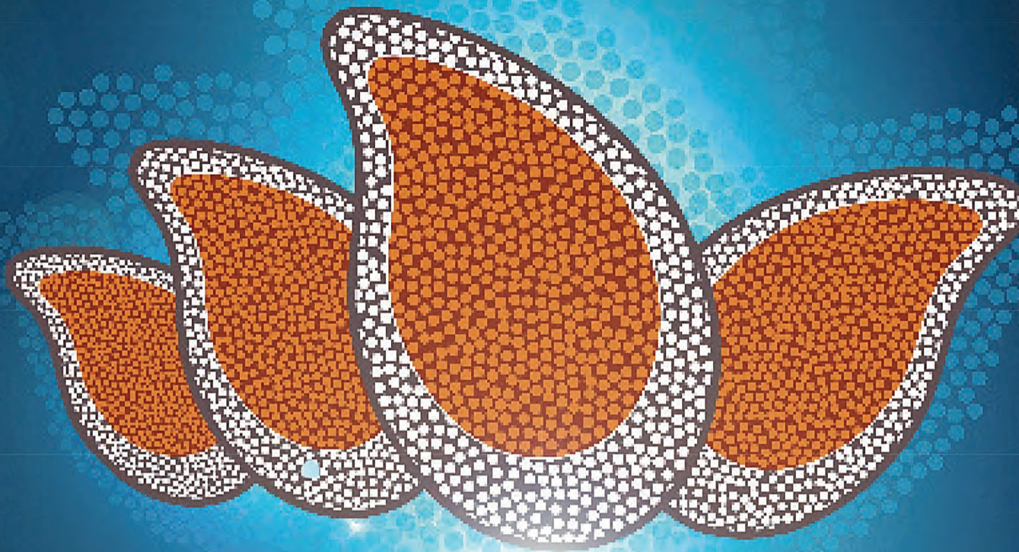

PROCEEDINGS OF THE 9TH INTERNATIONAL CONFERENCE ON MOLLUSCAN SHELLFISH SAFETY

SYDNEY, AUSTRALIA, 17 - 22 MARCH 2013

ICMSS 2013
TWENTY YEARS:

DEFINING FUTURE SHELLFISH SAFETY FRONTIERS THROUGH INNOVATION IN SCIENCE AND POLICY

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Preface

The 9th International Conference on Molluscan Shellfish Safety (ICMSS) was held in Sydney, Australia in March 2013. Attendees to the ICMSS included the cream of the international molluscan food safety community, with more than 200 registered delegates, including scientists, regulators and industry representatives from over 30 countries.

The ICMSS2013 provided a unique platform for developing and extending professional networks, discussing latest advances with colleagues and plenty of opportunities for talking face-to-face with authors of recent peer reviewed journal articles. The conference programme included topics related to a range of contaminants that shellfish may accumulate which are present in water and may cause illness amongst consumers of shellfish, including presentations on latest advances on harmful algal bloom research, toxin methods, viruses and bacteriological contaminants, risk assessment and management strategies, and for the first time at an ICMSS there were sessions dedicated to the impact of cyanotoxins in molluscs, a potentially emerging threat, and the safety of non-bivalve molluscs, such as abalone.

The ICMSS also included an exciting series of extracurricular events including: a Harbour cruise taking in key iconic sites such as the Opera House, the Harbour Bridge and surrounds; an extended afternoon and evening visit to one of the world's greatest oyster production areas, the famous Hawkesbury River; and an oyster and wine tasting event hosted at the infamous Sydney Fish Market. The programme provided a stimulating platform for out of session discussions and development of new collaborations and ideas for future advancement of the field.

To those companies and agencies who have supported our efforts through provision of funding and donations of food and wine, we sincerely thank you for your support and dedication to this important forum and to the need to improve on the provision of safe and nutritious molluscs to consumers.

Catherine McLeod and Gustaaf Hallegraeff
Co-conveners ICMSS 2013

Tribute to Phil Busby: 1947-2013

Phil Busby, in his capacity as Principal Advisor (Shellfish) at the Ministry of Agriculture and Forestry (MAF) in New Zealand, touched many in his productive career of putting quality assurance of molluscan shellfish on the world map. He retired from this position due to poor health on 31 October 2012 and passed away on 8 March 2013.

Phil initially wanted to become a plumber, but changed his mind to train as an Inspector of Health in the New Zealand Department of Health. He enjoyed many adventures doing field work in Wellington and Invercargill including rare trips to the mutton bird islands off New Zealand's southern coast. He gave up the field work to join the Department's Head Office to run the training course for health inspectors in 1983. Those who went through the course under Phil will never forget his no-nonsense marking style! From 1989 MAF took over running the New Zealand Shellfish Quality Assurance Programme and with it came Phil - initially on secondment from the Department of Health. He became responsible for all public health aspects of the New Zealand Shellfish Quality Assurance Programme, and in 1992 wrote the first New Zealand Shellfish Standard. The 1993 Karenia bloom in Hauraki Gulf and resulting Neurotoxic Shellfish Poisoning outbreak which led to New Zealand wide harvesting closures, catapulted him into the national and international spotlight. He did rise to the challenge, simply was always there, made a difference through persuasion and presence, and maintained his position of authority with grace, charm and flair. He effected major change through (sometimes polarising) debate and arguments, preferably over a few beers in a bar. Debates on human health significance of breve-toxins, gymnodimine, pinnatoxins, Phil has seen them all. He was responsible in 1996 for the requirement that Cawthron Institute acquire accreditation prior to phytoplankton being accepted as part of the New Zealand Marine Bio-toxin Management Programme. Phil organised twice yearly Seafood Safety workshops through what is now called Ministry of Primary Industry. The informal proceedings of these meetings capture the significant changes in focus as research and regulation on harmful algal blooms matured in New Zealand and tried to keep

up with each other. In 2001 he was responsible for the validation and approval of LC-MS methods for ASP and DSP marine biotoxins, in 2003 for introducing mandatory hydrolysis of DSP samples, and in 2004 the approval of a LC-MS Screen Test Method for Brevetoxins. Each of these initiatives represented international trend setting decisions and showed the resolve of Phil to provide novel outcome based solutions to improve public health protection. In 2004 Phil was asked to chair the Joint FAO/IOC/WHO Expert Workshop in Dublin on Biotoxins in Molluscan Bivalves and he went on to Chair the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Molluscan Bivalves in Oslo. His role was facilitated through his position as Vice Chair of the UNESCO Intergovernmental Oceanographic Commission Panel on Harmful Algae Blooms and Chair of the Panel Task Team on Biotoxin Monitoring and Management. The respect in which he was held by his national and international peers showed in his re-election in 2009 to both positions. In 2006 Phil drafted and put in place the Bivalve Molluscan Shellfish Regulated Control Scheme (affectionately known as the BMSRCS) comprising new shellfish safety regulations and specifications to take the New Zealand Shellfish Programme through the years ahead and provide access to markets throughout the world.

Thank you for all your contributions, Phil. You will be a hard act to follow!

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Table of Contents

Section I – Management and Regulation……………………………………………………………..1

Global bivalve production, marketing and safety issues
Iddya Karunasagar………………………………………………………………………......………...2

Salmonella spp. and fecal pollution indicators bacteria in zones of bivalve mollusks along the Mediterranean Coast of Egypt
Moustafa El-Shenawy, Mohammed El-Shenawy, Aida Farag, Mohammed Tawfeek, Mahmoud Abdel-Aziz, Samir Nasr, Hoda Baghdadi, Ola Saleh, J.M. Soriano and J. Mañes………………………………………………………………………………………...………..6

Molluscan shellfish safety issues in the Pacific islands: case studies from Fiji and New Caledonia
Timothy Pickering, Ruth Garcia-Gomez and Milika Sobey…………………………………….....10

Sanitary wastewater system assessments in Canadian bivalve molluscan shellfish harvest areas
C. Roberts, P. Klaamas, C. Leblanc and J. Stobo…………………………………....................15

Microbiological risk assessment (MRA) – useful or just feel-good?
John Sumner, Cath McLeod and Tom Ross…….…………………….………………………..................18

Dye tracing techniques used in NSW shellfish growing areas to assess pollution sources
Phil Baker…….…………………..…………………….................25

The use of fluorescent whitening compounds as signals of human sourced contamination
Phillip Geary, Craig Evans, Jasmin Kable and Motswadi Maswabi……....……………………......28

Integrated water quality forecasting system for shellfish harvesting areas
Nathan Crowell, Timothy Webster, Sean Butler, William Livingstone and Greg Rose………………………………………………………………………………………………..33

Adding value throughout the supply chain
Douglas McLeod……………………………………………………………………………….....37

Microbial source tracking to identify and manage sources of fecal contamination in shellfish growing waters
Stephen H Jones……………………………………………………………………………........39

Section II – Viruses and Microbial Contaminants………………………………………………44

Epizoic barnacles act as a reservoir for pathogenic bacteria on commercial shellfish beds: Implications for the shellfish industry.
Katie Clements, Luis Giménez, Davey L. Jones, James Wilson and Shelagh K. Malham…………………………………………..……………………………………..45

Evaluation and application of viral source tracking in New Zealand
Gail E Greening and Joanne Hewitt……………………..……………………………………...49

Improving the management of the risk of human enteric viruses in shellfish at harvest
Brenda Hay, Dorothy-Jean McCoubrey and Anthony Zammit……………………………………...53

Evaluation of the sanitary status of the dutch shellfish production waters over a 7 year period
I.E. Pol-Hofstad, S.A. Rutjes, A. Gerssen, M. Poelman and A.D. van der Linden……………………………………………………………………………………………..57

Detection and genotyping of norovirus from clinical and shellfish samples: epidemiological implications
Carmen F. Manso, Alba Monteagudo, Jesús L. Romalde………………………………….………..61

Quality programmes as a commercial opportunity
Helen Smale…………….……………………………………………………………………………66

Application of risk analysis for ensuring food safety after the Fukushima nuclear accident: Focused on molluscan shellfish safety
Hajime Toyofuku…………….……………………………………………………………………....73

Incidence of *Vibrio parahaemolyticus* in oysters along Northern Manila Bay, Philippines
Reivin T. Vinarao, Jessica L. Jones, Rosario J. Ragaza, Catharina H.M. Lüdeke, William Burkhardt, Junwell S. Cabigao and Rosa A. Bassig……………………………………..………………....87

