

# Workshop on Molluscan Shellfish Safety

**August 10-12, 2005**

**Grand Hotel Marriott Resort, Point Clear, Alabama USA**



2005 MOLLUSCAN SHELLFISH SAFETY



## Abstracts and Program



## 2005 MOLLUSCAN SHELLFISH SAFETY



On behalf of the Organizing Committee for The Workshop on Shellfish Safety we want to welcome each and every one of you from as far away as New Zealand and Japan to as close to home as Bon Secour, Alabama. This international workshop provides an opportunity to integrate scientific advancements presented at the International Conference on Molluscan Shellfish Safety with the Biennial Meeting of the Interstate Shellfish Sanitation Conference (ISSC). During this workshop, internationally renowned scientists will report on the most recent scientific and regulatory developments involving microbial pathogens. Most importantly, collaboration with ISSC will permit interaction between scientists, industry and government and familiarize the international community with the US programs.

The response to our call for abstracts was excellent, well beyond our expectations and we are very grateful for the quality of these abstracts and the resulting oral presentations and posters. Science addressing concerns for shellfish-borne illnesses due to vibrios and viruses, and respective controls will be highlighted. Common problems such as the increasing occurrence of *Vibrio parahaemolyticus* affecting shellfish in Asia, Europe, North and South America will be featured. Invited presentations will encourage mutual interest in new analytical methods including real time PCR and detection of Norovirus and Enterovirus, plus development of validated processing controls. International cooperation on such issues as discharge of ballast waters, global warming, and ecological adaptation will be critical in implementing effective controls. Roundtables will be held at end of each day's sessions to discuss topics of the day and address questions from the audience.

The workshop is organized into five sessions to address Epidemiology, Methods, Ecology and Distribution, Risk Management and Communications, and Research Needs and Future Directions. Within these sessions there is an emphasis on emerging issues, for example within Epidemiology there is a focus on emerging trends such as outbreaks associated with climate change or globalization. Within the Methods Session we will discuss emerging methods such as real time PCR particularly those that can be applied to risk assessments or address the transition from the research applications to regulatory analysis. We will focus on research that addresses major data gaps affecting our ability to conduct meaningful risk assessments for vibrios and viruses. Within Risk Management and Communication we will cover the effectiveness of various control measures in reducing pathogen levels including harvest controls, ballast water discharge controls, depuration, PHP, and education. Our final session is titled Research Needs and Future Directions. The speakers will address research needs based on risk management questions and we expect lively roundtable discussions on the effectiveness of current shellfish programs for controlling pathogens. We will end the workshop with a discussion of research needs and how we can expand opportunities for international cooperation.

We would like to thank you for your interest in this workshop and encourage you to participate in discussions of plans for future directions and opportunities for international cooperation.

Kay Bruening  
Angelo DePaola  
Tom Herrington  
Dorothy Leonard  
LaDon Swann

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# AGENDA

## First ISSC/ICMSS Joint Workshop and Integration of Science into Policy: Special Emphasis on Vibrios and Viruses

**Wednesday, August 10, 2005**

**7:30 a.m. Continental Breakfast**

**9:00 Welcome**  
Alabama (1<sup>st</sup> District) Congressman Jo Bonner

**9:15 Opening Remarks**  
Dot Leonard, Ocean Equities LLC

### **Session I: Epidemiology**

**Moderator: Spencer Garrett, NMFS, Office of Sustainable Fisheries, National Seafood  
Inspection Laboratory**

**9:40 Reporting Programs for Vibrios and Challenges of Meeting Risk Assessment Needs**  
John Painter, Center for Disease Control (CDC)

**10:00 *Vibrio parahaemolyticus* Gastroenteritis Associated With Consumption of Alaskan Oysters**  
Joe McLaughlin, Alaska Section of Epidemiology

**10:20 Break**

**11:00 *Vibrio parahaemolyticus* Outbreaks in Spain: Implications for the EU**  
Jaime Martinez-Urtaza, University of Santiago de Compostela, Spain

**11:20 The Role of Human Volunteer Studies in Risk Management:  
Evidence from Virus Infectivity Data Subjected to Quantitative Risk Assessments**  
Mark Sobsey, University of North Carolina

**11:40 *Vibrio parahaemolyticus* in Clinical and Shellfish Samples During the Diarrhea Outbreaks of  
2004 and 2005 in Southern Chile**  
Romilio Espejo, Nutrition & Food Technology Institute, Chile

**12:00 p.m. Lunch and Posters**

# AGENDA

## First ISSC/ICMSS Joint Workshop and Integration of Science into Policy: Special Emphasis on Vibrios and Viruses

**Wednesday, August 10, 2005 (continued)**

### Session II: Methods

**Moderator:** Gary Richards, United States Department of Agriculture (USDA), Agricultural Research Service, Delaware State University

- 1:40**      **Detection of Pathogens in Shellfish and the Environment Using Molecular Methods**  
Asim Bej, University of Alabama at Birmingham
- 2:00**      **Detection of Viruses in Shellfish-Challenges of Method Standardisation, Validation and Ring Trials**  
Rachel Rangdale, Center for Environment, Fisheries, and Aquaculture Science (CEFAS), United Kingdom
- 2:20**      **Development and Application of Quantitative Multiplex RT-PCR Assays for Calici- and Enteroviruses in Shellfish and Wastewater**  
Bill Burkhardt, Food and Drug Administration (FDA) Gulf Coast Seafood Laboratory
- 2:40**      **Rotavirus Virus Like Particles as Surrogates to Evaluate Virus Persistence in Shellfish**  
Soizick LeGuyader, IFREMER, France
- 3:00**      **Break and Posters**
- 3:40**      **Viral Methods Development and Applications**  
James Lowther, Center for Environment, Fisheries, and Aquaculture Science (CEFAS), United Kingdom
- 4:00**      **Quantitative (Real-Time) PCR Assessment of Post Harvest Processing of Oysters**  
Anita Wright, University of Florida
- 4:20**      **Detection of Noroviruses in Shellfish: Development of a Method for Regulatory Use in New Zealand**  
Gail Greening, Institute of Environmental Science and Research Ltd., New Zealand
- 4:40**      **Roundtable to discuss topics of the day and address questions from the audience and to formulate recommendations to facilitate efforts for harmonization.**
- 6:00 - 8:00 p.m.**      **Welcome Reception and Risk Assessment Demonstrations**

# AGENDA

## First ISSC/ICMSS Joint Workshop and Integration of Science into Policy: Special Emphasis on Vibrios and Viruses

**Thursday, August 11, 2005**

- 8:00 a.m.**      **Continental Breakfast**
- 8:40**            **Opening Remarks**  
Don Kraemer, FDA
- Session III: Ecology and Distribution**  
**Moderator: Geoff Scott, NOAA National Ocean Service**
- 9:00**            **The First Pandemic of *Vibrio parahaemolyticus* Infection: Emergence, Spread, and Evolution of the Pandemic O3:K6 Clone**  
Mitsuaki Nishibuchi, Kyoto University, Japan
- 9:20**            **Studies on the Two Genotypes of *Vibrio vulnificus* Biotype 1**  
Jim Oliver, University of North Carolina at Charlotte
- 9:40**            **Hepatitis A Transmitted by Food: An Outbreak in Italy**  
Luciana Croci, ISS, Italy
- 10:00**          **Microbiological Survey of Oysters in U.S. Retail Markets**  
Paul DiStefano, FDA
- 10:40**          **Break and Posters**
- 11:00**          **Ballast Water**  
Patricia Desmarchelier, Food Science Australia
- 11:20**          **Survey of PHP Oysters in the United States**  
John Bowers, U. S. FDA
- 12:00 p.m.**    **Lunch and Posters**
- 1:20**            **Tracking Sources of Microbial Contaminants**  
Jan Gooch, Center for Coastal Environmental Health and Environmental Research, National Oceanic and Atmospheric Administration (NOAA)
- 1:40**            **Virus Detection in South Carolina Shellfish Waters: Indicators Versus Pathogens**  
Brian Robinson, Center for Coastal Environmental Health and Environmental Research, National Oceanic and Atmospheric Administration (NOAA)



# AGENDA

## First ISSC/ICMSS Joint Workshop and Integration of Science into Policy: Special Emphasis on Vibrios and Viruses

**Thursday, August 11, 2005 (continued)**

### Session IV: Risk Management and Communications

**Moderator:** Chris Nelson, Bon Secour Fisheries

- 2:00**      **The Efficacy of High Pressure Processing for Viruses**  
David Kingsley, USDA, Delaware State University
- 2:20**      **The Colony Overlay Procedure for Peptidases to Detect and Enumerate Total Vibrionaceae in Molluscan Shellfish and Their Growing Waters**  
Gary Richards, USDA, Delaware State University
- 2:40**      ***Vibrio parahaemolyticus* Risk Management in British Columbia**  
Klaus Schallié, Canadian Food Inspection Agency, Canada
- 3:00**      **Break and Posters**
- 3:40**      **The FAO/WHO Risk Assessments on *Vibrio* spp. in Seafoods**  
Sarah Cahill, FAO, Italy
- 4:00**      **Vibrios, Viruses, Verdicts And Virtuosos**  
Dorothy-Jean McCoubrey, New Zealand Food Safety Authority, New Zealand
- 4:20**      **Development of Improved Risk Assessment and Management Tools for *Vibrio vulnificus*: What Makes Them Pathogenic for Humans?**  
Mark Strom, Northwest Fisheries Science Center, NOAA Fisheries
- 4:40**      **Brief History of Microbial Indicators in the National Shellfish Sanitation Program, USA**  
Bill Watkins, FDA
- 5:00**      **Roundtable to discuss topics of the day and address questions from the audience and to formulate recommendations to facilitate efforts for harmonization.**
- 7:00-10:00 p.m.**      **Seafood Festival, USS Battleship Alabama**  
PCR and phage demos  
UNC-Morehead City, EPA, FDA, UNC Chapel Hill, UAB

# **AGENDA**

## **First ISSC/ICMSS Joint Workshop and Integration of Science into Policy: Special Emphasis on Vibrios and Viruses**

**Friday, August 12, 2005**

- 8:00 a.m. Continental Breakfast**
- 8:40 Opening Remarks**  
Rob Wittman, ISSC
- Session V: Research Needs and Future Directions**  
**Moderator: Angelo DePaola, FDA Shellfish Laboratory**
- 9:00 Research Needs and Future Directions of the U.S. Environmental Protection Agency (U.S. EPA)**  
Joel Hansel, U.S. EPA
- 9:15 Research Needs and Future Direction of the European Union (EU) Shellfish Programme**  
Ron Lee, CEFAS
- 9:30 European Industry Concerns, Research Needs and Future Directions**  
Doug McLeod, Association of Scottish Shellfish Growers
- 9:45 NOAA Oceans and Human Health Program**  
Juli Trtanj, NOAA National Ocean Service
- 10:00 Break and Posters**
- 10:30 National Sea Grant College Program Shellfish Safety and Future Directions**  
Rick Wallace, Mississippi-Alabama Sea Grant Extension Program
- 10:45 Future Research Needs for Vibrios and Viruses in Molluscan Shellfish: FDA Perspective**  
Don Kraemer, FDA
- 11:00 *Vibrio Vulnificus* Education, The Gulf of Mexico Program and the United States Ocean Action Plan**  
Tom Herrington, FDA, Office of Seafood
- 11:15 Post Harvest Processing of Oysters to Reduce Risk**  
Mike Voisin, Motivati Seafoods, Inc.
- 11:30 Leveraging Resources Based on Risk Assessment**  
Jon Bell, Louisiana State University Agricultural Center
- 11:45 Research Needs and Future Direction in New Zealand**  
Helen Smale, NZ Industry Water Quality Committee, New Zealand
- 12:00 Roundtable to discuss topics of the day and address questions from the audience and to formulate recommendations to facilitate efforts for harmonization.**
- 12:30 p.m. Announcements and Adjourn**

## ***Vibrio parahaemolyticus* GASTROENTERITIS ASSOCIATED WITH CONSUMPTION OF ALASKAN OYSTERS**

Joseph B. McLaughlin, M.D.\*, M.P.H., Angelo DePaola, Ph.D., Cheryl A. Bopp, M.S., Karen A. Martinek, R.N., M.P.H., Nancy P. Napolilli, B.S., Christin G. Allison, B.S., Shelley L. Murray, B.S., Eric C. Thompson, B.S., Michele M. Bird, M.S., John P. Middaugh, M.D.

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**BACKGROUND:** *Vibrio parahaemolyticus* (*Vp*), the leading cause of seafood-associated gastroenteritis in the United States, is typically associated with consumption of raw oysters gathered from warm estuarine waters. We describe a *Vp* outbreak that occurred in Alaska during the summer of 2004.

**METHODS:** We conducted a retrospective cohort study among cruise ship passengers to determine illness attack rates and risk factors, active surveillance to identify additional case-patients not associated with the cruise ship, and an extensive environmental study to identify sources of *Vp* and factors that might have contributed to the outbreak.

**RESULTS:** Of 189 passengers, 132 (70 percent) were interviewed; 22 (17 percent) met our gastroenteritis case definition. Raw oysters were the only significant predictor of illness after multiple logistic regression analysis. Cruise ship case-patients consumed a median of one oyster. Active surveillance identified numerous additional case-patients. Pulsed-field gel electrophoresis patterns were highly conserved across clinical and oyster isolates. Seventy-four percent of environmental isolates were positive for the presence of the thermostable direct hemolysin gene (a known virulence factor).

**CONCLUSIONS:** This large *Vp* outbreak associated with consumption of raw Alaskan oysters demonstrated a low infectious dose and extraordinarily high proportions of pathogenic environmental isolates.

## ***Vibrio parahaemolyticus* OUTBREAKS IN SPAIN: IMPLICATIONS FOR THE EU**

Jaime Martinez-Urtaza

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Universidad de Santiago de Compostela  
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*V. parahaemolyticus* infections have increased globally during the last decade. From 1996, outbreaks of illnesses due to this organism have been detected in several Asia countries, Chile and the USA. By contrast, the risk of infections caused by *V. parahaemolyticus* in Europe has been considered low according to recent reviews of epidemiological information. Data obtained after an exhaustive survey of unreported cases at Spanish hospitals have shown that *V. parahaemolyticus* infections in Spain are more common than previously assumed. This organism was isolated from patients with gastroenteritis in Barcelona (1986, 1987 and 1999), Zaragoza (1993) and Madrid (1998 and 2000). In Galicia (NW Spain), 84 cases of *V. parahaemolyticus* infections were identified retrospectively from hospital records from 1997 to 2000. A single outbreak of 64 cases in 1999 was associated with oyster consumption. Most Spanish clinical isolates were serotype O4:K11 and pulsed-field gel electrophoresis (PFGE) analysis demonstrated these to be a unique clone distinct from Asian and American clinical strains. In July 2004, a second large *V. parahaemolyticus* outbreak of 80 illnesses occurred in A Coruña. The outbreak isolates belonged to the serotype O3:K6 and O3:K untypeable, had the *toxR*, *tlh*, and *tdh* genes, lacked the *trh* gene, and were positive for the group specific-PCR assay. Results from PFGE and arbitrarily primed PCR analyses unequivocally linked the outbreak isolates to the pandemic clone of *V. parahaemolyticus*. The epidemiological investigation associated with this outbreak identified boiled crab as the most probable vehicle of infection.

