBK
Date:	2/2/2012	2/3/2012	2/4/2012	2/6/2012	
Sample 6 Weight: 218.6 g HHP average 38354 - 38080 psi	A	+	-		Pass
	B	+	+		
	C	+	-		
	D	+	-		
	E	+	-		
	F	+	-		
	G	+	-		
	H	+	+	-	
	I	+	-		
	J	+	-		
Sample 7 Weight: 228.8 g HHP average 38057 - 38026 psi	A	+	+	-	Pass
	B	+	+	-	
	C	+	+	-	
	D	+	+	-	
	E	+	-		
	F	+	-		
	G	+	+	-	
	H	+	-		
	I	+	-		
	J	+	+	-	
Sample 8 Weight: 229.9 g HHP average 38279 - 38028 psi	A	+	+	-	Pass
	B	+	-		
	C	+	+	-	
	D	+	+	-	
	E	+	-		
	F	+	+	-	
	G	+	-		
	H	+	-		
	I	+	+	-	
	J	+	-		
Sample 9 Weight: 224.8 g HHP average 38241 - 38002 psi	A	+	-		Pass
	B	+	-		
	C	+	+	-	
	D	+	+	-	
	E	+	-		
	F	+	-		
	G	+	+	-	
	H	+	+	-	
	I	+	-		
	J	+	-		
Sample 10 Weight: 218.6 g HHP average 38088 - 37871 psi	A	+	+	-	Pass
	B	+	+	-	
	C	+	-		
	D	+	-		
	E	+	-		
	F	+	-		
	G	+	-		
	H	+	-		
	I	+	-		
	J	+	-		

Comments: Although 4 positive Vp tubes were allowed in the 10 HHP-PHT samples of a 1g/tube 10 tube MPN, there were 0 positive tubes.

Nicholls State University Environmental and Public Health Microiology Laboratory Room 208 Gouaux Hall Marilyn B. Kilgen PhD

Motivatit Seafood, Inc. Two Log Reduction *Vibrio parahemolyticus* Validation

Lot # 3 Control

Lot Number: Lot # 3

Date Harvested: 1/30/2012

Site Harvested: LA Area 15

Date processed at Motivatit: 1/31/2012

Oyster weight: 265.7

Initial V.P. Measurement

Homogenate Mass: 0.01, 0.001, 0.0001 g

Tube code: 9, 3, 0

Notes :

Samples	Process-NSU	APW	TCBS	TLH-AP Probe	Results
	Initial	Turbidity (+)	Green (+)	Brown - rust (+)	
Tech:	S Martinez	S Martinez	A Corbin	A Corbin S Martinez	
Date Processed at NSU:	1/31/2012	2/1/2012	2/2/2012	2/3/2012	
Controls:					MBK
V. parahaemolyticus (17802)		+	+	+	
V. vulnificus (27562)		+	+	-	
V. harveyi (14126)		+	Yellow	-	
V. alginolyticus (33840)		+	Yellow	-	
Homogenate Mass:	10MPN				
1 g	A	+	+	+	
	B	+	+	+	
	C	+	+	+	
	D	+	+	+	
	E	+	+	+	Total positives: 10
	F	+	+	+	
	G	+	+	+	
	H	+	+	+	
	I	+	+	+	
	J	+	+	+	
0.1 g	A	+	+	+	
	B	+	+	+	
	C	+	+	+	
	D	+	+	+	
	E	+	+	+	
	F	+	+	+	Total positives: 10
	G	+	+	+	
	H	+	+	+	
	I	+	+	+	
	J	+	+	+	
0.01 g	A	+	+	+	
	B	+	+	+	
	C	+	-		
	D	+	+	+	
	E	+	+	+	
	F	+	+	+	Total positives: 9
	G	+	+	+	
	H	+	+	+	
	I	+	+	+	
	J	+	+	+	
0.001 g	A	+	+	+	
	B	+	+	+	
	C	+	-		
	D	+	+	-	
	E	+	+	-	
	F	+	+	+	Total positives: 3
	G	+	+	-	
	H	+	+	-	
	I	+	+	-	
	J	+	+	-	
0.0001 g	A	+	+	-	
	B	+	+	-	
	C	-			
	D	-			
	E	-			
	F	+	+	-	Total positives: 0
	G	-			
	H	+	+	-	
	I	-			
	J	-			

Lot # 3 Hydrostatic High Pressure PHT Samples

Control Number : Lot # 3
Date Harvested : 1/30/12
Site : LA Area 15

Initial V.P. Measurement (from control)
Control Tube code : 9, 3, 0
Control Homogenate mass: 0.01, 0.001, 0.0001 g

Sample homogenate mass (from SOP chart): 0.1 g
Number of tubes per sample required: 7
Positive tubes allowed: 3

Date processed at Motivatit : 1/31/2012

Nicholls State University Environmental and Public Health Microbiology Laboratory Room 208 Gouaux Hall Marilyn B. Kilgen, Ph.D.

