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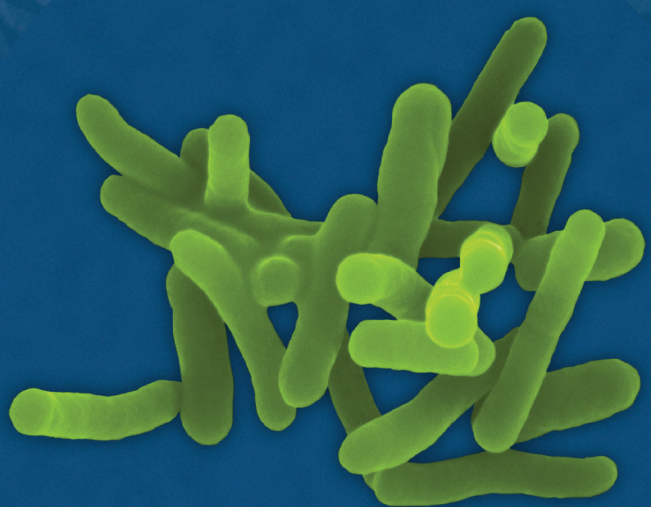
Risk assessment tools for *Vibrio parahaemolyticus* and *Vibrio vulnificus* associated with seafood

MEETING REPORT



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MICROBIOLOGICAL RISK ASSESSMENT SERIES

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Food and Agriculture Organization of the United Nations
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Declarations of interest

Two of the 17 meeting participants declared a potential interest relevant to the topic of the meeting. These related to patents for a specific element or component of methodologies for the detection of *Vibrio* spp. and other foodborne pathogens. However, these were not considered by FAO and WHO to present any conflict in light of the objectives of the meeting. Nevertheless, these declared interested and the Declaration on Interest forms of all the experts were brought to the attention of all meeting participants.

Abbreviations

AP-PCR	Arbitrarily primed polymerase chain reaction
APW	Alkaline Peptone Water
°C	Degree Celsius
CAC	Codex Alimentarius Commission
CCFH	Codex Committee on Food Hygiene
CFU	Colony forming units
CPC	Colistin-Polymyxin B-cellobiose
EO	Electrolyzed oxidizing
DGREA	Direct genome restriction enzyme analysis
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
GS-PCR	Group-specific PCR
HPP	High pressure processing
ICMSF	International Commission on Microbiological Specifications for Foods
ISO	International Organization for Standardization
JEMRA	Joint FAO/WHO expert meetings on microbiological risk assessment
KP	Kanagawa phenomenon
L	Litre
LAMP	Loop-mediated isothermal amplification
LOD	Limit of detection
MLST	Multi-locus sequence typing
Mpa	MegaPascal
MPN- PBS	Most probable number - phosphate buffered saline
ORF	Open reading frame
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
ppt	parts per thousand
RFLP	Restriction fragment length polymorphism
TCBS	thiosulphate citrate bile salts sucrose aga
TDH	Thermostable direct hemolysin
TRH	TDH-related hemolysin
USDA	United States of Amercia Department of Agriculture
USFDA	United States of Amercia Food and Drug Administration
VBNC	Viable but nonculturable
VPRA	*Vibrio parahaemolyticus* risk assessment
WHO	World Health Organization

Executive summary

There has been an increase in reported outbreaks and cases of foodborne disease attributed to pathogenic *Vibrio* species. As a result, there have been several instances where the presence of pathogenic *Vibrio* spp. in seafood has led to a disruption in international trade. The number of *Vibrio* species being recognized as potential human pathogens is increasing. The food safety concerns associated with these microorganisms have led to the need for microbiological risk assessment to support risk management decision making for their control.

V. parahaemolyticus is considered to be part of the autochthonous microflora in the estuarine and coastal environments in tropical to temperate zones. Food safety concerns have been particularly evident with *V. parahaemolyticus*. There have been a series of pandemic outbreaks of *V. parahaemolyticus* foodborne illnesses due to the consumption of seafood. In addition, outbreaks of *V. parahaemolyticus* have occurred in regions of the world where it was previously unreported. The vast majority of strains isolated from patients with clinical illness produce a thermostable direct haemolysin (TDH) encoded by the *tdh* gene. Clinical strains may also produce a TDH-related haemolysin (TRH) encoded by the *trh* gene. It has therefore been considered that those strains that possess the *tdh* and/or *trh* genes and produce TDH and/or TRH should be considered those most likely to be pathogenic.

V. vulnificus can occasionally cause mild gastroenteritis in healthy individuals following consumption of raw bivalve molluscs. It can cause primary septicaemia in individuals with chronic pre-existing conditions, especially liver disease or alcoholism, diabetes, haemochromatosis and HIV/AIDS. This can be a serious, often fatal, disease with one of the highest fatality rates of any known foodborne bacterial pathogen.

The 41st Session of the Codex Committee on Food Hygiene (CCFH) requested FAO/WHO to convene an expert meeting to address a number of issues relating to *V. parahaemolyticus* and *V. vulnificus* including:

- conduct validation of the predictive risk models developed by the United States of America based on FAO/WHO risk assessments, with a view to constructing more applicable models for wider use among member countries, including adjustments for strain virulence variations and ecological factors;

- review the available information on testing methodology and recommend microbiological methods for *Vibrio* spp. used to monitor the levels of pathogenic *Vibrio* spp. in seafood and/or water;
- conduct validation of growth rates and doubling times for *V. parahaemolyticus* and *V. vulnificus* in *Crassostrea virginica* (Eastern or American oyster) using strains isolated from different parts of the world and different bivalve molluscan species.

The requested expert meeting was held on 13-17 September 2010, and this report is the outcome of this meeting.

Rather than undertaking a validation exercise, the meeting considered it more appropriate to undertake an evaluation of the existing risk calculators with a view to determining the context to which they are applicable and potential modifications that would need to be made to extend their application beyond that context. A simplified calculator tool could then be developed to answer other specific questions routinely. This would be dependent on the availability of the appropriate data and effort must be directed towards this.

The development of microbiological monitoring methods, particularly molecular methods for *V. parahaemolyticus* and *V. vulnificus* is evolving rapidly. This means the identification of any single method for the purposes of monitoring these pathogens is challenging and also of limited value as the method is likely to be surpassed within a few years. Therefore, rather than making any single recommendation, the meeting considered it more appropriate to indicate a few of the monitoring options available while the final decision on the method selected will depend to a great extent on the specific purpose of the monitoring activity, the cost, the speed with which results are required and the technical capacity of the laboratory.

The meeting considered that monitoring seawater for *V. parahaemolyticus* and *V. vulnificus* in bivalve growth and harvest areas has limited value in terms of predicting the presence of these pathogens in bivalves. A linear relationship between levels of the vibrios in seawater and bivalves was not found and whatever relationship does exist can vary between region, the *Vibrio* spp. etc. Also, the levels of *Vibrio* species of concern in seawater tend to be very low. This presents a further challenge as the method used would need to have an appropriate level of sensitivity for their detection. Nevertheless, this does not preclude the testing of seawater for these vibrios; for example, in certain situations testing can provide an understanding of the aquatic microflora in growing areas. Monitoring of seafood for these pathogenic vibrios was considered the most appropriate way to get insight into the

levels of the pathogens in these commodities at the time of harvest. Monitoring on an ongoing basis could be expensive, so consideration could be given to undertaking a study over the course of a year and using this as a means to establish a relationship between total and pathogenic *V. parahaemolyticus* and *V. vulnificus* in the seafood and abiotic factors such as water temperature and salinity. Once such a relationship is established for the harvest area of interest measuring these abiotic factors may be a more cost-effective way of monitoring.

The meeting undertook an evaluation exercise rather than attempting to validate the existing growth models. The experts considered the JEMRA growth model for *V. vulnificus* and the FDA growth model for *V. parahaemolyticus* were appropriate for estimating growth in the American oyster (*Crassostrea virginica*). The JEMRA growth model for *V. vulnificus* was appropriate for estimating growth in at least one other oyster species, *Crassostrea ariakensis*. The FDA model for *V. parahaemolyticus* was also appropriate for estimating growth in at least one other oyster species, *Crassostrea gigas*, but was not appropriate for predicting growth in the Sydney rock oyster (*Saccostrea glomerata*). There was some evidence that the *V. parahaemolyticus* models currently used over predict growth at higher temperatures (e.g. > 25 °C) in live oysters. This phenomenon requires further investigation. Growth model studies were primarily undertaken using natural populations of *V. parahaemolyticus* as these were considered to be the most representative. Data were limited and inconsistent with respect to the impact of the strain on growth rate although recent studies in live oysters suggest differences exist between populations possessing *tdh/trh* (pathogenic) versus total or non-pathogenic populations of *V. parahaemolyticus*. There was no data to evaluate the performance of the growth models in any other oyster species or other filter feeding shellfish or other seafood and as such its use in these products could not be supported. If the models are used there should be a clear understanding of the associated uncertainty. This indicated a data gap which needs to be addressed before the risk assessments could be expanded in a meaningful manner.

1

Introduction

1.1 BACKGROUND

Based on the FAO/WHO risk assessment on *Vibrio vulnificus* in oysters and *Vibrio parahaemolyticus* in seafood, the Codex Committee for Food Hygiene (CCFH) has developed Guidelines on the Application of General Principles on Food Hygiene for the Control of Pathogenic *Vibrio* species in Seafood, which were adopted by the 33rd session of the Codex Alimentarius Commission in July 2010. However, in doing so the 41st session of the CCFH recognised the need to provide countries with tools to assist them in the implementation of the guidelines under the various conditions that exist in different regions and countries. Such tools are envisioned to support countries in their efforts to use risk based approaches in the selection of control measures appropriate for their seafood species, primary production and post-harvest practices. Such a tool has already been developed for application in the United States of America. However, as it is based on the conditions and data of the United States of America, its broader application could not be recommended without a review of its validity when applied to the non-United States of America scenarios. In light of this, the CCFH requested FAO/WHO to convene an expert meeting with the following terms of reference:

- conduct validation of the predictive risk models developed by the United States of America based on FAO/WHO risk assessments, with a view to constructing more applicable models for wide use among member countries, including adjustments for strain virulence variations and ecological factors;

- review the available information on testing methodology and recommend microbiological methods for *Vibrio* spp. in order to monitor the levels of pathogenic *Vibrio* spp. in seafood and/or water; and
- conduct validation of growth rates and doubling times for *V. parahaemolyticus* and *V. vulnificus* in *Crassostrea virginica* (Eastern or American oyster) using strains isolated from different parts of the world and different bivalve molluscan species.

1.2 *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS*

V. parahaemolyticus is a marine micro-organism native to coastal and estuarine waters. The organism was first identified as a foodborne pathogen in Japan in the 1950s (Fujino *et al*., 1953). By the late 1960s and early 1970s, *V. parahaemolyticus* was recognized as a cause of diarrhoeal disease worldwide, although most commonly reported in Asia and the United States of America. A recent history of seafood consumption is a consistent aspect of *V. parahaemolyticus* infection. Vibrios concentrate in the gut of filter-feeding molluscan shellfish, such as oysters, clams and mussels, where they multiply and cohere. Although thorough cooking destroys these microorganisms, oysters, mussels and other filter feeding shellfish are often eaten raw or lightly cooked and so tend to be the most common food associated with *V. parahaemolyticus* infection.

In Asia, *V. parahaemolyticus* is a common cause of foodborne disease. In general, the outbreaks are small in scale, involving fewer than 10 cases, but occur frequently. In Japan, during 1994-95 there were 11 364 reports of infection due to the organism. From 1996 to 1998, there were 1 699 outbreaks and 24 345 reported cases of *V. parahaemolyticus* infection, while from 1999 to 2005, 25 211 cases were reported and from 2006 to 2008, there were a total of 2 682 reported cases of infection (NIID, 2010). In general, outbreaks were more frequently reported in the summer months, with a peak in August. Boiled crabs caused one large-scale outbreak, involving 691 cases. The increased incidence from 1997 to 1998 has been attributed to an increased incidence of *V. parahaemolyticus* serovar O3:K6.

During 1997 and 1998, there were more than 700 cases of illness due to *V. parahaemolyticus* in the United States of America, the majority of which were associated with the consumption of raw oysters. In two of the 1998 outbreaks a serotype of *V. parahaemolyticus*, O3:K6, reported previously only in Asia, emerged as a

principal cause of illness for the first time. Subsequent studies on these strains have revealed their pandemic spread. The incidence of *Vibrio* spp. infection in the United States of America has continued to increase since approximately 2001 (CDC, 2011), with a summer time peak also being observed.

Illness outbreaks caused by *V. parahaemolyticus* were reported in Puerto Montt, Chile (where approximately 80 percent of the country's seafood is produced) in 2004 (1 500 cases) and reached a peak during the summer of 2005 with 3 725 cases. All reported cases up to 2006 were related to the serovar O3:K6. A decrease in the number of reported cases was observed after 2005 (477 cases in 2007) as well as a change in the causal serovar from O3:K6 to O3:K59. In 2007 approximately 40 percent of the clinical cases were related to the serovar O3:K59 while 98 percent of the clinical cases reported in summer 2008 (1 143) were associated with the pandemic strains serotype O3:K6 with the remaining cases being related to non pandemic *tdh*- and *trh*-negative strains. It was suggested that warmer than usual water temperatures were responsible for the outbreaks. In Chile, no *V. parahaemolyticus* infections were reported before the summer of 2004, their absence being explained by the low ocean temperatures which seldom reach above 16 °C. Table 1 provides an overview of the reported *V. parahaemolyticus* infections around the world over approximately the last 20 years.

TABLE 1. Available data on the incidence of *V. parahaemolyticus* infections.

Country	Period	No. of cases (outbreaks)	Attributed food	*tdh / trh* presence (where reported) (%)	Symptoms where known	Origin of data
Chile	2005	10 984				García *et al.*, 2009
	2006	~11 000				Fuenzalida *et al.*, 2006
	2007	1 008				García *et al.*, 2009
Chile (Region de los Lagos)	2004	1 500				García *et al.*, 2009
	2005	3 725				
	2006	1 083				
	2007	477				
	2008	1 153				
	2009	441				
China	1994-2005	10 790 (211)				Wang *et al.*, 2007

(cont.)

Country	Period	No. of cases (outbreaks)	Attributed food	*tdh* / *trh* presence (where reported) (%)	Symptoms	Origin of data
Denmark	1987-1992	3 10	Clinical infections		Wound infection Ear infection	Hornstrup and Gahrn-Hansen, 1993
	1980-2000	2			Gastroenteritis	Statens Serum Institut, Copenhagen, Denmark
	2006	2			Wound infection	Frank *et al*, 2006
Finland	2000 2001 2004 2005 2009 2010	2 1 1 1 1 2				Baker-Austin, 2012 (Personal communication)
France	1995-1998	6 1			Gastroenteritis Septicaemia	Geneste *et al.*, 2000
	1997	44			Gastroenteritis [1]	Lemoine, Germanetto and Giraud, 1999
	2001	100			Gastroenteritis	Hervio-Heath *et al*., 2005
	1995-2009	23			Gastroenteritis Septicemia Wound infection + septicemia Suppuration	Quilici & Robert-Pillot, 2011
Germany	2009	1				Baker-Austin, 2012 (Personal communication)
India (Calcutta)	1994-1996	201				Okuda *et al*., 1997
India (Kolkata)	2005-2007	57		50 *tdh*$^+$ 3 *trh*$^+$ 4 *tdh*$^+$/*trh*$^+$		unpublished data
Japan	1996-1998	24 345 (1 699)				NIID, 2010
Italy	2007	1	Patient had eaten fresh shellfish	100 *tdh* +ve		Ottaviani *et al*.,2008
Mozambique (Beira)	Feb. –May, 2004	42 (1)			Gastroenteritis	Ansaruzzaman *et al*., 2005
Mexico	2003	1(1)			Gastroenteritis	Cabanillas-Beltran *et al*., 2006
	2004	103(1)			Gastroenteritis	
Norway	1999	4				Unpublished data

(cont.)