Evaluation of spatial contamination patterns for norovirus and faecal indicator bacteria near to a coastal sewage discharge using *Mytilus edulis* as biosentinels
James B. Winterbourn, Katie Clements, James Lowther, Shelagh K. Malham, James E. McDonald and Davey L. Jones……………………………………………………………………………..94

Treatment time and environmental factors affect the reduction of Vibrio parahaemolyticus concentrations in relayed oysters (*Crassostrea virginica*)
Michael Taylor, Jong Yu, Cheryl Whistler, Vaughn Cooper, Thomas Howell and Steve Jones……98

Section III – Algae and Biotoxins……………………………………………………………..…102

Harmful algal blooms in the Australian region: changes between the 1980s and 2010s
Gustaaf M. Hallegraeff……………………………………………………..…………………103

Identification and seasonal occurrence of potentially toxic *Pseudo-nitzschia* diatoms in oyster growing estuaries of New South Wales, Australia
Penelope Ajani, Shauna Murray,, Gustaaf Hallegraeff, Steve Brett, Grant Webster and Leanne Armand……………………………………..…………………………………………..…..109

First reported closure of a classified mussel production area in Northern Ireland due to Domoic Acid
H.C. Higgins, H. McEneny, April McKinney and Richard Gowen…………..………………..….113

Alexandrium species in New South Wales (NSW) coastal waters: historical distributions and identification of high-risk zones.
Hazel Farrell, Penelope Ajani, Steve Brett, Anthony Zammit and Shauna Murray………………..116

Comparison of phycotoxin composition and distribution in toxigenic plankton from the north and south Atlantic
Bernd Krock, Urban Tillmann, Allan D. Cembella, Gustavo A. Lovrich and C. Marcela Bore…...120

Risk assessment from paralytic shellfish poisoning (PSP) due to bivalve consumption in Nha Trang city, Vietnam
Nguyen Thuan Anh………………………………………………………………………….....123

First report on the detection of yessotoxin from *Gonyaulax spinifera* in the Benguela current upwelling system
Chibo Chikwililwa, Christine Neumann, Detlef E. Schulz-Bull, Joanna J. Waniek, Norbert Wasmund and Bronwen Currie. ……………………………………………………………..126

Cyanobacterial toxin accumulation in shellfish, finfish and crustaceans: guidelines for consumer safety
Ian R. Falconer…………………………………………………………………………………..131

A new high resolution LC-MS method for identification, quantification and monitoring of palytoxin-like compounds in molluscs
Patrizia Ciminiello, Carmela Dell'Aversano, Emma Dello Iacovo, Ernesto Fattorusso, Martino Forino and Luciana Tartaglione……………………………………………………………..135

The patterns of hsp27 phosphorylation in cells exposed to mixtures of okadaic acid and palytoxin reveal synergistic effects
Gian Paolo Rossini, Gian Luca Sala and Mirella Bellocci………………………………..139

Risk and Mitigation of Food Safety Risks Associated with Abalone and Crustaceans
C. J. Foord………………………………………………………………………………………..144

Section IV – Speaker Abstracts…………………………………………………………….148

Section V – Poster Abstracts……………………………………………………………….233

Appendix A
ICMSS 2013 Industry Session:
Learning Lessons; PST Event in Tasmania……………………………………………………248

Appendix B……………………………………………………………………………………..258
FDA Dye Study Report

Section I – Management and Regulation

Global bivalve production, marketing and safety issues

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Abstract
Global bivalve molluscs production (capture + aquaculture) has increased substantially in the last fifty years, going from nearly 1 million tonnes in 1950 to about 14.54 million tonnes in 2011. The increase of bivalve mollusc production was driven by international demand since the early 1990s. Total bivalve trade has expanded continuously during the past three decades to reach US$ 2.1 billion in 2009. Most of the problems in international markets are related to hygiene, biotoxins, viruses, and other causes. FAO/WHO have been providing scientific advice to Codex Alimentarius Commission and the FAO/WHO risk assessments for pathogenic *Vibrio* spp. have led to the development of Codex Guidelines on the control of pathogenic *Vibrio* spp. in seafoods. FAO/WHO have been working on risk management tools for use by national authorities and this requires data from different geographical regions.Data collection requires appropriate methodology. To provide guidance on the methods to be used for detection and enumeration of pathogenic *Vibrio* spp. in seafood, performance characters of the methods and their application for various end uses have been discussed and documented. This could facilitate data collection from different parts and development of a risk management tool with wide geographical applications.

Keywords: aquaculture, international trade, risk assessment, Codex Guidelines, *Vibrio parahaemolyticus*

Introduction

Global bivalve molluscs production (capture + aquaculture) has increased substantially during the last fifty years, going from nearly 1 million tonnes in 1950 to about 14.54 million tonnes in 2011. While production by capture has marginally declined from about 1.9 million tonnes to about 1.77million tonnes in 2011, production by aquaculture increased from 8.3 million tonnes in 2000 to 12.77 million tonnes in 2011. 89.8% of aquaculture production came from Asia, 4.9% from Europe, 4.3% from the Americas and 0.9% from Oceania.China accounted for 82% of global bivalve aquaculture production.The increase of bivalve mollusc production was driven by international demand since the early 1990s.In 2009, global bivalve trade was valued at $2.1 billion. In terms of quantity, scallops accounted for 24% of export, while mussels contributed to 48%. Scallops are the most important species contributing to 46% of value, followed closely by mussels (26%). 465,535 tons of bivalves were exported in 2009. Major exporters (>20,000 tons) were China, Spain, Netherlands, New Zealand, UK, US, Canada, Thailand, Korea.Major importers were France, US, Japan, Italy, Spain, Korea, Belgium and Netherlands.EU was a major importer (301,670 tons), US imported 77,784 tons and Japan imported 55,756 tons. The bivalve importing countries have very stringent regulations regarding hygiene and biotoxin control. Bivalves like oysters are consumed raw in many countries, particularly in EU and therefore sanitary measures are very important in ensuring consumer safety.

Reasons for trade compliance standard failures

Data from trade standard compliance failures would be helpful in understanding problems associated bivalve safety management. In the EU, there is system of issuing “alerts”, whenever border inspection reveals non-compliance of regulatory requirements. Table 1 provides a summary of the alerts in theEU Rapid Alert System for Foods and Feeds (RASFF) for 2006-2012. The number of alerts has been ranging between 45-78 annually, the majority of alerts being due to microbiological causes followed by biotoxins. The microbiological issues are due to detection of *Escherichia coli* at unacceptable levels or due to detection of *Salmonella* or noroviruses.

Table 1. Alerts in the EU Rapid Alert System for Foods and Feeds during 2006-2012

Causes	Number of alerts						
	2006	2007	2008	2009	2010	2011	2012
Microbiological	17	33	34	31	50	23	31
Chemicals and residues	8	8	2	1	1	7	1
Biotoxins	24	16	6	8	18	11	7
Others	6	11	3	12	9	27	14
Total	55	68	45	52	78	68	53

Biotoxins detected at levels exceeding permitted levels include diarrhetic shell fish poison (DSP), amnesic shellfish poison (ASP), azaspiracid and lipophilic toxins.The numbers of trade compliance failures in Japan were lower, but the volume of imports into Japan were about 1/6th of that coming into EU. There were 11 violations in 2011, 4 due to biotoxins, 4 due to microbiological issues, two due to additives, one due to pesticide residue. In 2012 there were 17 violations and of these 10 were due to pesticide residues, 5 due to microbiological issues, 2 due to biotoxins.

FAO/WHO scientific support for issues in bivalve molluscan safety

The Codex Committee on Fish and Fishery products has been working on a standard for live and raw bivalve molluscs (Codex Stan 292-2008). For providing scientific support for the development of this standard, FAO/WHO carried out risk assessment of *Vibrio parahaemolyticus* and *V. vulnificus* (FAO/WHO, 2005; 2011). These risk assessments led to the development of Codex Guidelines on the application of general principles of food hygiene for the control of pathogenic *Vibrio* spp. in seafood(CAC/GL 73-2010) with an annex on the control measures for *Vibrio parahaemolyticus* and *V. vulnificus* in bivalve molluscs. This guideline recognises that effective control measures for *V. parahaemolyticus*and *V. vulnificus* at primary production typically require an evaluation in terms of the risk associated with environmental factors in the harvesting area and harvesting practices based on epidemiology and environmental conditions (i.e. air and water temperature and salinity).Predictive tools using these environmental monitoring parameters and growth rates as inputs have been elaborated based on the FAO/WHO risk assessments and, when validated, may be used to estimate corresponding *V. parahaemolyticus* and *V. vulnificus* levels and risk.

The presently available tool developed by the US isbased on an Excel spreadsheet in which the inputs are level of *V. parahaemolyticus* in oysters at harvest, the air temperature and the maximum time oysters are at unrefrigerated condition. Based on these inputs, the calculator would work out the predicted level of *V. parahaemolyticus* at consumption and using a set value for the proportion of these being pathogenic strains, the tool would work out the predicted risk of illness.

However, a Joint FAO/WHO Expert Meeting on Microbiological Risk Assessment (JEMRA) held in 2010 concluded that this*Vibrio parahaemolyticus* calculator tool may be used to estimate relative risk reductions, primarily because of the linear dose-response, associated with temperature controls (post-harvest refrigeration) in areas in which the strain virulence, initial concentration and growth rates of *V. parahaemolyticus* in the bivalve spp. of concern are similar to that indicated in data from the United States.The *Vibrio vulnificus* calculator tool is less likely than the *V. parahaemolyticus* calculator to be applicable to a broader region than the United States of America because of uncertainty about the dose-response relationship. The meeting concluded that to develop a tool that is applicable to particular regions and/or other products, or to answer other risk management questions, other than post-harvest refrigeration, it would be preferable to first modify the existing JEMRA risk assessment models, or develop a new model, that considers and evaluates the influence of other factors including salinity, strain differences, temperatures etc. A simplified calculator tool could then be developed to answer these other specific questions routinely. This is dependent on the availability of the appropriate data and effort must be directed towards this.