Investigations of the environmental circumstances existing during the outbreak episodes have revealed that the sudden increment of infections detected in 1999 and 2000 were coincident with the presence of exceptional oceanographic conditions (dominant downwelling periods and warm seawater temperatures), which suggests that changing environmental conditions could drive the increment of *V. parahaemolyticus* levels in the marine environment of Galicia. By contrast, no unusual oceanographic conditions were observed in the nearby dates of the 2004 outbreak. This observation, together with the pandemic nature of the isolates, directs the most probable origin of the contamination to the ballast water from ships.

The emergence of *V. parahaemolyticus* infections in the European continent is an important public health concern and stresses the urgent need for the revision of the current status of this organism in the European microbiological surveillance system for infectious gastroenteritis and its possible incorporation in the control programs for shellfish harvesting areas and ready-to-eat seafood in Europe.

# THE ROLE OF HUMAN VOLUNTEER STUDIES IN RISK MANAGEMENT: EVIDENCE FROM VIRUS INFECTIVITY DATA SUBJECTED TO QUANTITATIVE RISK ASSESSMENTS

Mark D. Sobsey

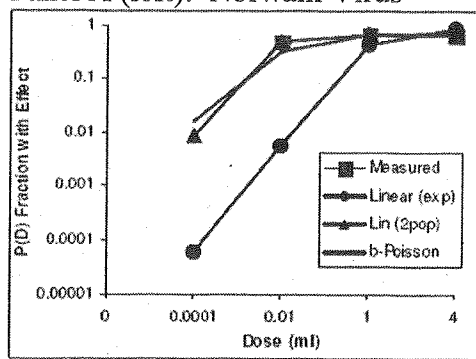
University of North Carolina  
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A limited number of human volunteer studies have been done to predict the risks of viral infection and illness from ingestion of raw mollusks. Two main types of studies have been done: those using safety-tested inocula of specific human enteric viruses and those using actual shellfish harvested from natural waters. In this presentation will briefly review the available data from both types of studies, the strengths and weaknesses of these data and the challenges faced in trying to use such data for microbial risks assessments and risk management decisions. Also to be discussed are the future potentials for such studies and the challenges presented by new regulations governing microbial inocula quality and safety, health protection and ethics in human volunteer studies.

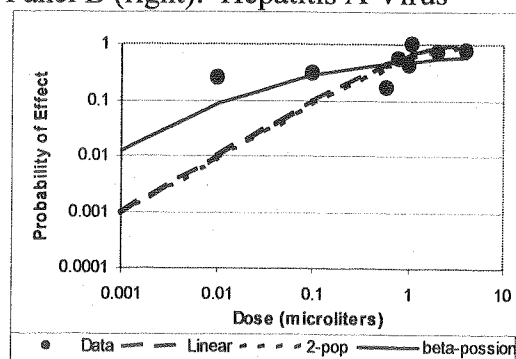
The presentation will include information and data analysis from human volunteer dose-response infectivity studies that have been done with safety-tested inocula for hepatitis A virus, Norwalk Virus, Rotavirus, Polioviruses, Echoviruses, Coxsackieviruses and Adenoviruses. The need to "mine" these previous data for human infectivity dose-response relationships and the development of risk estimates relevant to ingestion of raw shellfish will be discussed and examples will be presented. Specifically, historical data for Norwalk Virus and Hepatitis A Virus will be presented with new dose-response analyses. Examples of the dose-response analyses of these data are shown in the Figures below. Also to be presented are the results of human volunteer studies on risks of gastroenteritis from ingestion of raw oysters and clams that were done in the 1980s. The extent to which these data can be used for risk assessments and risk management decisions will be considered.

## Dose-Response Modeling of Virus Illness from Historical Human Volunteer Data:

Panel A (left): Norwalk Virus



Panel B (right): Hepatitis A Virus



# ***Vibrio parahaemolyticus* IN CLINICAL AND SHELLFISH SAMPLES DURING THE DIARRHEA OUTBREAKS OF 2004 AND 2005 IN SOUTHERN CHILE**

Romilio T. Espejo\*, Jessica Toro, Cristina Hernández, Loreto Fuenzalida, and Jaime Romero

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Large diarrhea epidemic outbreaks associated to seafood consumption have taken place the austral summers of 2004 and 2005 in the environs of Puerto Montt Chile (41 °S 72°W, approximately). The 2004 epidemic reached approximately 1.500 clinical cases and the epidemic of 2005 about 3.600. Samples of clinical cases and shellfishes were analyzed to understand the origin and spread of these epidemics. Our observations indicate that the epidemics were directly related to the presence of the O3:K6 serovar pandemic clone, in spite this clone was a minor component of a small *V. parahaemolyticus* population in shellfishes.

The epidemic in 2005 was not exclusive of the Puerto Montt area. About 11.000 clinical cases were reported in the whole country. Nevertheless, it is likely that seafood from the Puerto Montt region caused most of the cases because this region produces about 80 % of the seafood consumed in the large cities. Analysis of the stools from clinical patients indicated that both epidemic outbreaks were caused by the O3:K6 pandemic clone of *V. parahaemolyticus* that emerged in Southeast Asia in 1996. Before 2004, *V. parahaemolyticus* infection had not been reported in this region and their absence was thought to be due to the low seawater temperature that seldom reaches more than 16 °C.

Analysis of the shellfish samples obtained at the midst of the epidemics (January-March) showed that 53% contained *V. parahaemolyticus*. However the total load was low, ranging from 3 to 93 g<sup>-1</sup>, and only 4 of the 108 positive samples showed O3:K6 pandemic isolates. *V. parahaemolyticus* population in shellfish was mainly composed by non-pandemic strains, which could be differentiated into 14 clonal groups. None was *tdh* or *trh* positive. Only four

of the 14 clonal groups were found both summers, suggesting either temporal changes in population composition or a much larger diversity than that observed by us in these two years.

| Total and pandemic <i>V. parahaemolyticus</i> in clinical and shellfish samples |       |                 |                |
|---|-------|-----------------|----------------|
| Samples   | Total | <i>V. parah</i> | O3:K6 pandemic |
| Clinical  | 64    | 63              | 58 (90%)       |
| Shellfish   | 204   | 108             | 3 (3%)         |

Clonal groups were differentiated by a new method developed to allow rapid and simple comparison of the bacterial origin. This method permits direct genome restriction enzyme analysis (DGREA) with equal discriminatory index than RFLP-PFGE. It is based on digestion of bacterial DNA with a six base-restriction endonuclease, separation of the fragments with sizes ranging from 500 to 2.500 bp by polyacrylamide gel electrophoresis, and comparison of the fragments patterns after visualization by silver nitrate staining.



## DETECTION OF PATHOGENS IN SHELLFISH AND THE ENVIRONMENT USING MOLECULAR METHODS

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Detection of microbial pathogens in shellfish using molecular methods can provide rapid and reliable assessment for the microbiological safety of post-harvest-processed products (PHP) for the consumers. Recently the application of fluorogenic real-time PCR has progressed significantly, establishing its potential as an alternate method for the detection of *Vibrio vulnificus* and *V. parahaemolyticus* in shellfish and its surrounding waters. Detection of *V. vulnificus* in shellfish homogenate has been achieved by using SYBR Green I fluorescent dye or by dual-labeled fluorescent reporter dye on taqman probes. This detection assay targeting a 205 bp segment of the *Vibrio vulnificus* hemolysin gene includes a 5-h enrichment process, followed by the release of DNA by treatment with Instagene® matrix, and PCR amplification with appropriate reaction mixtures. The detection in seeded and natural oyster tissue homogenates or Gulf of Mexico water was achieved at an initial inoculum of <10 cfu per gram. A real-time <8h PCR assay for the detection of *V. vulnificus* in shellfish has been proposed for further validation and implementation, as an additional approach for monitoring this microorganism in shellfish.

Detection of *V. parahaemolyticus* in shellfish has been achieved by real-time PCR with Taqman probes targeting thermolabile hemolysin (*tlh*) for species identification, thermostable direct hemolysin (*tdh*) and thermostable-related hemolysin (*trh*) for hemolysin-producing pathogenic strains, and open-reading frame 8 (ORF8) for the detection of pandemic strains of O3:K6 serotype. A multiplexed PCR for all 4 targeted genes, using gene-specific Taqman probes and primers, was achieved after 8-16 h of enrichment of oyster homogenate and consisted of an initial level of <10 cfu/g of this microorganism. Detection for all targeted genes with an equivalent sensitivity was possible with  $10^6$  cfu non-*V. parahaemolyticus* background microorganisms. The application of multiplexed 4-loci Taqman PCR for the identification of total and pathogenic *V. parahaemolyticus* in shellfish distributed by the seafood industry remains promising.

In addition to the real-time PCR, the potential for comprehensive multigene-targeted natural and human-introduced pathogen detection in shellfish has been investigated by using DNA-microarray technology. The method has been tested for the presence of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in seeded and natural oysters. Identification of phage-displayed peptides and the application of a biosensor technology are currently being investigated for an "intact cell"-based detection of *V. vulnificus* or other pathogenic vibrios in shellfish. Validation and implementation of molecular-based detection methods for pathogenic microorganisms in PHP oysters warrant an active partnership among industry, ISSC and academic institutions for the benefit of consumer health and the seafood industry.

## **DETECTION OF VIRUSES IN SHELLFISH-CHALLENGES OF METHOD STANDARDISATION, VALIDATION AND RING TRIALS**

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The introduction of an internationally recognised reference method for detection and quantification of norovirus (NoV) and hepatitis A (HAV) viruses will facilitate the incorporation of viral standards into legislation and supporting monitoring programmes. The acceptance of such a method will also provide a benchmark for research and development, and comparative validation of alternative procedures for virus detection/quantification.

CEN, the European Committee for Standardization, was founded in 1961 by the national standards bodies in the European Economic Community and EFTA countries. In 2004 the working group (CEN/TC 275/WG 6/ TAG 4) on "Detection of viruses in food" was established with the aim of developing a horizontal method for NoV and HAV in foodstuffs, including shellfish. The timescale for production of a draft standard is 21 months with publication as a Technical Specification within 3 years. Thus far a consensus has been reached on a number of important aspects of method standardisation:

- use of a real time PCR (probe based) format
- extraction methods suitable for each matrix would be incorporated into the standard- for bivalve shellfish sample preparation would be targeted at the dissected digestive gland.
- acid extraction should be common to all matrices- based upon Boom silica bead/GITC method.
- use of controls - process (extraction) control (FCV, EMCV (Mengo), positive / negative RNA target control, PCR inhibition control.

In parallel, CEFAS in its role as the EU Community Reference Laboratory for bacteriological and viral contamination of bivalve molluscs has established virus proficiency testing for NoV and HAV. The scheme is open to any laboratory with an interest in virus testing of shellfish and is now in its fourth year. In 2004/5, samples of variously high, moderate and low titre tissue culture grown HAV HM174), and faecal material containing either or genogroup I (Luton (AF439541) and II (Farmington Hills (AY502023) NoV present either singularly or in combination were distributed to up to 16 laboratories in Belgium & Luxembourg, Denmark, Finland, France, Germany, Greece, Italy, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, Spain, The United Kingdom and The United States. Analyses of data have enabled assessments of method specific and laboratory performance and contributed toward method standardisation. A number of themes have emerged, specifically all extraction methods do not produce equivalent results for NoV, detection of GI is more problematic than GII and laboratories experience fewer difficulties in detection of HAV than NoV. In 2004/5 the ring trial samples comprised tissue culture grown HAV, and GI and GII from faecal material. Thus whilst challenging the ability of participants' to detect viral RNAs from mixed samples, issues associated with specificity, sensitivity and/or discrimination in a bivalve shellfish matrix were not fully addressed. The next distribution will comprise bioaccumulated bivalve *Crassostrea gigas*. The results of these ring trials and plans for future programmes are discussed.

## **DEVELOPMENT AND APPLICATION OF QUANTITATIVE MULTIPLEX RT-PCR ASSAYS FOR CALICI- AND ENTEROVIRUSES IN SHELLFISH AND WASTEWATER**

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Noroviruses have been implicated as the causative agents responsible for the majority of non-bacterial gastroenteritis in shellfish consumers approximately 23 million cases of gastroenteritis in the United States annually; of these cases, 9.3 million cases are attributed to ingestion of contaminated food. These viruses are transmitted via the fecal-oral route and are found in municipal wastewater. Bivalve molluscs can become vectors of these agents if the water in which they feed is contaminated by human fecal waste or improperly treated wastewater. While certain enteroviruses can be cultured by conventional tissue culture techniques, the use of RT-PCR based assays has increased detection sensitivity and reduced analysis time. Human noroviruses, which cause the vast majority of gastrointestinal illnesses, are placed into two genogroups, GI and GII. These genogroups are non-culturable, so their detection is based primarily upon non-quantitative RT-PCR assays. Advances in real-time quantitative RT-PCR (qRT-PCR) technology have allowed the recent development of several qRT-PCR assays for the rapid detection and enumeration of culturable Caliciviruses (San Miguel Sea Lion Virus, serotype 17), enteroviruses, and unculturable noroviruses. However, available Taqman style assay developed for norovirus requires separate simplex reactions to distinguish the GI and GII genogroups, while the SYBR Green based assay is unable to distinguish these two genogroups. This presentation describes a multiplex qRT-PCR assays developed on the Cepheid SmartCycler® system for the simultaneous the detection and enumeration of enteroviruses, norovirus genogroup I, and norovirus genogroup II as well as qRT-PCR assays for SMSV-17 and hepatitis A virus. Each of these assays has incorporated a novel quantitative internal control to prevent the reporting of false negatives due to inhibition or failure of qRT-PCR reactions. Applications of these assays include determining viral load in municipal wastewater and treatment plant efficiency, viral elimination from shellfish, shellfish-associated outbreak investigations, and determining efficiency of extracting viruses from foods.

# **ROTAVIRUS VIRUS LIKE PARTICLES AS SURROGATES TO EVALUATE VIRUS PERSISTENCE IN SHELLFISH**

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Bivalve molluscs may accumulate viral contaminants within their tissues during feeding, and outbreaks of viral gastroenteritis have been associated with oyster consumption, and even with depurated shellfish. Little is known about viral persistence following depuration or relaying. Data on virus persistence are needed to address how long viruses may persist in contaminated oyster bed so that early reopening of beds that might pose a risk to consumer can be prevented.