Country	Period	No. of cases (outbreaks)	Attributed food	*tdh / trh* presence (where reported) (%)	Symptoms	Origin of data
North America	1997	209 (1)				Drake *et al.*, 2007
	1998	120 (1)				
Peru	1993-2002	100				Gil *et al.*, 2007
Republic of Korea	2003	732(22)				Lee *et al.*, 2008a
	2004	300(15)				
	2005	663(17)				
	2006	566(26)				
Singapore	2009	19				Ministry of Health (MOH)
Spain	1995-1998	19			Gastroenteritis	INS, 1996
	1999	64(1)				Lozano-León *et al.*, 2003
Spain (A Coruna region)	July 2004	80(1)				Martinez-Urtaza *et al.*, 2005
Spain (Galicia region)	1997-2000	84				
Sweden	1992-1997	350(1)			Gastroenteritis[2]	Lindqvist *et al.*, 2000
United Kingdom (England and Wales)	1995-1999	115				PHLS, Colindale, United Kingdom
United Kingdom (Northern Ireland)	1990-1999	0				CDSC (Communicable Disease Surveillance Centre, NI, United Kingdom
United Kingdom	2004-2005	57				Baker-Austin *et al.*, 2010
United States of Amercia	1973-2006	1 393(45)				Iwamoto *et al.*, 2010
United States of Amercia	Annual est.	34 664				Scallan *et al.* 2011
United States of Amercia (Texas, Washington, New York)	1998	416				Daniels *et al.*, 2000
Vietnam (Khanh Hoa)	1997-1999	548				Tuyet *et al.*, 2002

Notes: 1. One outbreak associated with seafood imported from Asia. 2. One outbreak associated with consumption of crayfish imported from China.

Since *V. vulnificus* was first reported in the 1970s, it has been the subject of many research and review articles (Oliver, 1989; Strom and Paranjpye, 2000). This organism naturally inhabits warm estuarine and coastal environments and can infect humans via wound exposure or seafood consumption. Three biotypes of *V. vulnificus* have been reported (Bisharat and Raz, 1996; Bisharat *et al*., 1999): Biotype 1 accounts for nearly all human cases resulting from seafood consumption, Biotype 2 is associated with infections in cultured eels, and Biotype 3 has been limited to wound infections associated with handling fish cultured in inland ponds in Israel. Most of the studies of Biotype 1 *V. vulnificus* have been conducted in the United States of America, and outside of that country there is currently little epidemiological information as *V. vulnificus* is not a reportable disease in most countries and surveillance is limited. Table 2 summarises some of the available data on the incidence of infections caused by these organisms in different countries. While foodborne *V. vulnificus* infections are relatively rare, even in the United States of America where most of the reports come from (approximately 30–40 reported cases of primary septicaemia per year, with a slightly higher number of non-foodborne cases), they have the highest case fatality ratio among foodborne illnesses, which exceeds 50 percent (Hlady and Klontz, 1996; Mead *et al*., 1999). Individuals with pre-existing liver disease are at greatest risk of contracting primary septicaemia, with subsequent mortality, but other chronic illnesses and immune deficiency conditions are also associated with increased risk. Healthy individuals may be at risk for relatively mild gastroenteritis, but the risk for primary septicaemia in the absence of reported risk factors is considered negligible.

TABLE 2. Available data on the incidence of *V. vulnificus* infections in different countries.

Country	Period	No. of cases	Symptoms	Origin of data
China, Hong Kong SAR	2003-2005	29		Chung *et al*., 2006
Belgium	1985	1		Baker-Austin, 2012 (Personal communication)
Denmark	1989-1993	2		Høi *et al*., 1998a
	1994	11		Baker-Austin, 2012 (Personal communication)
	1995	3		
	1997	4		
Finland	2002	1		Baker-Austin, 2012 (Personal communication)

(cont.)

Country	Period	No. of cases	Symptoms	Origin of data
France	1995-2009	13	Wound infection + septicemia Suppuration Septicemia	Quilici and Robert-Pillot, 2011
Germany	1994 2002 2003 2006 2010	1 1 2 3 4		Baker-Austin, 2012 (Personal communication)
Germany (Mecklenburg-Vorpommern)	2003	2		Frank *et al*., 2006
Italy		1	Wound infection	Stabellini *et al*., 1998
Japan	1984-2008	37		Matsumoto *et al*., 2010
	2001	6 1	sepsis (foodborne) wound infection type	Matui, Ono and Inoue *et al*., 2004
Netherlands	1991 2008	1 1		Baker-Austin, 2012 (Personal communication)
Republic of Korea	2000-2005	85		Kim and Jang., 2010
Sweden	1994 1997 2001	1 2 1		Baker-Austin, 2012 (Personal communication)
Spain	1977-2002	4	Wound infection Septicemia	Tores *et al*., 2002
United States of America (Gulf Coast)	1988-2006	> 900		DaSilva *et al*., 2012
United States of America	1992-2007	459		Jones and Oliver, 2009
	2002-2007	180		
	2004	64 28	Septicemia wound infections	
United States of America	Annual estimate (domestic)	96		Scallan *et al*., 2011

Notes: Partial data on the incidence of *V. vulnificus* in different countries/regions

1.3 EXISTING RISK ASSESSMENTS AND TOOLS

Quantitative risk assessments have been developed for *V. parahaemolyticus* in oysters, finfish and bloody clams (FAO/WHO, 2011) and for *V. vulnificus* in oysters (FAO/WHO, 2005). The *V. parahaemolyticus* risk assessments follow the risk assessment structure as outlined by Codex in their guidelines for microbiological risk assessment: (1) hazard identification, (2) hazard characterization, (3) exposure assessment, and (4) risk characterization.

The risk assessment of *V. parahaemolyticus* in oysters is a quantitative product pathway analysis of the factors affecting *V. parahaemolyticus* presence in oysters and the flow of events leading to consumer illnesses. In this analysis, the key steps from harvest through post-harvest handling and processing to consumption were modelled. The evaluation of key factors such as (i) the likelihood of illness following exposure to pathogenic *V. parahaemolyticus* from consumption of raw oysters, (ii) levels of the organism in oysters at the time of consumption (taking into account different harvest and post-harvest practices which may vary in different geographic areas and at different times of year) and (iii) the impact of geographical conditions on the predicted risk were taken into consideration. The scope of this risk assessment was to determine the factors that contribute to the risk of becoming ill from the consumption of pathogenic *V. parahaemolyticus* in raw oysters and to evaluate the likely public health impact of different control measures, including the effectiveness of current and alternative microbiological standards.

The risk assessment for *V. vulnificus* in oysters was undertaken as one of five pathogen-commodity combinations addressed in the FAO/WHO risk assessment work on *Vibrio* spp. in seafood. Within that framework *V. vulnificus* was identified as one of the three *Vibrio* spp. responsible for most cases of human illness caused by vibrios, where seafood was the vehicle of transmission. In considering approaches to undertake a risk assessment on this pathogen in seafood, with limited available resources, it was decided to extend the *V. parahaemolyticus* models described in the USFDA "Draft Risk Assessment on the Public Health Impacts of *V. parahaemolyticus* in Raw Molluscan Shellfish" ("USFDA-VPRA") (USFDA, 2005) and the Joint FAO/WHO Risk Assessment of *V. parahaemolyticus* in raw oysters (FAO/WHO-VPRA) (FAO/WHO, 2011) to *V. vulnificus*. The general approach and many of the parameters used in the current *V. vulnificus* risk assessment are the same as those used in the draft USFDA-VPRA and FAO/WHO-VPRA. The first objective of this risk assessment was to determine the usefulness of adapting the USFDA-VPRA and FAO/WHO-VPRA models to assess the risk from *V. vulnificus* septicaemia associated with the consumption of raw oysters. Secondly, the risk assessment aimed to identify the most appropriate data, as well as gaps in the available dataset, for modelling purposes. In addition to estimating the risk of *V. vulnificus* septicae-

mia associated with the consumption of raw oysters, the risk assessment model was also developed with the objective of evaluating targeted mitigation levels aimed at reducing the risk of *V. vulnificus* septicaemia. For reasons of data availability, the risk assessment was limited to consideration of primary septicaemia cases associated with consumption of raw oysters from the Gulf Coast of the United States of America. Collection of data in different regions, in different bivalve species and for geographically diverse strains of pathogenic *V. parahaemolyticus* and *V. vulnificus*, is required to conduct validation of the existed predictive risk models, and further to modify/develop risk assessment models. There is the potential to expand these risk assessment approaches for consideration in other geographical areas and using different seafood commodities.

The existence of the aforementioned risk assessments facilitated the development of simplified and easy to use risk calculators by the USFDA for use in addressing the *Vibrio* problem in that country. This easy to use risk based approach is very desirable from a user's perspective. However, the value of the output is only as good as the information and original risk assessments upon which these calculators are based. Therefore, these aspects were addressed when considering the wider applicability of such tools. This is addressed in detail in Chapter 4 of this report.

2

Pathogenicity

2.1 VIRULENCE MARKERS OF *V. PARAHAEMOLYTICUS*

Clinical strains of *V. parahaemolyticus* isolated from ill patients usually produce a clear beta-type hemolysis on Wagatsuma agar due to production of a thermostable direct haemolysin (TDH). This phenomenon is called the Kanagawa phenomenon (KP). On the contrary, only a small portion of the environmental strains show a positive KP. Therefore, TDH encoded by the *tdh* gene has been considered a major virulence factor of *V. parahaemolyticus*. However, subsequent to the description of the KP, an outbreak due to *V. parahaemolyticus* was reported where none of the isolates carried the *tdh* gene. These strains carried the *trh* gene coding for a TDH-related haemolysin (TRH). TRH was judged to be associated with reported illnesses and thus considered to be another important virulence factor of *V. parahaemolyticus*.

Some strains harbor both *tdh* and *trh* genes. There are five sequence variants of the *tdh* gene (*tdh*1 – *tdh*5) and two sequence variants of the *trh* gene (*trh*1 –*trh*2) (Nishibuchi and Kaper, 1990; Nishibuchi and Kaper, 1995). KP-positive strains have *tdh*1 and *tdh*2 genes; the *tdh*2 gene is expressed at high levels and large amounts of TDH produced from the *tdh*2 gene are responsible for the KP (Nishibuchi, Kumagai and Kaper, 1991). Strains carrying, a single copy of the *tdh* gene only, the *trh* gene only, single copies of each of the *tdh* and *trh* gene have been isolated from patients at much lower frequencies than KP-positive strains. The *tdh* genes (*tdh*3, *tdh*4, or *tdh*5) or the *trh* genes (*trh*1 or *trh*2) contained in these KP-negative clinical strains and the *tdh*1 gene in KP-positive strains are expressed at low levels (<1/10 of *tdh*2 expression level) and produce small amounts of TDH or TRH (Nishibuchi and Kaper, 1990; Nishibuchi and Kaper, 1995; Lin *et al*., 1993; Okuda and Nishibuchi,

1998; Nakaguchi *et al.*, 2003). Therefore, these strains are considered to be pathogenic but probably exhibit reduced virulence.

Biological activities of purified TDH1 – TDH5 and TRH1 and TRH2 are similar (Yoh *et al.*, 1991; Shirai *et al.*, 1990; Kishishita *et al.*, 1992). It is possible that susceptibility of individuals to these toxins can vary and that there are those who are more susceptible to these toxins than others and who may respond to relatively small amounts of TDH or TRH produced from exceptional KP-negative strains and exhibit clinical symptoms.

Detection of *tdh*$^-$*trh*$^-$ strains among clinical strains has been the source of debate on the pathogenic roles of the *tdh*$^+$ and/or the *trh*$^+$ genes. Although a possibility that such strains carry important virulence genes but not the *tdh*$^+$ and *tdh*$^+$ genes cannot be ruled out, evidence has accumulated to explain the rare detection of *tdh*$^-$ *trh*$^-$ strains among clinical strains and support the pathogenic roles of the *tdh*$^+$ and/or the *tdh*$^+$ genes. Bhoopong *et al.* (2007) provided solid evidence for the possibility that has long been suscpected among clinical microbiologists: the colonies on thiosulphate citrate bile salts sucrose agar (TCBS agar) that are derived from clinical samples may consists of virulent (*tdh*$^+$ and/or the *tdh*$^+$) and avirulent (*tdh*$^-$*trh*$^-$) strains of *V. parahaemolyticus* and accidental isolation of an avirulent (*tdh*$^-$*trh*$^-$) strain(s) actually is causing a misleading interpretation of the avirulent (*tdh*$^-$*trh*$^-$) strain(s). Another explanation for the *tdh*$^-$*trh*$^-$ strains among clinical isolates was supported by the undeniable evidence provided by Kamruzzaman *et al.*, (2008) that the *tdh* gene can be deleted from a chromosome of *V. parahaemolyticus* by an active insertion sequence during propagation of bacterial cells.

Tables 3 and 4 summarize examples of distribution of the *tdh* and *trh* genes in clinical strains (Table 3) and environmental strains (Table 4). These tables show a range of *tdh* and *trh* gene carriage distributions geographically. The results for the strains listed as from the "World" are those strains collected in Nishibuchi's laboratory. A total of 3 922 clinical strains and 1 296 environmental strains were confirmed to belong to *V. parahaemolyticus* by the PCR method targeting the *toxR* gene (Kim *et al*, 1999). The clinical strains were isolated from the patients with diarrhoea in 15 countries or international travelers who arrived at Japanese quarantine stations between 1973 and 2009 (during 37 years). The environmental strains were isolated in 18 countries between 1983 and 2010 (during 28 years). All test strains were analyzed for the presence or absence of the *tdh* and *trh* gene by PCR methods (Tada *et al.*, 1992). A review of the data from literature as presented in Table 5 leads to very similar results in terms of the proportion of *tdh*$^+$, *trh*$^+$ and *tdh*$^+$/*trh*$^+$ strain among clinical and environmental strains.

Apart from the *tdh* and *trh* genes, a number of other possible virulence factors/genes have been proposed for *V. parahaemolyticus*. However, none of them have

been considered as major virulence factors due to a lack of consistency in association with illness. Recently, type III secretion systems (T3SS), of which there are two types, have received attention. In particular, those located in the pathogenicity islands associated with the *tdh* and *trh* genes are named T3SS2 are considered to be possible virulence markers. The T3SS2 were designated as ά and β, respectively (Caburlotto *et al*., 2009; Okada *et al*., 2009). An investigation into whether these markers could be better virulence markers of *V. parahaemolyticus* than the *tdh* and *trh* genes has been carried out by examining 36 $tdh^{-}trh^{-}$ strains from a collection of 296 $tdh^{-}trh^{-}$ clinical strains for the presence or absence of the *vscC2* genes representing T3SS2ά and T3SS2β and some other genes in T3SS2. However, only three strains were positive for T3SS2ά and no strains were positive for T3SS2β (Nishibuchi, personal communication). Considering this molecular epidemiological evidence and the biological actions of TDH and TRH, it has been concluded that, at the time of writing this report, and given limited understanding of other potential virulence factors, the *tdh* and *trh* genes are currently the most suitable virulence markers of *V. parahaemolyticus*.