Table 2. Performance characteristics of methods for detection/quantification of pathogenic *Vibrio* spp. (modified from FAO/WHO, 2014)

Performance criterion	Direct plating		Enrichment Presence/Absence			MPN		
			Selective plating		No Selective plating	Plate		Broth only
	No P.H[1].	P.H (NSM)[2]	Biochem[3]	Mol[4]	Mol	Biochem	Mol	Mol
Quantitative?	No (presum)[5]	Yes	No	No	No	Yes (presum)	Yes	Yes
Recovery of stressed cells	Depends (M.S)	yes	yes	yes	yes	yes	yes	yes
Sensitivity/ Specificity	Low/ Low	Low/ High	High/ Moderate	High/ High	High/ High	High/ Moderate	High/ High	High/ High
Variability	Low	Low	Low (not often estimated)			May be higher depending on MPN format		
Distinguish pathogenicity?	No	yes	No	yes	yes	No	yes	yes
Possibility of strain characterisation	yes	yes	yes	yes	No	yes	yes	no
Time to results	18-24 (presum)	36-48h	3-4 days (presum)	3-4d	1-2d	5-10d	4-5d	1-2d

[1]Probe hybridisation; [2]Non-selective medium; [3]Biochemical characterisation; [4]Molecular characterisation; [5]Presumptive

Monitoring of vibrio concentrations in the seafood itself is the only direct way to establish the levels in these commodities at the time of harvest and through the production chain. Such data is invaluable when undertaking a risk assessment. However, undertaking monitoring at a sufficient intensity to detect potential health risks on an ongoing basis can pose practical difficulties and will be expensive. Determination of other factors that may help predict concentrations of total and pathogenic *V. parahaemolyticus* and *V. vulnificus* in the seafood may enable countries to determine risk based largely on those factors. Where a potential risk has been identified to exist in a country, cost-effective programmes may incorporate monitoring of such factors to determine higher risk areas and seasons. Seawater temperature and salinity have been identified as two important abiotic factors for predicting the concentrations of *V. parahaemolyticus* and *V. vulnificus* in some oyster species in some parts of the world, but other ecological factors may be involved elsewhere. However, in applying risk assessments and risk calculators more widely, such relationships need to be confirmed for the species of interest (if different) and for the specific geographical and environmental situation. The collection of locally relevant data is therefore an important part of the potential application of the international risk assessments.

Based on the conclusions of the JEMRA meeting, the 42nd Session of CCFH asked FAO/WHO to (a)provide recommendations on a range of test methods for quantifying *V. parahaemolyticus*(total and pathogenic (e.g. tdh+, trh+)) and *V. vulnificus* in seawater and bivalves and facilitate performance evaluation of the proposed methodologies (b) develop data collection strategies (that would facilitate the collection of data) by countries to support the modification/development of models with a broader scope than those which currently exist (c) encourage the collection of data in different regions, in different bivalve species and for geographically diverse strains of pathogenic *V. parahaemolyticus* and *V. vulnificus* according to the data collection strategy and using recommended test methods (d) to modify/develop risk assessment models that could be used to address a range of risk management questions in a

number of different regions and products, when adequate data becomes available.

In response to this request, FAO/WHO convened an Expert Meeting in 2011 and looked at the end uses of *Vibrio* methodology and performance characters of presently available methods. Table 2 provides a summary of the conclusions on the performance characters. The choice of the method by a user would depend on whether the data required is qualitative or quantitative; does the use require information on pathogenic strains, cost, time and skill required to perform the assay, availability of reagents and other factors. The end uses considered were (a) harvest area monitoring (b) postharvest process verification (c) end product monitoring (c) outbreak investigation (d) growth studies. For most applications, MPN method with molecular characterisation directly from the broth appeared to have high value. Enrichment presence/absence method with molecular confirmation could be of high value for postharvest process verification or end product monitoring depending on the criteria applied. Direct plating on non-selective medium and probe hybridisation method could be of high value for harvest area monitoring and for performing growth studies. Based on the outputs of this Expert Meeting FAO/WHO have developed a Guidance document (FAO/WHO, 2014) that could be useful for national authorities, food testing and research laboratories

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Salmonella spp. and fecal pollution indicators bacteria in zones of bivalve mollusks along the Mediterranean Coast of Egypt

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Abstract
Shellfish (44 samples) and their water beds areas, including 44 samples each of surface and bottom sea water collected from eleven stations located along the Mediterranean coast of Egypt, were microbiologically examined during four seasonal sampling cruises, from March 2010 to February 2011. All samples were analyzed for total bacterial plate count, levels of total coliform, fecal coliform, and fecal streptococci as well as the prevalence of *Salmonella* spp. Moreover, temperature, salinity, pH and dissolved oxygen of the coastal water samples were also measured.
The overall incidence of *Salmonella* in the entire examined samples was 18%. Shellfish presented a higher incidence (32%) than surface (14%) and bottom (9%) waters. A seasonal pattern was noted for the isolation of *Salmonella* spp. where the highest incidence was detected in summer. Of the eleven studied stations, 7 of the 8 stations that have levels more than 320 cfu/100 g or ml of fecal coliform in meat or water in harvesting areas (the maximum level criteria in the European Communities regulations) were positive for the presence of salmonella supporting the view that the presence of high levels of fecal coliform may indicate the presence of Salmonellae.

Keywords: Bivalve mollusks; coastal water beds; *Salmonella* spp; fecal pollution.

Introduction
Waterborne salmonellosis is considered a persistent threat to human health in many developing countries. Increase in pollution of seawater by discharging of sewage effluents intensifies the occurrence of pathogenic organisms, mainly *Salmonella* spp. Shellfish are prone to contamination by fecal pathogens from sewage polluting the waters in which they grow (Orinigo *et al.,* 1990)According to the International Commission on Microbiological Specifications for Foods (Kfir *et al.*, 1993), a single oyster can filter up to 10 liters of water/hour, thus removing microorganisms and pollutants from the water into the mollusk leading to infectious diseases including salmonellosis (Halliday *et al.,* 1991).There is a considerable amount of epidemiological data available regarding the presence of *Salmonella* in seafood in general and in shellfish in particular (Panisello *et al.*, 2000). Rapid growth in population, as observed along the Delta coastal area, is often coupled with limited sanitary facilities. This results in an increased discharge of polluted water into the marine environment leading to pollution of seawater and shellfish with pathogenic organisms, including *Salmonella* (Norhana *et al.*, 2010).

In view of the above facts, this paper investigates some microbiological properties of the shellfishes and their water beds (surface and bottom water) in eleven stations along the Mediterranean coast of Egypt. These include the total viable count, the presence of the fecal pollution indicators bacteria (total coliforms, fecal coliforms , and fecal streptococci) as well as the prevalence of

Salmonella spp. Correlation between the presence of the fecal contamination indicators and the presence of *Salmonella* spp., in the examined samples/stations was another goal. Some environmental parameters of the coastal water samples were also measured.

Material and Methods

Sites investigated: - Eleven stations in the main harvested shellfish zones (Fig.1), located at the Mediterranean coast of Egypt were investigated. These include Salloum, Matrouh, Agamy, El-Max, Eastern Harbour, Sidi Gaber, Abu-Qir, Rashid, El-Burg, Damietta and Port Said.

Fig.1

Sampling: - Samples of surface and bottom waters as well as shellfish (*Donax trunculus*) were collected seasonally during four seasonal sampling cruises from March, 2010 to February, 2011. Water samples were collected in duplicate at depth/over of 50 cm of the surface/bottom of shellfish beds using the methods and guidelines adopted by ISO 9308/1(1992). All water samples were preliminary processed for microbiological analysis immediately in an on-site mobile microbiological laboratory. Shellfish samples were either hand-picked or purchased from fishermen, located in the same stations, kept in refrigerator of the Van Laboratory and were analyzed within 24 h of collection.

Microbiological analysis: - The membrane filtration technique was used in all the bacteriological analysis, the biochemical confirmation tests of the fecal pollution indicators and the calculation of the final bacterial counts (cfu) per 100 ml / g of sea water / meat, were done as described by ISO 9308/2(1990) and 7899/2(1984).Quantities of 0.1, 1.0,10 and 100 ml of water samples were used to count the total viable count and the three fecal pollution parameters. Shellfish were pooled, aseptically shucked to a sterile blender jar, and blended at high speed for 90 sec. The resultant slurries were used to prepare dilutions in all analyses. For determination of the total viable count, the direct pour plate's technique was used and plates were incubated at 32°C for 5 days. For detection of total coliforms, fecal coliform and fecal streptococci the membranes were fixed onto m-Endo-Les agar, m-FC agar and m-enterococcus agars and incubated at 37 ^{o}C for 24 h, 44.5 ^{o}C for 24 h, and 37 ^{o}C for 48 h respectively. For detection of *Salmonella* spp. the method adopted by FDA (2002) was applied. The suspected colonies were checked on chromogenic medium. Typical *Salmonella* sp. colonies were submitted to biochemical screening on triple sugar iron agar (TSI), lysine iron agar (LIA) and urea agar (UA). Colonies suspected to be *Salmonella* were analyzed by complementary biochemical tests (dulcitol, indole, malonate, Methyl Red Voges Proskauer, and citrate).Presumptive colonies were confirmed with the API 20E test (bioMe´rieux, Marcy l'Etoile, France), Hi *Salmonella* identification kit and Hi *Salmonella* Latex test kit. The confirmed colonies were serologically confirmed as *Salmonella* spp. using Difco-polyvalent 'O' (somatic) antisera.

Environmental parameters:- The environmental parameters of coastal waters including the water temperature, salinity, pH and dissolved oxygen were measured on site using multi parameters CTD YSI-Mode 650 DM.

Results and Discussion

A total of 132 samples including 44 samples of shellfish, as well as 44 samples each of surface and bottom waters collected from the studied stations were investigated. The obtained ranges of temperature, salinity, pH and dissolved oxygen were 16.1 – 29.8 ^{o}C, 7.8 – 38.4 ppt, 7.5 – 8.5 and 3.43 – 8.3 mg/L respectively.

The total aerobic bacterial counts ranged from 10^2 to 10^6 cfu/100 ml and from 50 to 10^6 cfu/100 gm in water and shellfish samples respectively. Coliform, fecal coliform, and fecal streptococci were found in seawater samples in levels ranging from <1 to 10^6, <1 to 10^5 and <1 to 10^3 cfu/100ml respectively, however levels detected in shellfish samples ranged from <1 to 10^6, <1 to 10^6 and <1 to 10^3cfu/100 g respectively. Heterogenic results were recorded in different countries. For example, in Japan, raw shellfish samples had aerobic

bacterial counts of 5.47 log CFU/g (Asai *et al.*, 2008)).In Singapore, the samples had counts that were 1.0 log cfu/g higher than those of samples from Japan (Yunle huang *et al.*, 2012).Our obtained results overlapped these results. The high total plate count / fecal contamination parameters detected in both seawater and shellfish samples in this work may be attributed to run-off of organic matter into the Nile, animal waste, agriculture fertilization with non-composted manures, farming systems with animal's house, untreated wastes/toilets discharging, and poor hygienic handling practices (FAO, 2010).