Rotavirus virus-like particles (VLPs) and MS2 bacteriophages were bioaccumulated in bivalve mollusks to evaluate viral persistence in shellfish during depuration and relaying under natural conditions . Using this non-pathogenic surrogate virus, we were able to demonstrate that about one log10 of virus-like particles was depurated after one week in warm sea-water (22°C). Phages MS2 were depurated more rapidly (about two log10 in one week) than were virus-like particles, as determined using a single compartment model and linear regression analysis. After relaying in the estuary, under the influence of the tides, virus-like particles were detected in oysters for up to 82 days following seeding with high levels of VLPs (concentration range between 1010 and 109 particles per g of pancreatic tissues) and for 37 days for lower contamination levels (105 particles per g of pancreatic tissues).

| Days of relaying | Log10 of VLPs in oyster tissues |       |       |
|------------------|---------------------------------|-------|-------|
|                  | Exp A                           | Exp B | Exp C |
| 0                | 10.5                            | 9     | 5     |
| 7                | 10                              | 9     | 5     |
| 14               | 10                              | 8     | 4.5   |
| 21               | 8.5                             | 8     | nd    |
| 30               | nd                              | nd    | 4     |
| 37               | nd                              | nd    | 4     |
| 41               | 7.5                             | 6.5   | <4    |
| 49               | 7                               | 6     | nd    |
| 70               | nd                              | 5     | nd    |
| 82               | 5                               | <4    | nd    |
| 89               | <4                              | nd    | Nd    |

Table 1 : Reductions of VLPs in oysters under relaying condition.

After one week depuration, oysters were placed on the shore under influence of tides for a natural relaying. Samples were collected and analyzed by ELISA in duplicates.  
nd: analysis not done.

This is the first study that clearly demonstrates that a virus surrogate (viral-like particles) can be used in field experiments, and that they persist in shellfish for several weeks in natural environment. The data presented here also suggest that after contamination by human enteric viruses shellfish may be unsafe for human consumption for quite a long period of time.

## **VIRAL METHODS DEVELOPMENT AND APPLICATIONS**

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Norovirus is the most important cause of outbreaks of acute gastroenteritis among people of all ages worldwide, and the principal agent of bivalve shellfish-associated gastric illness. The ability to detect norovirus (NV) in bivalve shellfish is and will in the future be of paramount importance in protecting both public health and consumer confidence related to shellfish. Until recently procedures for the detection of NV in shellfish have relied upon the use of conventional nested RT-PCR. Real-Time PCR technology offers potential improvements however, in terms of sensitivity, rapidity, quantification and ease of interpretation of test results. The high sequence diversity within and between NV strains and genogroups for some time confounded the design of broadly-reactive Real-Time PCR primers, however increases in the volume of NV sequence data available, and in particular the publication of a number of complete genome sequences has allowed the identification of regions of the genome suitable for the design of broadly-reactive primers, and a number of TaqMan™ assays for NV detection have been published in the last two years. As European Community Reference Laboratory (CRL) for monitoring bacteriological and viral contamination of bivalve molluscs, CEFAS has assessed the performance of several broadly-reactive TaqMan™ primer/probe sets in comparison to a conventional nested PCR-based method for routine NV testing. We have also used quantitative Real-Time PCR to carry out investigations into the methodology currently in use for extraction of viral RNA from shellfish and generation of cDNA template for the TaqMan™ assay, and to identify and implement improvements to the methodology. These studies have enabled us to rationally develop a complete protocol for the testing of shellfish using a Real-Time PCR format, and this data along with that generated by other European laboratories should facilitate the development of a Europe-wide standard for the detection of NV and other viruses in bivalve shellfish.

## QUANTITATIVE (REAL-TIME) PCR ASSESSMENT OF POST HARVEST PROCESSING OF OYSTERS

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U.S. Gulf Coast states are currently mandated by FDA to perform post harvest processing on 25 percent of oyster shellstock in order to reduce cases of *V. vulnificus* disease that are related to the consumption of raw oysters. The University of Florida has partnered with the Florida Oyster Dealers Association, Florida Sea Grant, and USDA in order to establish a laboratory in Apalachicola, FL for the purpose of providing evaluation of post harvest processes. This laboratory will provide on-site certification that is necessary to validate and verify processes and can be used as a model by the seafood industry for compliance with FDA guidelines. Related research also compared and improved the methods used for evaluation of these processes, including standard microbiological analysis, DNA and PCR probe assays for confirmation species, as well as various PCR and microtiter formats for Most Probable Number (MPN) determinations. The use of Quantitative PCR (QPCR) offered a high throughput alternative to current methods for detection of *Vibrio* spp. in processed oysters. QPCR can be more rapid, precise, and efficient compared to standard DNA probe confirmation of MPN, while providing equivalent results. Potentially, QPCR offers greater sensitivity than current methods for determination of processes that truly reduce *V. vulnificus* to “non-detectable” levels.



## DETECTION OF NOROVIRUSES IN SHELLFISH: DEVELOPMENT OF A METHOD FOR REGULATORY USE IN NEW ZEALAND

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Noroviruses (NV) have been detected in imported oysters and in bivalve shellfish from New Zealand shellfish growing areas. This is a problem for regulators, the shellfish industry and the general public. There is an urgent need for a sensitive assay that detects noroviruses in contaminated shellfish. Recovery of NV from shellfish is complex and there is no standard international method. Methods for NV detection in shellfish have been non-quantitative and insensitive for detection of low virus numbers, but real-time RT-PCR has allowed progress in this area. Our goal is to implement a method that is sensitive, timely, robust, reproducible and detects a wide range of noroviruses.

Different recovery procedures, including protease digestion, acid adsorption, alkaline elution, PEG concentration, ultracentrifugation, and magnetic beads were compared. The methods were evaluated on oysters seeded with high concentrations of a GII/3 NV strain prepared from faecal specimens and titrated in conventional and real-time RT-PCR assays. Seeding was carried out by injection of titrated NV into the gut of freshly dissected oysters. A specific NV real-time RT-PCR assay was used to compare NV recovery from oysters by the different methods.

No virus concentration method was found to be significantly more efficient for virus recovery. The protease digestion method gave the greatest recovery of all methods when seeded with high levels of norovirus. Following further comparison trials with the acid adsorption and alkaline elution methods, the protease digestion method was selected for evaluation and standardisation. Studies to validate the protease digestion method, and to determine the limit of detection and limit of quantitation in shellfish contaminated with low levels of norovirus, are in progress.

Table 1. Comparison of protease digestion, acid adsorption and alkaline elution methods for NV recovery from oysters (3 replicate oyster samples per method). NV quantities recovered were determined from standard curves generated in real-time RT-PCR assays. Results show no. of replicates positive for NV.

| Method                               | NV Inoculum                  |                              |
|--------------------------------------|------------------------------|------------------------------|
|                                      | 2 x 10 <sup>3</sup> NV RTPCR | 1 x 10 <sup>4</sup> NV RTPCR |
| Protease digestion                   | 2/3                          | 3/3                          |
| Alkaline elution + PEG concentration | 0/3                          | 3/3                          |
| Acid adsorption + PEG concentration  | 0/3                          | 3/3                          |

The key factors required for a virus detection method are sensitivity, specificity and reproducibility. The protease digestion method, combined with a real-time generic norovirus RT-PCR assay, were chosen for further evaluation and validation as a method for New Zealand regulatory use. This combination provides efficient virus recovery, is sensitive and reproducible, of lower cost than labour-intensive methods and can be completed in 1-2 days.

# THE FIRST PANDEMIC OF *Vibrio parahaemolyticus* INFECTION: EMERGENCE, SPREAD, AND EVOLUTION OF THE PANDEMIC O3:K6 CLONE

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Infection by *Vibrio parahaemolyticus* is usually sporadic and an outbreak has usually been limited to a small community scale. Starting with a collaborative study in India in 1996, we found increase in the number of *V. parahaemolyticus* infection in various Asian countries and the United States. These infections were caused by *V. parahaemolyticus* O3:K6 strains possessing the *tdh* gene but lacking the *trh* gene. In addition, these strains were shown to be clonal and distinct from O3:K6 strains isolated before 1995 by the DNA fingerprint analysis using an arbitrarily primed PCR. We thus named this new clone as a pandemic clone. This is the first emergence of the pandemic in the history of *V. parahaemolyticus* infection. We developed a PCR method to detect the strains belonging to the pandemic clone (GS-PCR). The GS-PCR detects two bases in the *toxRS* operon that are unique to the pandemic strains. This PCR allowed detection of serovariants of the pandemic strains and further spread of the pandemic strains to various parts of the world.

We carried out phylogenetic analysis of 60 strains isolated in Asia between 1983 and 2001. They belonged to the pandemic clone and related groups. Comparison of the nucleotide substitutions in eleven genes distributed in two chromosomes allowed us to confirm clonality of the pandemic strains including serovariants and to speculate evolution of the clone. A 22-kb chromosomal insert unique to the pandemic clone was identified by a subtractive hybridization method. This sequence was shown to be associated with a growth characteristic under a certain condition by a mutagenic analysis.

The pandemic strains have been isolated from molluscan shellfish in southern Thailand and Japan, from the sediments in Japan, and from the environmental water in Bangladesh. The question to be answered is how the pandemic strains were transferred across the international borders.

## STUDIES ON THE TWO GENOTYPES OF *Vibrio vulnificus* BIOTYPE 1

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Although *V. vulnificus* is commonly found in molluscan shellfish in relatively high numbers, the incidence of disease is relatively low, leading to the hypothesis that not all strains of *V. vulnificus* are equally virulent. We performed a DNA sequence analysis of six clinical and four environmental isolates and found that the strains could be divided into two groups which correlate with clinical ("C-type") or environmental ("E-type") origin. We subsequently designed PCR primers that distinguish between the two groups, and typed 55 randomly selected strains. We found that 90% of the C-type strains were clinical isolates, while 93% of environmental isolates were classified as E-type (Rosche, T.M., Y. Yano, and J.D. Oliver. 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. Microbiol. Immunol. 49:381-389). Thus, the C-type genome appears to be a strong indicator of potential virulence.

Presence of capsule is critical to virulence of this pathogen. However, encapsulated ("opaque") strains frequently mutate to a non-encapsulated ("translucent") morphotype which is avirulent. In studying this switching phenomenon as temperatures increase from 23 to 40°C, we observed that C-type strains undergo relatively little capsule switching as compared to E-type strains. Thus, it is possible that C-type strains are virulent because they maintain their anti-phagocytic capsule when ingested, whereas E-type strains lose capsule and are cleared.

In what we believe to be the first such studies, we have conducted *in situ* gene expression studies by placing cells of *V. vulnificus* into membrane diffusion chambers suspended into natural estuarine environments. Two genes, *wza* and *wzb*, are known to be necessary for capsule production. We found that encapsulated cells continue to express these genes *in situ* for at least 24h, providing the first evidence that cells which produce capsules *in vitro* also express capsule genes in the environment. During these studies we further noted that, along with encapsulated (*wzb*+) and non-encapsulated (*wzb*-) morphotypes, a third "intermediate" capsular morphotype (*wzb*+) type exists. RT-PCR studies indicate that such cells exhibit greatly reduced expression of *wzb*, with significantly less capsule production *in vitro* compared to the opaque morphotype, and thus appear as translucent colonies. Whether these intermediate-type cells are virulent is not yet known.

Genes for an alternate sigma factor (*rpoS*) and an elongation factor (*tufA*) were found to be constitutively expressed by both E and C-type strains in cold estuarine waters as these cells enter the viable but nonculturable state (VBNC) state. However, while expression of the *V. vulnificus* hemolysin gene, *vvhA*, is constitutive in a C-type strain, an E-type strain exhibited only transient expression of this putative virulence factor. *In situ* studies in warm estuarine waters revealed that, as *V. vulnificus* enters the starvation-survival state, the cells continue to express *vvhA*, *rpoS* and *tufA* for at least 108h, and *katG* for over 24h, suggesting a need for the continued expression of these genes during this state.

We have also identified major differences in low temperature (5°C) survival of C and E-type strains at various salinities. At the same time, quantitative differences in isolation of the two genotypes on CPC agar following APW enrichment were revealed. Such results may help us understand why C and E-type strains differ in their isolation frequencies.

## HEPATITIS A TRANSMITTED BY FOOD: AN OUTBREAK IN ITALY

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Hepatitis A transmission occurs by the fecal-oral route, either from person to person or via food, water or fomites contaminated with virus-containing feces. Worldwide different patterns of hepatitis A virus (HAV) endemicity can be identified, and it is considered intermediate in the Southern and Eastern Europe and low in the Northern and Western Europe.

Italy is considered a country at low endemicity for hepatitis A, characterized by low annual incidence rates (around 5/100,000 inhabitants) and a shift in the average age of infection towards adulthood, when the clinical illness is more frequent and more severe. However, available data from the statutory notification system suggest that the epidemiological pattern is not homogenous across the country. In Southern Italy, especially in two regions (Campania and Puglia), HAV infection shows an intermediate level of endemicity with annual incidence rates up to 30/100,000.

Communities with intermediate level of endemicity typically present recurrent outbreaks: these episodes usually, but not always, have periodic intervals and persist for several years during which the overall diseases rates reach 50 to 200 cases/100,000 inhabitants per year. Consistently with this, Southern Italy also experienced outbreaks of hepatitis A presenting a very similar pattern. In 1996 and 1997 two large episodes (5,673 and 5,382 cases, respectively) were reported in Puglia region, accounting for an annual incidence rate of 138 and 132 cases/100,000 respectively. Both outbreaks were caused by the consumption of raw (or improperly cooked) shellfish and sustained over time through person-to-person transmission.

During 2004, a large outbreak of hepatitis A occurred in Campania region, with 882 cases reported from January to August. Regional and local epidemiological units together with ISS, the Italian National Health Institute, carried on the activities for the identification of the infection source, the route of transmission, and the implementation of appropriate control measures. The surveillance system was implemented with the institution of an on-line database (EPOS) shared by the different public health professionals involved, who were able to produce real-time analysis and epidemic curves. A case-control study was conducted among residents of the town with the highest attack rate. Viral identification and genotyping were carried out on patients sera cared for in two regional hospitals and on seafood samples sold in Campania. The epidemiological study and microbiological investigation each identified shellfish consumption (traditionally eaten raw or slightly cooked in this region), as the most important infection source.

## MICROBIOLOGICAL SURVEY OF OYSTERS IN U.S. RETAIL MARKETS

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In order to assess regional and seasonal trends, and to assess correlation between indicator organisms and microbes of current concern, the Interstate Shellfish Sanitation Conference (ISSC), the U.S. Food and Drug Administration (FDA) and state shellfish program partners are initiating a nationwide, year-long market survey of raw shell stock oysters in the U.S. Nine states (e.g. WA, CA, LA, AL, FL, VA, MA, IL, CO) will collect raw oyster samples twice a month from retail outlets. These samples will be representative of harvest areas on the Pacific, Atlantic and Gulf Coasts. Samples will be shipped to FDA laboratories for microbiological analysis.