Motivatit Seafood, Inc. Two Log Reduction *Vibrio parahemolyticus* Validation

	Process-NSU	APW	TCBS	Probe	Results		Process-NSU	APW	TCBS	TLH-AP Probe	Results
Sample	Initial	Turbidity (+)	Green (+)	Brown - rust (+)	Pass / Fail	Sample	Initial	Turbidity (+)	Green (+)	Brown - rust (+)	Pass / Fail
Tech:	S Martinez	S Martinez	S Martinez	A Corbin S Martinez	MBK	Tech:	S Martinez	S Martinez	S Martinez	A Corbin S Martinez	MBK
Date:	2/8/2012	2/9/2012	2/10/2012	2/13/2012		Date:	2/8/2012	2/9/2012	2/10/2012	2/13/2012	
***V. parahaemolyticus* (17802)**		+	+			Sample 6	A	+	-		
***V. vulnificus* (27562)**		+	+			Weight:	B	+	-		
***V. harveyi* (14126)**		+	yellow			257.2 g	C	+	-		
***V. alginolyticus* (33840)**		+	yellow				D	+			
Sample 1	A	+	-			HHP	E	+	+	-	Pass
Weight:	B	+	-			average	F	+	-		
236.7 g	C	+	-			38241 -	G	+	-		
	D	+	+	-		38002	H				
	E	+	-		Pass	psi	I				
HHP average	F	+	-				J				
38288 -	G	+	-			Sample 7	A	+	-		
38033	H					Weight:	B	+	-		
psi	I					219.3 g	C	+	-		
	J						D	+	-		
Sample 2	A	+	-			HHP	E	+	-		Pass
Weight:	B	+	-			average	F	+	-		
228.1g	C	+	-			38195 -	G	+	-		
	D	+	-			37979	H				
	E	+	-		Pass	psi	I				
HHP average	F	+	-				J				
38337 -	G	+	-			Sample 8	A	+	-		
38064	H					Weight:	B	+	-		
psi	I					198.1 g	C	+	-		
	J						D	+	+	-	
Sample 3	A	+	-			HHP	E	+	-		Pass
Weight:	B	+	-			average	F	+	-		
232.7 g	C	+	-			38295 -	G	+	-		
	D	+	-			38041	H				
	E	+	-		Pass	psi	I				
HHP average	F	+	-				J				
38169 -	G	+	-			Sample 9	A	+	-		
37956	H					Weight:	B	+	-		
psi	I					214.4 g	C	+	-		
	J						D	+	-		
Sample 4	A	+	+	-		HHP	E	+	-		Pass
Weight:	B	+	-			average	F	+	-		
200.2 g	C	+	-			37963 -	G	+	-		
	D	+	-			37829	H				
	E	+	-		Pass	psi	I				
HHP average	F	+	-				J				
38250 -	G	+	-			Sample 10	A	+	-		
38010	H					Weight:	B	+	-		
psi	I					252.7 g	C	+	-		
	J						D	+	-		
Sample 5	A	+	-			HHP	E	+	-		Pass
Weight:	B	+	-			average	F	+	-		
222.3 g	C	+	-			38288 -	G	+	-		
	D	+	+	-		38033	H				
	E	+	+	-	Pass	psi	I				
HHP average	F	+	-				J				
38354 -	G	+	-								
38088	H										
psi	I										
	J										

Comments: Although 3 positive Vp tubes were allowed in the 10 HHP-PHT samples of a 0.1g/tube 7 tube MPN, there were 0 positive tubes.

Nicholls State University

SCOPE OF SERVICES

Two Log Reduction of *Vibrio parahaemolyticus* in Gold Band ® Oyster Products Using Hydrostatic High Pressure Post Harvest Processing.

The Contractor shall confirm the validation of reduction of *Vibrio Parahaemolyticus (Vp)* to National Shellfish Sanitation Program (NSSP) acceptable levels in oysters post harvest processed with hydrostatic high pressure (HHP) using the Avure Quintus Food Press QFP 320l - 400 hydrostatic ultrahigh pressure processor at Motivatit Seafoods, Inc., Houma, LA. Specifically, this assay will be substituted for biochemical analysis or other NSSP acceptable methods for confirmation of *V parahaemolyticus* following enrichment in alkaline peptone water (APW) as per the Bacteriological Analytical Manual (BAM) protocol for sample preparation of shellstock for *Vibrio* species analysis (FDA 2007).

All validations will be conducted using NSSP approved methods and will be conducted in labs which have been evaluated and meet NSSP criteria.

Test of Effectiveness of Relaying as a Post Harvest Process for reducing levels of *Vibrio vulnificus* and *V. parahaemolyticus* in Shellstock Oysters

William Walton, Chris Nelson, Mona Hochman & John Schwarz

Introduction

Laboratory observations by Kaspar and Tamplin (1993) of pure cultures of *Vibrio vulnificus* suggested that elevated salinities of 25 ppt or higher reduced *V. vulnificus* numbers. Motes and DePaola (1996) showed that oysters relayed to high-salinity offshore waters (>32 ppt) showed a significant reduction in *V. vulnificus* numbers after two (2) weeks.

Less well known, though, is the amount of time necessary for shellstock oysters, harvested and transported under refrigeration and placed back into the environment (relay) to reestablish "ambient" levels of *V. vulnificus* and *V. parahaemolyticus* consistent with other oysters in the same area. Current regulatory requirements in the Gulf States require rapid refrigeration post harvest for any oysters to be sold live, in the shell, for raw consumption. Shellstock which have been harvested and rapidly refrigerated in compliance with a *Vibrio vulnificus* Control Plan, are referred to as "white tag" shellstock. Oysters refrigerated less quickly are given a different tag and must be shucked by a certified dealer or post-harvest processed. These are referred to as "non-matrix", "restricted use" or "green tag" shellstock.

The present time temperature requirements which are imposed during the summer months are very restrictive and severely limit the harvest time for many, if not most, oyster harvesters. Should relaying prove effective in reducing *V. vulnificus* and *V. parahaemolyticus* levels it could be considered as a viable control for reducing *V. vulnificus* illnesses.

Given the fact that green tag shellstock are expected to be more common than white tag shellstock under the newest requirements, any post-harvest process that would reduce levels of *V. vulnificus* and *V. parahaemolyticus* in green tag shellstock to levels equivalent to those of the white tag product could be useful to the Gulf oyster industry. An argument may be made that, although the relayed product cannot be labeled as having either of these organisms reduced to non-detectable levels, the re-harvested product should be allowed for sale as "white tag" shellstock as if it had been placed under refrigeration in accordance with a state's *Vibrio vulnificus* Control Plan when it was originally harvested. The objective of the proposed research, therefore, was to test the effectiveness of the relay of oysters (*Crassostrea virginica*) to a monitored, inshore site and to a remote, higher salinity site as a means of reducing *Vibrio* levels.