The overall incidence of *Salmonella* in the total examined samples was 18 %(24/132) of which six surface water samples out of 44 (13.6%), four bottom water samples out of 44 (9%) and four-teen shellfish samples out of 44 (32%) were found to be positive for the presence of *Salmonella* spp. Our findings are in agreement with several others regarding the incidence levels of *Salmonella* in shellfish samples (Amagliani, 2012); moreover our obtained ranges were slightly lower than those reported by others (Kfir *et.al*, 1993).

On a regional basis, the highest incidence of *Salmonella* occurred in the central part of the coast rather than western area. *Salmonella* spp. was detected in both water and shellfish samples in 4 stations namely El-Max, Eastern Harbour, Abo-Qir and El-burg, whereas, the bacterium was detected only in shellfish samples in three stations, namely Sidi-Gaber, Rashid, and port-Said. Moreover, no *Salmonella* spp. were detected in either waters or shellfish samples in the remaining four stations including salloum, Mersa Matrouh, Al-Agamy, and Damietta. The highest incidence of *Salmonella* coupled with the highest levels of the fecal pollution indicators bacteria as well as the total plate count was recorded in El-Max station. This may be ascribed to the location of this site at the drainage canal connecting the heavy polluted lake Mariout to the open sea.

From a seasonal perspective, the highest incidence of *Salmonella* (n= 13), in the total examined samples collected during whole year (n= 132), was recorded in summer (10%), followed by fall and spring (n= 4each equal 3%) however the least incidence occurred in winter (n= 3 equal 2.3%). Moreover, the highest levels of fecal indicator bacteria were also recorded in summer. Two contradicting results were recorded regarding the seasonal incidence of *Salmonella*, where some researchers reported the highest incidence of both *Salmonella* and coliform bacteria in summer (Rhodes and Kator, 1988), while others reported that winter is the highest incidence season (Wilson and Moore 1996, Bouchriti *et al.,* 1995). Generally, differences in *Salmonella* isolation between summer and winter may be due to differences in water temperature, with colder waters reducing the presence of bacteria and warmer waters increasing possibility of bacterial survival (Rhodes and Kator, 1988). Our findings support the view that weather related factors may be responsible for the higher isolation of *Salmonella* and coliform bacteria in summer, where the recorded temperatures were in the range of 28.3- 29.8 °C. Additionally,other factors may contribute as agriculture runoff and higher fecal contamination rates specially that animals harbor *Salmonella* in their intestines (Clegg *et al.* 1983).

The FDA imposed a limit of 320/100 g or ml of fecal coliform in meat or water in harvesting areas (Fiandrino *et al.*, 2003, Lee *et al.*, 2003, Centers for Disease Control and Prevention 2000). In this study, the obtained numbers/counts of coliforms and/or fecal coliforms were determined and contrasted with the presence of *Salmonella* spp. in the examined samples. Of the 11 tested sites, 8 stations having counts of fecal coliform higher than ranges set by the FDA (namely El-Max, Eastern Harbour, Sidi Gaber, Abu-Qir, Rashid, El-Burg, Damietta and Port Said), were positive for the presence of *Salmonella* spp. with the exception of Damietta station which was negative for the pathogen. The remaining 3 stations, (namely Salloum, Matrouh, and Agamy), recorded coliform counts within the permissible limits, were negative for *Salmonella*.

From these findings, it can be proposed that, as the coliform numbers increased, over the permissible limits adopted by FDA, higher percentages of salmonella-positive samples were detected. Conclusively, high fecal coliform presence could be used as a viable predicator of *Salmonella*

presence and hence support the decision makers regarding the coastal water and shellfish safety.

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Molluscan shellfish safety issues in the Pacific islands: case studies from Fiji and New Caledonia

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Abstract
Bivalve mollusc aquaculture constitutes the most valuable aquaculture industry among Pacific island countries, however it is for round pearl production from blacklip oyster *Pinctada margeritifera.* Large-scale commercialised fisheries or aquaculture production and export of molluscs for food is conspicuous more by its absence. A major constraint is lack of capacity to assure food safety of edible bivalve molluscs. Major rivers in Fiji support robust fisheries for freshwater mussel *Batissa violacea,* which is a reliable livelihood opportunity for village women with few alternative sources of regular income. Periodically there are cases of shellfish poisoning which leads to this fishery being closed for a time. This shellfish has export potential to Pacific islanders living in Australia and New Zealand, however export licence applications are routinely declined because of the uncertain phyto-sanitary status. Lack of capacity in shellfish food safety thereby results in economic losses for Fiji. Meanwhile in New Caledonia a successful trial of scallop spat collection has led to aquaculture of two species of scallops, *Bractechlamys vexillum* and *Mimachlamys gloriosa.* For two years now, local communities have been farming these scallops commercially in submerged cages. A simple and user friendly depuration system, combined with periodical water quality control protocols, has been implemented in order to avoid food safety concerns. There is also a Pacific oyster *Crassostrea gigas* farm operating in New Caledonia. The approaches being taken toward shellfish safety, to enable these new seafood industries to become established in New Caledonia, are here described.

Keywords: shellfish safety, Pacific islands, Fiji, New Caledonia, bivalve aquaculture

Introduction

Bivalve mollusc aquaculture is the most valuable aquaculture industry in the Pacific islands region (Ponia, 2010), however this is for an inedible product; round pearls of the blacklip pearl oyster *Pinctada margeritifera*. Commercialised fisheries or aquaculture for production, value-adding and export of bivalve molluscs as *food* is conspicuous by its near absence. The reason is mainly due to lack of capacity to address shellfish safety issues for human consumption. This point will be illustrated by describing and analyzing case studies from two places in the Pacific; New Caledonia and Fiji Islands.

The significant edible bivalve molluscs of New Caledonia and Fiji Islands

In both places, bivalve molluscs are popular as food. In New Caledonia there is aquaculture production of Pacific oyster *Crassostrea gigas*, and pilot-scale trials of scallop *Bractechlamys vexillum* and *Mimachlamys gloriosa.* In Fiji there are fisheries for Freshwater mussel *Batissa violacea*, Giant clam *Tridacna* spp., and Ark shell *Anadara* spp., which are important for food security and rural incomes. Blacklip pearl *Pinctada margeritifera* is considered good-eating in sashimi form, but supply is restricted and consists of discards from pearl farm operations.

A high number of Pacific oysters are consumed in New Caledonia, and most of these are imported from Australia, New Zealand or France. There is one local Pacific oyster farm, "Huitres de Dumbea" at Dumbea near Noumea. To stock this farm, oyster spat are brought all the way from

France. The production of local oysters is valued between USD 225,000 and USD 617,000 per annum (Direction des Affaires Maritimes, rapport 2012, Nouvelle-Calédonie).

Capture-based aquaculture of scallops is now at a pilot commercial phase of development in New Caledonia. The spat being collected are of the species *Bractechlamys vexillum* and *Mimachlamys gloriosa.* Five local communities are involved in spat collection activities at sites in Poum (Northern Province) and Ouano Bay (Southern Province). The collection gear is buoy-supported vertical ropes approximately 10 m in length, with onion or potato bags suspended every 2 m.
During 2012 the price for scallops delivered to restaurants and hotels in Noumea was USD 1.09 per piece (Direction des Affaires Maritimes, rapport 2012, Nouvelle-Calédonie). Community members are paid USD 0.10 per piece for collected spat that has been grown out to ca. 1cm size. The total amount of spat collected in 2012 was 12,000 pieces, and at the time of writing the farm was stocked with 9,000 scallops with a value of USD 9,810 (Nadine Sepharad, pers. comm.).
In Fiji Islands, the freshwater mussel *Batissa violacea* (*kai* in Fijian) is an important fishery in the major rivers of the two largest islands. The Fiji *kai* resource is in the top three of the Pacific islands region's most significant freshwater fisheries, with catches estimated at between 4000T and 7000T per annum (Gerhke *et al*., 2012). Of this catch, 25% is sold commercially in markets with a value of USD 600,000 – 900,000 per annum.

Socio-economic importance

Capture-based culture of scallops is an important new initiative to diversify New Caledonia's aquaculture sector, which is dominated by production of shrimp *Litopenaeus stylirostris*. The potential socio-economic benefits of scallops stem from the fact that the rural areas north of Noumea are economically depressed, that alternative livelihoods to mining and shrimp are badly needed, and that scallop spat collecting is an accessible entry point for rural indigenous (Kanak) people to engage in aquaculture.

The socio-economic importance of *kai* in Fiji Islands stems from the fact that, with no fisheries management controls whatsoever, this fishery continues to be both productive and resilient to fishing pressure. The activity of fishing for *kai* is largely the domain of village women, and it represents an important source of income for them. *Kai* is one of the main sources of livelihood for those rural communities in close proximity to the lower reaches of the major river systems. The women will often spend three to five days collecting *kai* and one to two days marketing their catch. Weekly income ranges from USD40-100/week and revenue earned makes a significant contribution to meeting household needs, paying school fees and contributions to village projects.

Human health issues

The scallop farm site in New Caledonia is pristine with values of *E. coli* and *Enterococcus* spp. below 5 BCFU/100ml and 15 BCFU/100ml respectively for 2012 and 2013. Methods applied for the determining these two values are NF EN ISO 9308-3 and NF EN ISO 7899-2 respectively, under the local regulatory framework "Arrêté N 2010-3055 du 15/09/2010 – deliberation N 23/CP/2010" regarding physical, chemical and microbiological values in aquaculture sea waters. The Dumbea oyster farm site, on the other hand, is at a river estuary surrounded by residential suburbs. Counts of *E. coli* in seawater samples can be high at times (<15 BCFU/100ml following the reference method NF EN ISO 9308-3). For both types of bivalve there is currently only a single farm each, so monitoring of water quality need only be done at a single site.

In Fiji Islands, the river terraces in lower reaches support dense human populations with no sewerage systems. Livestock are grazed and pigs are raised right down to river banks, despite local-government regulations that require at least a 5 m buffer strip. Almost all of the major rivers in Fiji support *kai* fisheries. There are many points of collection along each river, ranging from up-river and pristine to down-river and highly urbanized or degraded. In Ba and Labasa there are sugar mills that discharge waste into *kai* rivers during the cane-crushing season.