The significance of KP-positive strains is being recognized globally due to the emergence of the pandemic clone. The epidemiology of the diarrhoea outbreaks caused by the different serotypes of *V. parahaemolyticus* changed abruptly after 1996. The emergence of a particular clone with serotype O3:K6 led to increased diarrhoeal disease outbreaks, initially in Southeast Asia and subsequently worldwide (Okuda *et al*, 1997; Matsumoto *et al*, 2000; Nair *et al*., 2007). Before the spread of the pandemic clone, infections caused by *V. parahaemolyticus* were usually associated with a variety of different serotypes. In contrast almost 100 percent of the worldwide isolates with O3:K6 were indistinguishable by the DNA fingerprinting methods such as arbitrarily primed PCR (AP-PCR), restriction fragment length polymorphism-pulsed field gel electrophoresis (RFLP-PFGE), mulit-locus sequence typing (MLST), and direct genome restriction enzyme analysis (DGREA) and hence are considered to constitute a clone (Matsumoto *et al*., 2000; Wong *et al*., 2007; Fuenzalida *et al*., 2006; González -Escalona *et al*., 2008). The increase in diarrhoeal disease outbreaks in Southeast Asia (Matsumoto *et al*., 2000) and the large outbreaks in southern Chile where temperature is low and *Vibrio* load is small (Harth *et al*., 2009), could be best explained by a higher infectious capacity of the pandemic strain. Serotype variants of the pandemic clone have been recognized in various parts of the world, but their DNA fingerprinting patterns confirm they belong to the pandemic clone (Nair *et al*., 2007). Genetic markers for the pandemic strains include altered nucleotide bases in the *toxR* gene – detectable by a PCR named as group-specific PCR (GS-PCR) (Matsumoto *et al*., 2000), an open reading frame (ORF8) in a lysogenic filamentous phage (Nasu *et al*., 2000) and gene sequences in 16-kb or 23-kb chromosomal inserts specific to the pandemic clone (Hurley *et al*., 2006; Nishioka *et al*., 2008).

TABLE 3. Distribution of genetic markers in clinical strains of *Vibrio parahaemolyticus*

Virulence level	Presence of the gene[a]:	% of the strains with the indicated genotype among the isolates from: World (15 countries), 1973-2009[b]	United States of America 1979-1995[c]	United States of America 2006-2007[d]	France, 1995-2009[e]	Southern Thailand, 1999[f]	Vietnam, 1997-1999[g]	Chile, 2004-2010[h]
Virulente	*tdh*⁺ only	83.7	23.9	11.7	70	89	84.5	92.9
Less virulent	*trh*⁺ only	2.4	6.82	15.6	25	1.26	0.57	0.5
Less virulent	*tdh*⁺, *trh*⁺	6.4	63.6	43.5	5	2.52	0.57	2.2
Barely virulent	*tdh*⁻, *trh*⁻[i]	7.5	5.68[j]	27.3	0	7.26	14.3	2.2
Virulent (Pandemic)	*toxR* (GS-PCR)	63	ND[k]	(5.2)[l]	35	76.3	48.9	90.1

[a] Based on the results of conventional PCR.
[b] Personal communication with Nishibuchi. Explained in detail in the text.
[c] West Coast (Okuda *et al*., 1997)
[d] July 2006 – November 2007 (Jones, personal communication)
[e] Quilici and Robert-Pillot (2011)
[f] Laohaprertthisan *et al*. (2003)
[g] Tuyet *et al*. (2002); Chowdhury *et al*. (2004)
[h] Personal communcation with Romilio Espejo
[i] Isolation of non-pathogenic *tdh*⁻ and *trh*⁻ strains from clinical samples can be accounted for at least in part by mixture of heterologous genotypes or by an insertion-mediated tdh deletion during bacterial growth (explained in detail in the text).
[j] 3 (60 percent) of the 5 isolates were from extraintestinal infections.
[k] ND: not determined.
[l] Serotype (O3:K6) rather than GS-PCR was used as an indicator of the pandemic strains.

TABLE 4. Distribution of the genetic markers in environmental strains of *Vibrio parahaemolyticus*

Virulence level	Presence of the gene[a]:	% of the strains with the indicated genotype among the isolates from: World (18 countries), 1983-2010[b]	Thailand, 1998-1999[c]	France, 1994-2009[d] 1999[e]	France, 1994-2009[d] 1994-2001[f]	France, 1994-2009[d] 2008-2009[g]	Chile[h]
Virulente	*tdh*⁺ only	6.7	0.34	0	0	0	9
Less virulent	*trh*⁺ only	2.9	0.34	4.9	3.2 and 15	15.2	0
Less virulent	*tdh*⁺, *trh*⁺	2.1	0	0	0	0	2.1
Barely virulent	*tdh*⁻, *trh*⁻	88.3	99.2	95.1	96,8 and 85	84.8	91
Virulent (Pandemic)	*toxR* (GS-PCR)	8.2[i]	0.34	ND[j]	ND	ND	90.1

[a] Based on the results of conventional PCR.
[b] Personal communication with Nishibuchi. Explained in detail in the text.
[c] A total of 114 (54 shellfish, 30 shrimp, and 30 fish) samples were examined (Vuddhakul *et al*., 2000).
[d] Personal communication with Hervio-Heath
[e] Hervio-Heath *et al*., 2002
[f] Robert-Pillot *et al*., 2004
[g] Deter *et al*., 2010a.
[h] Year(s) unknown. Personal communication with Romilio Espejo
[i] 5.2 percent for *tdh*⁺ strains, 3 percent for tdh- strains
[j] ND: not determined.

TABLE 5. Data from published literature on *tdh* and *trh* genetic marker distribution in clinical strains from different countries.

Location	Strains	*tdh*		*trh*		*tdh*$^+$/*trh*$^+$		*tdh*$^-$/*trh*$^-$		Reference
		No. positive	%	No. positive	%	No. positive	%	No. positive	%	
Chile	44	42	95.4	0	0	2	4.6	0	0	González-Escalona *et al.*,2005
China	24	21	84	3	12	0	0	0	0	Vongxay *et al.*,2008
France	11	5	46	4	36	1	9.09	1	9.09	Robert-Pillot *et al.*, 2004
France	20	14	70	5	25	1	5	0	0	Quilici and Robert-Pillot, 2011
India (Kolkata)	57	50	87.7	3	5.3	4	7	0	0	[1]Murthy, personal communication
Italy	1	1	100	0	0	0	0	0	0	Ottaviani *et al.*, 2008
Mexico	132	103	78.1	0	0	0	0	29	21.9	Cabanillas-Beltrán *et al.*, 2006
Mozambique	42	42	100	0	0	0	0	0	0	Ansaruzzaman *et al.*,2005
Peru	92	81	81	11	11	0	0	0	0	Gil *et al.*, 2007
United States of America	27	11	40.7	0	0	11	40.7	5	18.5	Bej *et al.*,1999
United States of America (Washington)	26	0	0	0	0	26	100	0	0	DePaola *et al.*, 2003a
United States of America (New York)	9	9	90	0	0	0	0	0	0	DePaola *et al.*, 2003a
Thailand	434	396	81	9	2	29	6	0	0	Suthienkul *et al.*, 1995
Total	919	775	84.33	35	3.81	74	8.05	35	3.81	

[1] Virulence characteristics of *Vibrio parahaemolyticus* isolated from acute diarrheal patients admitted into the Infectious Diseases Hospital, Kolkata, India during 2005-07 (From S. K. Bhowmick, Ph. D thesis to be submitted)

2.2 *V. VULNIFICUS* VIRULENCE MARKERS

In 1994, Aznar and colleagues reported that sequence determination of rRNA gene polymorphisms of *V. vulnificus* could be used to identify two groups, which they termed A and B. Later, Nilsson *et al.* (2003) showed that these two groups were associated with clinical (B) or environmental (A) isolation. In a study reported by Rosche, Yano and Oliver (2005), 55 *V. vulnificus* strains were typed using a virulence marker ("*vcg* gene") which had been identified in clinical strains by Warner and Oliver (1999). Of these, 95 percent of the strains possessing the "C-type" genomic structure were human clinical isolates, while 94 percent environmental (oyster, water) isolates exhibited the "E-type" structure. Subsequent virulence studies (Personal communication J. Oliver, September 2010) found that 88 percent of the C-type strains were virulent (LD_{50} levels of $\leq 3.5x10^3$) whereas 38 percent of E-type strains were virulent in a mouse model. A multiplex system employing the *V. vulnificus* hemolysin A gene (*vvhA*) for the identification of this species, with simultaneous determination of the C/E genotype (*vcg* gene) has been reported (Warner and Oliver, 2008a). This method is rapid (3 hours) and is substantially simpler than the earlier method reported for 16S rRNA sequencing to determine genotype. A number of subsequent studies (e.g. Cohen *et al.*, 2007; Rosche, Binder and Oliver, 2010; Sanjuan *et al.*, 2009) have now reported these "two genomic types" in all *V. vulnificus* strains examined and confirmed a high correlation between the vcg PCR and 16S sequencing methods.

It was subsequently reported that the distribution of C and E-genotypes of *V. vulnificus* in seawater and oysters (*C. virginica*) varied dramatically (Warner and Oliver, 2008b). Of 292 *V. vulnificus* isolates taken from waters at 32 sites from the east and Gulf coasts of the United States of America, approximately equal numbers of the two genotypes were represented (46.9 percent E-genotype, 53.1 percent C-genotype). In contrast, of 880 *V. vulnificus* isolates taken from 85 oysters harvested from these same waters, 84.4 percent were the E-genotype while only 15.6 percent were the virulent (C-genotype). Further, of these 85 oysters, only one had more C- than E-genotype isolates. Such a low level of the more virulent genotype likely help explains the relatively small number of cases reported each year in the United States of America. A variety of labs have reported a similar distribution of C and E-genotypes of *V. vulnificus* in seawater and oysters (Warner and Oliver, 2008b).

While the overall numbers of *V. vulnificus* in seawater demonstrate a seasonal response, the percentage of the C-genotype in both water and oysters appears to be favored by warmer waters. Similar results were recently reported by Han *et al.* (2009) although they reported a higher overall percentage of C-genotype strains in oysters during the summer months. Further, salinity may play a role in C/E distribution, as C-genotype strains are not seen in east coast United States of America waters above 20ppt (Figure 1; Oliver, unpublished).

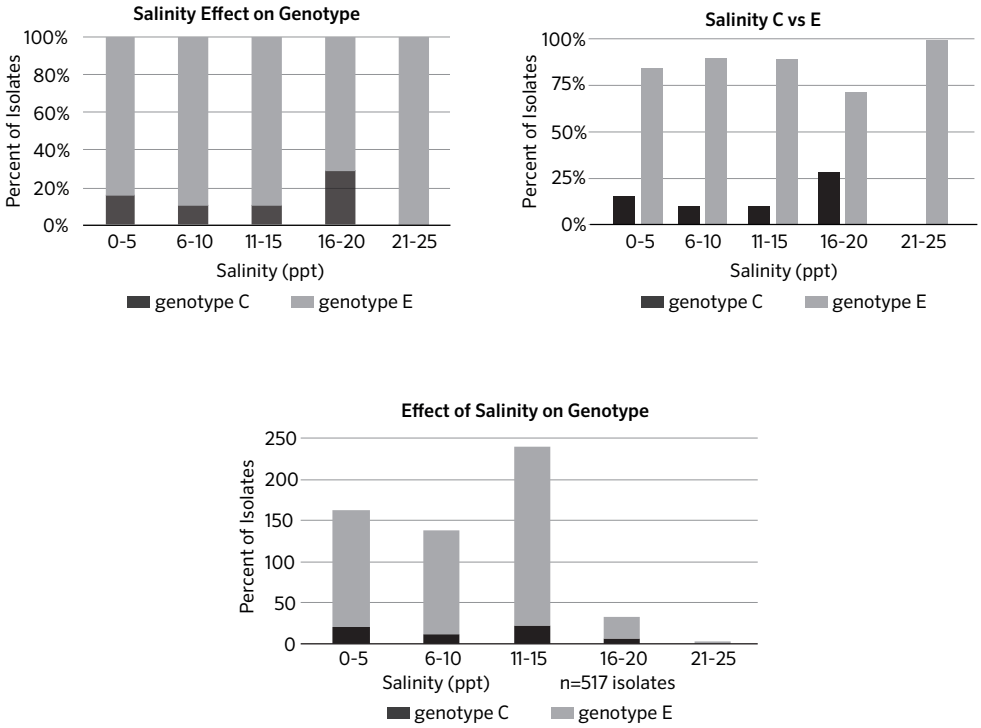

FIGURE 1. Effect of salinity on the genotype distribution among *V. vulnifcus* isolates from sea water (Oliver, unpublished data).

3

Factors relevant to the fate of *V. parahaemolyticus* and *V. vulnificus*

Tools to estimate the risk of infections of *V. vulnificus* and *V. parahaemolyticus* in oysters have already been developed for application in the United States of America. These tools are based on data on the concentraion of *V. parahaemolyticus* and *V. vulnificus* and the proportion of pathogenic *V. parahaemolyticus* strains at the point of harvest inferred from previous studies carried out in the coastal areas of the United States of America. The main environmental factor identified affecting the abundance and the seasonality of *V. vulnificus* and *V.parahaemolyticus* in this area has been seawater temperature.

The distribution and population dynamics of *V. parahaemolyticus* and *V. vulnificus* in coastal environments are the result of complex interactions with a number of diverse biotic and abiotic parameters which vary with the oceanic and climatic conditions prevailing in the different regions. The application of the United States of America tools in the non-United States of America scenarios requires a revision of the particular conditions prevailing in different areas of the world. This is required to evaluate if the ecological factors associated with *Vibrio* found in the United States of America can be extrapolated to another region where different patterns exist according to local particularities.

Other approaches include the elaborate statistical models have been applied to the interrelationships between the abundance of *V. vulnificus* and *V. parahaemolyticus*

and the ecological factors to enable prediction of the risk of their presence or to infer the abundance of these organisms in a particular area. Since the abundance of *V. vulnificus* and *V. parahaemolyticus* is a critical parameter to estimate the risk of infections, the development of the predictive models based on environmental variables may be a practical tool for a rapid and inexpensive estimation of the load of *V. vulnificus* and *V. parahaemolyticus* in shellfish in areas were the surveillance is limited or absent.

The number of studies describing the ecological conditions and environmental factors associated with the distribution of *V. vulnificus* and *V. parahaemolyticus* in natural environments is limited and most of them are restricted to some specific regions of the world. The disparity of sampling programs (samples sizes, sampling periods, frequency) and of statistical analyses, results in important limitations in comparison of the data and in the inference of any general pattern of association between the main environmental variables and the distribution of *Vibrio* spp. These constraints are even more significant for the analysis of pathogenic populations due to the extremely low occurrence of pathogenic strains in the environment. Due to the absence of clear scientific evidence supporting a distinctive ecological preference of pathogenic strains, it has been assumed for the purposes of this work that the levels of pathogenic strains are influenced by the same environmental conditions as total *V. vulnificus* and *V. parahaemolyticus* populations.