Levels of total and pathogenic strains of *V. parahaemolyticus* and *V. vulnificus* will be measured. DNA probe and real time PCR (qPCR) assays will be used to target the *tlh* and *vvh* genes of *V. parahaemolyticus* and *V. vulnificus*, respectively for species identification. Virulence of the vibrios will be assessed by targeting the *tdh* and *trh* genes for *V. parahaemolyticus*, and allelic typing of the 16S rRNA genes in *V. vulnificus*.

The presence of enterohemorrhagic *E. coli* (EHEC), Salmonella, and toxigenic *V. cholerae* will be determined using qPCR assays targeting appropriate genes. Oysters will also be tested for viral pathogens, including norovirus and enterovirus. Finally, indicators of bacterial quality, including fecal coliforms, *E. coli*, male specific bacteriophage (MSB) and aerobic plate count, will be enumerated using standard methods.

Results will provide a baseline for the exposure to pathogens among consumers of shellfish from various regions and seasons. These results can be compared to past surveys (i.e. a 1998-1999 *Vibrio* study) or future surveys to assess the impact of changes in risk management regimes. These data will be useful for validation of the exposure assessment component in risk analysis, including further validation of FDA's *V. parahaemolyticus* Risk Assessment. Data will also be used to examine the correlation between noroviruses and indicators such MSB and fecal coliforms. Finally, data from this study will assist in examining equivalency between the U.S. shellfish safety program (water quality based) and the European Union shellfish safety program (shellfish quality based).

## BALLAST WATER

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The potential for translocation of marine life via water held in ballast tanks of ships is a prime concern for maritime and quarantine organisations. Microorganisms that may be translocated include human pathogens, pathogens for higher marine life or organisms threatening biodiversity in indigenous marine flora and fauna. *Vibrio* species are of particular concern as they present risks to public health if ingested on contaminated seafood.

We undertook an assessment of the risk of introduction of *V. cholerae* into Australian waters via ballast water. Of the pathogenic vibrios toxigenic *V. cholerae* was considered most significant due to the potential severity and epidemic nature of cholera and its restricted epidemiology and ecology in coastal waters. In contrast, *V. parahaemolyticus* and *V. vulnificus* are autochthonous members of our coastal waters and cause sporadic human illness and thus not exotic. At the time epidemic strains of *V. parahaemolyticus* had not been reported. The translocation of *V. cholerae* in ballast water has been proven and implicated in other continents. The direct link with an impact on public health is less certain.

A large volume of ballast water is moved around our seaboard and could be discharged into coastal waters; however, much of this water is not taken up in cholera endemic regions. Toxigenic and non-toxigenic *V. cholerae* had been isolated from Australian rivers and estuaries over several years in limited areas and resident shellfish had been contaminated. Indigenous cholera cases in these regions were sporadic and the availability of health care and a high standard of hygiene and sanitation limited epidemic spread. This suggested that the bacterium could establish in these ecosystems if introduced although the public health impact would be minimal.

Representative microcosms were used to demonstrate survival of *V. cholerae* in ballast water was influenced by salinity and water temperature. Various treatments were assessed, in particular heating. While these may be effective in eliminating higher organisms they would have little impact on vibrios. A survey of ballast water from international locations was undertaken using culturable and non-culturable molecular methods. Low levels of non-toxigenic non-O1 *V. cholerae* in water and a *ctx* positive plankton sample were detected. It was concluded the risk of introduction of this bacterium via ballast water was minimal.



## SURVEY OF PHP OYSTERS IN THE UNITED STATES

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Reduction of the risk of illness due to *V. vulnificus* (Vv) and *V. parahaemolyticus* (Vp) in raw oysters can be achieved by a variety of post-harvest processes (PHP) such as high hydrostatic pressure (HHP), mild heat treatment and rapid freezing. These and other treatment methods are currently being validated for effectiveness in reducing vibrio densities. Effectiveness can be evaluated as either: (a) assurance that a process achieves a reduction of a specified magnitude (e.g. 5 or 6 logs), or (b) assurance that vibrio densities after treatment are below the limit of detection of a suitably chosen method or otherwise below a specified limit (e.g., 30/g). An appropriate level of effectiveness for risk-reduction has yet to be fully determined but FDA has determined that product may be labeled “for added safety” only if it has been treated to reduce Vv to a nondetect level. Absolute assurance that a given level of effectiveness will always be achieved is not a feasible objective.

Evaluation and verification of the degree of effectiveness of any PHP is important. However, the current focus on stringent standards and a high level of assurance that a PHP achieve the standard consistently, for each and every sample processed, potentially over-shadows the fact that large reductions in (average) risk to the consumer may be possible even in the absence of a consistent reduction below a given standard for each and every sample processed. That is, the (average) risk to the consumer will be determined by the average vibrio levels achieved by a PHP and not by the maximum vibrio level after treatment.

To illustrate, data on the distribution of Vv and Vp densities in PHP oysters are evaluated and compared to data on the distribution of Vv and Vp densities in oysters collected at retail absent any PHP. Also, based on FDA and FAO/WHO risk assessments, estimates of risk are determined corresponding to the distribution of levels after PHP and this is compared to estimates of risk in the absence of PHP.

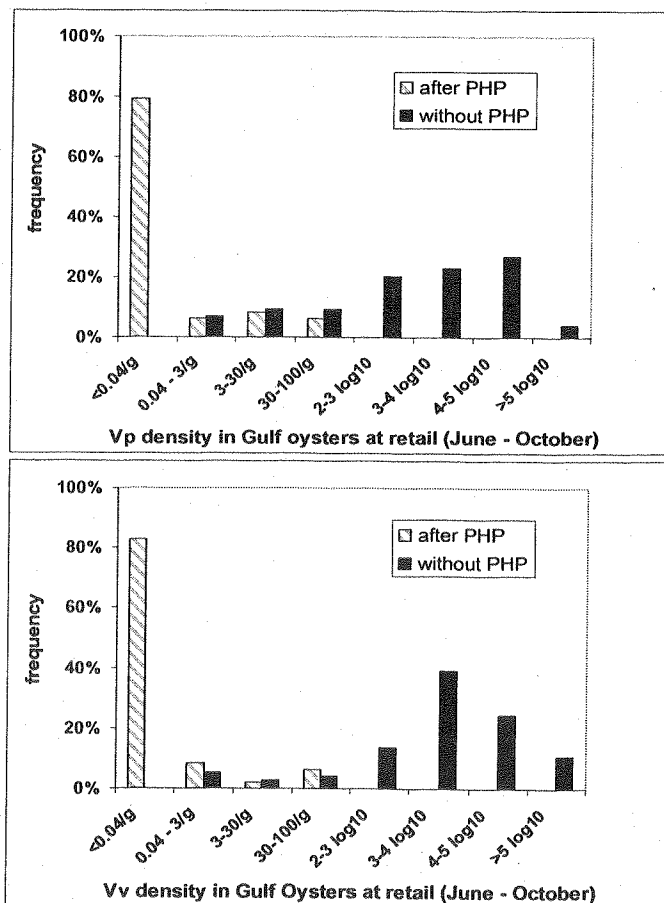


Figure 1. Histogram of the distribution of Vp (top) and Vv (bottom) in retail oysters from June to October with and without PHP.

## TRACKING SOURCES OF MICROBIAL CONTAMINANTS

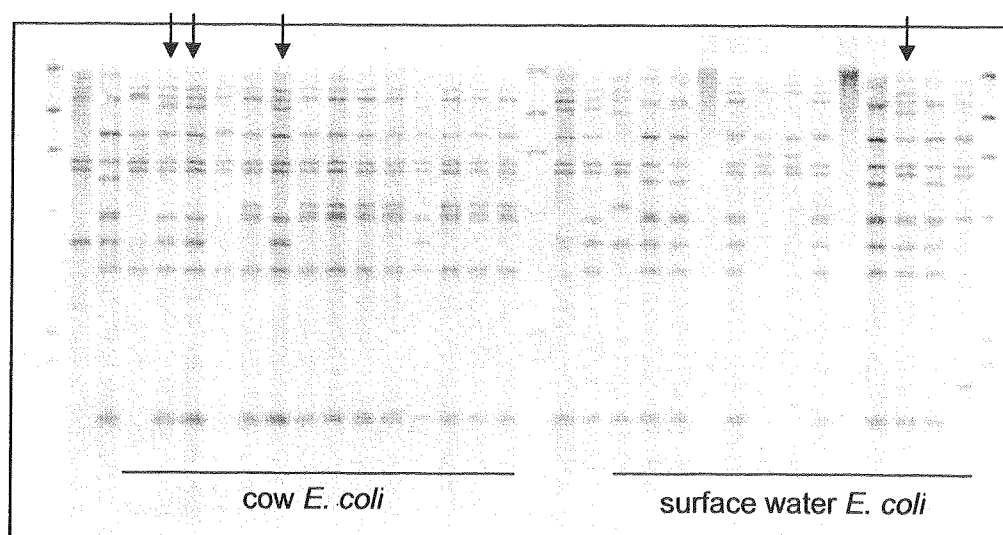
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Microbial source tracking (MST) techniques are being used to differentiate point from nonpoint sources of fecal pollution in contaminated water bodies and to assist managers in developing total maximum daily loads (TMDLs). The methods are designed to identify host-specific characteristics of fecal microorganisms, allowing source identifications (e.g. human or non-human). MST efforts to date have focused on library-dependent comparisons of phenotypic or genotypic fingerprints of fecal indicator bacteria. Methods are also under development to identify species-specific microorganisms or host-specific molecular markers from environmental samples, without the need to maintain extensive libraries or databases for comparison. Each MST method has been found to have advantages and limitations during field applications and methods comparison studies. Additional research is needed to optimize existing techniques and to develop improved, library-independent approaches.

### Ribotyping *E. coli*

matching patterns observed for cow and surface water *E. coli*



*E. coli* isolates from cow feces (library source) were run with the unknown bacterial isolates from a contaminated water sample collected near the cattle farm. Unknown isolate patterns are compared to source patterns in the library. A software program calculates a maximum similarity index for each unknown isolate pattern.

## **VIRUS DETECTION IN SOUTH CAROLINA SHELLFISH WATERS: INDICATORS VERSUS PATHOGENS**

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In South Carolina estuaries, increased runoff due to urbanization has caused shellfish harvesting beds to close because the consumption of oysters from these areas may pose a risk to human health. In our lab, a composite methods list has been used to assess shellfish waters. Enteric viral indicators (coliphages) and pathogens (Norovirus) have been used and compared to concentrations of bacterial indicators including fecal coliforms and enterococci. A 2005 winter study of 11 tidal creek systems tested 39 water samples for the above indicators and pathogens. These areas were surrounded by various levels of development and many of the systems harbor oyster populations. All samples contained fecal coliforms and enterococci at an average of 1,230 cfu/100ml and 475 cfu/100ml respectively. Thirty-three samples tested positive for somatic coliphage averaging 122 pfu/100ml and 30 samples were positive for F+ coliphage averaging 2 pfu/100ml. F+RNA coliphage isolates were genotyped as group I and IV, indicative of animal fecal pollution. Only three samples tested positive for Norovirus. The presence of Norovirus did not correlate with the presence of fecal coliforms, enterococci, or coliphages. Higher concentrations of bacterial and viral indicators were found in headwaters, with decreasing concentrations observed in increasing stream orders. Future studies will involve the detection of additional viral pathogens such as enteroviruses, adenoviruses, and rotaviruses; and detection of pathogens in oyster tissue as well as the surrounding waters.

## THE EFFICACY OF HIGH PRESSURE PROCESSING FOR VIRUSES

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Noroviruses (NV) and hepatitis A virus (HAV) can be transmitted to humans by shellfish consumption. Depuration and relaying of oysters and clams are of limited use as an intervention strategy for shellfish-borne viruses. Our research has focused on high pressure processing (HPP) as an intervention. Different viruses display variable sensitivity to pressure but results obtained with a norovirus surrogate, feline calicivirus (FCV; strain KCD), and for HAV strain HM-175, suggest that HPP can be used as an intervention for uncooked shellfish. In DMEM tissue culture media, reduction of HAV titer was observed with treatments between 300 and 450 megapascal (MPa; approx 3,000-4,500 atm or 45,000-60,000 psi), with a room temperature 5-min treatment at 450 MPa being sufficient to completely inactivate 7-log<sub>10</sub> pfu/ml. Treatment of HAV-contaminated oysters with a 1-min 400-MPa treatment at 9°C inactivated more than 3-log<sub>10</sub> pfu of HAV within oyster meats. A 5-min room temperature treatment at 275 MPa is sufficient to inactivate 7-log<sub>10</sub> pfu/ml of FCV. Investigation of the effects of treatment duration and temperature on FCV was determined. At room temperature, plotting log N/N<sub>0</sub> against treatment time at 200 MPa and 250 MPa, FCV pressure inactivation curves showed a rapid decline followed by tailing, consistent with nonlinear Weibull and log-logistic functions. Different temperature treatments of FCV at constant time and pressure indicate that inactivation above, and particularly below, room temperature result in enhanced inactivation compared to room temperature. For example, a 200-MPa 4-min treatment at -10°C and +50°C reduced the titer of FCV by 5.0 and 4.0 log<sub>10</sub> respectively; while at 20°C, the same treatment only reduced the titer by 0.3 log<sub>10</sub>. Recently, work with murine norovirus-1 (MNV-1), a virus closely related to human norovirus, has been undertaken. Preliminary results confirm that MNV in DMEM media is sensitive to pressure and inactivation of MNV-1 is enhanced by >3-log<sub>10</sub> when treatments are performed at 5°C as compared with 20°C. Research geared toward inactivating MNV-1 within oysters, potentially the first demonstration of norovirus inactivation directly in shellfish tissues by high pressure processing, is ongoing. This work lays the groundwork for a recently-funded human volunteer study to demonstrate inactivation of non-propagable human norovirus strain 8FIIB-contaminated oysters by HPP. Collectively, this work suggests optimism for the success of HPP as a virus intervention strategy for commercial shellfish.

# THE COLONY OVERLAY PROCEDURE FOR PEPTIDASES TO DETECT AND ENUMERATE TOTAL VIBRIONACEAE IN MOLLUSCAN SHELLFISH AND THEIR GROWING WATERS

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Since 1925, molluscan shellfish harvesting has been regulated in the United States based on sanitary surveys of shellfish growing waters. Surveys have relied on the use of coliform standards which have effectively eliminated outbreaks of typhoid fever and other bacterial illnesses. Only the *Vibrionaceae* have not been controlled by the implementation of coliform or fecal coliform standards. The *Vibrionaceae* are indigenous to marine waters, and subsequently, can not be predicted based on the levels of fecal coliform contamination. *Vibrionaceae* contain members of the genera *Vibrio*, *Aeromonas*, *Plesiomonas*, and *Photobacterium*.