Table 1. Water classification system, with control points for New Caledonia shellfish farming, based on Lee *et al*. (2008).

	Total coliforms (100 ml water)		Faecal coliforms (100 ml water)		
Classification	Geometric Mean	90 % compliance[1]	Geometric mean	90 % compliance[1]	Treatment required
Approved areas	≤70	≤230	≤14	≤43	None
Restricted areas	≤700	≤2300	≤88	≤260	Purification or relaying in an approved area
Prohibited areas	No sanitary survey or conditions for approved/restricted areas not met[2]				Harvesting not permitted

[1] Values for 5-tube decimal dilution test – different 90 percent compliance values are given for the 3-tube MPN and mTEC membrane filtration tests.

[2] Aspects other than the concentration of contaminants may be used to declare an area prohibited

The water quality of lower reaches of several major rivers in Fiji is known to be poor. Bacterial levels in the flesh of *kai* on sale at Suva Market can be unacceptably high. For example, Hatha *et al*. (2005) reported a total coliform load of between 1.1 x 10^4 to 1.1 x 10^5 per 100 g of *kai* flesh in samples purchased at Suva Market . Consumers do routinely place *kai* in a bucket of water for 24 hr before they are cooked and eaten, to allow *kai* to purge themselves of sand and grit, however this does little to reduce bacteria. Cases of shellfish poisoning as a result of eating *kai* are regularly reported in the newspapers, resulting in short-term bans on commercial sales of *kai* imposed by the Department of Health. In one instance (The Fiji Times, 2010a) the Health Department reportedly conducted tests for typhoid in *kai*, and the publicity given to this resulted in a slow-down in sales by *kai* vendors, who depend upon this commodity for an average income of around USD 60 per week. The human health issues surrounding *kai* in Fiji not only relate to the risks to consumers but also to periodic loss of income to *kai* fishers.

A market for frozen *kai* exists overseas, particularly among Pacific islanders now living in New Zealand and Australia. The relevant competent authorities regularly receive requests for export certification to send shelled and cooked or frozen *kai* to these markets. Such requests are routinely declined because of uncertainty about the health status of the *kai* being shipped. There is potential for *kai* product development to add value, for example smoked, chowder, canned, or cooked by Fijian recipes (The Fiji Times, 2010b). Investment in value-adding will continue to be deterred however, if food safety cannot be assured.

Responses to health issues

In New Caledonia, microbiological tests are carried out monthly at each farm site to determine the levels of total coliforms, faecal coliforms, and vibrios, following the current EU legislation (EEC, 1991; EEC, 1997). HAACP principles were used to develop a response plan, whereby threshold levels of bacteria in these tests (see Table 1) trigger use of a depuration protocol for harvested shellfish. The depuration protocol to be applied, if control point thresholds are exceeded, consists of maintaining the scallops or oysters in a chlorinated salt water tank for 48 hours. Chlorine compounds are used to disinfect the seawater used for depuration. For the purposes of depuration, 2 to 3 mg/l free chlorine is normally used for a contact time of up to an hour.

Regulatory oversight is provided by the New Caledonian Veterinary Services (DAVAR), who are in charge of the water quality control protocols. For this "fledgling" industry the cost of water quality monitoring are born entirely by the provincial government.

As strategies to communicate the "safe to eat"

message, Pacific oyster sales are either through one clearly recognisable retail outlet in Noumea, or are supplied direct to chefs at hotels and restaurants. Consumers have confidence in the DAVAR protocols.

In Fiji Islands the main strategy to protect human health is to ban commercial sales of *kai* every time there is a reported case of shellfish poisoning. Applications for permits to commercially export frozen or cooked *kai* overseas are routinely declined. "Un-official" export does take place in personal carry-on luggage, as gifts for relatives living overseas (Fiji Department of Agriculture, pers. comm.).

Suitable bacterial testing facilities do exist in Fiji, however un-depurated *kai* would fail in the majority of cases (Hatha *et al*., 2005). There is currently no infrastructure or capacity to operate and monitor shellfish depuration facilities in Fiji. There is no QA system in place for shellfish, or any supporting legislation to enable one.
The policy options for Fiji Islands are (i) to establish and monitor depuration facilities for harvested shellfish; (ii) establish a reputable QA system for edible shellfish in Fiji, and (iii) establish certification and labelling to build local consumer confidence.

These options raise issues of cost, however. The number of sites that will need monitoring is much larger in Fiji than in New Caledonia. It is certain that depuration will be needed for *kai* from almost all fishing sites. It will be difficult for consumers to differentiate between depurated and un- depurated *kai*. While depuration may be established and operated at a local level (for example, at specific village sites) there is ample scope for there to be "free loaders" on any investment in depuration (for example, via counterfeit labelling).

Conclusion

In New Caledonia the establishment of a shellfish QA system has enabled economic growth, by providing consumers with confidence about the shellfish they eat. This justifies a higher price for shellfish. The product can comply with export requirements, if necessary. The confidence of consumers then justifies investment in commercial aquaculture of bivalves.

In Fiji Islands, lack of shellfish QA causes economic losses. The risks to consumers result in interruption of *kai* fishers' livelihoods due to bans on commercial sales. Lack of consumer confidence locally holds the price of *kai* down low. Would-be exporters of *kai* find that they are unable to comply with export requirements. It is difficult to justify investment in commercial aquaculture or value-adding of bivalves.

Fiji Islands clearly faces practical problems to implement shellfish QA. If solutions to these problems can be found, such as public- or private-sector investment in technical transfer of protocols used in other places, then this would greatly increase the economic contribution of bivalve mollusk resources.

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Sanitary wastewater system assessments in Canadian bivalve molluscan shellfish harvest areas

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Abstract
Sanitary wastewater systems discharging to bivalve molluscan shellfish harvesting areas are assessed to determine appropriate shellfish area classification under the requirements of the Canadian Shellfish Sanitation Program. The objective of the technical assessments includes predicting the extent of impacts under reasonably-common failure scenarios. Assessments include: reviewing the operating history and status of the collection and treatment systems; the collection of samples of raw influent, post-treatment/pre-disinfection effluent and final disinfected effluent to determine representative fecal coliform concentrations; hydrometric drogue studies of receiving waters; and detailed hydrometric modelling to predict system failure impacts under varying operating and environmental conditions. The assessment results provide a greater range of shellfishery management options in adjacent harvesting areas, with increased confidence in wastewater impact prediction.

Keywords: shellfish, sanitation, classification, wastewater, modelling

Introduction

Environment Canada's Marine Water Quality Monitoring program supports the department's responsibilities under the Canadian Shellfish Sanitation Program (CSSP). The CSSP provides reasonable assurance that Canadians and consumers in other countries that Canadian shellfish such as mussels, oysters and clams are wholesome and safe to eat. (Canadian Food Inspection Agency *et al*., 2012)

The CSSP is a partnership between three federal ministries and agencies; the Canadian Food Inspection Agency, Environment Canada, and Fisheries and Oceans Canada, with expert advice provided by Health Canada. The CSSP also involves the cooperation of Provincial fisheries, aquaculture, and environment ministries. (Canadian Food Inspection Agency *et al*., 2012).

Environment Canada (EC) evaluates the environmental quality of shellfish growing areas and assesses the potential impacts of pollution sources. Based on the results of these surveys, EC recommends the appropriate classification of shellfish harvest areas to Fisheries and Oceans Canada.

Wastewater Treatment in Canada

There are over 3700 sanitary wastewater systems in Canada (WSER, 2010). Of those, some 320 discharge to marine waters in the vicinity of shellfish harvesting areas on the Pacific and Atlantic coasts, including Quebec.

Table 1. Summary of wastewater systems discharging in the vicinity of Canadian shellfish areas (Environment Canada, 2013)

Treatment Level		Treatment Type	
Primary	7%	Mechanical	41%
Secondary	84%	Lagoon	45%
Tertiary	1%	Other	14%
Other	8%		
Primary	7%		

Historically, the regulation of most municipal wastewater systems has been a Provincial government responsibility. In 2012, the Government of Canada implemented new Wastewater System Effluent Regulations (WSER) under the federal Fisheries Act. The WSERs will require that systems achieve effluent quality standards primarily attained through secondary-level treatment or better. Wastewater systems

deemed to be high-risk based on several environmental and water use factors are required to meet the new requirements by 2020 (WSER, 2010).

Approach

The initial assessment step is a review of the system infrastructure and performance. In addition to the treatment level and type of process at the wastewater treatment plant (WWTP), it is imperative that components of the collection system are identified, including: pumping or lift stations, sanitary sewer or combined sewer overflows, bypass points, and all discharge points and outfalls. Anomaly detection and contingency systems are also identified to determine common failure scenarios and the ability of the wastewater authority to react and manage such incidents.

Samples of wastewater are collected on a number of occasions from key points in the treatment process: raw influent, post-treatment/pre-disinfection, and final disinfected effluent. Samples are analyzed for fecal coliform (FC), and at selected plants, for male-specific coliphage (MSC). The frequency of sampling varies from site to site, but is intended to capture sewage characteristics over different environmental conditions. Periods of high and low flow are targeted, as well as seasonality particularly for long-retention systems such as lagoons. Generally, sampling occurs on 5-10 occasions, with 3-5 replicate samples collected at each point. The raw influent FC concentrations are used to estimate untreated discharges pumping station overflows or treatment plant bypasses. The post-treatment concentrations provide an estimate for disinfection system failures. The final effluent concentrations demonstrate normal operation of the system and complement routine receiving water quality monitoring in the adjacent shellfish growing area. MSC results are used as an indicator to estimate the log reduction values of viruses in sewage across the treatment and disinfection stages.

Concurrent with the sampling effort, a hydrometric study of the receiving waters is often conducted to determine tidal effects on effluent plume travel. GPS-equipped drogues are launched from outfall locations and tracked over various tidal cycles to determine the potential extent of contamination. The GPS track data retrieved from the drogues are then processed and used to verify model predictions.

Wastewater System Impact Modelling

Infrastructure information collected from wastewater system operators, influent and effluent quality data, hydrographic data (tidal ranges, river flow, bathymetry) and other environmental data (temperature, salinity, meteorological data) are input to modelling software. Environment Canada uses a suite of estimation tools to predict the extent of wastewater impacts depending on the pollution source and receiving environment. These tools range from rudimentary dilution models to sophisticated three-dimensional hydrologic models.