3.1 WATER TEMPERATURE

Seawater temperature has been reported as one of the principal environmental factors increasing the abundance of *V. vulnificus* and *V. parahaemolyticus* in many areas of the world.

The positive effect of seawater temperature on the abundance of *V. parahaemolyticus* has been observed in temperate regions with low and moderate temperatures and clear seasonal pattern, such as France (Deter *et al*., 2010b), Spain (Martinez-Urtaza *et al*., 2008), the Gulf of Mexico (DePaola *et al*., 2003b) or Chesapeake Bay of the United States of America (Parveen *et al*., 2008). Conversely, seawater temperature has been shown to have a minimal influence on *Vibrio* abundance in tropical areas where the values of water temperature remain almost constant around the year (Deepanjali *et al*., 2005).

A similar pattern of association of abundance with seawater temperature has been observed for *V. vulnificus*. A significant influence of temperature was observed in North Carolina, the United States of America, with seawater ranging from 7-32ºC (Blackwell and Oliver., 2008), whereas no influence has been detected in tropical waters (24-34ºC) in Mangalore, India (Parvathi *et al*., 2004).

3.2 SALINITY

Salinity has also been shown to play an important role in the ecology of *V. vulnificus* and *V. parahaemolyticus* and has been identified as the primary factor governing the distribution of *V. parahaemolyticus* in areas with values of salinity closer to oceanic waters (35ppt). The highest incidence of *V. parahaemolyticus* characteristically occurred in periods and areas with lower values of salinity (Martinez-Urtaza *et al*., 2008). However, the effect of salinity is less significant in regions where this parameter reaches values favourable for the survival of *Vibrio* or shows minimal variations throughout the year (Parveen *et al*., 2008).

V. vulnificus occupies an ecological niche similar to *V. parahaemolyticus* and its distribution is also governed by variations in salinity. *V. vulnificus* does not tolerate high salinity, and its distribution is mostly restricted to brackish water environments of temperate and tropical areas (Parvathi *et al*., 2004; Rivera *et al*., 1989).

According to the current available information, the distribution of *V. parahaemolyticus* in relation to the variations in salinity and seawater temperature could be summarized according to the following pattern:

- Areas of moderate salinity (from 1 to 25 ppt) and temperate or warm waters (e.g. Gulf of Mexico, Chesapeake Bay, the United States of America): seawater temperature is the major factor influencing the abundance.
- Areas with salinity close to oceanic waters (from 25 to 35 ppt) and temperate waters (e.g. Atlantic coasts of Europe): *V. parahaemolyticus* is detected in areas and periods of lowest salinity, whereas seawater temperature influences the concentration.
- Tropical areas with minor changes in seawater temperature (e.g. India), no influence of salinity and temperature has been reported.

In accordance with this, the probability of *V. parahaemolyticus* detection declines in waters with values of salinity above 30 ppt, whereas the concentration is influenced by the seawater temperature over a wide range above 12°C.

The combined effect of seawater salinity and temperature on the distribution and concentration of *V. vulnificus* and *V. parahaemolyticus* in coastal habitats of different regions have been used to infer the response of these population in relation to variations of these variables. The knowledge of these basic relationships can be used to assess the risk of *V. vulnificus* and *V. parahaemolyticus* in a specific area and to develop mathematical models for application as predictive tools to estimate the load of *V. vulnificus* and *V. parahaemolyticus* at specific temperatures and salinity. After field validation of the models, they could be used as dynamic

tools to forecast the load of *V. vulnificus* and *V. parahaemolyticus* in near real time using data of salinity and temperature available from remote sensing systems.

There is a variety of post-harvest practices that are frequently applied in various places around the world and these have a potentially significant impact on risk. Therefore, it is important that any further development of the tool take these into consideration.

3.3 OTHER ENVIRONMENTAL FACTORS

In addition to seawater temperature and salinity, some additional abiotic and biotic factors have been identified modulating the presence and abundance of *V. vulnificus* and *V. parahaemolyticus* in coastal water around the world. However, the effects of these variables are not conclusive and, in some cases, have been reported in a particular study effecting a specific area. It is likely a range of interacting and not mutually independent factors causing fluctuations in the prevalence and concentration of these species in the natural environment and marine animals at any one period of time.

Chlorophyll has been reported to positively influence the abundance of *V. parahaemolyticus* in the coasts of France, although a contrary effect has also been reported for northwest Spain (Deter *et al*., 2010b; Martinez-Urtaza *et al*., 2008). Turbidity has been identified as an important factor explaining the dynamics of total and pathogenic *V. parahaemolyticus* populations on the coast of Mississippi, the United States of America, in different studies (Zimmerman *et al*., 2007; Johnson *et al*., 2010). The presence of *V. parahaemolyticus* in seawater from the French Atlantic coast could be attributed to the average temperature over the 7-day period prior to sampling and turbidity (Deter *et al*., 2010b).

Bacteriophages abundant in shellfish and seawater have been proposed as potential agents that influence the concentration of *V. parahaemolyticus*. Bacterial lysis during the phage replication cycle would result in a reduction in viable bacterial populations. *V. parahaemolyticus* specific phages, VP93 and VP58.5, have recently been described in Chile and are suggested as a potential cause of the rise and decline, respectively, of infections associated with the pandemic *V. parahaemolyticus* O3:K6 in Southern Chile (Zabala, Garcia and Espejo, 2009; Bastías *et al*., 2010). Lytic bacteriophage VP93 may have initially favoured the growth of the pandemic strains among similar phage-sensitive strains, while temperate phage VP 58.5 may have resulted in the decline by killing pandemic cells and conferring increased ultraviolet sensitivity on the lysogenised phage resistant cells in the environment (Garcia *et al*, 2013).

3.4 SEAFOOD OF CONCERN

V. parahaemolyticus occurs in a variety of fish and shellfish, including clams, shrimp, lobster, crayfish, scallops, crabs, oysters and mussels. Oysters, mussels and clams are likely the most common food associated with *Vibrio* infection in some countries (Hlady, 1997; EC, 2001; FAO/WHO, 2011). Apart from bivalves, there have been reports of *V. parahaemolyticus* infections associated with the other types of seafood. One such report was a case-control study of sporadic *Vibrio* infections in two coastal areas of Louisiana and Texas, the United States of America, conducted in 1992-93, in which crayfish consumption was reported by 50 percent (5/10) of the persons with *V. parahaemolyticus* infection (Bean *et al*., 1998).

Outbreaks of *V. parahaemolyticus* gastroenteritis aboard two Caribbean cruise ships were reported in 1974 and 1975 (Lawrence *et al*., 1979). The outbreaks were most likely caused by contamination of cooked seafood with seawater from the ships' seawater fire systems. In 1972, an estimated 50 percent of 1200 persons attending a shrimp feast in Louisian, the United States of America became ill with *V. parahaemolyticus* gastroenteritis and samples of uncooked shrimp tested positive for the organism (Barker and Gangarosa, 1974). Three outbreaks occurred in Maryland, the United States of America in 1971 (Dadisman *et al*., 1972). Steamed crabs were implicated in two of the outbreaks after cross-contamination with uncooked crabs. The third outbreak was associated with crabmeat contaminated before and during canning. In Japan, *V. parahaemolyticus* gastroenteritis has also been associated with consumption of finfish (FAO/WHO, 2011). In China, where the rate of *Vibrio* infections is very high, a range of fish and seafood have been linked with *V. parahaemolyticus* infections. (Liu *et al*., 2004; Wang *et al*., 2007). However, in these cases the fish/seafood was often subject to some cooking although it may not have been enough to completely kill the organisms. Food preparation practices are diverse worldwide, and their preparation with wine, vinegar and spices is also common. In Central and South America ceviche made from raw fish has been linked to outbreaks (Cabanillas-Beltran *et al*., 2006; Gil *et al*., 2007).

In the case of *V. vulnificus,* the illnesses reported in the United States of America have been almost exclusively related to oysters (FAO/WHO, 2005). The consumption of raw shellfish is likely linked to illnesses in other parts of the world. For example, in Japan, raw oysters are eaten only in winter (December- February) and most infections occur during June-November with a peak in July and a mud shrimp, Upogebia major, is a common source of infection in summer months. (Inoue *et al*., 2008).

For the purposes of this work it was concluded that bivalves were the seafood products of greatest concern. However, considering that these may be consumed in different ways around the world, the inclusion of a step in the risk assessment

which would allow consideration of these different practices e.g. partial or inadequate cooking, addition of mild acids, condiments etc. would be a valuable addition in terms of extending the applicability of the tool.

3.5 PREHARVEST AND POST HARVEST PRACTICES

The effects of post-harvest treatment technologies, alone or in combination, were discussed at the JEMRA meeting in August in 2002. It was concluded that these may all have the effect of reducing the numbers of pathogenic vibrios but the effectiveness will vary according to the conditions of use, and there may be a need to balance between obtaining the maximum possible reduction in bacterial content and retaining consumer-acceptance of either the product or the process. Reports on the effectiveness of depuration vary greatly and this may again depend on the conditions of use - some reports indicate that proliferation of vibrios may occur during this process. The general opinion of the expert consultation was shown on a qualitative/semi-quantitative basis in Table 6.7 of the expert consultation report[1]. Below is a summary of some new data regarding post-harvest treatment technologies.

3.5.1 Depuration

According to the Codex Code of Recommended Practice for Fish and Fishery Products, depuration means the reduction of microorganisms to a level acceptable for direct consumption by the process of holding live bivalve molluscs for a period of time under approved, controlled conditions in natural or artificial sea water suitable for the process, which may be treated or untreated. Depuration is practiced in a number of countries for a range of bivalve molluscan species (e.g. clams, oysters, mussels, scallops) and this process may be performed in static, flow through or recirculating systems using water treated with Ultra Violet (UV) light, chlorine, iodine or ozone (Lee, Lovatelli and Ababouch, 2008b). While depuration has been reported to be effective in removal of human enteric bacteria, the reported efficacy for removal of autochthonous bacteria such as *Vibrio* spp. has not been consistent.

The depuration process has a long history as a postharvest treatment for reducing total microbial populations in shellfish. However, depuration at ambient temperatures has been reported as ineffective for reducing *Vibrio* contamination in oysters (Colwell and Liston, 1960, Vasconcelos and Lee, 1972). Similarly, Chae *et al.* (2009) reported that depuration of oysters (*Crassostrea virginica*) at 22° C had limited effects on reducing *V. parahaemolyticus* or *V. vulnificus* in oysters with populations reduced by 1.2 and 2.0 $\log_{10}$ MPN/g, respectively, after 48 h of depuration at

[1] http://www.who.int/foodsafety/publications/micro/aug2002.pdf

22° C. Decreasing water temperature to 15° C increased the efficacy of depuration with reductions increased to 2.1 and 2.9 $\log_{10}$ MPN/g, respectively, after 48 h of depuration at 15 °C. However, depurations at 10 and 5 °C were less effective than at 15 °C in reducing the *Vibrio* spp. in oysters. Extended depuration at 15 °C for 96 h increased reductions of *V. parahaemolyticus* and *V. vulnificus* in oysters to 2.6 and 3.3 $\log_{10}$ MPN/g, respectively (Chae *et al*., 2009). Su, Yang and Häse (2010) reported that depuration with refrigerated seawater for 96 h reduced *V. parahaemolyticus* populations by >3.0 $\log_{10}$ MPN/g in oysters harvested in the winter while 144 h of depuration at 5 °C was required to achieve a 3.0-$\log_{10}$ reduction in oysters harvested in the summer. Depuration with refrigerated seawater at 5 °C for up to 144 h caused no significant fatality in the Pacific oyster and could be applied as a postharvest treatment to reduce *V. parahaemolyticus* contamination in Pacific oysters during summer (Su, Yang and Häse 2010).

Eyles and Davey (1984) reported that depuration using UV treated seawater did not produce a substantial reduction in *V. parahaemolyticus* levels in the Sydney rock oyster (Craassostrea commercialis). Tamplin and Capers (1992) found that depuration using recycled UV treated water at above 23 °C was ineffective. They suggested *V. vulnificus* was able to multiply in oyster tissues under these conditions and was released to the surrounding seawater at a greater rate than the reduction in the seawater by the UV treatment in contrast to treatment at 15°C when *V. vulnificus* was not isolated from seawater.

Nordstrom and others (2004) were able to achieve better reduction in *V. parahaemolyticus* levels after overnight tidal submersion, compared to intertidal exposure, in Hood Canal, Washington State, the United States of America, which indicated that intertidal exposure could purges vibrios from shellfish tissues.

Ren and Su (2006) examined the effects of electrolyzed oxidizing (EO) water depuration on reducing *V. parahaemolyticus* and *V. vulnificus* in laboratory-contaminated oysters and found that both species could only be reduced by approximately 1.0-$\log_{10}$ unit after 8 h at room temperature.

3.5.2 Refrigeration storage

Chilled storage can achieve certain reductions of *Vibrio* spp. in oysters. Thompson and Vanderzant (1976) reported that populations of *V. parahaemolyticus* in shucked oysters decreased from 411 000 to 0.36 MPN/g after 7 days of storage at 31°C. Limthammahisorn, Brady and Arias (2009) observed one to two logs decrease in viable *V. vulnificus* cells after transferring oysters to refrigeration temperatures. Liu, Lu and Su (2009) reported that populations of inoculated *V. parahaemolyticus* in oysters decreased by 2.45, 1.71, and 1.45 $\log_{10}$ MPN/g after 1 month of storage at

10, 20, and 30°C, respectively, and after 4 months of storage, the levels of *V. parahaemolyticus* in oysters were reduced by 3.83 (10°C), 3.14 (20°C), and 2.28 (30°C) $\log_{10}$ MPN/g, further at the end of 6 months of study, the levels of *V. parahaemolyticus* were reduced by 4.55, 4.13 and 2.53 $\log_{10}$ MPN/g in oysters stored at 10, 20, and 30°C , respectively.

When *V. vulnificus*-contaminated oysters were incubated at 35°C for 10 h, cells increased by a half $\log_{10}$ unit. After oysters were placed at 4°C for 168 h cell numbers declined moderately by two $\log_{10}$ units. Similarly, when *V. vulnificus* was incubated at 25°C for 12 h, cell numbers slightly increased but after cold shock at 4°C for 168 h, cell numbers decreased by 1.5 $\log_{10}$ units. When *V. vulnificus* contaminated oysters were acclimated at 15°C for 36 h, *V. vulnificus* numbers remained constant and slightly decreased only a half $\log_{10}$ unit, after downshift to 4°C for 168 h (Limthammahisorn *et al*., 2009).

3.5.3 Freezing

Frozen storage is a method commonly used to preserve product quality by inhibiting growth of bacteria and certain degrees of reductions of *V. parahaemolyticus* in oyster meat can be achieved by freezing.

Muntada-Garriga *et al*. (1995) reported that viable cells of *V. parahaemolyticus* (10^{5-7}cfu/g) inoculated in oyster homogenates were completely inactivated by freezing at -18 and -24°C for 15–28 weeks depending on initial populations of the microorganism and freezing temperatures. Freezing combined with frozen storage for 30 days at –30 °C and –15°C is projected to result in a 1.2 and 1.6-$\log_{10}$ reduction of *V. parahaemolyticus* numbers in oysters, respectively. A similar decline (2 to 3-log10) of *V. parahaemolyticus* (natural population and dosed with pathogenic O3:K6 serotype) was observed in oysters frozen 35 days at –20°C (USFDA, 2005).