We developed an enzyme assay known as the colony overlay procedure for peptidases (COPP) that detects and quantifies *Vibrionaceae* family members in seawater and shellfish based on the presence of the enzyme phosphoglucose isomerase and its lysyl aminopeptidase activity. This procedure is relatively simple, rapid, and inexpensive compared to other *Vibrio* testing methods. Dilutions of oyster homogenates or seawater are spread on plates of tryptic soy agar plus 0.5% added NaCl and incubated at 37°C overnight. Countable plates are overlaid for 10 min with a cellulose acetate membrane containing a commercially available synthetic substrate, L-Lys-7-amino-4-trifluoromethylcoumarin. The membrane is then observed under UV light. Fluorescent foci corresponding with bacterial colonies indicate the presence of *Vibrionaceae* family members. Total *Vibrionaceae* counts may be determined by multiplying the total number of fluorescent foci detected by the dilution factor. High levels of total *Vibrionaceae* may serve as an indicator for the possible presence of pathogenic *Vibrio* species and signal a point when shellfish beds should be closed to harvesting or when additional, more sophisticated testing for specific pathogens is warranted.

In a monthly survey of oysters and seawater in the Delaware Bay over a one-year period, we detected levels of *Vibrionaceae* exceeding  $10^6$  per gram of oyster and  $10^3$  per ml of seawater during the summer months. *Vibrio vulnificus* was particularly prevalent in oysters, with counts exceeding  $10^5$  per gram from May – August, while *Vibrio parahaemolyticus* levels exceeded  $10^4$  per gram in May and July. The COPP assay may find utility in identifying peak periods when *Vibrionaceae* are at their highest levels in East, West, and Gulf Coast oysters and growing waters and allow the correlation of total counts with the incidence of *Vibrio* outbreaks in those areas. The COPP assay is equally applicable in the United States and the European Union where shellfish are currently regulated based on coliform levels in seawater and shellfish meats, respectively.

## ***Vibrio parahaemolyticus* RISK MANAGEMENT IN BRITISH COLUMBIA**

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In 1997 the Pacific Coast of North America experienced an unprecedented outbreak of *Vibrio parahaemolyticus* (Vp) illnesses attributed to the consumption of raw and lightly cooked oysters. Several hundred confirmed and clinical cases were reported, mostly following consumption of oysters from British Columbia (BC) and Washington State. In late 1997 and early 1998 regulators and stakeholders from the US and Canada worked together to develop a strategy to address Vp illnesses which eventually evolved into the Vp Interim Control Plan (ICP) section of the US Shellfish Model Ordinance.

This framework was modified and refined in BC to further minimize risk to consumers of raw oysters. The BC Vp Advisory Committee was established to address the Vp issue. The committee includes representatives from government regulators (federal, provincial and regional) and stakeholders (processors, growers and restaurant association.) The primary focus of the strategy is to minimize consumer exposure to oysters with high levels of Vp through the implementation of monitoring and stringent post-harvest time and temperature controls.

This presentation will include:

- a brief history of the 1997 Vp illness outbreak and development of the ICP;
- the 2000 project conducted to track Vp levels in intertidal oysters over a tide cycle;
- the specific components of the BC Vp risk management strategy including:
  - environmental monitoring of Vp levels in oysters by the CFIA;
  - implementation of molecular methods for detecting pathogenicity;
  - Vp monitoring at harvest sites by stakeholders;
  - post-harvest time / temperature controls;
  - record keeping under QMP / HACCP;
  - mandatory cook advisory on shucked oysters;
  - enhanced illness reporting and traceback;
  - illness statistics from 1997 to present; and
  - new developments / update for 2005, including a HACCP guidance document for BC regulators and industry.

The number of reported Vp illnesses implicating BC oysters has remained at a low level since the implementation of the modified strategy.

## THE FAO/WHO RISK ASSESSMENTS ON *Vibrio* SPP. IN SEAFOODS

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*Vibrio parahaemolyticus*, *Vibrio vulnificus* and choleraogenic *Vibrio cholerae* have been identified as the species responsible for most cases of human illness caused by *Vibrio* spp.. The FAO/WHO *Vibrio* spp. risk assessment therefore addresses all three hazards focussing on *V. parahaemolyticus* in raw oysters, finfish consumed raw and bloody clams, *V. vulnificus* in raw oysters and choleraogenic *V. cholerae* O1 and O139 in warm-water shrimp in international trade. The assessments considered information on *Vibrio* spp. in seafood that was being generated and was available at regional and national levels, and this formed the substantive basis for their development. The assessments illustrate how different approaches were used to reflect the national capacity to generate data, the pathogen of concern and the commodity of concern.

The risk assessments of *V. vulnificus* in oysters and *V. cholerae* in warm water shrimp are presented as examples of the FAO/WHO risk assessments and illustrate the different purposes of risk assessment, some of the possible approaches and the impact of relevant data.

The risk assessment of *V. vulnificus* in raw oysters successfully investigated how an established *V. parahaemolyticus* model (FDA, 2001) could be adapted to a different pathogen. Despite the fact that data were only available from the USA, this risk assessment was undertaken because *V. vulnificus* illness has one of the highest mortality rates of any foodborne disease and has emerged as an important issue in a number of countries and regions including the USA, New Zealand, Japan, Republic of Korea, and Europe. The work clearly demonstrated the utility of adapting the framework and parameters for the *V. parahaemolyticus* risk assessment to another *Vibrio* spp.. Additional retail study data served to validate the exposure assessment predictions. The dose-response relationship was established using exposure predictions together with the reported frequency of illness. The risk assessment also evaluated the establishment of target levels (3/g, 30/g and 300/g) as an example of a management intervention and estimated that substantial reductions in risks were associated with target levels of 3/g and 30/g.

The *V. cholera* risk assessment was undertaken to estimate the likelihood of contracting cholera from the consumption of warm-water shrimp (cooked and uncooked) in international trade. No previous risk assessments had been carried out on this pathogen-product combination. A range of approaches were employed, including qualitative, semi-quantitative and quantitative techniques, to undertake a risk assessment using the available data. In all cases, based on the available data, the risk of acquiring cholera from imported warm-water shrimp was estimated to be very low.

These two examples illustrate the flexibility of risk assessment as a food safety management tool and the range of approaches that can be applied therein. They provide a good basis from which countries can develop their own risk assessments, an overview of the currently available data and the data gaps and identify some of the issues to be addressed when undertaking a risk assessment.

## VIBRIOS, VIRUSES, VERDICTS AND VIRTUOSOS

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It is now 11 years since we held the first International Molluscan Shellfish Safety Conference in Sydney, Australia. Public health issues of major concern at the time were Vibrios, particularly Vibrio vulnificus, and viruses.

In 2005, as scientists, health professionals and industry how have we measured up? The statistics may tell us some of the story:

|   | 1994 | 2004 |
|---|------|------|
| Vv cases USA                            | 11   | 19   |
| Virus outbreaks associated with seafood | 7    | 3    |
| Restricted harvest areas in NZ          | 1    | 4    |

Epidemiologists and virologists can no more stop seafood illness or environmental degradation than economists can stop inflation. However, the power of science is surely its capacity to change things, and to promote a better environment in which to live. Environmental science without decision making or policy context is a waste of time.

HACCP and Risk Assessment were supposed to be the new weapons against illness. Risk assessment tools quickly make us realize that Vibrios and viruses will take two entirely different management approaches. Vibrios, being natural pathogens are going to force society to focus on how much disease is acceptable to a population.

Viruses, however take us back to the root of shellfish sanitation programmes. Our shellfish sanitation forbears were able to prevent typhoid, why can we not prevent viral illnesses associated with shellfish consumption? We find ourselves once again dealing with environmental issues – specifically sewage contamination. To combat typhoid four groups were necessary to ensure public health reforms; politicians, professional groups, lobby groups and the press.

This knowledge must reach decision makers (whoever they are) and in a timely fashion, in a form they understand, and in a manner which encourages a positive response.

Our challenge therefore is not only to collect the science and data, but to provide clear information and explicit requirements to the policy analysts who act as brokers with the political decision makers, the press and the public. This conference has a wealth of professional skills which could be used to produce guidelines for the policy makers e.g. providing clear information on the appropriate sewage treatment systems. Let us hope 2114, this conference will have made an impact on the statistics.



## DEVELOPMENT OF IMPROVED RISK ASSESSMENT AND MANAGEMENT TOOLS FOR *Vibrio vulnificus*: WHAT MAKES THEM PATHOGENIC FOR HUMANS?

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*Vibrio vulnificus*, a Gram-negative halophilic bacterium common to estuarine and marine waters, colonizes shellfish and the intestinal contents of fish. Food borne infections caused by this opportunistic pathogen through ingestion of raw or undercooked shellfish are life threatening, with a high mortality rate. In addition to the human health costs, outbreaks of *V. vulnificus* infections cause a significant economic impact on the shellfish industry. In spite of considerable efforts to modify harvesting, transportation and storage practices during the warmer summer months, to develop post harvest treatment methods to eliminate the pathogen, and to educate the at-risk population about the risks of raw oyster consumption, the annual number of clinical cases and mortality has remained largely unchanged. It has long been recognized that there is a wide range of virulence, as measured in various animal models. Moreover, human clinical infections with this pathogen usually arise from a single strain, even though an individual oyster can harbor hundreds to thousands of strains as determined by clamped homogeneous electric field gel electrophoresis. Clearly, management of the shellfishery would be aided by a method to assess risk that could identify potentially virulent strains from other environmental strains that are less likely to cause human disease. However until recently, efforts to develop genotypic and/or phenotypic methods to differentiate strains have been largely unsuccessful.

We and others have shown that *V. vulnificus* can be divided into two or more groups, type A and type B, based on a 17 nucleotide polymorphism between two alleles of the 16S rRNA gene, of which there are nine copies in the *V. vulnificus* genome. We have demonstrated a high correlation between human clinical isolates and the type B genotype. We have recently extended the original T-RFLP assay used to differentiate the 16S rRNA type to a rapid assay using real-time PCR. This test has the potential to be used in monitoring programs that would allow fishery or public health managers to better gauge risk of shellfish harboring this organism. Type A and B strain differentiation can also be used to focus basic molecular pathogenesis research towards the identification of *V. vulnificus* virulence determinants important for human infection. Taken together, research based on this data should replace assumptions about the virulence of *V. vulnificus* with empirical data.

In our laboratory, research on *V. vulnificus* and *V. parahaemolyticus* is largely supported by the NOAA West Coast Center for Oceans and Human Health. The NOAA Oceans and Human Health Initiative is poised to leverage resources that can be used to address critical gaps in risk assessment and risk management for marine pathogens and shellfish safety.

## **BRIEF HISTORY OF MICROBIAL INDICATORS IN THE NATIONAL SHELLFISH SANITATION PROGRAM, USA**

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Microbial indicators of fecal contamination have been studied and employed to assess the sanitation of waters and foods since 1880. The National Shellfish Sanitation Program (NSSP), initiated in 1925, places high importance on water quality as the first critical control point in the production of safe molluscan shellfish, because shellfish can accumulate pathogens, toxins, and chemicals from their surrounding waters. The NSSP is a voluntary safety program implemented by individual states, with federal oversight, and enforced collectively through the Interstate Shellfish Sanitation Conference (ISSC). State authorities classify shellfish growing areas, control the harvest of molluscan shellfish, and certify dealers. Classification of shellfish growing areas requires periodic shoreline surveys to identify and assess all actual and potential sources of contamination and periodic water quality testing to verify classifications.

The NSSP requires state authorities to determine indicator bacteria levels in shellfish waters. Beginning in 1925 the NSSP indicators were coliform bacteria determined by fermentation testing. However, uniform water standards for classifying areas were not formally prescribed until after 1941. Even so, from the inception of the NSSP most states applied water standards that approximated a Most Probable Number (MPN) of 70 coliform bacteria per 100 ml. Following a U.S. Public Health Service report in 1941, the NSSP formally prescribed a median or geometric mean water MPN of 70 coliform bacteria per 100 ml for approved areas in 1946. In 1958, the NSSP prescribed a water standard for restricted areas, and in 1959 90<sup>th</sup> percentile values for approved and restricted area classifications were added. The 90<sup>th</sup> percentile values allow for variability inherent to the test method, and establish a two-part standard for the classification of shellfish areas. In 1961 fecal coliforms were proposed as an alternative to coliforms, and based on numerous studies, the NSSP formally added fecal coliforms as an indicator in 1974. A median or geometric mean MPN values of 14 fecal coliform bacteria per 100 ml was prescribed for approved areas, with 90<sup>th</sup> percentile values per 100 ml for also included. In 1986, values for restrict areas were prescribed.

It has long been recognized that bacterial indicators do not readily index the potential occurrences of viruses sometimes present in fecal contamination. Nonetheless, the requirements and restrictions established by the NSSP, based in part on coliform and fecal coliform bacterial water quality standards, have proven to be substantially effective for producing commercial shellfish that are safe for consumers. In 2005, for the first time, a proposal has been made to introduce a viral indicator in the NSSP as a means to re-open areas following atypical sewage contamination events. The proposal will be deliberated at the biennial meeting of the ISSC in August 2005.

## RESEARCH NEEDS AND FUTURE DIRECTIONS OF THE U.S. ENVIRONMENTAL PROTECTION AGENCY (U.S. EPA)

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The U.S. EPA has a long history of scientific research in the area of microbial contamination in ambient waters. In the next 5 to 10 years, we are planning a series of studies/actions that will benefit molluscan shellfish safety in the future.

First, U.S. EPA has recently published a guide document on microbial source tracking (EPA/660-R-05-064, June 2005). This document serves as a general guide to interested resource managers concerning the basis for microbial source tracking, the different approaches that are available, and a decision matrix for assisting in the determination of what approaches, in general, should be used under certain conditions. We expect that resource managers from various programs, including shellfish management, will begin to use this guide document and the results from these managers will be used to further refine the document as necessary.

As part of the National Epidemiological and Environmental Assessment of Recreational Water Study, U.S. EPA is collecting bacterial indicator data using techniques that can provide results in about two hours. We believe that these techniques will have utility to the shellfish program if new indicators are established or in the event of a pollution event or outbreak, in assisting in determining when water quality conditions have returned to acceptable levels.

U.S. EPA is currently developing a methodology for developing Clean Water Act Section 304(a) human health criteria for microbes. This methodology will be used to assist the Agency in the development of new recreational water criteria. As new recreational criteria are developed, this methodology could be used in coordination with the U.S. Food and Drug Administration to generate new criteria for shellfish harvesting waters.