For sophisticated modelling, Environment Canada uses the MIKE software suite developed by the DHI Group. MIKE is used worldwide in coastal zone engineering applications to model contaminant transport. (DHI Group, 2013)

To evaluate various failure scenarios, parameters of sewage characteristic considered by the model include: 90th percentile of daily flow or peak monthly flow from system (depending on data availability); FC and MSC concentrations (raw influent, post-treatment/ pre-disinfection, final effluent); decay rates (FC and viruses); and, outfall diffuser characteristics. Environmental parameters include: tidal ranges and periods of peak tidal amplitude; tributary flow and contaminant concentration; water temperature (during harvest season); salinity; and wind (velocity and predominant direction during harvest season).

Model iterations are run for various failure scenarios (WWTP bypass, overflow at key lift stations, loss of disinfection, etc.). Once the most significant failure source condition is determined, iterations are run for peak tidal periods, and under selected wind conditions. The final selected scenario is then overlaid on marine water quality survey data which represent normal conditions (red and green areas). The combination results in a recommended shellfish growing area classification which is then implemented, managed and enforced by regulatory agencies.

To implement conditional harvesting management scheme, a supporting conditional area management plan is developed which identifies the roles and responsibilities of each CSSP agency and the wastewater system operator to ensure timely detection, notification and response to a failure and in closure of an area. Processors also have a key role in control of shellfish product harvested from these areas.

Conclusions

The current approach used by Environment Canada appears to be effective in assessing sanitary wastewater system impacts on bivalve molluscan shellfish areas. To date, there has been no evidence that contaminated shellfish have been harvested from areas within conditional management zones during normal operation of a wastewater system. Nor has there been evidence of contaminated shellfish being harvested from areas beyond conditional management boundaries when a failure has occurred and the conditional area is under closed status.

Predicted impact areas correlate well with hydrometric drogue study data. The paths and extent of drogue tracks over flooding and ebbing tidal excursions have matched well with the modelled currents and contamination transport limits.

Model results can be useful in planning dye dispersion studies to identify areas where dye plumes may travel. In addition, the empirical dye study results can be used to calibrate model predictions under matching conditions.

Two-dimensional animations of plume travel over several tidal cycles have proven very effective as a risk communication tool to fishery stakeholders. Particularly effective is illustrating contamination build-up during a failure event and that eventually, contamination levels will subside so that normal harvesting can resume after a safe period.

The higher-resolution modelling has resulted in a greater degree of confidence in shellfish area classification and management. In some areas, the classification can be refined and impact areas estimated by coarser models can be reduced. This reduces disruption to the shellfish industry while ensuring that food safety objectives are met and risks to consumers are minimized.

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Microbiological risk assessment (MRA) – useful or just feel-good?

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Abstract
In this paper we question the extent to which risk managers make use of risk assessment-based estimates. It often seems that risk managers consider it 'safer' to maintain testing regimes and conservative approaches based on the idea of 'zero tolerance'. Justification for conservative approaches has traditionally been based on the fact that it is the risk managers who bear responsibility for public health risk.
The paper reviews examples of risk assessors heeding, or ignoring, the advice of risk assessments and concludes that risk communication can be an essential component of risk management.

Keywords: risk management, risk communication, seafood safety, *Vibrio parahaemolyticus*, *Vibrio vulnificus*,

Introduction

For more than three decades HACCP has been the food safety management system of choice. Within it, Principle 1 requires a hazard analysis, involving an assessment of the severity of the hazard and the likelihood it will occur: in other words a risk assessment. However, HACCP plans often pay lip service to risk with the insertion of subjective terms such as 'serious' (for severity) and 'rare' (for occurrence) in a HACCP worksheet. It was not until 1994, when Notermans and colleagues published *"The HACCP concept: specification of criteria using quantitative risk assessment"* that HACCP and quantitative microbial risk assessment (MRA) were aligned (Notermans *et al*., 1995). Later, Canadian workers published on risk analysis (Todd & Harwig, 1996), and on quantitative risk assessment (QRA) as a tool for emerging pathogens (Lammerding & Paoli, 1996), and completed the first QRA on risks associated with consumption of hamburgers contaminated with *E. coli* O157:H7 (Cassin *et al*., 1998).

Work by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1998), Codex Alimentarius Commission (CAC, 1999), the International Commission on Microbiological Specifications for Foods (ICMSF, 1998) and the World Trade Organisation coalesced in rules for world trade (the Sanitary-PhytoSanitary ("SPS") Agreement and Technical Barriers to Trade ("TBT") Agreement (WTO 2007a, b). Those rules mandated that the only basis for restriction of international trade in foods was demonstration of unacceptable risk to human, plant or animal health in an importing nation due to imports from other nations.

In 1999, the United Nations Food and Agriculture Organisation (FAO) organised an expert consultation on the trade impact of *Listeria* in fish products, setting the scene for a series of risk assessments known as the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA), intended to provide advice to Codex. In 2001 JEMRA established a team to work on the risks to human health from vibrios in seafoods which, over the next decade, published assessments on *V. vulnificus* risk from raw oysters (FAO/WHO 2005a), *V. cholerae* risk from warm-water shrimp in international trade (FAO/WHO, 2005b) and *V. parahaemolytius* in seafood (FAO/WHO, 2011).

Now entering its second decade, MRA occupies a potentially important position in food safety management: due diligence, public health protection, import testing and entry to the World

Trade Organisation are all reasons for undertaking MRA. But how widely are risk estimates used by regulators at local, national and international level? In this paper we review some of the risk assessments completed during the past two decades in the context of the extent to which risk managers have employed the risk estimates produced by the risk assessors.

Vibrio cholerae in warm-water shrimp

Risk assessments

In 1997, the European Union imposed a ban on imports of prawns from Bangladesh because of perceived public health risk from presence of *V. cholerae*, a unilateral ban not followed by other major trading blocs. Using qualitative, semi-quantitative and quantitative models, a JEMRA *Vibrio* team estimated risks of contracting cholera from consuming warm-water shrimp in seven major importing countries (FAO/WHO, 2011). From the qualitative framework, the team estimated risk of cholera from consumption of shrimp raw (as sushi/sashimi), or cooked, as very low. To generate a risk rating and an estimate of predicted illness the *Vibrio* team then used a spreadsheet software tool called Risk Ranger. The tool, and its applications and limitations, are described in Ross and Sumner (2002) and FAO/WHO (2011). Using the tool, risk ratings were low, ranging between 25 (Japan and Spain) and 21 (USA, Italy, UK, France and Germany), the difference reflecting higher consumption *per capita* in the former countries. Annual illnesses were estimated at 1-2 cases/decade of cholera caused by consumption of warm-water shrimp in Japan, USA and Spain and approximately one case every 25 years for the other countries.

In a quantitative assessment, the *Vibrio* team modelled the steps from port-of-entry in the importing country to the point of consumption within that country. A Beta-Poisson dose-response model developed using data for the El Tor biotype of *V. cholerae* (Levine *et al*., 1988) was used and mean risk was evaluated using Monte Carlo simulation. The median predicted likelihood of cholera following consumption of imported warm-water shrimp varied between countries; for Japan it was 1-5 cases every five years, while for Germany it was 2 cases/century. These estimates accorded with the public health records from eleven major importing countries which, in 2000 for example, totalled 88 cases of cholera (from all sources) of which 71 were acquired abroad. The *Vibrio* team concluded that: *"... predictions of low risk by each of the approaches taken is supported by absence of epidemiological evidence that imported warm-water shrimp have ever been incriminated in a cholera outbreak in any developed nation in the world".*

Risk management

Despite the JEMRA team's key finding that cholera and warm-water shrimp are not linked epidemiologically, risk management approaches by importing nations remain based on port-of-entry (PoE) testing. Acceptance criteria vary from not detected/g (Australia) to not detected in 25g (e.g. USA, and others). In addition, despite the JEMRA assessment stressing that only toxigenic strains of *V. cholerae* cause illness, only the USA tests for choleragenic strains; other jurisdictions test for 'generic' *V. cholerae* and reject consignments on this basis.

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Table 1. Predicted and reported annual illnesses due to *V. parahaemolyticus* following consumption of oysters in Japan, Australia, New Zealand and Canada.

	Annual cases predicted by model	Epidemiological evidence for *V. parahaemolyticus* illness from oysters
Australia	91	2 cases in 18 years; two large outbreaks from other seafood sources
New Zealand	0	No cases during 1997–2002 from oysters; several outbreaks from other seafood sources
Japan	66	13 cases during 1998–2004
Canada	186	212 cases in decade 1997–2006 in British Columbia

Vibrio parahaemolyticus in oysters

Risk assessments

In 1997-98 in the USA there were four outbreaks and 683 cases of illness caused by *V. parahaemolyticus* following consumption of raw oysters (FDA, 2005). In 1999, a risk assessment undertaken by the Center for Food Safety and Applied Nutrition (CFSAN), the U.S. Food and Drug Administration (FDA) and the U.S. Department of Health and Human Services estimated that consumption of raw oysters was likely to cause 2,826 illnesses in USA each year, mainly (2,596 cases) in states bordering the Gulf of Mexico. It also explored 'what-if' scenarios for risk reduction with interventions focusing on reducing the temperature of oysters as soon after harvest as possible to reduce growth of *V. parahaemolyticus* (FDA, 2005). The JEMRA team also assessed the risk of contracting illness from *V. parahemolyticus* in oysters consumed raw in USA, Canada, Japan, Australia and New Zealand using the USA model. As may be seen from Table 1, the model over-predicted illnesses for Australia, Japan and Canada, reflecting differences in growing conditions and in species – *Crassostrea gigas* (Pacific oyster) and *Saccostrea glomerata* syn. *commercialis* (Sydney rock oyster).

Risk management

How risk managers mitigate the likelihood of illness from vibrios (*V. vulnificus* and *V. parahaemolyticus*) in oysters is illustrated by various approaches. The Canadian Food Inspection Agency requires harvesters to establish critical limits in their HACCP plan to *"... ensure shellfish are not exposed to sources of contamination or conditions allowing microbiological pathogens to grow to unacceptable levels during harvesting, holding, and transporting from the harvest area to the processing establishment";* in British Columbia this translated to icing oysters within one hour of harvest. In California by contrast, health authorities banned importation of oysters from Louisiana during the warmer months of spring and summer, citing recent deaths associated with consumption of oysters from Louisiana, supplier of 70% of California's oysters (Anon., 2003a). Banning of oyster harvesting in the summer months has also been the risk management option in Japan because of high counts of total *V. parahaemolyticus* in seawater (Ken Osaka, pers. comm.). In these examples we see two diametrically opposing responses: one to manage *via* a risk-based HACCP plan, the other to eliminate the risk by not allowing potentially contaminated product to be consumed.