Long-term storage (4 to 6 months) of halfshell the United States of America Gulf oysters at -20 °C was reported to reduce low levels of *V. vulnificus* (1 000 cells per g of oyster) in oysters to undetectable levels (Andrews, 2004). Certain oyster producers have utilized this technology to deliver high quality frozen oysters to consumers for raw consumption.

However, no studies have been conducted to determine if frozen storage could be used as a postharvest process to achieve greater than 3.52-$\log_{10}$ reductions of *V. parahaemolyticus* in oysters, as recommended by the United States of America National Shellfish Sanitation Program's postharvest processing validation–verification interim guidance for *V. vulnificus* and *V. parahaemolyticus* (National Shellfish Sanitation Program. 2005). A combination of vacuum packaging and freezing

decreased *V. vulnificus* levels in oysters by 3- to 4-$\log_{10}$ CFU/g within 7 d postfreezing, and levels continued to drop throughout frozen storage up to day 70, complete elimination was never achieved although this combination controlled *V. vulnificus* levels more effectively than did freezing with conventional packaging (Parker *et al.*, 1994).

3.5.4 High hydrostatic pressure

High pressure processing (HPP) is increasingly being used for minimising the risk of pathogens in seafood. *Vibrio* spp. have been reported to be sensitive to high hydrostatic pressure. The inactivation depends on the food matrix, strains and their physiological state. At 241 megaPascal (Mpa) pressure, 11 min was required to achieve 6 $\log_{10}$ reduction of pandemic *V. parahaemolyticus* O3:K6 and 5 min was required for *V. vulnificus* in PBS (Koo *et al.*, 2006). In oysters, a 5 $\log_{10}$ reduction in numbers of *V. vulnificus* was achieved with 250 Mpa for 120 s, but treatment at 300 Mpa for 180 s was required to obtain a comparable reduction in pandemic O3:K6 *V. parahaemolyticus* (Cook, 2003). HPP treatment has been reported to assist in oyster shucking, due to inactivation of the adductor muscle (Su and Liu, 2007) and treatments of 240-275 Mpa has been reported to be optimal for shucking Pacific oysters without any change in appearance (He *et al.*, 2002).

3.5.5 Mild heat treatment

Andrews, Park and Chen (2000) developed low temperature pasteurization for shellstock oysters by placing the oysters in 55 °C water to achieve an internal temperature of 48–50 °C for 5 min. The authors reported that the process reduced *V. parahaemolyticus* in oysters (1.2 x 10^5 MPN/g) to non-detectable levels (≤0.3MPN/g).

3.6 CONCLUSION

Although a limited of number of studies are available globally reporting the ecological factors influencing the presence and population abundance of *V. vulnificus* and *V. parahaemolyticus* in the environment, from studies available there is a clear consensus about the primary influence of seawater salinity and temperature on the distribution and population abundance of *V. vulnificus* and *V. parahaemolyticus* in coastal habitats. However, the impact of temperature in tropical areas in not significant.

The probability of *V. parahaemolyticus* detection declines in waters with values of salinity above 30 ppt, whereas seawater temperature influences the concentrations over a wide range of temperatures above 12°C. Other environmental factors are being identified as important in some areas of the world, although additional data

would be needed to determine any conclusive relationship and the interrelationships among parameters in natural environments. New information on the impact of ocean anomalies appears to be quite significant and deserves close attention in the future.

However, when outbreaks of *V. parahaemolyticus* and *V. vulnificus* have occurred they are usually associated with seawater temperatures above 15°C (*V. parahaemolyticus*) and 20°C (*V. vulnificus*).

4

Evaluation and application of existing risk assessment tools

4.1 INTRODUCTION

Typically, risk assessments are used to make decisions about levels of risk and the best approach to manage that risk if it considered unacceptably high. In microbial food safety, risk is considered to result from exposure to a pathogen or its toxins in a food and the risk assessment attempts to relate the level of the pathogen or toxin in the food to the probability of illness. Thus, risk assessment comprises estimation of exposure to the hazard, called "exposure assessment" and characterization of the relationship between the amount or dose of the hazard that is ingested and the probability of illness, or some other measure of harm (FAO/WHO, 2003). The latter process is described as "dose response assessment" or "hazard characterization".

Assessment of exposure requires data describing the level and frequency of the hazard in foods at the point of consumption, but this data is rarely available. Instead, data describing pathogen levels in foods at some point in an earlier stage in the farm-to-fork chain is often available. Change in those frequencies and levels of contamination between that point of contamination and the point of consumption is estimated from knowledge of the conditions to which the food is subject between those points and how they affect the final hazard level in the product. Typically, this will involve proxy measurements of storage temperature, composition of the food, etc. and interpretation of their effects on microbial growth or inactivation over time using 'predictive microbiology' models (Ross and McMeekin, 1994).

Data that relate the dose ingested to probability of illness is scarce. Feeding trials involving people are both dangerous and are now considered unethical, but limited data from older studies exist. Data are sometimes available from animal models, but the results are not always easily or reliably extrapolated to human health responses. Foodborne disease outbreaks can, in principle, provide data but this is an ad hoc process, and obtaining data on the doses consumed after the outbreak commences is also difficult. Another alternative to is to estimate the exposure of a defined population to a hazard, as described in the previous paragraph. If there is good epidemiological data for the incidence of the illness in a population, the relationship between dose and probability of infection may be estimated from the microbiological assessment of the remaining food and the number of cases, and a dose-response model generated. A similar approach was used and described in detail in FAO/WHO (2004) to develop a dose-response model for *Listeria monocytogenes*. The predicted exposure of the United States of America population to *L. monocytogenes* in all foods was equated to the reported number of cases of listeriosis to derive a dose response relationship.

Even in the absence of a dose-response relationship, risk management decisions can be made by estimating the relative risk increase or decrease due to potential risk management actions. This approach still requires at least knowledge of the relationship between changes in dose and corresponding changes in the probability of infection. Thus, in risk assessments, models are needed that relate:

i) proxy measurements to microbial loads in foods and
ii) microbial dose ingested via food to probability of infection or change in exposure to relative increase or decrease in probability of illness.

This overall approach, and the role of various models, can be conceptualized as shown in Figure 2.

Often risk management decisions will be made by establishing an acceptable level of risk for the consumer and determining how that can best be achieved. As risk per se cannot be measured the models developed to allow risk estimation are used 'in reverse' to estimate acceptable levels of contamination in the food, either at consumption, or at some earlier point in the chain that correspond to that level of risk. The models can also be used to provide advice on how those levels can consistently be achieved by control of processes, acceptable levels of initial contamination, etc. Thus, practical actions that can be measured are used to manage risk, and those measurable properties become proxy measures of risk.

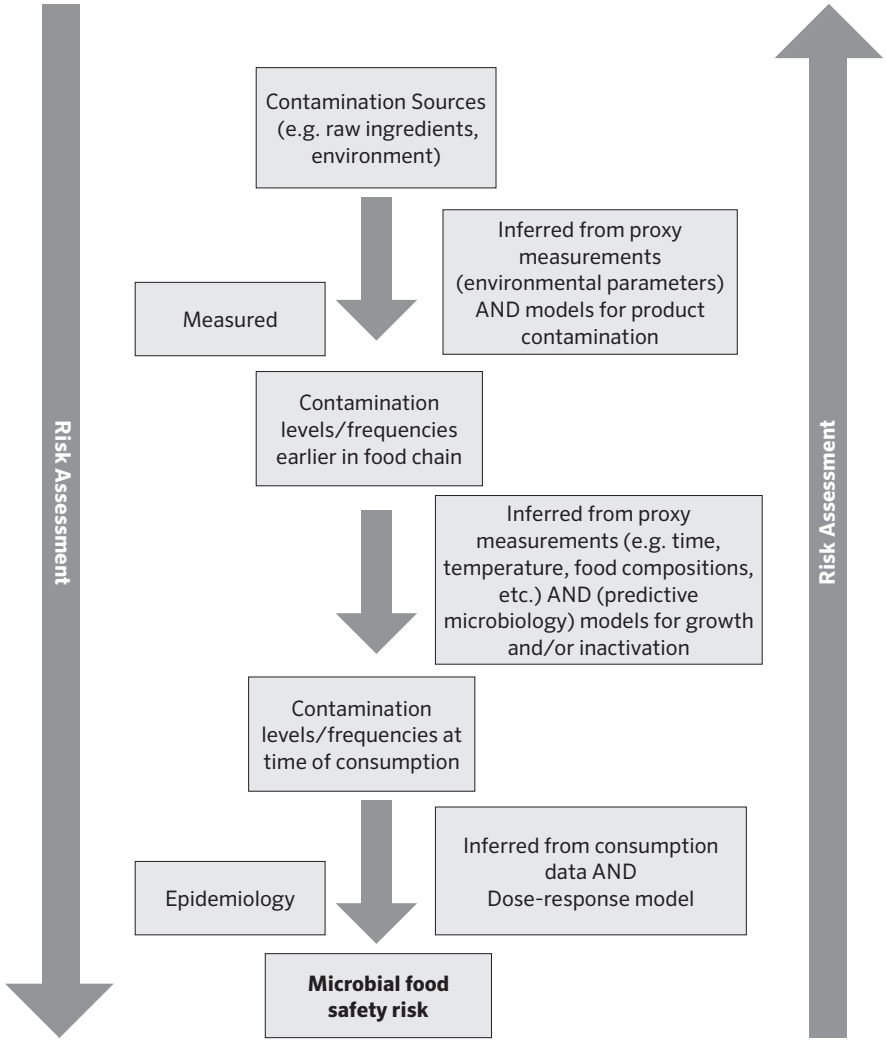

FIGURE 2. Overview of the role of models and data in microbial food safety risk assessment and risk management. In 'risk assessment' information about risk affecting factors ('inputs') is gathered and synthesised to infer the risk, i.e., moving "down" the flow chart so that the inputs determine the risk. In "risk management', a target risk is determined/specified, and the conditions of the food, or food chain, that are needed to consistently be at or below that level of risk are deduced by using the process 'in reverse'. That is, the required end result is used to determine acceptable levels of the inputs.

In assessing the reliability of a particular risk assessment model or its applicability to another situation, it is appropriate to evaluate the reliability and 'fitness of purpose" of the overall risk assessment model and its component models in those other situations, and deal with other risk management questions.

In this section the *Vibrio* Calculator tool is described and its component models are evaluated for their general applicability to other regions, other species of shellfish, etc. and other potential risk management questions from its original setting.

4.1.1 Structure and purpose of the *Vibrio* Calculator

The *V. parahaemolyticus* calculator tool is based on the USFDA *V. parahaemolyticus* risk assessment model, USFDA-VPRA (USFDA, 2005), while the *V. vulnificus* calculator tool is a simplified version of the full stochastic (Monte Carlo) risk assessment model described in FAO/WHO (2005). The *V. vulnificus* model itself is based on the USFDA *V. parahaemolyticus* model and was developed to assess whether it was possible to apply the USFDA model to other regions. The USFDA model is based on US data and the oyster *Crassostrea virginica*. Both calculators were developed to facilitate interactive assessment and evaluation of selected types of risk mitigation scenarios in a user-friendly manner that is accessible and of value to risk managers.

The full models include many discrete sets of information (i.e., inputs) that are used to estimate the risk. However, two variables were considered to be most relevant to management of the risk, namely initial contamination level and limitation of increase of *Vibrio* by control of the time taken for the product to be cooled to a temperature below which *Vibrio* cannot grow. This was achieved by using the full model to systematically estimate the risk as a function of those various inputs (water temperature, air/oyster temperature, maximum time unrefrigerated and time to cool). A regression model was constructed to estimate the risk as a function of the inputs and this was then translated into an Excel spreadsheet to enable risk to be estimated from time to cool and initial temperature.

Thus, the *Vibrio* Calculators are simplifications of the full stochastic model for *V. parahaemolyticus* and *V. vulnificus* that answer a specific risk management question, namely how quickly must the product be cooled to achieve the risk reduction target.

The Calculators infer- initial contamination levels of either *Vibrio* spp. at harvest from measured water temperature at the growing region. This is inferred by a mathematical model based on measured levels in oysters at harvest and measured water temperatures in Chesapeake Bay and the Gulf of Mexico coast of the United

States of America. The oysters are assumed to be cooled and the rate of cooling inferred from differences between water and air temperature. The temperatures and times experienced during cooling are related to microbial growth, based on predictive models, as decribed in Sections 4.3.1 and 4.3.2.

A Beta-Poisson dose-response model for *V. parahaemolyticus* was derived by USFDA (2005) using human clinical feeding trial studies and epidemiological surveillance data. FAO/WHO (2005) described the development of a Beta-Poisson model to relate dose of *V. vulnificus* to probability of illness. Thus, the *Vibrio* risk predictor tools rely on:

- models to relate water temperature to contamination levels at harvest
- models to predict the increases in levels of the two *Vibrio* spp. with time and temperature
- models to relate numbers of *V. parahaemolyticus* and *V. vulnificus* ingested to the probability of an infection in the consumer.

The validity and broader applicability of each of those sub-models is considered more fully in Sections 4.2 to 4.4 below.

4.1.2 Shellfish contaminated by *Vibrio* spp.

Several seafood species have been implicated in cases of *V. parahaemolyticus* and *V. vulnificus* infections. In the United States of America, nearly all cases of *V. vulnificus* primary septicaemia are due to consumption of raw oysters from the Gulf coast (FAO/WHO, 2005). In Japan, oysters are not the primary source, since raw oysters are eaten only in winter (December-February) and most infections occur during June-November with a peak in July. A mud shrimp Upogebia major has been reported the common agent associated with *V. vulnificus* infections in Japan (Inoue *et al*., 2008). While Gulf Coast oysters are the main source of *V. parahaemolyticus* infections in the United States of America, but a variety of seafood have been implicated in outbreaks in other countries. In Thailand and other countries in Southeast Asia, clams (*Anadara granos*) are suspected to be involved, but based on the FAO/WHO risk assessment the risk of transmission via clams is low since they are generally consumed after cooking (FAO/WHO, 2011). Undercooking or cross contamination could explain the presence of *V. parahaemolyticus* in these products. In Japan finfish species consumed raw are the primary sources of *V. parahaemolyticus* infections (FAO/WHO, 2011).

The survival of *V. parahaemolyticus* and *V. vulnificus* has been studied in several bivalve species. Growth of *V. parahaemolyticus* in the United States of America Gulf Coast oysters at 26 °C has been recorded by Gooch *et al*. (2002). However, this organism did not grow in Sydney rock oysters (*Crassostrea commercialis*) even

at 30 °C for 7 days (Eyles, Davey and Arnold, 1985), an observation confirmed by Fernandez-Piquer *et al*., (2011). In the oysters *Crassostrea commercialis*, *V. parahaemolyticus* numbers increased slightly during first four days of storage in water at temperatures between 20 and 25 °C, after which the counts decreased (Son and Fleet, 1980). In Indian clams (*Meritrix casta*) held in seawater at ambient temperature (~26 °C), *V. parahaemolyticus* survived for over 30 days (Karunasagar *et al*., 1987). In the ark shell clam (*Tegillarca granosas*) that is popular in China, Republic of Korea and Japan, *V. parahaemolyticus* levels decreased by about 2 $\log_{10}$ units during storage at 0-5 °C. More recent studies (Parveen *et al*., 2008; Fernandez-Piquer *et al*., 2011) have involved inoculation of live oysters with *V. vulnificus* or *V. parahaemolyticus* and have shown that growth is possible in oysters at least in the range 13-30°C.