Finally, and in concert with the actions above, the U.S. EPA is developing a systematic plan for selecting acceptable shellfish growing waters for obtaining shellfish for epidemiological-based feeding studies and water quality samples to examine a number of candidate indicator organisms. The risk based information from these studies would provide critical data for future shellfish harvesting waters criteria. We expect the field site selection portion of the study to begin in 2006.

U.S. EPA believes that all of these actions will fill critical information needs and lead to improved microbial criteria and public health protection. We look forward to working with all interested parties to initiate this research agenda.

## RESEARCH NEEDS AND FUTURE DIRECTION OF THE EUROPEAN UNION (EU) SHELLFISH PROGRAMME

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There is a need for further development of the current system of public health protection in relation to shellfish based on sound science. With regard to further research, there are a number of key areas where effort needs to be focussed.

**Viruses** Standard methods for detection/quantification of Norovirus and Hepatitis A need to be developed in order to allow the introduction of viral standards into EU legislation and the application of associated monitoring programmes. The development/acceptance of such standard methods need to be underpinned by validation exercises, the development of proficiency testing schemes for viruses and the establishment of stable reference materials. Quantification by real-time PCR needs further development and validation.

Practical application of such standard methods needs clarification of the human health risk of PCR-positive results. Regarding the use of direct detection/quantification of viral pathogens in monitoring programmes, it will be necessary to determine the spatial and temporal variability of occurrence, uptake and depuration kinetics in the environment and identification of appropriate sampling strategies. It will be important to re-assess the relationship between indicators and pathogens and to evaluate the possible use of surrogate variables in monitoring programmes. This could lead to the possibility of active management of harvesting areas with improved public health protection. Real-time PCR will enable further work to be undertaken on the quantification of viral removal during depuration.

**Vibrios** There is a need for the validation of both conventional and molecular methods for use within the European context. Application of such methods, together with improved epidemiological information, would allow re-assessment of the public health significance of pathogenic vibrios in Europe and consideration of the need for statutory standards and monitoring programmes and better assessment of risks from imports.

**Other pathogens** Methods for the detection and quantification of *Campylobacter*, *Giardia* and *Cryptosporidium* need to be validated with respect to use with shellfish. Subsequently an assessment should be undertaken of the public health significance of the occurrence of these organisms in shellfish.

**Source tracing** To target remedial and protective measures for faecal contamination of shellfisheries effectively, there is a need for further development of the best potential candidates for source tracing and/or a re-evaluation of the current state of the art and identification of potential new candidates.

**Imports** Specific risks from shellfish imported into the EU need to be assessed, particularly regarding Hepatitis E, pathogenic *Vibrio* spp and *Salmonella* Typhi/Paratyphi from third world countries where these are endemic/epidemic and the potential for spread of new Norovirus variants. Increasing global trade compounds all these matters.

**International harmonisation** Many of these issues would be best progressed in the framework of increased harmonisation of public health controls on bivalve mollusc production.

## EUROPEAN INDUSTRY CONCERNS, RESEARCH NEEDS AND FUTURE DIRECTIONS

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This presentation will address shellfish safety issues, including prioritisation of research requirements, from the perspective of the cultivation sector in Europe. ‘Life threatening’ issues facing European producers include:

- **Water quality**, in terms of microbiological standards and related concerns :
  - Reality, where there are true contamination issues – need for improved waste water treatment;
  - Perception, where there are minimal contamination issues, but regulators perceive concerns - the ‘psychological BSE effect’;
  - Proposals such as the ‘phage standard for depuration (basically cooking molluscs in the depuration facility);
  - Harvesting Area classification, with different national approaches ploughing up the sought after ‘level playing field’.
- **Biotoxins** :
  - Apparent increasing frequency;
  - The mouse bioassay;
  - Increasing regulatory burdens;
  - The continuing saga of scallops and ASP.

The scientific community has the potential to play a critical role in contributing to the resolution of these issues and associated problems; or alternatively it can pursue internal research interests in isolation from the real world, distanced from socio-economic policy objectives such as employment, wealth creation, gender equality, rural development, coastal management, etc. The choice is there for scientists – involvement, or the ivory tower!

**Research priorities** must include improved faecal tracking methods, direct assessment of viral contamination, development and accreditation of chemical test methods for biotoxins and accelerated removal of biotoxins.

**Future directions** for the industry, in collaboration with the scientific community and regulators, must include optimisation of consumer protection through the increased application of credible risk assessment combined with enhanced real time monitoring. Future sustainability for the industry lies in enhanced quality, as the potential for expanding crude production volumes is limited – but imports from third countries to satisfy increasing consumer demand must satisfy EU criteria in order to protect consumer health and prevent inequitable competition.

## **NATIONAL SEA GRANT COLLEGE PROGRAM SHELLFISH SAFETY AND FUTURE DIRECTIONS**

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Sea Grant is comprised of 30 Sea Grant colleges and institutions conducting integrated research, education and outreach. Sea Grant's mission is to enhance the practical use and conservation of coastal, marine and Great Lakes resources to create a sustainable economy and environment. Sea Grant provides the science for management decisions and is a bridge between government, academia, industry, scientists and citizens.

### **Sea Grant Research Programs and Initiatives**

- Gulf Oyster Industry Program
- Hazardous algal blooms
- Marine biotechnology

### **Sea Grant Research**

- Sea Grant has funded "Seafood Safety" research for the past 30 Years
- Past 10 Years Approximately \$4 million
- Majority of recent funding through the Gulf Oyster Industry Program

### **Gulf Oyster Industry Program**

- Post Harvest treatment
- Harmful Alga Blooms and impacts on safety
- Education Programs for "At Risk"
- Diagnostic tools for toxin and bacteria
- HACCP processes
- Support of state and ISSC education programs

### **Sea Grant and Seafood Safety**

Sea Grant has provided education and training to over 5,000 seafood processing plants on FDA regulations, resulting in 20,000 to 60,000 fewer seafood-related illnesses a year and saving \$115 million annually.

### **Sea Grant Future**

- Continue funding at state and national level
- Gulf Oyster Industry Program will continue at approximately \$1 million per year
- Continue or expand education and outreach efforts

## **FUTURE RESEARCH NEEDS FOR VIBRIOS AND VIRUSES IN MOLLUSCAN SHELLFISH: FDA PERSPECTIVE**

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Research prioritization by a public health agency should be thought of from a risk management perspective. When considering the foodborne pathogens of greatest concern for molluscan shellfish, frequency and severity of illness are important factors. In the U.S. this strategy results in a focus on *V. parahaemolyticus*, *V. vulnificus* and norovirus.

The most defensible approach to meeting the risk management goal of reducing the incidence of illness is risk assessment – including modeling the effectiveness of risk mitigation strategies (i.e., what we call “what-if” scenarios). This approach results in the prioritization of research that: 1) facilitates conduct of a risk assessment; 2) fills information gaps to reduce uncertainty; 3) facilitates risk mitigation modeling (“what-if” scenarios); or facilitates risk management activities.

For *V. vulnificus*, the key research needs are: improved information on virulence potential of strains; seasonal and regional trends of key genotypes; and improved information on host susceptibility.

For *V. parahaemolyticus*, the key research needs are: improved information on incidence of pathogenic *Vp*; improved information on impact of post-harvest handling and processing; improved consumption information; improved dose response information; and, improved risk management tools.

For norovirus, the key research needs are: improved information about contamination of shellfish from environmental sources; improved information about surrogates; improved information about levels in foods; improved information about dose-response; and, improved information about the effectiveness of potential control measures.

## **VIBRIO VULNIFICUS EDUCATION, THE GULF OF MEXICO PROGRAM AND THE UNITED STATES OCEAN ACTION PLAN**

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Congress enacted the Oceans Act of 2000, which created the U.S. Commission on Ocean Policy. On September 20, 2004, the White House formally received the U.S. Commission on Ocean Policy Final Report. On December 17, 2004, the President submitted to Congress his formal response as the "U.S. Ocean Action Plan". and established a Cabinet-level "Committee on Ocean Policy" to coordinate the activities of executive branch departments and agencies regarding ocean-related matters "to advance the environmental and economic interests of present and future generations of Americans." The Committee will advise the President and, as appropriate, agency heads on the establishment or implementation of policies concerning certain ocean-related matters. The Plan also recognized the leadership that the Gulf States have demonstrated in forming a Gulf of Mexico Alliance to address common needs in the Gulf of Mexico.

The Gulf Alliance is a Memorandum Of Agreement amongst the five gulf state governors to work more closely together on Gulf issues in the marine and estuarine systems.

A meeting was held of the Gulf of Mexico Alliance and Bush Administration in June, 2005 to establish the framework of a Gulf Alliance Plan of Action and to explore and better define partnership opportunities between the Gulf states and federal partners. There were five priority areas for discussion for the June meeting:

1. Reductions in nutrient loading
2. Improving Gulf water quality, with emphasis on healthy beaches and shellfish beds
3. Restoration of coastal wetlands
4. Identification of Gulf habitats to inform management
5. Gulf of Mexico environmental education (e.g., *Vibrio vulnificus*)

These discussions lead to the formation of five federal/state working groups in preparation of a detailed workplan for incorporation into the Gulf Alliance Plan of Action to be announced at the State of the Gulf of Mexico Summit in November 2005.

The Gulf of Mexico Program has long been a supporter of *Vibrio vulnificus* education through projects and grants to the Interstate Shellfish Sanitation Conference and Gulf States. The new Gulf Alliance Plan of Action will have an impact on future efforts.



## POST HARVEST PROCESSING OF OYSTERS TO REDUCE RISK

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United States, Gulf Coast oyster processors have taken the lead in developing new Post Harvest Processing technologies to ensure alternatives to traditional raw oysters for certain at-risk consumers. Through three basic methods of post-harvest processing, processors throughout the United States, Gulf Coast are providing greater choices and wider availability of oyster products with non-detectable levels of *Vibrio vulnificus*: a naturally occurring bacteria which may adversely impact consumers with certain underlying health conditions.

While different technologies are employed, each post-harvest process (PHP) is designed to allow at-risk consumers to enjoy uncooked oysters. Not insignificantly, these processes were developed and successfully marketed by private entrepreneurs in response to consumer demand, and without a government mandate or directive.

Existing post-harvest treatment processes take variations of three forms: (IQF), low heat pasteurization or (HCP), and (HPP) treatment. Although these technologies currently account for a small percentage of all domestic raw oyster sales in the United States, on-going marketing and educational efforts geared toward the at-risk consumers are expanding acceptance and knowledge regarding these PHP oyster products. Such efforts will clearly open the market for these products, thereby creating less risk for the at-risk consumers of raw oysters in the future. Specific processes and other facts regarding each will be discussed.

## LEVERAGING RESOURCES BASED ON RISK ASSESSMENT

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Complex epidemiological and ecological factors associated with the environmental *Vibrio* species have served as the impetus for recent risk assessment efforts. The first iterations of the FDA *V. parahaemolyticus* and the WHO/FAO *V. vulnificus* risk assessments identified a variety of data gaps, data limitations, and assumptions. Clearly, focusing research efforts on these data restrictions will help inform future risk assessment iterations. With this in mind, a research team consisting of investigators from North Carolina State University, Louisiana State University, and the FDA Dauphin Island research facility designed a study, the purpose of which was to improve our understanding of the ecology of pathogenic *Vibrio* spp. in raw molluscan shellfish and the efficacy of representative control strategies. Recently funded by the USDA-CSREES National Research Initiative, the underlying hypothesis of the study is that there are strain to strain differences among the pathogenic *Vibrio* spp. with respect to their ecology, prevalence, and virulence. The objectives of the project are to (i) characterize industry practices by means of survey instruments; (ii) quantify the total levels of *V. vulnificus* and *V. parahaemolyticus* in shellfish throughout harvesting, packing, and refrigerated storage; (iii) investigate potential associations between environmental/ecological factors and total and pathogenic strains of *V. parahaemolyticus* and *V. vulnificus*; (iv) investigate strain to strain differences in virulence, growth, and survival; and (v) investigate the relationship between mitigation strategies and the prevalence/levels of pathogenic *Vibrio* spp. in Gulf oysters. Using recent risk assessment documents as guidance in designing the study, we will be able to provide relevant and important information to inform future risk assessment iterations.

## RESEARCH NEEDS AND FUTURE DIRECTION IN NEW ZEALAND

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This paper provides a brief outline of recent research and development along with the rationale underpinning the priorities. It is observed that research priorities in terms of food safety, should clearly derive from a logical cascade of sought outcomes thus:

1. Assuring the safety of shellfish for human consumption
2. The issues that regulations and regulatory authorities face in achieving point 1.
3. The issues that industry faces in its practices and interface with regulatory authorities in achieving point 1.

Research is defined to include the chemical and physical as well as behavioural sciences, emphasising the need to understand behaviours leading to pollution of growing waters, the causative agents and events leading to bacterial and viral contamination and biotoxin blooms, thus enhancing predictability.

A case is made that a balanced portfolio of research is required, short, medium and long term, i.e. resolving immediate and foreseeable needs as well as some element of future gazing.

In the New Zealand situation a considerable portion of research is directly funded by industry. This could risk a short term focus yet it is noted that to date at least, industry has driven the implementation of more specific, accurate and precise biotoxin test methodologies, although those methods are more expensive than the traditional mouse bioassay.



# **POSTER PRESENTATIONS**

## OYSTER X-RAY IRRADIATION: *Vibrio vulnificus* RESPONSE AND CONSUMER DIFFERENCE TESTING

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*Vibrio vulnificus*, although a natural inhabitant of estuarine and ocean environments, can cause serious illness and death in susceptible persons when consumed along with raw half-shell oysters.

Objectives of this study were 1) establish the effect of x-ray irradiation and dose needed to reduce *Vibrio vulnificus* to nondetectable levels; 2) determine the effect of x-ray irradiation on shelf life of shucked oysters; and 3) determine consumer's ability to differentiate between x-ray irradiated and control oysters.

Live in-shell and shucked oysters, with naturally incurred *Vibrio vulnificus*, were exposed to 0-3 kGy dose x-ray radiation to determine the response of *V. vulnificus* and aerobic spoilage bacteria to this process. Consumer volunteers at the 2004 Boston Seafood Show and the Biloxi Crawfish Festival were asked to determine differences between treated (1 kGy) and untreated oysters by triangle difference testing.

Naturally incurred *Vibrio vulnificus* was reduced from  $10^6$  cfu/g in half shell oyster meat to nondetectable levels (<3 mpn/g oyster meat) at an x-ray exposure of 0.75 kGy and aerobic and psychrotrophic spoilage bacteria were reduced by 2 - 3 logs ( $\log_{10}$ ) with an extension of shelf life of at least 1 week over untreated product.

Aerobic and psychrotrophic spoilage bacteria in shucked oysters were reduced by 2-3 logs with a 12-15 day extension of shelf life.