Listeria monocytogenes in ready-to-eat (RTE) seafood

Risk assessment

In 2003 the CFSAN, FDA and the U.S. Food Safety and Inspection Service (FSIS) undertook a risk assessment of *L. monocytogenes* in a wide range of foods. On a per-serving basis smoked seafood and cooked crustaceans were estimated to cause 6.2 and 5.1 listerioses /billion servings, translating to 1.3 and 2.8 listerioses /annum in USA. At that time, epidemiological evidence linking listeriosis with smoked seafood and cooked crustaceans was confined to one outbreak in which three healthy people aged 83, 37 and 10 years in Australia became ill with symptoms limited to the gastrointestinal tract (Misrachi *et al*., 1991; Mitchell, 1991; Eyles, 1994). The illnesses, in 1991, followed consumption of New Zealand smoked mussels which had been illegally repackaged with use-by dates over 3 months beyond their original, and had *L. monocytogenes* >10^6 CFU/g.

Risk management

Since the mid-1990s, USA authorities have considered *L. monocytogenes* in RTE foods to be an adulterant and have applied a 'zero tolerance' policy requiring the absence of organism in 5 x 25g samples from a lot/consignment. In the 2003 risk assessment USA regulators make reference to other countries which did not apply zero tolerance e.g. Germany, Denmark and Canada. Nonetheless the risk assessment team, which contained representatives from regulatory authorities, maintained the adulterant/zero tolerance concept. The assessment also set arbitrary levels for perceived risk on per serving and cases/annum bases; which placed smoked seafood and cooked crustaceans in high-risk and moderate-risk categories, respectively. The judgement that an estimated four listerioses/annum from consumption of smoked seafood and cooked crustaceans in a

Table 2. Microbiological criteria for *Listeria monocytogenes* in ready-to-eat foods (Codex Alimentarius Commission, 2007)

Product	Point of application	n	c	m
Ready-to-eat foods in which growth of *L. monocytogenes* will not occur	From the end of manufacture or port of entry (for imported products), to the point of sale	5	0	100 cfu/g[a]
Ready-to-eat foods in which growth of *L. monocytogenes* can occur	From the end of manufacture or port of entry (for imported products), to the point of sale	5	0	25 g (< 0.04 cfu/g) [b]

Where n = number of samples that must conform to the criterion; c = the maximum allowable number of defective sample units in a 2-class plan; m=a microbiological limit which, in a 2-class plan, separates acceptable lots from unacceptable lots.
a This criterion is based on the use of the ISO 11290-2 method. (AS5013 24.2)
b Absence in a 25g analytical unit. This criterion is based on the use of ISO 11290-1 method (AS5013.24.1)

population of 300 million is 'high' or 'moderate' may be considered ultra-conservative by some.
Zero tolerance is still applied in many importing nations despite the recommendation of the Codex Alimentarius Commission (2007), which makes a significant distinction between foods that support the growth of *L. monocytogenes* and those that do not. The Codex guidelines state that products should contain <100/g *L. monocytogenes* at the time of consumption and that products which do not support the growth of this organism should be subjected to quantitative testing, rather than qualitative testing for the presence/absence in 25g. However, in foods that do support growth of *L. monocytogenes* a 'zero tolerance' policy is mandated, because of the potential to grow to levels during normal use that lead to an unacceptably high probability of illness, and due to the severity of listeriosis. The microbiological criteria specified by Codex are presented in Table 2.

Positive risk management

Use of risk assessment to rescind a regulation

Perhaps influenced by the USA assessment of smoked seafood and cooked crustaceans as high-risk foods, the Health Ministers of the Australia New Zealand Food Standards Council (ANZFSC) requested a review of microbiological limits for *L. monocytogenes* in fish and crustacea. As part of the review the Australia New Zealand Food Authority (ANZFA) included consideration of *L. monocytogenes* in cooked crustacea (ANZFA, 2002). While no epidemiological links were found between this hazard:pathogen pairing and no risk estimate was generated, a new regulation was promulgated in which *L. monocytogenes* was required to be absent in 5x25g samples. Following representations from the Australia prawn industry Food Standards Australia New Zealand (FSANZ) undertook a survey of *L. monocytogenes* in domestically-produced (n=230) and imported (n=139) prawns (Marro *et al*., 2003), in which the prevalence was 2% and 5%, respectively; no positive sample had a count greater than the limit of detection (50 cfu/g). The data, together with information on processing and storage, were used for a through-chain quantitative risk assessment (Anon., 2002) with the prediction of <1 case of listeriosis per annum in Australia, reducing to <1 case per millennium if the prawns were consumed the day of purchase, thereby mitigating the possibility of increase to an infectious dose.
The standards setting body (FSANZ) rescinded the regulation, a case where the risk estimate proved pivotal for a risk management decision.

Use of risk assessment tools by risk managers

In USA, a risk assessment tool to aid in management of *V. vulnificus* infection risk due to shellfish is used. The tool is based on a model that links *V. vulnificus* numbers in oysters to the date of harvest, which is related to the water temperature at harvest and temperature:time relationships during processing (Da Silva *et al*., 2012). For risk management purposes, the risk manager can manipulate harvest parameters and devise strategies to reduce the expected number of cases. As a target, risk managers were encouraged to find ways to reduce the number of cases by 50% of the baseline value.

Recently adopted Codex and EFSA approaches to risk management of *L. monocytogenes* in RTE foods consider that levels of 100 CFU/g in foods that do not support the growth of *L.*

monocytogenes do not pose an unacceptable risk to the health of consumers. A predictive model for the combined effects of temperature, water activity, pH, storage atmosphere and a range of organic acids on the probability of growth of *L. monocytogenes* in seafoods has been developed (Mejlholm *et al*., 2009) and validated (Mejlholm *et al.,* 2010) for a range of foods. This model, part of the 'Seafood Spoilage and Safety Predictor' software (http://sssp.dtuaqua.dk) is a powerful tool for management of the risk of *L. monocytogenes* is RTE foods, including lightly preserved molluscan seafoods.

The future – where might the risk managers take us?

Paralytic Shellfish Poisoning (PSP) in bivalve molluscan shellfish

It is suggested that there are ~2000 seafood-associated human cases of PSP globally per annum with a 15% mortality rate (Hallegraeff, 1993). A maximum permitted level of 0.8 mg kg^{-1} shellfish meat for saxitoxins in bivalves, which has been in place since 1958 in North America (Wekell *et al*., 2004) and is widely accepted as providing adequate public health protection, is recommended in the draft Codex standard for bivalves. This level is currently adopted by most shellfish-producing countries.

In 2004 a joint FAO/WHO/IOC expert consultation established a Lowest Observable Adverse Effect Level (LOAEL) for saxitoxins and applied a safety factor of 3 to arrive at an acute reference dose for saxitoxins in bivalve shellfish of 0.7µg kg^{-1} body weight (Toyofuku, 2006). In 2009, the European Food Safety Authority (EFSA) also assessed the risk of the saxitoxin group but derived an acute reference dose of 0.5 µg.kg^{-1} body weight and a maximum guidance level of 0.075 mg STX-eq kg^{-1} shellfish meat (Anon., 2009), more than ten-fold lower than the historical USA limit.

The maximum levels derived by the FAO/WHO/IOC *ad hoc* Expert Consultation and EFSA are significantly lower than the existing historical limit of 0.8 mg/kg though these limits have still not been adopted into international food safety policies, possibly because of the variation in toxicity of different saxitoxin congeners. However, in December 2012 in New Zealand 26 people were hospitalised following consumption of bivalve shellfish containing predominantly high potency congeners (e.g. saxitoxin) at levels close to the historical limit of 0.8 mg.kg^{-1}. Where risk management must account for wide variations in toxicity a conservative approach is appropriate.

Viruses in bivalve molluscan shellfish

In 2008 FAO/WHO coordinated an expert meeting to develop scientific advice on viruses in food in response to the increasing recognition of viruses as an important cause of foodborne disease, noting that norovirus is the most common cause of foodborne viral gastroenteritis worldwide and that hepatitis A virus continues to pose a significant threat. A key conclusion was that the highest priorities are noroviruses and hepatitis A virus in shellfish, fresh produce and prepared foods. It was recommended that intervention strategies should be focused on these virus:food combinations and that specific guidance should be developed for each commodity to minimise foooborne viral disease (FAO/WHO 2008). Following this, EFSA issued two scientific opinions on food borne viruses, including one focused on norovirus in oysters and recommended:

- the use of real time-PCR as a means of detection and quantification of norovirus in oysters;
- determination of a safe limit for norovirus genogroups I and II in oysters; and
- development of treatment regimes that can be relied upon to reduce norovirus counts in oysters.

The EFSA Panel recommended that *'risk managers should consider establishing an acceptable limit for norovirus in oysters to be harvested and placed on the market'*, and that testing of oysters should be used to verify compliance with the limit. It was further noted that testing of oysters for norovirus should be considered by both food safety operators (industry) to demonstrate compliance with their HACCP plan and by competent authorities (Anon., 2012). So, will the future see microbiological criteria for norovirus integrated into food control plans? EFSA suggest that such a move *'would reduce the*

number of contaminated oysters placed on the market and therefore the risk for consumers to become infected', but at what cost?

Final thoughts

Zero tolerance (with its implied zero risk) and the precautionary principle are anathema for risk assessment because they consider all hazards so serious that they may not be present at any concentration. However, for pathogens with relatively high ID_{50}, foods that preclude their growth can represent a low risk to public health. In the case of toxins in foods, the zero tolerance concept is yet more indefensible because, as laboratory detection becomes more sensitive, we can expect food which today is acceptable to become 'unacceptable' for human consumption, e.g. farmed seafood containing veterinary residues. As this paper was in preparation an Italian court convicted six scientists of manslaughter for underestimating the likely effect of an earthquake which, when it came, killed 309 people in L'Aquila. The case prompts the question: will the six years in jail and the $10 million in costs and damages incurred by the six scientists prove a deterrent to the use of risk assessment, including MRA?