4.2 EVALUATION OF THE MODELS FOR *VIBRIO* CONCENTRATION AT HARVEST

As noted in Section 4.1.1, the usefulness of the USFDA-VPRA model to assess the risk from *V. parahaemolyticus* and *V. vulnificus* associated with the consumption of oysters is being validated for wide use among FAO/WHO member countries. It is recommended that the guidelines be reconsidered and that region-specific risk assessments be performed to account for potential ecologic variation, in terms of the adjustment of ecological factors such as temperature and salinity.

As noted in Section 4.1.1, the calculator tools for *V. parahaemolyticus* and *V. vulnificus* are developed by simulating and summarising the results of more complex risk assessments developed by USFDA and FAO/WHO so as to more quickly generate the results predicted by the more complex models. Figure 3 depicts the overall complexity of the full models and shows that the simplified forms of the models are summaries of the full models designed to respond to questions about a specific and restricted range of risk management options.

The primary utility of the approach is to make the process of investigating specific risk management options faster since the complex model does not have to be re-simulated every time an alternative option is to be investigated. The simplification however comes at the expense of more detailed understanding of risk affecting factors and their relative contributions. In the simplified model it is assumed that the only factors able to be manipulated to reduce the risk from pathogenic *Vibrio* in oysters are the time taken to cool the oysters, which in turn is a function of the temperature at which cooling takes place. The simplifying assumptions adopted need to be remembered when the model is being used to ensure that they are relevant to the specific situation for which the model is being used.

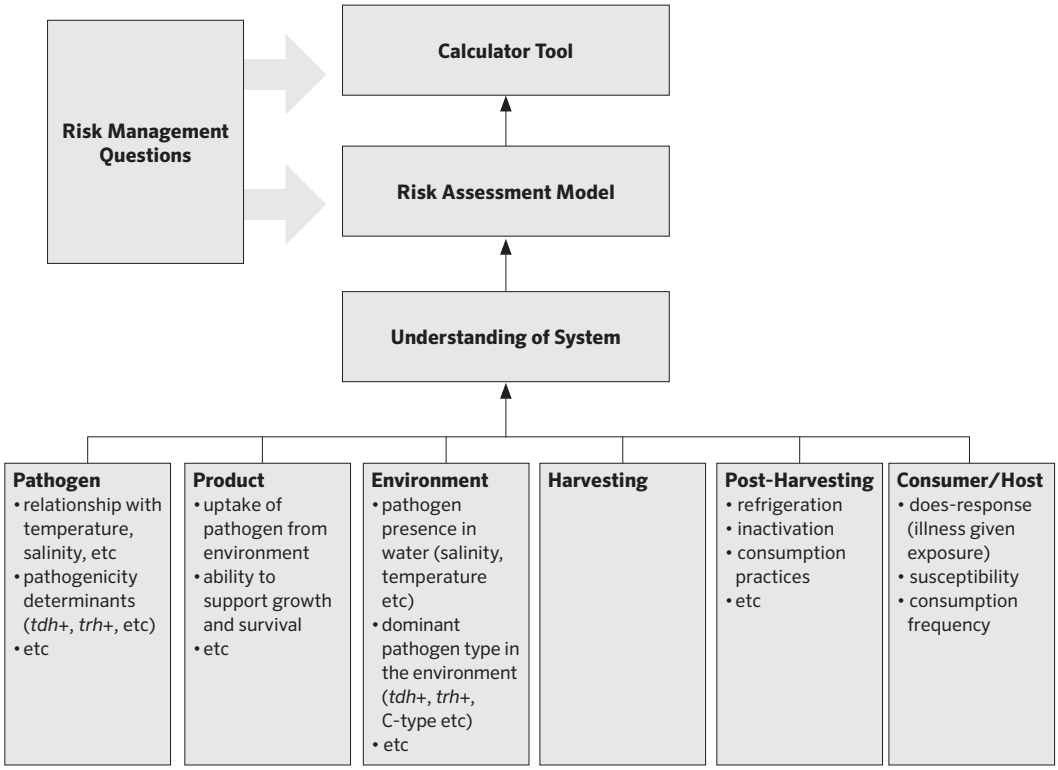

FIGURE 3. Illustration of the complexicity of the risk assessment model and of the use of such models to respond to risk management questions.

A discussion of assumptions and simplifications in the models follows, beginning with the applicability of models that relate *Vibrio* concentration in oyster to harvest water temperature.

4.2.1 *V. parahaemolyticus*

4.2.1.1 Temperature

V. parahaemolyticus is reported to grow (both culture and food) at temperatures from 5 °C to 43 °C (ICMSF, 1996) with a maximum growth rate at 37 °C (ICMSF, 1996; Miles *et al.*, 1997). However, growth is very slow in the range 5-10 °C; it is usually accepted that growth rates are very low or negligible in seafood at these temperatures. At temperatures less than 5 °C, populations of *V. parahaemolyticus*

decline due to inactivation, albeit very slowly (Gooch *et al*., 2002). *V. vulnificus* also has a maximal growth rate at 37 °C (Kelly, 1982) and growth can occur at temperatures ranging from 9 to 31°C (O'Neil, Jones and Grimes, 1992). At temperatures below 15 °C the bacterium can enter a viable-but-non-culturable (VBNC) state (Oliver, 1993; see also Section 5.3). It does not survive, though inactivation is slow, at temperatures less than 8.5°C (Kaspar and Tamplin, 1993).

Unusual variations in seawater temperature may explain the sudden emergence of *V. parahaemolyticus* outbreaks in new areas. Positive correlations between water temperature and the concentration of *V. parahaemolyticus* in waters, oysters, and blue mussels have been reported in studies in the United States of America, Japan, France and Germany (Cook *et al*., 2002b; DePaola *et al*., 1990, 2003b; Duan and Su, 2005; Chowdhury *et al*., 1990; Lhafi and Kuhne, 2007; Igbinosa and Okoh, 2008; Martinez-Urtaza *et al*., 2008; Parveen *et al*., 2008; Deter *et al*., 2010b). Deepanjali *et al*. (2005), however, noted that water temperature in tropical coastal regions of India (range 25–35 °C) was always near optimal for *Vibrio* growth and survival and thus did not significantly influence the total *V. parahaemolyticus* levels in oysters. According to Igbinosa and Okoh (2008), the direct relationship between *V. parahaemolyticus* and water temperature seems to determine its geographical distribution in the United States of America, Europe and Asia. Therefore, in temperate climates, as opposed to tropical regions, water temperature is a major factor effecting the concentration of *V. parahaemolyticus* in estuarine environments, which ultimately influences the load of *V. parahaemolyticus* in the oysters or other shellfish harvested in the particular areas of concern.

Seawater temperature plays a key role in determining the levels of *V. parahaemolyticus* and *V. vulnificus* at the time of harvest and variations in regional water temperatures at oyster or shellfish harvesting areas could require different control measures. When establishing risk management programs relating for postharvest processing, the importance of harvest water temperature should be considered.

Three linear correlations between *V. parahaemolyticus* in oysters and sea water temperature at the time of harvest have been presented (USFDA, 2005; Ogawa *et al*., 1989; Sobrinho *et al*., 2010); and provide a useful working sub-model; albeit with variation (that can be taken into account in full microbiological risk assessment). A comparison of those linear correlations is shown in Table 6. There are data for other regions and shellfish, e.g. in mussels (Martinez-Urtaza, 2008); and in oysters (Ristori *et al*., 2007); that could be analysed to further assess the generality of the "straight line" submodel relating water temperature to *Vibrio* levels in shellfish.

TABLE 6. Slope and independent term of linear correlations for sea water temperature and $\log_{10}$ *V. parahaemolyticus* in oysters

Slope	% Difference in slope	Intercept term	% Difference in independent term	Species	Reference
0.0998	0	- 0,625	0	Oyster	USFDA (2005) / United States of America
0.103	(+) 3.2	- 0.934	49.44	Oyster	Ogawa (1989) / Japan
0.175	(+) 75.3	- 0.944	51.04	Oyster	Sobrinho *et al.* (2010) / Brazil

From Table 6 it is clear that different linear correlations exist for the same type of products; with differences both in the slope and intercept term. Whereas the difference in the intercept term are relatively minor, the difference in slope for the model fitted to the Brazilian dataset is significant and leads to a ~50-fold difference in the predicted $\log_{10}$ (*V. parahaemolyticus*) levels in oysters at 25°C compared to the other two relationships.

4.2.1.2 Salinity

Various studies (Cook *et al.*, 2002a; DePaola *et al.*, 2003b) have found a significant relationship between salinity and the abundance of total *V. parahaemolyticus* (Cook , Bowers and DePaola, 2002a; DePaola *et al.*, 2003b). In other studies (Deepanjali *et al.*, 2005; DePaola *et al.*, 1990; Kaneko and Colwell, 1975; Ristori *et al.*, 2007) in which the salinities observed were well below theoptimal salinity of 23 ppt reported in the USFDA *V. parahaemolyticus* risk assessment (USFDA, 2005), significant relationships have not been found. These differences between studies are likely a consequence of the differences in the natural range of variation of salinity levels in the regions studied.

A positive influence of water salinity on the occurrence of vibrios in water has often been detected when the range of variation of salinity levels is broad enough and the sample size sufficient (Cook *et al.*, 2002a; DePaola *et al.*, 2003b; Zimmerman *et al.*, 2007; Martinez-Urtaza *et al.*, 2008; Parveen *et al.*, 2008). In particular, a wide range (5.6–34 ppt) of salinities was observed in the Cook, Bowers and DePaola, (2002a) and Parveen *et al.* (2008) studies compared to the other studies. Taken together, these reports provide perspectives regarding the salinity conditions regulating the dynamic of *V. parahaemolyticus* in different regions and are of practical use in modification of the USFDA predictive risk model and its modification, if appropriate.

4.2.1.3 Other factors

Temperature, and more specifically temperature over time could have an impact on the ecology of *V. parahaemolyticus* populations, and hence on observed initial populations. There are a number of publications on possible ecological impacts due to temperature such as its influence on the growth of phyto and zooplankton. Available data seems to be incomplete at this stage and a notable modelling effort would be necessary to identify the relevant factors to reduce the ecological complexity to a manageable model.

4.2.2 *V. vulnificus*

4.2.2.1 Temperature

V. vulnificus is an estuarine bacterium with an optimal growth rate at 37°C (Kelly, 1982). However, the growth can occur at temperatures ranging from 8 to 43°C (ICMSF, 1996) and at temperatures below 15°C the bacterium can enter to a viable but non culturable stage (Oliver, 1993) and can survive poorly below 8.5°C (Kaspar and Tamplin, 1993).

For *V. vulnificus*, there is also a relationship between concentration in water and temperature and this, based largely on the data of Motes *et al*., (1998), was modelled in the FAO/WHO (2005). The model developed was also consistent with the other United States of America data (M. Tamplin, unpublished data). Unlike the *V. parahaemolyticus* data, a quadaratic model was required to describe the data and a significant effect of salinity was also noted. At 25°C predicted *V. vulnificus* levels are similar to those for *V. parahaemolyticus* from the Sobrinho *et al*. (2010) model, but at 15°C they are similar to the *V. parahaemolyticus* levels predicted from the USFDA (2005) and Ogawa (1989) models.

Extrapolation of the above correlations to other species of filter feeding shellfish eaten raw in some countries (e.g. mussels, clams, sea-urchins) has not been considered systematically but given the variability between models described above, extrapolation does not appear to be justified.

For *V. vulnificus* there is a quadratic correlation to adjust *V. vulnificus* density in oysters (MPN/g) versus sea water temperature in the range of 10-32°C utilized in current *V. vulnificus* (FAO/WHO, 2005). There are some papers that suggest a linear regression (FAO/WHO, 2005); however, dispersion is large.

4.2.2.2 Salinity

Understanding the ecological factors (including temperature and salinity) contributing to the incidence and abundance of *V. vulnificus* would be very important in predicting the risk of illness associated with seafood consumption. According

to the data available about ecological factors on *V. vulnificus*, the salinity in the coastal environmental has played an important role in its incidence and population levels. Evidence shows that salinity is negatively correlated with *V. vulnificus* concentrations (Rivera, Lugo and Hazen, 1989; Tamplin *et al*., 1982; Parvathi *et al*., 2004). *V. vulnificus* could not be detected when the salinity was either lower than 1 ppt (Roberts *et al*., 1982) or higher than 30ppt (Rivera, Lugo and Hazen, 1989). Other research has also indicated that the levels of *V. vulnificus* were high when the salinity of the water was low and temperature was high, which indicates that low salinity and warm temperature strongly influence the presence of *V. vulnificus* (Parvathi *et al*., 2004). Negative correlations have also been observed between salinity and the incidence of lactose fermenting *Vibrio* spp. isolated from seawater (Oliver, Warner and Cleland, 1982; Oliver, Warner and Cleland, 1983). However, the correlation between salinity and the abundance of *V. vulnificus* in seawater does not always follow the same pattern.

The possible application of one explicit correlation to other species, eaten raw in some countries (e.g. mussels, clams, squid, octopus, sea-urchins) has not been searched systematically either, however, it could not be expected it fit with the existing ones.

4.3 EVALUATION OF THE *VIBRIO* GROWTH RATE MODELS

4.3.1 *V. parahaeomolyticus* model

In the USFDA (2005) Risk Assessment, a mathematical model is used to model the growth, over time, of *V. parahaemolyicus* in oysters at various temperatures. It is derived from the model of Miles *et al*. (1997), which was based on growth rates from four strains of *V. parahaemolyticus* determined at ~25 temperatures in the range 9-56°C and 15 water activity levels in the range 0.936 to 0.998 (~ 0.5 to 9.6 percent NaCl). The growth rates were determined in tryptone soy broth, adjusted with NaCl. Miles *et al*. (1997) compared predictions of their model to other published observations in broth and several seafood, but not in live oysters. To make the predicted growth rates better match actual growth rates in oysters, the data of Gooch *et al*. (2002) were used to calibrate the Miles *et al*. (1997) model. Gooch *et al*. (2002) reported the growth rates of *V. parahaemolyticus* in oysters, but only at 26°C, and the observed growth rate was ~4 times slower than that predicted by the Miles *et al*. (1997) model. More recent data presented by Fernandez-Piquer *et al*. (2011) for *V. parahaemolyticus* growth in live oysters include a greater range of temperatures but also indicate that the Miles *et al*. (1997) growth rate model overpredicts *V. parahaemolyticus* growth in live oysters.

Miles *et al.* (1997) reported a strong effect of salt on *V. parahaemolyticus* growth rate in broth. In the range of relevance to seawaters from which oysters are harvested from 0.5 percent to 3.5 percent NaCl, corresponding to a water activity range of 0.993 to 0.980, the growth rate variability is expected to be <˜35 percent. The USFDA (2005) model also assumes that the growth rate observed will be that which would occur at water activity = 0.985, equivalent to approximately 27 ppt NaCl.

Thus, the USFDA (2005) model for growth rate of *V. parahaemolyticus* in oysters makes several assumptions that may not be applicable to all growing/harvesting regions. Similarly, different species of oyster are grown in different nations. An evaluation of the applicability of the *V. parahaemolyticus* growth rate model to growth of other strains of *V. parahaemoyticus* and other species of live oysters was also undertaken.