Approach and Methodology

To determine experimentally the minimum amount of time for *Vibrio* loads within green tag oysters to return to levels no higher than those detected in white tag oysters from the same original harvest area, we conducted a field study at the Point aux Pins Oyster Farm, in Sandy Bay, AL and at a remote location at the west end of Dauphin Island, AL with two independent replicate runs. For each run, three (3) time zero (t_0) samples of 15 oysters each were taken from shellstock oysters on board a harvest vessel in Moss Bay, LA (Area 17) immediately after harvest and refrigerated with gel ice until sampling on both August 14, 2011 and September 18, 2011. During the same harvesting trip, oysters from the same area were segregated into two additional

groups aboard the vessel: one group that was refrigerated within one hour so that the internal temperature of the oysters putatively reached 55ºC or less within 6 hours (referred to as 'white tag') and one to remain unrefrigerated on the boat deck for the duration of the harvesting trip (referred to as 'green tag'). A temperature recording device was placed with both groups of oysters to record the approximate internal temperature of the oysters. Surface water temperature and salinity data were also collected at the original harvest site using a refractometer and immersion thermometer. After the harvesting vessel returned to the dock, the t_0 samples and the white and green tag oysters were loaded onto a refrigerated conveyance and brought to Bon Secour Fisheries, Inc in Bon Secour, AL.

At Bon Secour Fisheries, three (3) samples of 15 oysters each were taken from each of the white (t_{0WT}) and green tag (t_{0GT}) groups. All time zero samples were shipped overnight to the Texas A&M University, Seafood Safety Laboratory in Galveston, TX for bacteriological analysis (9 samples total per run). Within 24 hrs of arrival at Bon Secour Fisheries, green tag oysters were deployed in floating shellfish aquaculture cages (OysterGro™) at each of two sites: the Point aux Pins Oyster Farm in Sandy Bay, AL (~30º 23.007 N, 88º 23.007' W) and the northern side of the west end of Dauphin Island, AL (both within conditionally approved areas for shellfish harvest). On day 2 of the first deployment on Dauphin Island, the site was relocated from the middle of Dauphin Island (~30º 15.171' N, 88º 10.135' W) to a site further west (~30º 14.135' N, 88º 17.247' W) to increase the likelihood of sustained high salinity water.

Oysters were stocked into replicate plastic mesh (12 mm) bags at ~150 oysters/bag and held in the oyster cages. Samples of oysters (n = 15) were collected from each of five randomly selected bags at each site at 2 d (t_{2d}), 7 d (t_{7d}), and 14 d (t_{14d}) after relay from each site (5 samples/site/time period x 2 site x 3 time periods, or 30 samples total). All oysters were immediately chilled on board and refrigerated prior to shipping. All samples were then shipped priority overnight to the Texas A&M Laboratory in Galveston, TX for bacteriological analysis. Water temperature and salinity were measured at each relay site with a handheld YSI at each sampling period.

Once received by the Seafood Safety Lab, oyster samples were immediately processed according to the procedures in Chapter 9 of the FDA Bacteriological Analytical Manual for enumeration using a DNA alkaline phosphatase-labeled gene probe. Each oyster sample consisted of 15 oysters: 3 used to check the internal meat temperature, and 12 shucked for enumeration of *V. vulnificus* and *V. p*. The 12 shucked oysters (meat and liquor) were blended with an equal weight of Alkaline Peptone Water (APW), which was then used to create a 6-dilution, 3- replicate serial dilution in APW. Following 18-24 hours incubation, positive dilutions were streaked onto modified cellobiose-Polymixin B-Colistin (mCPC) agar and thiosulfate-citrate-bile salts-sucrose (TCBS) agar, which are specific for the growth of *V. vulnificus* and *V. parahaemolyticus*, respectively.

Following 18-24 hours incubation of the agars, suspect colonies of *V. vulnificus* (yellow with a "fried egg" appearance on CPC) and *Vp* (dark green on TCBS) were isolated into 96-well culture plates, and allowed to resuscitate for 4 hours. At this point, the isolated colonies were replicated onto T_1N_3 (1% tryptone, 3% NaCl) and Vva (*Vibrio vulnificus* agar) agar plates. From there, the colonies were lysed onto Whatman #541 filters, and the DNA prepared with Proteinase K.

Finally, the filters were run through the AP-labeled gene probe procedure for *Vibrio vulnificus*, and the tlh(+)tdh(-)forms of *V. parahaemolyticus*. Positive results for all the gene probes appear as dark brown/purple colonies, as opposed to tan/yellow colonies indicating negative results. Upon comparing the positives to the original serial dilutions, results were determined using an MPN (Most Probable Number) per gram format.

Results

Upon initial harvest on Aug. 14, 2011 at 8:05 AM, the water temperature was 28.5º C and salinity was 20 psu. Upon initial harvest for the second run on Sep. 18, 2011, the water temperature was 25.6º C and salinity was 22 psu.

Temperature and salinity for the deployment sites of green tagged oysters (Table 1) were broadly similar across sites. Temperature dropped at the end of the second run at both sites. Though salinity was expected to be higher at the Dauphin Island sites, this was not consistently observed, though all salinities observed exceeded 21.2 psu.