Sensory triangle difference tests by 160 volunteers (Boston) and 79 (Biloxi) resulted in confirmation that consumers, many of whom work in the seafood industry, could not distinguish between control and irradiated oysters ( $p < 0.001$ ).

X-ray processing is a safe and effective post harvest treatment method for vibrio remediation in oyster products.

Acknowledgement: This project was funded by the MASGC, Project Number R/AT-4-GOIP.

**COMPARISON OF BACTERIA UPTAKE AND DEPURATION RATES BETWEEN  
THE SUMINOE OYSTER *Crassostrea ariakensis* AND THE AMERICAN OYSTER  
*Crassostrea virginica*.**

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Deterioration of the habitat, overfishing, and eutrophication has resulted in a decline of the native oyster *Crassostrea virginica* along the mid-Atlantic coast of the United States. In an effort to improve water quality and restore socioeconomic benefits, coastal managers are considering introducing the Suminoe or Asian oyster *Crassostrea ariakensis*. To date, research has focused on the ecological and socioeconomic implications of this initiative, yet few studies have assessed its potential impact on public health and water quality. Consequently, there is a direct and immediate need for a comparative analysis between bacterial uptake and depuration rates of *C. virginica* and *C. ariakensis*. We are conducting a suite of experiments to assess the removal rates of *Vibrio* sp. and fecal coliforms (specifically *E. coli*) in each of the two oyster species using both molecular (quantitative polymerase chain reaction, QPCR) and traditional microbiological methods (multiple tube fermentation and directed substrate technology). We will determine the removal rates of bacteria during active filtration (feeding), and assess retention of the bacteria during both depuration and post harvest storage of these two species. Preliminary results of the pilot and full-scale experiments will be discussed.

## PREVALENCE OF ENTERIC VIRUSES AND F-SPECIFIC RNA BACTERIOPHAGE IN NEW ZEALAND SHELLFISH: PRELIMINARY RESULTS

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The ability of bivalve molluscan shellfish to concentrate pathogenic bacteria and enteric viruses from sewage-contaminated waters has been known for many years. Consumption of contaminated shellfish presents a risk to human health. There is little information on the prevalence of enteric viruses and F-specific RNA (FRNA) bacteriophage in New Zealand shellfish. A 2 year study is in progress to determine virus prevalence and to establish whether there is a relationship between presence of enteric viruses and FRNA bacteriophage in New Zealand shellfish.

The study commenced in January 2004. Sixteen sites, including sites known to be impacted by human sewage, were selected from around New Zealand. Both recreational and commercial shellfish harvesting areas are included. Shellfish samples are collected monthly or bimonthly and analysed for presence of adenoviruses, enteroviruses, noroviruses, FRNA bacteriophage and the standard bacterial indicators, faecal coliforms and *Escherichia coli*. Enteric viruses are recovered from shellfish tissue by the acid adsorption method and then detected by conventional and real-time PCR methods. ISO standard methods are used for the detection and enumeration of FRNA bacteriophage and *E. coli*.

A total of 208 shellfish samples have been analysed over a period of 15 months. One or more enteric viruses were detected in 112 (53.8%) of samples and FRNA bacteriophage were detected in 116 (55.8 %) of samples. To date, adenoviruses have been detected in 42 (20.2%) samples, enteroviruses in 30 (14.4 %) samples and noroviruses in 39 (18.7%) samples.

Enteric viruses have been detected in shellfish samples collected from all but one site during the study. Shellfish from areas of expected high contamination consistently showed presence of more than one enteric virus, and high levels of FRNA bacteriophage and *Escherichia coli*. In areas of expected low contamination FRNA bacteriophage or viruses were infrequently detected

The analysis of samples will be completed in February 2006. Preliminary data suggests that there is no correlation between the presence of enteric viruses and FRNA bacteriophage in shellfish harvested from recreational and commercial growing areas.



## **APPLICATION OF REAL-TIME RT-PCR FOR DETECTION OF NOROVIRUSES IN SHELLFISH**

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Foodborne viral disease is now a major public health problem, with noroviruses as the main causal agent and shellfish as a major vehicle of infection. Detection methods for noroviruses in shellfish have been insensitive and non-quantitative, but real-time RT-PCR has allowed progress in this area.

Specific and generic real-time RT-PCR probe-based assays were established to evaluate virus recovery methods, investigate norovirus persistence in shellfish under various conditions and to detect noroviruses in naturally contaminated shellfish. Norovirus contamination levels in shellfish were determined using standard curves prepared for each assay. A specific real-time RT-PCR assay was used to compare the efficiency of norovirus recovery from shellfish by different methods, to study persistence following heat and acid treatments, and to determine norovirus persistence in naturally-growing shellfish. A generic RT-PCR was used for detection of noroviruses in shellfish implicated in gastroenteritis outbreaks.

The results of these studies have assisted the regulators and the industry to formulate guidelines following contamination events.

## **A PRELIMINARY INVESTIGATION INTO THE USE OF F+ RNA BACTERIOPHAGE MOLECULAR PROBES IN DETERMINING THE ORIGIN OF SHELLFISH CONTAMINATION**

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*Escherichia coli* is used as an indicator of sanitary quality of bivalve molluscan shellfish in Scotland as required by EU Directive 91/492/EEC. *E. coli* are found in both human and animal faeces and methods currently used do not distinguish between bacteria of human and animal origin. Faecal source tracing methods can form an important part of shellfish water quality management, enabling targeted remediation action to improve water quality and consumer health protection.

Previously genotyping of F+RNA bacteriophage using synthetic, dioxygenin labeled oligonucleotide probes has suggested that strains of F+RNA bacteriophage from human wastes are typically members of group II and III whilst groups I and IV are generally found in animal faeces.

In this study, we have attempted to enumerate and speciate the male specific F+RNA bacteriophage content in shellfish harvested from Scottish harvesting areas using oligoprobes. Additionally, the bacteriophage content of animal faecal material, including horse, sheep, goat, cow, pig and birds has been assessed. Results indicate that speciation of F+RNA bacteriophage may provide an indication of the source of faecal contamination in shellfish harvesting areas and could be used to improve our understanding of shellfish contamination.

## **LATERAL FLOW IMMUNOCHROMATOGRAPHIC TESTS FOR PSP, ASP AND DSP**

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Jellett Rapid Testing, in collaboration with the National Research Council of Canada (NRCC), develops and manufactures simple, robust and accurate rapid diagnostic tests for PSP, ASP and DSP toxins. This collaborative research group also develops simple and efficient sample preparation methods for use with the Jellett Rapid Tests. The Rapid Test for PSP, now incorporated into the National Shellfish Sanitation Program of the USA, is also used in or being assessed by regulatory laboratories all over the world including Canada, the UK, Ireland, Portugal, New Zealand, Australia, China, and elsewhere. The Rapid Tests for ASP are used by fishermen, shellfish growers and processing plants in many countries. Distributed testing using the Rapid Tests can enhance shellfish safety by allowing more sampling and access to remote areas. The economics of community-based monitoring will be discussed as well as a pilot project in Washington State, where Rapid Tests used in phytoplankton monitoring have provided about one week early warning of impending shellfish toxicity in razor clam beds. The recently developed Rapid Test for DSP detects OA, DTX1 and DTX2 approximately equally and in preliminary work, can detect these toxins at about 0.1 mg/kg shellfish tissue using a simple and efficient sample preparation method. Comparative testing of naturally contaminated shellfish tissues and phytoplankton with the Rapid Test for DSP and LCMS will be presented.

## TRACKING BACTERIAL POLLUTION SOURCES FOR MANAGING SHELLFISHING WATERS IN NORTHERN NEW ENGLAND, USA

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Microbial Source Tracking (MST) methods have been used in coastal New Hampshire and southern Maine shellfishing waters and tributaries for tracking bacterial pollution since 2001. Despite long-term efforts to eliminate pollution, bacteria remain the most significant limitation for shellfish harvesting in the area. The main MST method used is ribotyping of *Escherichia coli*. *E. coli* strains isolated from surface water and oyster samples have been ribotyped and their patterns compared to those of strains from known source species, including humans (sewage, wastewater effluent, human feces) and ~35 species of birds, pets, livestock and wild animals. Studies have included research to test method assumptions and surveys to identify pollution sources in areas of concern. Results show humans, pets (Figure 1), different kinds of wild animals and livestock to be significant types of pollution sources in different areas of the region. Use of *E. coli* ribotyping has become progressively more focused and integrated into more comprehensive efforts to open more shellfish harvesting areas by eliminating pollution sources.

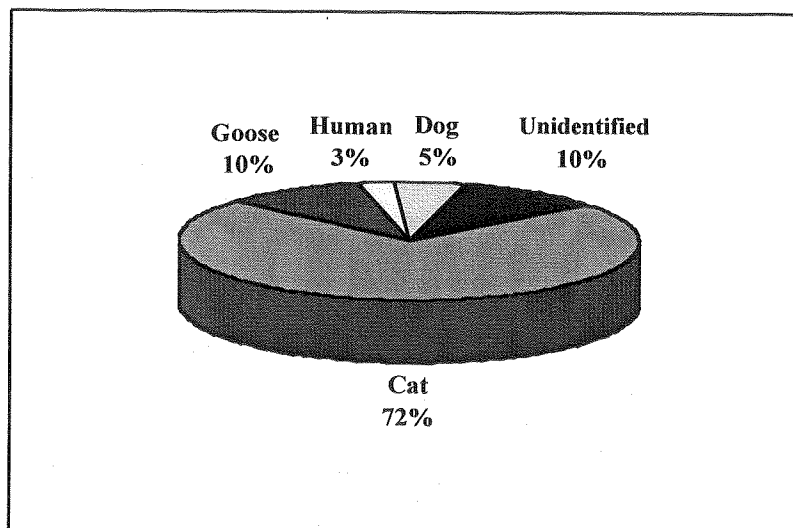


Figure 1. Types of source species identified in storm water pipe effluent in Seabrook, NH.

## ASSESSMENT OF ENTEROVIRUSES FOUND IN FINAL EFFLUENT AND RIVER WATER BY CELL CULTURE AND MOLECULAR ASSAYS

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**Background:** Assessment of water quality is critical in preventing shellfish and water-borne illnesses, in addition to tracking pollution sources during hydrographical surveys. Environmental factors such as temperature, ultraviolet light, disinfectants, and the presence of humic acids may inhibit viral detection by both cell culture and molecular assays. One of the concerns with the inhibition of RT-PCR detection is that it may result in false negative or deceptively low readings, which could lead to an underestimation of public health risks. Another concern arises from the fact that molecular assays, such as RT-PCR are unable to differentiate between inactive and infectious viruses. This could lead to an overestimation of the risk of viral infection. In this study we examined human enteroviruses (i.e. groups of polioviruses, echoviruses, and coxsackieviruses) in order to assess culturable and genomic RNA units of viruses in polluted water.

**Results:** In this study human enteroviruses in contaminated water were examined by cell culture and molecular assays. Water was collected from the final effluent of a sewage treatment plant and at two sites located up- and down-river from the plant. Viruses in the samples were concentrated 600-fold, and then inoculated into a Buffalo Green Monkey (BGM) cell culture assay. Enteroviruses were confirmed by a combination of BGM cytopathology and RT-PCR of the cell lysates. Eleven of 12 samples were positive for culturable enteroviruses, with the level ranging from 33 to 192 most probable numbers (MPN) in 100 L of water. In order to achieve a rapid examination using direct RT-PCR, and to avoid a week-long cellular incubation, the 600-fold sample concentrates were processed further by solvent extraction, PEG-precipitation, and RNA extraction. The two assays demonstrated consistent results for two-thirds of the samples tested (seven of the 11 culture-positive samples were also positive by direct RT-PCR). However, the water sample with the highest level of culturable viruses did not contain the greatest number of genomic viral RNA units. The ratios of culturable to total viral particles in the water samples ranged from 1:2 to 1:91. This study determined that direct RT-PCR detected viruses at  $< 1$  culturable MPN/reaction in contaminated water. This suggests that when using a molecular assay such as fluorogenic RT-PCR there can be wide-ranging variations in the ratio of total to culturable viral units. Hence, caution should be exercised when conducting risk assessment using data that are strictly derived from molecular assays without regard for actual infective viral units as confirmed through a cell culture assay.

# RECIRCULATION SYSTEM CAPABLE OF FILTRATION, CIRCULATION AND OXYGENATION FOR SANITATION OF MOLLUSCAN SHELLFISH IN STOCKING AND DEPURATION PLANT

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In accordance to the new directive of the European Community as a protection of the consumer, molluscan shellfish are continuously checked and monitored by sanitary and veterinary service. All products must be sold in a sealed package containing its own unmovable sanitary certificate and traceability report which must contain: (i) name of the company SDC (shellfish depuration center) with licence number assigned by Health Ministry, (ii) and minimum time of depuration.

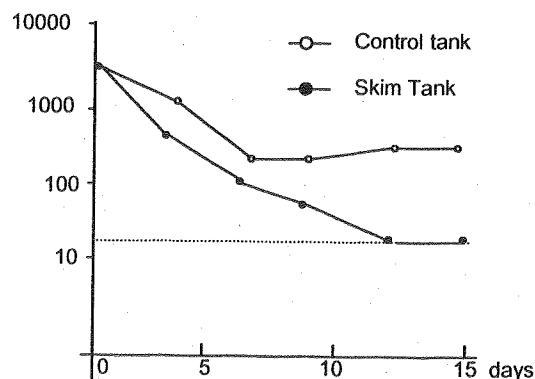
The needs of making available for the company operating in stocking and depuration of shellfish, a simple, economical and reliable system, led to research and development of Skim.

SKIM is a very compact depuration and stocking system capable of water oxygenation, water circulation and foam fractionation. The efficiency is granted by an high water capacity, mixed with micronized bubbles under pressure in counter cyclonic flow.