Specifically, data were available to assess growth rates of different strains of *V. parahaemolyticus* in two oyster species (Asian oysters: *Crassostrea ariakensis*; American oysters: *Crassostrea virginica*). Summary data are presented in Figures 4a, b and suggest that there are systematic differences in growth rates according to *V. parahaemolyticus* strain and oyster species. As noted above, the Sydney rock oyster (now *Saccostrea glomerata*) has been reported in two independent studies not to support the growth of *V. parahaemolyticus*.

4.3.2 *V. vulnificus* model

The *V. vulnificus* growth model (FAO/WHO 2005), which was based on growth rate data from two studies (Cook, 1994; Cook, 1997) using Gulf Coast oysters and data on minimum growth temperature from Kaspar and Tamplin (1993) was evaluated at the expert consultation. The evaluation included two steps: 1) A refitting of the growth rate equation to the original data using a "square root" model, which is a widely used approach to describe microbial growth rates (Grijspeerdt and De Reu, 2005), as opposed to the linear approach currently used in the model); and 2) comparing the growth rate predictions with observed growth rates in American oysters (*Crassostrea virginica*) and Asian oysters (*Crassostrea ariakensis*) harvested in the Chesapeake Bay and Gulf Coast of the United States of America.

The *V. vulnificus* square root growth rate model compared to the FAO/WHO growth rate model predicts slower growth rates from 13°C to approximately 27°C, after which the square root model predicts increasingly greater growth rates. The impact of the slower growth rate predicted by the square root model is most evident at approximately 20°C where the difference between the two models is greatest. At this temperature the FAO/WHO model estimates that *V. vulnificus* levels would

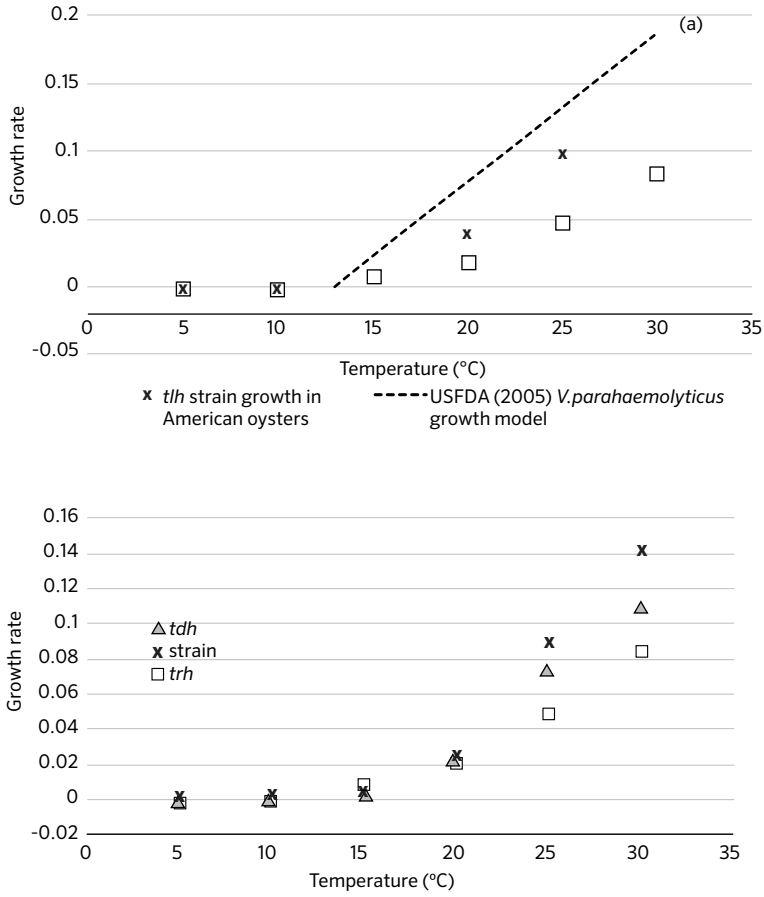

FIGURE 4a, b. Effect of oyster species (upper figure) and *V. parahaemolyticus* strains (lower figure) on growth rate of *V. parahaemolyticus* in oysters. Systematic differences in growth rate can be observed. The upper figure also shows the USFDA growth rate model predictions indicating that the model is highly conservative.

increase by 1 log (i.e., a tenfold increase) in approximately 13 hours, while the square root model estimates that this would take approximately 22 hours. At this temperature (20°C), there are six growth rate observations in oysters, with two observations clustered at the higher growth rate estimated by the FAO/WHO (2005) model and four at the lower growth rate estimated by the square root model. The two models; however, are bounded by the observed data with the existing FAO/WHO model tending to provide a better approximation to the upper bounds of the

observed data at this temperature. The variability in the observed data increases significantly after approximately 25°C, with the growth rate of most of the oyster species beginning to slow down or "plateau off" at or above this temperature. At this temperature the difference between the two models is much less significant and still bounded by the observed data. The variability in the observed data is most significant at 30°C, but there are two observations in *Crassostrea virginica* and *Crassostrea ariakensis* which follow the expected and predicted increasing trend in growth rate. Both these observations are reasonably well approximated by both models.

Given the observed data and its variability coupled with the FAO/WHO model effectively capturing the upper limit of the observed growth rates (tending towards being conservative or "fail safe") at temperatures between 15°C to 25°C, it can be concluded that this model is appropriate for estimating the growth of *V. vulnificus* in American oysters (*Crassostrea virginica*). Its ability to estimate growth in at least one other oyster species (*Crassostrea ariakensis*) also appears to be supported by the available data, although there is only one data set available to make this comparison. In addition, when the growth rate in *Crassostrea ariakensis* is compared to *Crassostrea virginica* it does tend to represent the upper range across all the

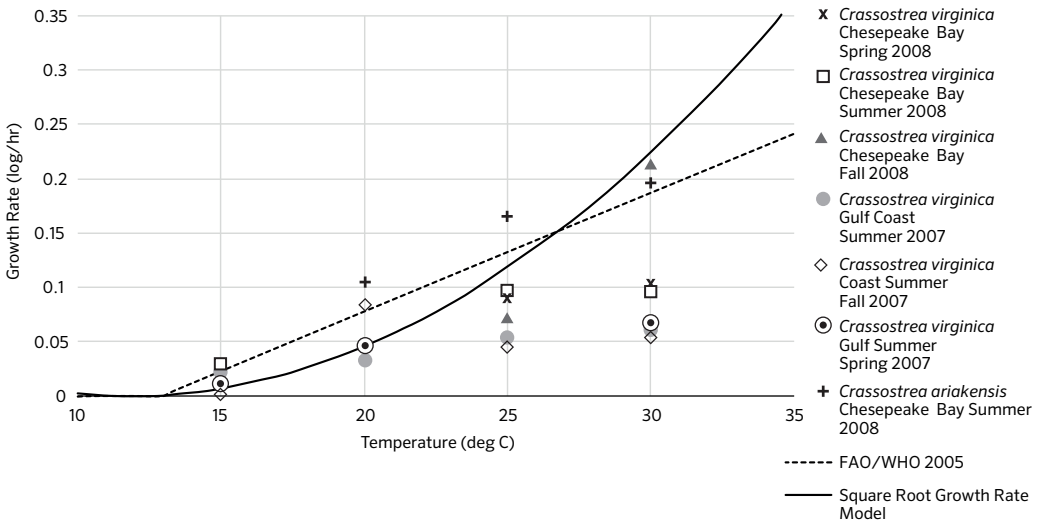

FIGURE 5. Comparison of *V. vulnificus* growth rate model (FAO/WHO, 2005; dashed line) with observed growth rates in oysters (*Crassostrea virginica* and *Crassostrea ariakensis*) and also compared with an alternative square root growth model (solid line)

temperatures, and while this is far from conclusive, it may hint at the potential for different growth rates in different oyster species. There is no data currently available to evaluate the model performance at estimating growth in any other oyster species or other filter feeding shellfish. It would therefore be prudent to use the current model in other oyster species or filter feeding shellfish (clams, mussels) only with a great degree of caution, recognizing that the model has been developed and compared to data from only one species of oyster, and one set of observations from an additional species.

4.4 EVALUATION OF DOSE RESPONSE MODELS

The existing dose-response model for *V. parahaemolyticus* was developed by calibrating an estimate of the dose-response from human clinical trial data to an estimate of the average number of oyster-associated *V. parahaemolyticus* infections occurring annually in the United States of America (USFDA, 2005). This re-calibration of the dose-response relationship was adjusted for under-reporting estimated at 20-fold (Mead *et al*., 1999). In the *V. parahaemolyticus* prediction model, there is an assumption that only *tdh*$^+$ strains are pathogenic and capable of causing illness. It is further assumed that all *tdh*$^+$ *V. parahaemolyticus* are equally virulent.

The existing dose-response model for *V. vulnificus* is based entirely on the United States of America epidemiological data. No feeding trial data exists due to the high case fatality ratio discussed earlier. The dose-response is an ecological relationship between group-level mean exposure and mean risk. Exposure is the retrospectively estimated mean number of total *V. vulnificus* per serving based on all facets of the exposure assessment, from pre-harvest levels through post-harvest handling. All *V. vulnificus* strains are assumed equally virulent. It is assumed that 7 percent of the population is susceptible to infection and there is no variation in susceptibility among the at-risk population.

The common feature of both dose-response assessments is that they are based on epidemiological data specific to the United States of America. Neither dose-response assessment has been validated. Lack of validation against independent epidemiological data is important and significant as a review of data conducted by the experts indicates that some of the existing assumptions pertaining to pathogenic strains are not accurate in light of recent scientific data.

When epidemiological data is used for dose-response estimation rather than validation (a common practice in microbiological risk assessment) there is the possibility of introducing compensating biases between two or more model parameters.

For example, insofar as the dose and slope parameter of the exponential dose-response model enter in a multiplicative fashion, any error in the definition of the dose (e.g. inappropriate definition of pathogenic) can be compensated for in the estimate of the dose-response parameter. An adequate fit to the epidemiological data is obtained but the estimated relationship does not extrapolate (i.e., is externally invalid) due to the inappropriate definition of dose. A good fit to the epidemiological data can lead one to believe that risk predictions will extrapolate when such is not the case.

This generic issue of model-building and parameter estimation in the dose-response component appears to be an important factor limiting the applicability of the predictive tools that have been developed in the United States of America to a wider context. However, where the dose-response is linear over the range of exposures most likely to occur (e.g., several logs lower than the ID50) the risk prediction model is also linear and could be applied to determine the relative effectiveness of control measures.

4.5 CONCLUSIONS AND RECOMMENDATIONS

4.5.1 Key Points / Conclusions

4.5.1.1 Calculator tools

The *V. parahaemolyticus* calculator tool may be used to estimate the relative risk reductions primarily because of the linear dose-response, associated with temperature controls (post harvest refrigeration) in areas in which the strains virulence, shellfish species, initial concentration and growth rates of *V. parahaemolyticus* in the bivalve species of concern if they are similar to the United States of America.

The *V. vulnificus* calculator tool is less likely to be applicable to a broader region outside the United States of America due to differences in environmental, harvesting and post harvesting parameters, but more significantly due to the basis of the dose-response relationship that is derived completely from the United States of America epidemiological data coupled with estimated exposure levels. Specific shellfish species might also influence the risk estimate.

To develop a tool that is generally applicable across many regions and other products, and to answer other risk management questions, other than post-harvest refrigeration, it would be preferable to first modify the existing JEMRA risk assessment model, or develop a new model, that considers and evaluates the influence of other factors including salinity, strain differences, temperatures etc. Simplified calculator tools could then be developed to answer those specific questions routinely.

4.5.1.2 Vibrio Concentration in Shellfish Models

It is known that *Vibrio* levels in oysters are affected by temperature and salinity, and other measurable properties of shellfish growing areas (e.g. turbidity, dissolved oxygen), but the influence of these other factors is currently poorly defined. The effect of temperature and salinity on *Vibrio* levels in oysters should be considered for specific regions before applying the Calculator tools.

While there is evidence that the *Vibrio* concentration in the shellfish model in the Calculator tools does provide realistic predictions of contamination levels in shellfish in some regions, there is also evidence that it is not accurate in other regions.

The extent of variability of factors other than temperature, and their effect on contamination levels in oysters should be considered for specific regions before applying the Calculator tools.

It is currently unknown whether the relationship between temperature and microbial load is the same for all species of shellfish.

4.5.1.3 Growth models

The FAO/WHO growth model for *V. vulnificus* and USFDA model for *V. parahaemolyticus* are appropriate for estimating growth in the United States of America oysters, *Crassostrea virginica*. There is insufficient data to reliably evaluate the performance of the growth models in any other oyster species, other filter feeding shellfish or other seafood. The limited data available suggests that there are systematic differences in growth rate related to oyster species and also between strains of *V. parahaemolyticus*.

The FAO/WHO growth model for *V. vulnificus* is appropriate for estimating growth in at least one other oyster species (*Crassostrea ariakensis*).

The USFDA model for *V. parahaemolyticus* is appropriate for estimating growth in at least one other oyster species (*Crassostrea gigas*) but is not appropriate for predicting growth in at least one species (Sydney rock oyster, *Saccostrea glomerata*).

There is some evidence that the *V. parahaemolyticus* model currently used overpredicts growth at higher temperatures (e.g., > 25°C) in live oysters. This phenomenon requires further investigation.

4.5.1.4 Simplified Modelling Tools

For a specific purpose in specific circumstances, a simplified model derived from a complex model can work well. However, where there are additional factors that do

not satisfy the assumptions inherent in the simplified model tools there may be a need to use the full "preharvest to consumption" model.

4.5.2 Recommendations

Generally, it is recommended to develop more widely applicable models.

Suggestions for improvement for the *V. parahaemolyticus* model:

- *V. parahaemolyticus* prediction model as it currently exists is a linear model and therefore may be useful to estimate relative change in risk (percent reduction in risk) for different countries with more virulent strains, provided that the ranges of doses in that country is much less than the ID_{50} for the more virulent strain (ie., in the linear range of the dose response relationship).
- country-specific assessments (e.g., in Chile) should be undertaken and use an ecological dose-response approach like *V. vulnificus* DR-assessment (but prospectively); studies should be undertaken in other regions to obtain estimates of dose by sampling at retail;
- dose-response models incorporating multiple strains and variations in virulence should be developed using data on relative expression of hemolysins plus differences in prevalence of pathogenic strains in clinical versus environmental isolates

Suggestions for improvement for the *V. vulnificus* model:

- Conversely, the use of the *V. vulnificus* prediction tool outside of the United States of America is not advisable at this time (dose-response nonlinear so not useful as relative risk reduction, different countries might have different prevalence of C-type vs E-type strains in the environment, there may be differences in the proportion of population susceptible, etc).
- The United States of America *V. vulnificus* dose-response assessment should be revisited using an approach that incorporates the effect of (a) seasonality of the C vs E type & (b) relative virulence of the C vs E type (e.g., for (a) try logistic regression of type C vs type E versus water temperature or season; for (b) 60-fold difference by comparison of prevalence in clinical versus environmental strains).
- The concept of incorporating distributions of susceptibility to infection among the at-risk population should be investigated (e.g., assess the reliability of extrapolation to a country with different prevalence of predisposing conditions).

5

Methods for isolation, identification, and determination of pathogenic potential of shellfish-associated *V. parahaemolyticus* and *V. vulnificus*

5.1 INTRODUCTION

Cases of *V. parahaemolyticus* and *V. vulnificus* infection have been reported throughout the world but are unevenly distributed between continents. The following is a review of currently available methods and a brief overview of validation and performance of these methods with recommendations for the best fit methods which can be employed for monitoring and controlling seafood safety and/or collection of data. Their availability and feasibility will vary in different parts of the world, and their use must take into account the differences between epidemiological and environmental conditions associated with these variations (e.g. host susceptibility for development of infection; variability in virulence of *V. parahaemolyticus* and *V. vulnificus* strains, environmental conditions, etc).