Table 1. Observed water temperatures and salinities at deployment sites in Mississippi Sound (AL)

Day	Sandy Bay		Dauphin Island	
	Temp (ºC)	Salinity (PSU)	Temp (ºC)	Salinity (PSU)
August Run				
2	29.1	25.3	30.2	22.6
7	29.8	26.9	30.8	27.6
14	28.2	27.5	30.2	29.0
Sept. Run				
2	28.5	21.2	27.1	27.8
7	26.8	22.3	27.4	26.8
14	22.6	23.7	21.6	22.1

Though there appeared to be differences among the time zero samples in terms of *V. vulnificus* and *V. parahaemolyticus* abundances, neither of these effects was significant due to the high variation in initial numbers and low replication (ANOVA, df = 2, 3, $p = 0.18$ and $p = 0.41$, respectively). Still, there is a clear trend of the abundances being highest in the green tagged oysters (with corresponding increased variation), with little apparent difference between the oysters iced immediately after harvest and the white tagged oysters.

Table 2. Time zero abundances of *Vibrio vulnificus* and *V. parahaemolyticus* in MPN/g

	***V. vulnificus* (± SD)**	***V. parahaemolyticus* (± SD)**
Immediately Iced (t_0)	11,240 (± 6,562)	318 (± 308)
White Tagged (t_{0WT})	11,730 (± 10,734)	1,066 (± 1,391)
Green Tagged (t_{0GT})	67,600 (± 42,426)	3,761 (± 3,785)

Interestingly, there was a strong interaction between deployment site and deployment day for *V. vulnificus* abundance (ANOVA, df = 3,8, $p < 0.001$). Despite the interaction (Fig. 1), the pattern was consistent within each site, even using the most conservative estimate of time zero abundance of estimates from the immediately iced oysters as the time zero reference; abundance on day 2 were significantly higher than any other day (Tukey's HSD, $p < 0.001$) with no significant differences among any of the other days within a site (Tukey's HSD, $p \geq 0.965$). Additionally, the abundances on day 2 at Sandy Bay were significantly greater than the day 2 abundances of *V. vulnificus* at the Dauphin Island site (Tukey's HSD, $p < 0.001$).

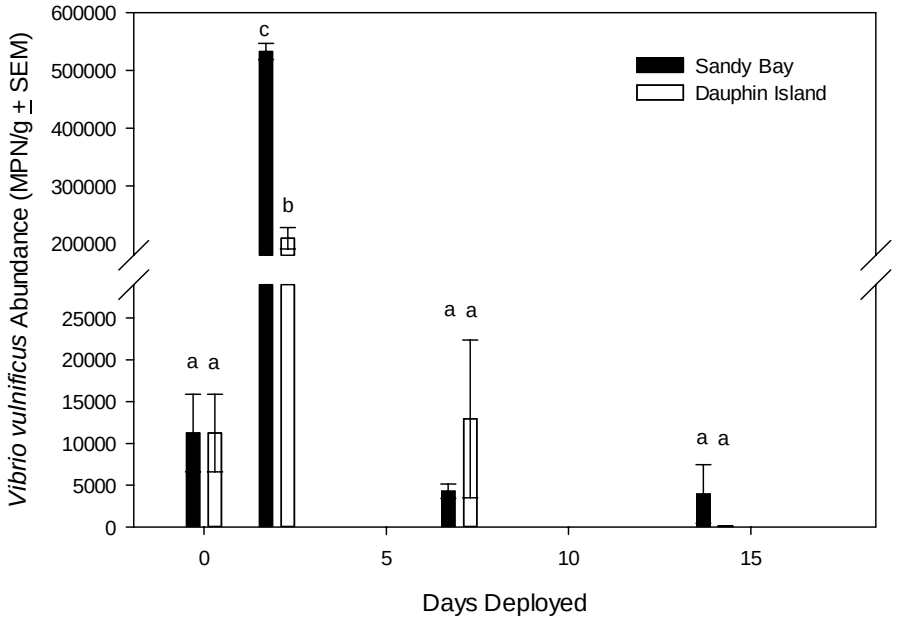


Figure 1. Relationship between days deployed and the abundance of *Vibrio vulnificus* (MPN/g) in green-tagged oysters deployed at two sites in coastal Alabama, Sandy Bay and Dauphin Island in 2011. Different letters indicate significant pair-wise differences.

For *V. parahaemolyticus*, data were rank-transformed to meet the assumptions of normality. In this case (Fig. 2), there was only a significant effect of day (ANOVA, df = 3, 8, $p < 0.001$), with no significant effect of sites or day*site interactions. The abundance of *V. parahaemolyticus* was greatest on day 2, but was not statistically greater than abundance on day 7 (Tukey's HSD, $p = 0.13$). Day 7, turn, was more abundant (but not significantly) than on day 14 (Tukey's HSD, $p = 0.19$). The abundance was lowest at time zero (using estimates again from immediately iced oysters as the time zero reference), but this did not differ significantly from abundance on day 14 (Tukey's HSD, $p = 0.53$).