100 m<sup>3</sup>/h<sup>1</sup> flow rate, bacterial removal, concentration of suspended material, clear water, healthy shellfish are achieved with 1.5 kw/h electric energy consumption only. Test trial carried out at 13 ± 0.5° C, and salinity 35.5 ppt in tanks stocked with 15 kg/m<sup>3</sup> of cockles contaminated at level of 2850 E.coli/100g. After 3 hours of treatment, there was nine fold decrease in bacteria. After 12-15 hours the bacteria concentration stabilized below 18 E.coli/100g.

| Parameter                       | Site          | Foam  | Water | Concentration Factor |
|---------------------------------|---------------|-------|-------|----------------------|
| Total Suspended Solids (mg/l)   | Fish pond 1   | 4542  | 17.8  | 255.2                |
|                                 | Fish pond 2   | 2574  | 13.7  | 188.3                |
|                                 | Fish nursery  | 2521  | 4.4   | 573.0                |
|                                 | Harbor        | 1578  | 8.9   | 177.3                |
|                                 | Oyster tank 1 | 74    | 1.1   | 68.5                 |
|                                 | Oyster tank 2 | 126   | 1.4   | 90.0                 |
|                                 | Mean          |       |       | 225.4                |
| Dissolved Organic Carbon (mg/l) | Fish pond 1   | 6813  | 208.4 | 32.7                 |
|                                 | Fish pond 2   | 2504  | 116.8 | 21.4                 |
|                                 | Fish nursery  | 12995 | 270.6 | 48.0                 |
|                                 | Harbor        | 5816  | 305.0 | 19.1                 |
|                                 | Oyster tank 1 | 2919  | 244.4 | 11.9                 |
|                                 | Oyster tank 2 | 2394  | 204.8 | 11.7                 |
|                                 | Mean          |       |       | 24.1                 |

Table 1. Concentration of total suspended solids and dissolved organic carbon in waste water and foam condensate, and concentration factor (CF = foam/water) from Hussenot, 2004



Mean concentration of E.coli in wet flesh of cockles during two purification treatments, from Glize, 2003

<sup>1</sup> i.e; 440 gal US/min

# REAL TIME PCR AND STANDARD CULTURE METHODS FOR ESTIMATING TOTAL AND PATHOGENIC *Vibrio parahaemolyticus*-MPN IN ALASKAN OYSTERS

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*Vibrio parahaemolyticus* (Vp) is a naturally occurring estuarine bacterium that is often isolated from warm seawater. It was therefore unexpected that the largest Vp outbreak of 2004 in the US was associated with consumption of raw Alaskan oysters, harvested from cool waters. As a side study to the outbreak investigation, environmental samples collected along the Alaskan coastline were analyzed for total and pathogenic Vp. Serial 10-fold dilutions were enriched overnight at 35°C in alkaline peptone water (APW) using a 3-tube most probable number (MPN) format. For the standard culture method, turbid APW tubes were streaked to TCBS agar for colony isolation, subsequent biochemical identification, and virulence characterization of suspect colonies. Aliquots of the APW enrichments were boiled and used as template in a multiplex real time PCR (qPCR) assay for detection of the *tlh* gene for species confirmation, and the *tdh* and *trh* genes for virulence determination. An internal amplification control was included in each PCR reaction to determine matrix inhibition and potential false negative results.

All of the samples and tubes that were positive by culture for total or pathogenic Vp were also positive by qPCR. Many of the standard culture MPN tube combinations for both total and pathogenic Vp gave highly improbable results with more positive tubes in 0.1g portions than in the 1.0g portions (Table 1). Application of a 1-dilution MPN versus traditional 3-dilution MPN resulted in greater Vp densities when improbable MPN codes were determined.

**Table 1.** Tube-by-tube comparison of standard culture- and qPCR-MPN methods demonstrating the frequency of improbable MPNs (in bold).

| Sample | qPCR    |                 |                 | Standard Culture |                 |
|--------|---------|-----------------|-----------------|------------------|-----------------|
|        | Vp-MPN  | <i>tdh</i> -MPN | <i>trh</i> -MPN | Vp-MPN           | <i>tdh</i> -MPN |
| 872    | 3-1-1-1 | 3-1-0-1         | 3-1-1-1         | 0-0-1-0          | 0-0-0-0         |
| 873    | 3-1-0-0 | 3-1-0-1         | 3-1-0-1         | 0-1-0-0          | 0-0-0-0         |
| 888    | 3-3-1-0 | 3-3-1-0         | 3-3-2-0         | 2-2-1-0          | 2-2-1-0         |
| 889    | 3-3-2-0 | 3-3-2-0         | 3-3-2-0         | 1-3-1-0          | 1-3-1-0         |
| 936    | 3-3-1-0 | 3-3-1-0         | 3-3-1-0         | 0-2-0-0          | 0-2-0-0         |
| 937    | 2-3-1-0 | 2-3-1-0         | 2-3-1-0         | 1-1-0-0          | 1-1-0-0         |
| 1022   | 2-0-0-0 | 1-0-0-0         | 0-1-0-0         | 2-0-0-0          | 0-0-0-0         |
| 1023   | 1-0-0-0 | 1-0-0-0         | 1-0-0-0         | 1-0-0-0          | 1-0-0-0         |
| 1047   | 1-1-0   | 1-1-0           | 1-1-0           | 0-1-0            | 0-0-0           |
| 1048   | 2-0-0   | 1-0-0           | 1-0-0           | 0-0-0            | 0-0-0           |
| 1053   | 0-0-0   | 0-0-0           | 0-0-0           | 0-0-0            | 0-0-0           |
| 1054   | 3-0-0   | 3-0-0           | 3-0-0           | 0-0-0            | 0-0-0           |
| 1072   | 0-0-0-0 | 0-0-0-0         | 3-3-0-0         | 0-0-0-0          | 0-0-0-0         |
| 1073   | 0-0-0   | 0-0-0           | 1-3-2           | 0-0-0            | 0-0-0           |
| 1229   | 1-0-0   | 1-0-0           | 1-0-0           | 0-0-0            | 0-0-0           |

\*All other samples were negative at all dilutions by both methods.

Fewer improbable MPNs were observed with qPCR and nearly all tubes that were positive for total Vp were also positive for pathogenic Vp. However, some APW tubes gave positive qPCR results for *tdh* and/or *trh* in the absence of *tlh* signal. Findings from the environmental portion of the outbreak investigation of a more pathogenic Vp population were confirmed by the use of qPCR. These findings suggest a unique ecology of Vp in Alaskan waters and higher risk of illness than in other regions of the world with warmer waters.

**USING REMOTE SENSING FOR THE INVESTIGATION OF *Vibrio parahaemolyticus* IN GULF COAST OYSTERS (*Crassostrea virginica*) AND OVERLYING WATERS FOR RISK ASSESSMENT**

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*Vibrio parahaemolyticus* (Vp), a commonly occurring bacterium indigenous to the marine and estuarine environment, is a frequent cause of food borne gastroenteritis affecting approximately 3000 people annually in the US, mostly due to consumption of raw or undercooked oysters. Water temperature has been shown to account for 50% of variability in Vp density; however, the factor(s) responsible for the other 50% are still unknown. Considering the highly dynamic nature of the coastal and estuarine areas where oysters are produced and harvested, *in situ* environmental sampling is not always feasible as it is resource intensive and sampling is limited both spatially and temporally. Therefore a remote sensing approach can be taken to supplement environmental sampling and thus be integrated into risk assessment models being developed by the FDA for predictions of risk associated with consumption of raw or undercooked shellfish. This study investigates possible environmental factors (i.e. sea surface temperature, chlorophyll, and turbidity) recorded from both *in situ* measurements and via remotely sensed satellite imagery, which may account for increased Vp density determined by DNA probe colony hybridization methods and real-time polymerase chain reaction (qPCR) assays. Statistical analysis shows significant correlations between Vp density and the environmental factors, suggesting these factors (other than temperature) may further explain Vp density variability and can be used for revision of the current FDA risk assessment model based solely on water temperature. Furthermore, *in situ* measurements significantly correlate with the respective remotely sensed data, supporting the notion that remotely sensed SST data are reliable and can be used in place of *in situ* sampling, as well as, imported into the FDA risk assessment model for real-time prediction of risk. A public access Vp monitoring website could be developed and used as an aid in making informed and timely decisions for intervention when the predicted risk may be high. This surveillance could reduce potential illness and thus increase consumer/retailer confidence in shellfish.



## HIGH POWER ULTRASONIC TREATMENT OF BALLAST WATER ORGANISMS

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Non-native, invasive, and/or pathogenic species have been, and are continuing to be transported in ballast water, resulting in ecological and economic damage. From a public health standpoint, ballast transfer of toxicogenic dinoflagellates and the pathogenic bacterium *Vibrio cholerae* are particular concerns. Treatment of ballast water organisms with high power ultrasound was the goal of this work. With the goal of establishing scaling parameters for a treatment system, the response of several relevant organisms to high power ultrasound was determined. A particular emphasis of this investigation was the robust determination of both the electrical and ultrasonic power efficiency, factors that are frequently lacking in published accounts on biological effects of ultrasound, yet are critical for engineering of a useful large-scale system.

The organisms investigated in this study are relevant to ballast water treatment and include the bacteria *Escherichia coli*, *Vibrio cholerae*, and *Enterococcus avium*, the cladoceran *Ceriodaphnia dubia*, rotifers of the genera *Brachionus* and *Philodina*, and the crustacean *Artemia franciscana*. The high power ultrasonic transducer was powered by linear amplification of a ~19 kHz AC signal with continuous measurement and logging of AC power as a function of phase angle between the two sine waves of current and voltage. Ultrasonic power transmitted into the medium by the titanium horn was measured calorimetrically. Ultrasonic killing of the bacteria was measured by plate counts, while animal survival was scored visually.

In bacterial batch experiments, higher intensity and larger horn size were correlated to kill rate, with decimal reduction times of the order of 1-10 minutes. Free radical quenching by cysteamine in bulk solution was found to have no effect on the bacterial death rate, indicating that mechanical destruction of the cells by cavitation is the likely mechanism, which was confirmed by microscopic examination. Zooplankton were killed by high power ultrasound much more easily than bacteria, with decimal reduction times of 3-9 seconds. Given large volumes and high flow rates when dealing with ballast water, high power ultrasound alone is unlikely to be a useful control for bacterial contamination, but may prove useful against zooplankton.

## COLONIZATION OF SHELLFISH BY PATHOGENIC *Vibrios*: FIRST EVIDENCE FOR A SPECIFIC MOLECULAR INTERACTION

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Bacteria belonging to the *Vibrio* genus are natural inhabitants of the estuarine environment. Several *Vibrio* species accumulate in shellfish and pose a significant threat to human health. While there is considerable evidence that *Vibrio* species such as *V. vulnificus* and *V. parahaemolyticus* have developed mechanisms to colonize shellfish with a higher avidity than other non-indigenous bacteria, little is known about the specific interactions of these microorganisms with shellfish at the cellular or molecular level.

We are characterizing bacterial surface structures called pili, to determine their role in persistence of *Vibrio sp.* in oysters, as well as in mammalian pathogenesis. Pili are thin fiber-like structures often involved in attachment of a bacterium to a variety of surfaces. Initial studies on the role of type IV pili in *V. vulnificus* have demonstrated they contribute to adherence to human epithelial cells, biofilm formation, and virulence. Using an uptake model, we have also shown that the pili are involved in persistence of the bacterium in oysters. We are currently examining the function of homologous pili in *V. parahaemolyticus*. If these factors prove to be responsible for the bacterium's ability to colonize oyster tissue, they may present a unique and specific target(s) for compounds designed to interfere with this attachment, leading to methods to reduce or eliminate the organisms from oysters.

## COMPARISON OF THE 16S rRNA GENE SEQUENCES OF *V. parahaemolyticus* AND *V. alginolyticus*

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*V. parahaemolyticus* and *V. alginolyticus* are two *Vibrio* species commonly detected in fishery products and their identification is often hampered by phenotypic (SAC- *V. alginolyticus* strains) and genetic similarity (as far as 99.7% in 16S rRNA gene). Aim of this study was the comparison of 16S rRNA sequences of 52 strains belonging to these species in order to detect possible significant commonalities and/or differences in the sequence.

A large number of bacterial strains, isolated from national or imported fishery products, seawater (Adriatic Sea) and clinical samples and presumptively belonging to Vibrionaceae family were subjected to identification by means of traditional or miniaturized biochemical tests (API systems). The PCR detection of *toxR* gene was used to confirm the identifications of *V. parahaemolyticus*; no molecular identification was available for *V. alginolyticus* strains. Fifty two bacterial strains randomly selected among those identified either as *Vibrio parahaemolyticus* or *Vibrio alginolyticus* (putative identification) and two reference strains (*V. parahaemolyticus* ATCC 43996 and *V. alginolyticus* ATCC 17749) were subjected to amplification and single strain sequencing of 16S rRNA gene. The sequences were aligned with reference sequences from GeneBank database (CLUSTAL X), were manually edited with the Bioedit program and gaps were removed from the final alignment. Phylogenetic trees were generated with the F81 model of substitution using both neighbor-joining (N.J.) and Maximum Likelihood (M.L.) tree building methods. The evolutionary model was chosen as the best-fitting nucleotide substitution model, according to the Hierarchical Likelihood Ratio Test (HLRT) implemented in the Model Test V3.0 software. The nucleotide substitution model parameters were estimated by M.L. using a N.J. tree (Jukes-Cantor distance) as base tree. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed with a bootstrap analysis using 1000 replicates for the N.J. tree. All calculations were performed with PAUP\*4.0 software.

As expected for this conserved gene, homology among the sequences of the strains belonging to the two species was high. Clustering of the sequences in two clades, a *V. parahaemolyticus* and *V. alginolyticus* clade, confirmed the biochemical and molecular identifications performed. In both clades, however, some isolated 16S rRNA sequences appeared to intermingle with the other species.

The results obtained, confirmed the phylogenetic closeness of the two species. Furthermore, the intermixing evidenced in this highly conserved sequence (subjected to neutral selection) could suggest the hypothesis of genetic exchange between these two species, an exchange that could also include sequences responsible for pathogenic characters. Further studies are advisable to verify this hypothesis.

## ASSESSMENT OF WASTE WATER TREATMENT PLANTS' ABILITY TO REDUCE BACTERIAL AND VIRAL LOADS USING REAL-TIME MULTIPLEX RT-PCR

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Discharges from wastewater treatment facilities can adversely affect the sanitary quality of recreational and shellfish growing waters. The impacts of these discharges are assessed by monitoring the effluent of these plants with bacterial indicator organisms, fecal coliforms or enterococci. Enumeration of these bacterial groups may not be a reliable index of the presence of enteric viral pathogens. This study examined wastewater from four municipal WWTP over a 10-month period to determine levels of bacterial and viral indicator organisms, enteroviruses, and noroviruses (GI and GII) in raw and treated wastewater. Conventional, pre-established methodologies were utilized to determine fecal coliform and male-specific bacteriophage (MSB) concentrations, while a real-time multiplex RT-PCR assay developed in our laboratory was used to enumerate the entero- and norovirus levels. Our findings demonstrated that fecal coliform, *E. coli*, and Enterococci densities were reduced up to 5 logs (99.999 %) by wastewater treatment using chlorine and/or UV disinfection. In contrast, the enteric viral stimulant MSB was reduced by up to 3 logs. Enterovirus levels ranging from  $1 \times 10^2$  to  $2 \times 10^4$  / 100 ml in raw untreated wastewater were reduced by  $> 2$  logs to  $< 2 \times 10^1$  to  $7 \times 10^2$  / 100 ml. Noroviruses were found in 93% and 64% of the raw and treated wastewater, respectively. This study supports previous reports that bacterial indicator organism may not index the presence of enteric viruses in wastewater and determined that MSB are more reliable in assessing the virological quality of wastewater.

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