The following discussion is applicable to seafood and seawater samples.

5.2 SAMPLING STRATEGY

The size of the sample to be analysed will likely be dependent on the sample (seawater, seafood) type. Pooled (composite) sampling is typically performed, with replicate sampling recommended. Limits of detection, which vary in all enrichment, culture, and identification methods, is a critical aspect which must be carefully considered when selecting among these methods. The time when samples are collected (seasonality) as well as the frequency of sampling will be application dependent. Finally, appropriate controls must always be employed at each step of the isolation/identification process.

5.3 ISOLATION, IDENTIFICATION, AND DETERMINATION

5.3.1 Presence/Absence (Qualitative Analysis)

There may be occasions when rapid screening may be of value (e.g. "are there any *tdh*$^+$ isolates present in a pooled sample?"). This might facilitate decision as to whether further testing should be conducted.

Such a method may involve tissue homogenization followed by 6 h enrichment in alkaline peptone water (APW), rapid deoxyribonucleic acid (DNA) extraction of enriched samples, and a real time polymerase chain reaction (PCR) run to determine presence of the appropriate target gene (e.g. *tdh*). Data are typically obtained within 10-12 h.

5.3.2 Enrichment

The standard procedure is a single step enrichment in APW, with variations in salt content and temperature being common (e.g. 35-37 vs 41.5°C for frozen or live samples, respectively). Incubation time is typically 18 h.

An optional method involves a two-step process with a second 6 h enrichment using material taken from the initial enrichment (ISO 21872-1, 2017).

The most probable number (MPN) method is often used for quantification if desired, with weight or volume varying according to the application.

Increasingly common is the use of enriched samples for subsequent direct molecular determination of the target organism, bypassing isolation/culture as a first step.

5.3.3 Isolation media

Following appropriate preparation, samples can be appropriately diluted, or concentrated using filtration or other methods. Spread plates (typically 0.1 ml) can be employed, or filters can be placed onto the solid media, depending on the level of detection required.

***Vibrio* spp.** – thiosulphate citrate bile salts sucrose agar (TCBS) has historically been the isolation medium of choice for vibrios, but it is now known to be quite inhibitory for this group, and provides little differentiation (only sucrose reaction). In addition, some pathogenic vibrios do not grow on this medium. Thus, TCBS is not especially suitable for environmental samples.

ChromagarVibrio™ has the advantage of allowing simultaneous isolation and differentiation (by colony color) of *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*. However, a concern with this medium is that it is proprietary, with only a single supplier.

V. parahaemolyticus – Isolates on ChromagarVibrio™ form distinctively pigmented (purple) colonies which are generally easily distinguished from background and other *Vibrio* spp. Other chromogenic media are currently being evaluated (e.g. ChromID™; bioMérieux). TCBS may also be employed, but ChromagarVibrio™ appears to be less inhibitorry for *V. parahaemolyticus* than TCBS, providing a higher probability of isolating this species (Di Pinto *et al*., 2011) and pathogenic species.

V. vulnificus – Colistin-Polymyxin B-cellobiose (CPC) medium (Massad and Oliver, 1987), or one of its several variants (Warner and Oliver, 2007; Høi, Dalsgaard and Dalsgaard, 1998b), is the medium of choice. However, in some high salinity environments false positives have occasionally been observed. ChromagarVibrio™ is also commonly employed, on which *V. vulnificus* forms distinctive, blue-pigmented, colonies. However, some *V. vulnificus*-like colonies appear on this medium, and some *V. vulnificus* strains have been reported not to grow.

5.3.4 Identification

Biochemical methods have certain limitations for identifying vibrios from environmental sources, and hence there is a movement towards molecular methods. While culture-based methods are useful for estimating the total number of vibrios they are not so reliable when it comes to determining pathogenicity.

In contrast, molecular methods (e.g. PCR) are rapid, precise, and able to discriminate strains based on virulence. A number of PCR variations exist, and there is

no individual method which can be recommended due to variations in sensitivity, costs, and applications. In contrast, there is general consensus regarding the appropriate gene targets for both bacterial species. However, it is recognized that these target genes sometimes occur in non-target *Vibrio* or related species. For example, occasional strains of *V. alginolyticus* and *Aeromonas* have been detected which possess *trh*. Similarly, strains of *V. hollisae* and *V. alginolyticus* have been detected possessing the *V. parahaemolyticus toxR* gene.

5.3.5 Total *V. parahaemolyticus* or *V. vulnificus*:

Colony hybridization – Probes for *V. parahaemolyticus* (*tlh*) and *V. vulnificus* (*vvhA*; haemolysin gene unique to this species) are available, but probes are only produced by one company, limiting this method as an option. The thermolabile haemolysin encoded by the tlh gene of *V. parahaemolyticus*, while not a virulence factor, is used as a reliable marker for this species.

PCR (conventional) – For *V. parahaemolyticus*: *tlh*, *toxR*, and a 0.76 kb fragment of pR72H (used by French National Ref. Lab for Confirmation). For *V. vulnificus*: *vvhA* is universally employed for identification of this pathogen.

PCR (real time) – employs the same target genes as conventional PCR.

LAMP (loop mediated isothermal amplification) – employs primers for the same targets.

5.3.6 Pathogenic *V. parahaemolyticus* and V. *vulnificus*

To detect pathogenic strains of *V. parahaemolyticus*: *tdh* and *trh* genes are targeted (see Section 2.1). In addition, the pandemic strain can be detected with several genes, including a 23kb fragment, ORF8, GS-PCR for the *toxR* gene, and T3SS (Nair *et al*., 2007; Chen *et al*., 2011). Multiplex systems for *tlh*, *tdh*, and *trh* exist. Real time PCR uses the same genes. Note that there is not a 100 percent agreement between *tdh* and the Kanagawa phenomenon on Wagatsuma agar. Different levels of expression of *tdh* are also recognized.

For *V. vulnificus*, two options exist: 16S rRNA and the "virulence-correlated gene" *vcgC/E* (see discussion below). A rapid multiplex system employing *vvhA* for the identification of this species, with simultaneous determination of the C/E genotype (*vcg* gene) has been reported (Warner and Oliver, 2008a). Real time PCR uses the same genes. LAMP employs primers for the same targets.

5.3.7 Growth and survival studies

Such studies should be conducted on the *V. parahaemolyticus* and *V. vulnificus* cells naturally occurring in seafood, as opposed to examining these species

following their inoculation into or uptake by the seafood under investigation. Methods should be quantitative (e.g. MPN-PCR, quantitative direct platings or colony hybridization) and employ recommended media (e.g. CPC or derivatives, ChromagarVibrio™), and accepted species confirmation procedures (e.g. PCR or colony hybridization of isolated colonies). Incubation temperatures and times should be appropriate to provide the growth/survival conclusions desired. In all cases, recommended methods should be used to allow comparisons between investigations.

5.3.8 VBNC state

It is generally recognized that *V. parahaemolyticus* and *V. vulnificus*, in response to low water temperatures (and possibly other stresses), enter into a viable but non-culturable (VBNC) state wherein the cells remain viable, but are no longer culturable on routine media (Oliver, 2005). While such cells would not be detected in any of the above isolation/culture procedures, and thus would contribute to an underestimation of the actual number of *V. parahaemolyticus* or *V. vulnificus* cells present, there is little indication that cells in this state play a significant role in the epidemiology of *V. parahaemolyticus* or *V. vulnificus* infections. The DNA of such cells may be detected by the various PCR methods.

5.4 METHOD PERFORMANCE AND COMPARABILITY

In 2010 the Codex Alimentarius Commission adopted "Guidelines on the Application of General Principles of Food Hygiene to the Control of Pathogenic *Vibrio* species in Seafood[2]" together with its annex on "Control Measures for *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Bivalve Molluscs". Among the key recommendations were for seafood producing countries to investigate local *Vibrio* populations for prevalence, abundance and behaviour (growth rates in seafood at various temperatures) of pathogenic strains such as *tdh* and *trh*-positive *V. parahaemolyticus*. This information should be used to fill critical gaps in risk assessment that have implications on global trade of seafood products. Knowledge on method performance parameters will be essential in determining appropriate application of conventional or molecular detection methods. Unfortunately, most existing regulatory methods are all based on culture and isolation of the target organism and are not reliable for determining presence or levels of pathogenic *V. parahaemolyticus*.

The current models for method validation and approval by internationally recognized organizations such as AOAC and ISO require multi-laboratory collabora-

[2] http://www.fao.org/fao-who-codexalimentarius/sh-proxy/jp/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXG%2B73-2010%252FCXG_73e.pdf

tive studies comparing a reference method to a proposed method. This approach is extremely expensive and typically requires years to complete. Additionally, molecular detection assays are targeting an ever-growing list of genes. The effort required to validate a method is largely attributed to inclusion of a reference method that is often not "fit for purpose" and the requirement to conduct the entire analysis starting with an inoculated food matrix. A definitive sample is needed to address the most vital questions about how the molecular detection method is performing over a defined range of target levels.

Another important obstacle in the evaluation of performance of molecular detection methods is the lack of sufficiently well characterized and diverse strains and defined reference material including the sample matrix. The availability of appropriate reference material would provide an objective means to compare the results from the diverse molecular methods currently used for detection and characterization of *V. parahaemolyticus* by the international shellfish safety community. While this approach is not a substitute for a formal validation, it would allow a laboratory to simultaneously demonstrate both method performance and analyst proficiency.

There was a consensus that the international shellfish safety community needs to calibrate molecular methods for detection and quantification of pathogens and that it would be helpful if laboratories were using common materials to evaluate method performance.

5.5 CONCLUSIONS AND RECOMMENDATIONS

5.5.1 Methods

Isolation and identification of *V. parahaemolyticus* and *V. vulnificus* from seawater is not routinely recommended for regulatory purposes as the levels of *V. parahaemolyticus* and *V. vulnificus* in seawater generally do not highly correlate with levels of these pathogens in seafood (especially molluscan shellfish). Such sampling may be indicated in certain geographical regions, and additionally may provide general information and correlation values.

A standard procedure for sample collection, transportation and conservation should be implemented. Samples of fresh seafood should not be frozen and should be analyzed for *V. parahaemolyticus* and *V. vulnificus* within 24 hours.

It is recommended that samples be prepared as appropriate to the seafood under study, and that the appropriate isolation media and identification methods be

employed. A rapid screening for presence of *V. parahaemolyticus* and *V. vulnificus* by PCR at the enrichment steps would help enhance the detection of the bacteria (especially the pathogenic strains; see above).

5.5.2 Method performance

Molecular detection methods including conventional PCR, real-time PCR and LAMP are generally accurate for identification of *V. parahaemolyticus* and characterization of virulence attributes when applied to pure cultures. Real time PCR and LAMP provide greater sensitivity than conventional PCR for detection of *V. parahaemolyticus* or its virulence genes for examination of shellfish enrichments. In some cases, the assay design effects detection of genes with variable sequences such as in *trh*. Analyst proficiency appeared to vary according to the training in molecular techniques and is likely to be associated with the complexity of the assay with regard to laboratory manipulations steps. Low levels of contamination can be problematic in sample preparations and this was more evident in assays with greater sensitivity.

RECOMMENDATIONS

1. Establish an internationally recognized system for developing criteria and protocols for evaluation of method performance parameters of molecular detection methods for pathogenic *Vibrio* spp. in seafood (explore similar approach for other pathogen/commodity pairs).
2. Examine appropriateness of coupling methods performance and analyst proficiency evaluation.
3. Develop a collection of appropriate reference material including bacterial strains and sample matrix.
4. Explore the development of a PCR network patterned after Global Food Net.

6

Conclusions and recommendations

The 41st Session of the CCFH requested FAO/WHO to undertake an expert meeting to address a number of issues relating to *V. parahaemolyticus* and *V. vulnificus*. Such a meeting was implemented on 13-17 September 2010. The response to the questions posed by the committee is summarized in the report from the 42nd Session of the CCFH[3].

V. parahaemolyticus has been recognized as an important seafood-borne pathogen since its discovery in 1950. Emergence and spread of the pandemic clone starting year 1996 reminded us of the significance of *V. parahaemolyticus* as a foodborne pathogens. These and genetic rearrangement due to active insertion sequences indicate the genes in *V. parahaemolyticus* are unstable.

Nevertheless, the *tdh* and *trh* genes remain as important virulence markers. Considering this molecular epidemiological evidence and biological actions of TDH and TRH, we believe the *tdh* and *trh* genes are most suitable virulence markers of *V. parahaemolyticus* at the moment. It is recommended for the purpose of dose-response models the inclusion of tdh^+ and trh^+ pathogenicity types would be appropriate. Given the information provided, trh^-, tdh^- types are of lower public health importance and therefore not considered a priority for inclusion in dose response

[3] Progress Report on the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA) and Related Matters, 42nd Session of CCFH. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FShared%2BDocuments%252FArchive%252FMeetings%252FCCFH%252Fccfh42%252Ffh42_03e.pdf

models. The *tdh*$^+$ and/or *trh*$^+$ pathogenicity types are considered to present the greatest risk, although there appear to be different degrees of virulence.

There is increasing evidence for a high correlation between the rRNA B-type/ vcg C-type of *V. vulnificus* with human disease. Further, there is indication that elevated temperature favors the B/C genotype. Thus, it is recommended that this evidence be considered when performing risk assessment calculations. It must be noted, however, that these data are exclusively from the United States of America studies at this point. The seasonal and geographical variation of these types have to be further studied before we can fully understand their significance.

Discussions highlighted the large number of variables that exist in relation to seafood, practices during harvest and post harvest, consumption to be considered future modelling. If a risk assessment model is to be commissioned it would be useful to have a database to compile information from around the world on the various variables relevant for the model.

For projects of this scope the accumulation of large amounts of data make it difficult to assess it all in the course of one meeting so it might be important to explore different mechanisms of assessing this data. While a web-based workspace was established in advance of this meeting and did prove to be an interesting option for sharing data and comments on the available information, more time may be required for such electronic discussions in order to make optimal use of such forum.

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There has been an increase in reported outbreaks and cases of foodborne disease attributed to pathogenic *Vibrio* species. As a result, there have been several instances where the presence of pathogenic *Vibrio* spp. in seafood has led to a disruption in international trade. A number of *Vibrio* spp. are increasingly being recognized as potential human pathogens. The food safety concerns associated with these microorganisms have led to the need for microbiological risk assessment for their control.

This report provides the review of risk assessment of existing tools for *V. parahaemolyticus* and *V. vulnificus* in oysters and different bivalve molluscan species, the available information on testing methodology and recommend microbiological methods to monitor the levels of pathogenic *Vibrio* spp. in seafood and/or water. Such tools are envisioned to support countries in their efforts to use risk-based approaches in the selection of control measures appropriate for their seafood species, primary production and post-harvest practices.

This volume and others in this Microbiological Risk Assessment Series contains information that is useful to both risk assessors and risk managers, the Codex Alimentarius Commission, governments and regulatory authorities, food producers and processers and other institutions and individuals with an interest in *Vibrio* spp. and its control.

For further information on the joint FAO/WHO activities on microbiological risk assessment and related areas, please contact

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