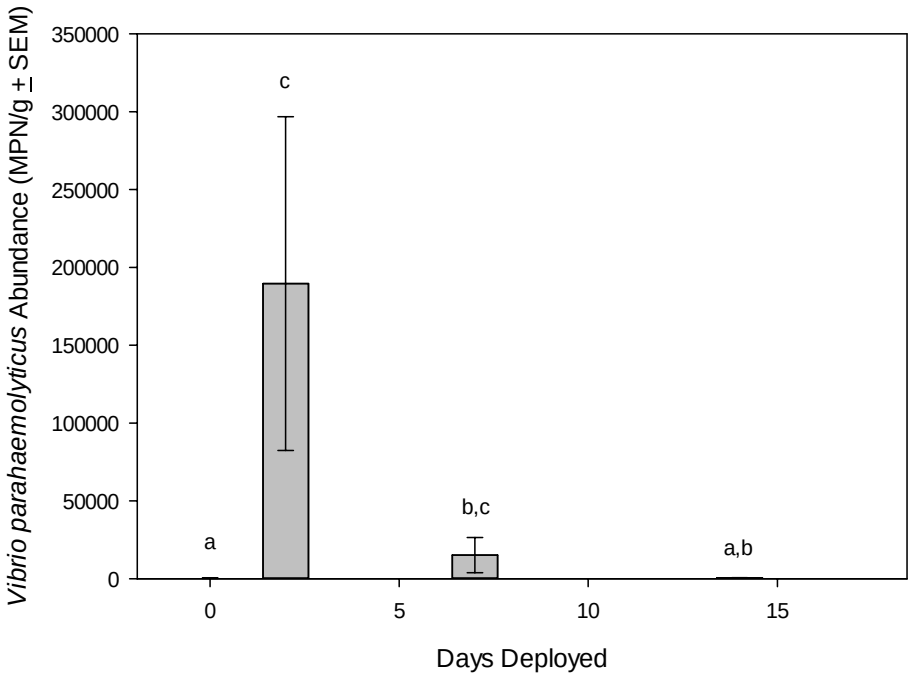


Figure 2. Relationship between days deployed and the abundance of *Vibrio parahaemolyticus* (MPN/g) in green-tagged oysters deployed in coastal Alabama in 2011. Different letters indicate significant pair-wise differences.

There was noticeable mortality across the deployments, with an average survival of 83.4% (± 3.13 SD). There was no difference observed between the two sites (ANOVA, df = 1, 10, $p < 0.18$).

Conclusions

Based on this preliminary study, relaying of green tagged oysters to relatively high salinity waters (approved for harvest) shows some promise as a means of reducing both *V. vulnificus* and *parahaemolyticus* abundance. Using the most conservative estimate of initial (time zero) abundances (within oysters immediately iced upon harvest), deployment was able to reduce *V. vulnificus* abundance to levels equivalent to those upon harvest within 7 days. Similarly, again using the most conservative estimate of initial abundances, deployment was able to reduce *V. parahaemolyticus* abundance levels equivalent to those upon harvest within 14 days at the two tested sites.

There are several important caveats to note. First, deployment of green tagged oysters actually led to very pronounced spikes in the abundances of both species of *Vibrio* by day 2. While deployment may be able to reduce abundances over time, it is not a linear decrease and no assumptions should be made about the dynamics in abundance during the first days of deployment of green tagged oysters.

Second, over the deployments, mortality approached 20%. These losses need to considered in any evaluation of the feasibility of this approach as a means of 're-claiming' green tagged oysters by deploying them in the field.

Third, the intent of the research was to determine if the risk associated with 'green tagged' oysters could be reduced back to that of oysters upon harvest, allowing a subsequent 'second harvest' of the oysters with the opportunity to maintain them as 'white tagged' oysters. Therefore, oysters treated in this manner would not be considered in any way free of all *Vibrios*.

Finally, this study was undertaken as an initial proof of concept and was minimally replicated. Any conclusions need to consider the very low power of the test. The results, however, suggest that this approach shows promise as an effective means of re-claiming green tag oysters.

References

Kaspar, C. W., & Tamplin, M. L. (1993). Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Applied and Environmental Microbiology,* 59, 2425-2429.

Motes, M.L. & DePaola, A. (1996) Offshore suspension relaying to reduce levels of *Vibrio vulnificus* in oysters (*Crassostrea virginica*). *Applied and Environmental Microbiology,* 62**,** 3875-3877.

Day 0

Comments

Harvest Information
Harvest Area: Area 17, Moss Bay, LA
Harvest Date: 8/14/11; 0805
Water Temp: 28.5°C
Salinity: 20.0 ppt

Boat

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_0B-1	8/16/11	348.71	8.63	11,000.0	200.0	0
t_0B-2	8/16/11	244.29	10.13	1,100.0	16.0	0
t_0B-3	8/16/11	288.21	5.30	2,400.0	150.0	0
t_0B-4	8/16/11	337.01	5.37	11,000.0	43.0	0
t_0B-5	8/16/11	340.26	9.87	7,500.0	93.0	0
	Average:	311.70	7.86	6,600.0	100.4	0.0

White Tag

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_0W-1	8/16/11	336.24	8.50	11,000.0	7,500.0	0
t_0W-2	8/16/11	330.59	9.60	24,000.0	140.0	0
t_0W-3	8/16/11	275.20	9.70	46,000.0	1,400.0	0
t_0W-4	8/16/11	288.90	5.77	4,600.0	280.0	0
t_0W-5	8/16/11	283.41	10.20	11,000.0	930.0	0
	Average:	302.87	8.75	19,320.0	2,050.0	0.0

Green Tag

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_0G-1	8/16/11	231.08	10.13	46,000.0	3,800.0	0
t_0G-2	8/16/11	257.87	8.73	46,000.0	290.0	0
t_0G-3	8/16/11	227.29	7.07	46,000.0	11,000.0	0
t_0G-4	8/16/11	326.78	8.33	110,000.0	2,100.0	0
t_0G-5	8/16/11	246.91	11.23	240,000.0	15,000.0	0
	Average:	257.99	9.10	97,600.0	6,438.0	0.0

Day 2

Comments

Green Tag - Sandy Bay

Harvest Information
Harvest Area: Sandy Bay
Harvest Date: 8/17/11
Water Temp: 29.1°C
Salinity: 25.3 ppt

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)	
t_2SB-1	8/18/11	207.25	10.63	24,000.0	24,000.0	0	
t_2SB-2	8/18/11	250.68	9.03	24,000.0	3,600.0	0	
t_2SB-3	8/18/11	221.95	9.67	2,400,000.0	460,000.0	0	
t_2SB-4	8/18/11	178.09	11.57	240,000.0	15,000.0	0	2 dead in bag
t_2SB-5	8/18/11	186.05	10.93	46,000.0	2,800.0	0	1 dead in bag
	Average:	208.80	10.37	546,800.0	101,080.0	0.0	

Green Tag - West Dauphin Island

Harvest Information
Harvest Area: DI (Myers)
Harvest Date: 8/17/11
Water Temp: 30.2
Salinity: 22.6

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_2DI-1	8/18/11	247.10	9.13	110,000.0	46,000.0	0
t_2DI-2	8/18/11	286.07	9.97	150,000.0	11,000.0	0
t_2DI-3	8/18/11	223.38	11.13	210,000.0	46,000.0	0
t_2DI-4	8/18/11	286.50	11.53	24,000.0	46,000.0	0
t_2DI-5	8/18/11	189.01	10.93	460,000.0	2,400,000.0	0
	Average:	246.41	10.54	190,800.0	509,800.0	0.0

Day 7

Comments

Green Tag - Sandy Bay

Harvest Information
Harvest Area: Sandy Bay
Harvest Date: 8/22/11
Water Temp: 29.8°C
Salinity: 26.9 ppt

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)	
t_7SB-1	8/23/11	261.20	5.43	11,000.0	240,000.0	0	1 dead in bag

t_7SB-2	8/23/11	289.06	10.40	240.0	210.0	0	
t_7SB-3	8/23/11	247.35	9.97	1,100.0	2,400.0	0	
t_7SB-4	8/23/11	315.96	8.20	11,000.0	150.0	0	
t_7SB-5	8/23/11	323.44	8.53	2,400.0	1,100.0	0	
	Average:	287.40	8.51	5,148.0	48,772.0	0.0	

Green Tag - West Dauphin Island

Harvest Information
Harvest Area: West Dauphin Island
Harvest Date: 8/22/11
Water Temp: 30.8°C
Salinity: 27.6 ppt

				V.v.	*V. p.*		
	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	Probe Results (MPN/g)	tlh Probe Results (MPN/g)	tdh Probe Results (MPN/g)	
t_7DI-1	8/23/11	247.82	13.70	750.0	150.0	0	
t_7DI-2	8/23/11	378.42	13.07	240.0	93.0	0	
t_7DI-3	8/23/11	359.79	12.50	1,100.0	150.0	0	
t_7DI-4	8/23/11	352.56	10.43	460.0	150.0	0	
t_7DI-5	8/23/11	320.31	12.93	15,000.0	2,400.0	0	2 dead in bag
	Average:	331.78	12.53	3,510.0	588.6	0.0	

Day 14

Comments

Green Tag - Sandy Bay

Harvest Information
Harvest Area: Sandy Bay
Harvest Date: 8/29/11
Water Temp: 28.2°C
Salinity: 27.5 ppt

				V.v.	*V. p.*		
	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	Probe Results (MPN/g)	tlh Probe Results (MPN/g)	tdh Probe Results (MPN/g)	
t_{14}SB-1	8/30/11	256.36	8.20	14,000.0	240.0	0	1 dead in bag
t_{14}SB-2	8/30/11	258.44	8.83	1,100.0	460.0	0	
t_{14}SB-3	8/30/11	318.13	7.73	2,400.0	150.0	0	3 dead in bag
t_{14}SB-4	8/30/11	290.70	8.43	16,000.0	460.0	0	
t_{14}SB-5	8/30/11	284.51	11.47	3,800.0	210.0	0	
	Average:	281.63	8.93	7,460.0	304.0	0.0	

Green Tag - West Dauphin Island

Harvest Information

Harvest Area: West Dauphin Island
Harvest Date: 8/29/11
Water Temp: 30.2
Salinity: 29.0

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)	
t_{14}DI-1	8/30/11	253.80	6.93	21.0	240.0	0	
t_{14}DI-2	8/30/11	233.70	9.43	9.2	1,100.0	0	1 dead in bag
t_{14}DI-3	8/30/11	297.37	4.80	240.0	93.0	0	1 dead in bag
t_{14}DI-4	8/30/11	179.86	8.23	93.0	240.0	0	1 dead in bag
t_{14}DI-5	8/30/11	153.19	7.97	15.0	240.0	0	3 dead in bag
	Average:	223.58	7.47	75.6	382.6	0.0	

Day 0

Comments

Harvest Information
Harvest Area: Area 17, Moss Bay, LA
Harvest Date: 9/18/2011
Water Temp: 25.6 C
Salinity: 22 ppt.

Boat

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_0B-1	9/20/11	249.92	4.93	2,400.0	21.0	0.0
t_0B-2	9/20/11	230.47	5.03	4,600.0	150.0	0.0
t_0B-3	9/20/11	317.02	6.27	24,000.0	1,100.0	0.0
t_0B-4	9/20/11	307.41	5.87	2,400.0	1,200.0	0.0
t_0B-5	9/20/11	317.30	6.50	46,000.0	210.0	0.0
	Average:	284.42	5.72	15,880.0	536.2	0.0

White Tag

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_0W-1	9/20/11	328.13	4.23	2,900.0	93	0.0
t_0W-2	9/20/11	213.80	4.50	4,600.0	43	0.0
t_0W-3	9/20/11	251.29	6.10	7,500.0	93	0.0
t_0W-4	9/20/11	238.54	5.83	4,600.0	93	0.0
t_0W-5	9/20/11	224.01	5.00	1,100.0	93	0.0
	Average:	251.15	5.13	4,140.0	1,084.6	0.0

Green Tag

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_0G-1	9/20/11	280.08	6.00	15,000.0	150.0	0.0
t_0G-2	9/20/11	304.25	6.77	24,000.0	93.0	0.0
t_0G-3	9/20/11	248.66	6.40	110,000.0	2,400.0	0.0
t_0G-4	9/20/11	323.28	3.97	15,000.0	380.0	0.0
t_0G-5	9/20/11	258.12	5.10	24,000.0	2,400.0	0.0
	Average:	282.88	5.65	37,600.0	1,084.6	0.0

Day 2

Comments

Green Tag - Sandy Bay

Harvest Information
Harvest Area: Sandy Bay
Harvest Date: 9/21/11
Water Temp: 28.5°C
Salinity: 21.2 ppt

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)	
t_2SB-1	9/22/11	261.45	9.70	46,000.0	46,000.0	0.0	2 dead in bag
t_2SB-2	9/22/11	170.52	7.57	29,000.0	110,000.0	30.0	4 dead in bag
t_2SB-3	9/22/11	261.58	9.10	2,400,000.0	20,000.0	0.0	
t_2SB-4	9/22/11	254.08	7.70	75,000.0	46,000.0	0.0	2 dead in bag
t_2SB-5	9/22/11	275.49	10.93	46,000.0	46,000.0	3.0	2 dead in bag
	Average:	244.62	9.00	519,200.0	53,600.0	6.6	

Green Tag - West Dauphin Island

Harvest Information
Harvest Area: West End D.I.
Harvest Date: 9/21/11
Water Temp: 27.1°C
Salinity: 27.8 ppt

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)	
t_2DI-1	9/22/11	361.93	5.83	460,000.0	29,000.0	300.0	1 dead in bag
t_2DI-2	9/22/11	262.22	7.23	24,000.0	110,000.0	0.0	
t_2DI-3	9/22/11	287.91	9.40	150,000.0	75,000.0	0.0	
t_2DI-4	9/22/11	334.67	11.00	46,000.0	46,000.0	0.0	1 dead in bag
t_2DI-5	9/22/11	256.03	8.80	460,000.0	210,000.0	0.0	1 dead in bag
	Average:	300.55	8.45	228,000.0	94,000.0	60.0	

Day 7

Comments

Green Tag - Sandy Bay

Harvest Information
Harvest Area: Sandy Bay
Harvest Date: 9/26/11
Water Temp: 26.8°C
Salinity: 22.3 ppt

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)
t_7SB-1	9/27/11	377.85	6.23	3,000.0	2,700.0	0.0

t_7SB-2	9/27/11	355.68	2.03	240.0	2,400.0	0.0	1 dead in bag
t_7SB-3	9/27/11	261.59	6.43	460.0	7,500.0	0.0	1 dead in bag
t_7SB-4	9/27/11	323.04	4.53	11,000.0	24,000.0	0.0	
t_7SB-5	9/27/11	277.83	4.80	2,400.0	2,400.0	0.0	
	Average:	319.20	4.80	3,420.0	7,800.0	0.0	

Green Tag - West Dauphin Island

Harvest Information
Harvest Area: West Dauphin Island
Harvest Date: 9/26/11
Water Temp: 27.4°C
Salinity: 26.8 ppt

				V.v.	*V. p.*		
	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	Probe Results (MPN/g)	tlh Probe Results (MPN/g)	tdh Probe Results (MPN/g)	
t_7DI-1	9/27/11	346.17	2.60	460.0	11,000.0	0.0	
t_7DI-2	9/27/11	374.32	6.20	21.0	2,400.0	0.0	
t_7DI-3	9/27/11	304.90	5.20	1,100.0	350.0	0.0	
t_7DI-4	9/27/11	353.65	4.60	240.0	1,100.0	0.0	
t_7DI-5	9/27/11	305.88	4.10	110,000.0	2,400.0	0.0	
	Average:	336.98	4.54	22,364.2	3,450.0	0.0	

Day 14

Comments

Green Tag - Sandy Bay

Harvest Information
Harvest Area: Sandy Bay
Harvest Date: 10/3/11
Water Temp: 22.6°C
Salinity: 23.7 ppt

				V.v.	*V. p.*		
	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	Probe Results (MPN/g)	tlh Probe Results (MPN/g)	tdh Probe Results (MPN/g)	
t_{14}SB-1	10/4/11	283.44	3.53	93.0	1,100.0	0.0	
t_{14}SB-2	10/4/11	303.12	2.93	93.0	1,100.0	0.0	
t_{14}SB-3	10/4/11	266.05	3.50	280.0	460.0	3.6	
t_{14}SB-4	10/4/11	307.75	4.40	150.0	210.0	0.0	
t_{14}SB-5	10/4/11	242.82	4.45	1,500.0	460.0	0.0	1 dead in bag
	Average:	280.64	3.76	423.2	666.0	0.7	

Green Tag - West Dauphin Island

Harvest Information

Harvest Area: West Dauphin Island
Harvest Date: 10/3/11
Water Temp: 21.6°C
Salinity: 22.1 ppt

	Date Tests Began	Weight per 12 Oysters (g)	Oyster Temp (°C)	*V.v.* Probe Results (MPN/g)	*V. p.* tlh Probe Results (MPN/g)	*V. p.* tdh Probe Results (MPN/g)	
t_{14}DI-1	10/4/11	231.66	3.30	23.0	460.0	0.0	
t_{14}DI-2	10/4/11	261.64	7.40	460.0	460.0	3.0	1 dead in bag
t_{14}DI-3	10/4/11	344.20	4.50	11.0	240.0	0.0	
t_{14}DI-4	10/4/11	199.66	4.03	93.0	2,400.0	0.0	1 dead in bag
t_{14}DI-5	10/4/11	226.47	5.07	43.0	93.0	0.0	
	Average:	252.73	4.86	126.0	730.6	0.6	

Auburn University

Scope of Work
William C. Walton, co-PI

Test of Effectiveness of Relaying as a Post Harvest Process for reducing levels of *Vibrio vulnificus* and *V. parahaemolyticus*in Shellstock Oysters

As a co-PI, Walton will oversee field deployment of oysters (including maintenance and sample collection) and lead the analysis of the results. This will include arrangement of boat time and provision of the grow-out gear. Additionally, he will work closely with the other PIs to coordinate sampling and deployments, as well as authorship of the final report.

Bon Secour Fisheries, Inc.

Executive Summary:

The objective of the proposed research is to test the effectiveness of the relay of oysters (*Crassostrea virginica*) to a monitored, inshore site and to a remote, higher salinity site as a means of reducing *Vibrio* levels.

Laboratory observations by Kaspar and Tamplin (1993) of pure cultures of *V. vulnificus* suggested that elevated salinities of 25 ppt, or higher, reduced *V. vulnificus* numbers. Motes and DePaola (1996), showed that oysters relayed to high-salinity offshore waters (>32 ppt) showed a significant reduction in *V. vulnificus* numbers after two (2) weeks.

Less well known is the amount of time necessary for shellstock oysters, harvested and transported under refrigeration and placed back into the environment (relay), to reestablish "ambient" levels of *V. vulnificus* and *V. parahaemolyticus* consistent with other oysters in the same area. Current regulatory regimes in the Gulf States require rapid refrigeration post harvest for any oysters to be sold live, in the shell, for raw consumption. Shellstock which have been harvested and rapidly refrigerated in compliance with a *Vibrio vulnificus* Control Plan, are referred to as "white tag" shellstock. Oysters refrigerated less quickly are given a different tag and must be shucked by a certified dealer or post harvest processed. These are referred to as "non-matrix", "restricted use" or "green tag" shellstock.

The present time temperature requirements which are imposed during the summer months are very restrictive and severely limit the harvest time for oyster harvesters. Should relaying prove effective in reducing *Vv* and *Vp* levels it could be considered as a viable control for reducing *Vv* illnesses.

Given the fact that green tag shellstock should be less costly to harvest and more available than white tag shellstock, post harvest processes which will reduce levels of *V. vulnificus* and *V. parahaemolyticus* in green tag shellstock to levels equivalent to those of the white tag product could be useful to the Gulf oyster industry. An argument may then

be made that, although the relayed product cannot be labeled as having either of these organisms reduced to non-detectable levels, the product should be allowed for sale as "white tag" shellstock as if it had been placed under refrigeration in accordance with a State's *Vibrio vulnificus* Control Plan when it was originally harvested.

Approach and Methodology:

To determine experimentally the minimum amount of time for *Vibrio* loads within green tag oysters to return to levels no higher than those detected in white tag oysters from the same original harvest area, we propose to conduct a field study at the Point aux Pins Oyster Farm, in Grand Bay, AL and at a remote location at the west end of Dauphin Island, AL. Three (3) time zero (t_0) samples of 15 oysters each will be taken from shellstock oysters on board the harvest vessel immediately after harvest and refrigerated with gel ice. During the same harvesting trip, oysters from the same area will be segregated into two groups aboard the vessel: one group to be refrigerated within one hour after which the internal temperature of the oysters will reach 55ºC or less within 6 hours. white tag) and one to remain unrefrigerated for the duration of the harvesting trip (green tag). A temperature recording device will be placed with both groups of oysters to record the approximate internal temperature of the oysters. After the harvesting vessel returns to the dock the t_0 samples as well as the white and green tag oysters will be loaded onto a refrigerated conveyance and brought to Bon Secour Fisheries, Inc. Three (3) samples of 15 oysters each will be taken from each of the white (t_{0WT}) and green tag (t_{0GT}) groups. All time zero samples will be shipped overnight to the Texas A&M University, Seafood Safety Laboratory in Galveston, TX for bacteriological analysis (9 samples total). Surface temperature and salinity data will also be gathered at the original harvest site using a refractometer and immersion thermometer. Green tag oysters will be deployed in the shellfish aquaculture cages at each of the two sites. Three samples of oysters from each of these sites will be taken at 2 days (t_{2d}), 7 days (t_{7d}), and 14 days (t_{14d}) after relay (18 samples total). At each of these three time points samples will be shipped overnight to the Texas A&M Laboratory in Galveston, TX for bacteriological analysis.

Water temperature and salinity will be measured at each relay site with a handheld YSI at deployment, and a continuous salinity/temperature logger (Star-Oddi) will be deployed with the oysters at each site, recording data every 5 minutes.

We propose two independent trial runs during Summer 2011. Data will be generated on the initial harvest levels of *V. vulnificus* and *V. parahaemolyticus* and in both green tag and white tag shellstock. Also measured will be the degree to which the levels in the green tag product are reduced by relaying to the two sites. Comparing salinity data from the original harvest site with those recorded at the two relay sites, while also measuring reductions in *V. vulnificus* and *V. parahaemolyticus*, should indicate the degree to which relay into a higher salinity regime is important in reducing *Vibrio* levels.

Project Deliverables:

This study will determine if *V. vulnificus* and *V. parahaemolyticus* levels in Gulf oysters can be lowered significantly by relay.This will be accomplished by comparing *Vv* and *Vp* levels in oysters at harvest (t_0), and after compliance with established time temperature controls for both white tag (t_{0WT}) and green (t_{0GT}) oysters, to those levels found after 2 days (t_{2d}), 7 days (t_{7d}) and 14 days (t_{14d}) after relay. Should *Vibrio* levels be reduced significantly this study will provide adequate data to validate relaying as a viable equivalent to the *V. vulnificus* and *V. parahaemolyticus* time-temperature requirements of the NSSP.