This example highlights an apparent paradox: if we accept the idea of risk assessment then we must also accept that sometimes the hazard will be present in a food and lead to undesirable affects for consumers. The challenge is to achieve consensus with all stakeholders on an acceptable level of risk that optimises the benefits to all. To build risk management strategies around 'zero-tolerance' means that there will be a cost e.g. in terms of cost of the foods due to increased testing, or their reduced availability, or increased processing and additives to make the foods safer. Risk managers generally appear to be loth to explain the costs and benefits of alternative risk management approaches, and to allow consumers to take some responsibility for their own level of risk. This approach, however, is the third domain of the risk analysis paradigm - risk communication. Increasingly, there are examples of use of risk communication as part of a risk management strategy e.g. advice brochures about foods to avoid due to the risk of listeriosis for pregnant women; advise to restaurant patrons in southern states of USA with certain illnesses, about risks of consumption of raw oysters at certain times of year. Without active risk communication as part of the risk management strategy, risk assessors and managers will have their hands tied.

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Dye tracing techniques used in NSW shellfish growing areas to assess pollution sources

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Abstract
The New South Wales (NSW) Food Authority – Shellfish Program has successfully employed a practical and cost effective dye tracing technique that has led to the positive identification of pollution sources impacting shellfish growing waters. Fluorescing dyes have long been utilised for hydrological and geological studies generally relying on a positive visual identification or water sample collection for in field fluorometer use to assess stream and groundwater flows and determine leaks from pipes and sewage systems. The technique utilised by the NSW Food Authority to assess pollution sources utilises activated carbon to adsorb dye which can be eluted in a laboratory for testing via spectrofluorophotometers. The advantage of this technique compared to either visual identification or a standard grab water sample is that it maximises the possible detection of tracer dyes and minimises the number of samples and overall sampling effort, in turn reducing overall cost. Activated carbon sample packets can be left unmonitored in the environment to ensure longer term exposure to possible dye detection, thus increasing the chance of recording a positive reading.
This technique has successfully been utilised in NSW shellfish growing waters which has led to the remediation of known pollution sources impacting shellfish growing waters.

Keywords: Dye Tracing, Charcoal, Carbon, Pollution Source Tracking

Background
There are 71 shellfish harvest areas across 27 estuaries and embayments in NSW with all areas being formally classified as either Approved for direct harvest or Restricted with mandatory depuration prior to human consumption (ASQAP, 2009) . One of the largest threats to the shellfish industry is the urban sprawl encroaching on existing shellfish harvest areas resulting in an increased risk from potential pollution sources. The technique outlined in this presentation is a cost effective means of assessing such pollution risks arising from an increase in population throughout shellfish harvest areas.

Dye tracing using fluorescing dyes has been a well established method in hydro-geological studies, stream flow studies and pipework studies and is now being employed as a method to evaluate potential sources with the potential to impact shellfish growing areas.

There are 5 commonly used fluorescing dyes. Fluorescein, Pyranine, Eosine, Sulphorhodamine B and Rhodamine WT. Each dye will only fluoresce under a particular wavelength of light which makes a positive identification of a particular dye a reliable result.

An evaluation study of fluorescing dyes was completed (Smart & Laidlaw, 1977) to determine the most suitable dye for water tracing and considered varying factors including sensitivity and minimum detectability, the effect of water chemistry on dye fluorescence, photo-chemical and biological degradation rates, adsorption losses, toxicity to both man and the environment. The outcomes from this report showed that the red dyes were more accurate than the green dyes due to general lower background interference levels. Pyranine was found to be strongly affected by pH over the range encountered in natural waterways. Fluorescein was found to display a high photo and chemical decay rate, particularly in the presence of Chlorine which most reticulated water is treated with and fluorescein is also used by local government authorities and plumbers for pipework assessments which may compromise any elevated

results. The red dyes of Sulforhrodamine B and Eosine have been found to have undesirable biological effects at high dosages in the environment and displays carcinogenic properties and its use should be discouraged. Rhodamine WT has been found to have no specific health effects, has a lower impact from photo and biological degradation and a moderately high resistance to soil adsorption. Due to these properties Rhodamine WT is the preferred tracer dye of choice employed by the NSW Food Authority.

The charcoal adsorption method outlined in this presentation relies on the use of activated carbon (charcoal) as a medium to adsorb trace amounts of dye. Charcoal is extremely porous, has an incredibly large surface area and effectively traps dye within its structure. Charcoal used in this method is activated carbon that is contained within fibreglass screening, commonly used as fly screen for windows that is heat sealed on three sides to form a pouch approximately 8cm square where 5g of laboratory grade activated carbon is placed inside of before being fully sealed. The benefits of this method are multiple and include the ability to remotely assess the presence of dye without physically being in the field throughout the duration of the study, this is particularly effective in remotely accessible areas. The charcoal adsorption method can be used to cost effectively assess suspected failing on-site sewage management systems, reticulated sewerage networks, suspected broken pipes, pipes discharging unknown wastes and effluent holding ponds on farmland.

Methodology

Methodology employed includes placing charcoal packets inside existing oyster infrastructure for example mesh oyster baskets or containers that allow a free pass of water flow but also provide some shadowing of sunlight to prevent potential photo-degradation of the dye. Sample sites are best located within existing currents and channels where water movement is maximised if testing within estuarine river systems, however if testing is being conducted inside an embayment or off-shore area then sample sites are best situated immediately adjacent to the system being investigated. If infrastructure is not suitable at the chosen sample site then floating mesh oyster baskets can be used and anchored to a sample site with a suitable anchoring system. The number of charcoal packets to be used is determined by the number of samples required from each site. NSW Food Authority collects samples following 24hr, 3 days and one week submersion at each site after the dye has been added to the system, this allows a short, medium and longer term assessment of the pollution source.

Using this approach, a minimum of four charcoal packets per site are required. It is also wise to include a spare charcoal packet per site in case a packet is compromised in some way. All charcoal packets for all samples are submersed at the designated sample site for one week prior to dye being deployed to obtain a background reading. Following one week submersion at the sample site one packet per site is removed to act as a background sample. Dye is then added to a sewage system or water body being assessed. If assessing onsite sewage systems, house owners are asked to run taps and flush toilets in order to get flow running through the system.

Collection of the charcoal packets requires strict sampler awareness to ensure clean uncontaminated samples. A new pair of disposable latex gloves are to be used at each sample site to handle each separate charcoal packet to prevent cross contamination. Packets are to be removed from the sample container and rinsed gently in the water to remove any built up detritus. Samples are then wrapped in aluminium foil to prevent photo-degradation. The foil wrapped sample is then placed into a zip-lock plastic bag with a label written on the outside with a black permanent marker pen. Coloured marker pens are not to be used as some; especially red markers may have fluorescing compounds that will compromise the sample. The foil wrapped charcoal packets inside the zip-lock bags are then placed into a cooler and kept out of sunlight. Contained ice inside bottles can be used to keep temperatures down and prevent algal and fungal growth on the charcoal medium. The use of ice bricks or packaged blue ice packs are not to be used as they may contain fluorescing dyes and compromise the sample.

Once packed samples are then shipped to the laboratory for analysis.

Analysis

The NSW Food Authority does not conduct analysis in house but uses independent 3rd party laboratories for analysis. The following is a breakdown of the laboratory methodology employed by the University of Newcastle which is a similar method used by Aley (2002). A standard elution solution of 5% aqua ammonia and 95% isopropyl alcohol is used to flush each sample packet individually. Potassium hydroxide is added until a super saturated layer is visible at the bottom of the container. Preparation of elution solutions use dedicated glassware which has never been used in contact with dyes or dye solutions. The eluting solution is poured over the charcoal inside a disposable sample beaker. The beaker containing the charcoal and elution solution is capped and allowed to stand for 60mins. Following this the solution is poured off the charcoal into another beaker. A 3ml sample of the elutant is withdrawn using a disposable pipette and placed into a rectangular polystyrene cuvette. The cuvette is then placed inside the spectrofluorophotometer for analysis.

Cost

Perhaps one of the largest benefits of using this technique is the low overall costs needed to set-up, analyse and receive results. Activated charcoal used is approx $15/kg for activated coconut charcoal. If using 5g per sampling packet, a 1kg amount of charcoal will be enough to produce 200 sample packets, this equates to 75c per packet.

Fly screen or pet mesh used to make the sampling packet pouches is available from standard hardware shops and is sold in various lengths. A 2m x 1m (6ft x 3ft) length of screen is approx $30 and will be large enough to produce 320 sample packet pouches. This equates to 9.3c per sample packet pouch.

Rhodamine WT dye used was imported from the United States at a cost of $1042 for 40L (5gal) and is enough to conduct between 20-40 individual tests, pending volumes of dye used. This equates to $26-$52 per test.

Analysis is under taken by a 3rd party laboratory at a cost of $100 per sample with a saving of 20% when 15 or more samples are submitted for analysis which can bring the costs down to $80 per sample.

Overall the cost per sample when all of the individual components are added together is approx $80.84 taking into account savings on more than 15 samples being analysed.

To save costs on long term investment the purchasing of a personal fluorometer may be an option. The Turner Designs fluorometer used by the University of Newcastle costs approx $5000 which will pay for itself after just 50 samples. Consumables will be the only cost to consider which will be approx $26-$52 for the dye and 0.84 cents for the sample packets.

Summary

The NSW Food Authority has successfully employed the charcoal packet method for positive dye detection on a number of on-site sewage management systems which has led to local government area municipal councils taking remediation actions on failing systems showing that the dye tracing using the charcoal packet method is a low cost effective means of evaluating pollution sources with the potential to impact shellfish growing areas.

Acknowledgements

Dr Steven Lucas - Newcastle University

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The use of fluorescent whitening compounds as signals of human sourced contamination

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Abstract
In sanitary surveys of estuaries, faecal contamination is typically assessed by microbial indicators which are ubiquitous in the environment and cannot be used to distinguish between sources of faecal contamination. The ability to distinguish between human and animal sources of contamination is particularly important where estuaries are used for commercial aquaculture and there is a need to know the fate or pathway of contaminants from domestic on-site wastewater management systems (OWMS). Chemicals associated with human metabolism and behaviour and which are also present in faecal material (such as caffeine, faecal sterols, and pharmaceuticals) have been used to characterise and identify human faecal contamination with limited success. One such group of chemicals are fluorescent whitening compounds (FWCs) which are present in wastewaters containing laundry washing products. These compounds have been used to a limited extent to assist in identifying human contaminant sources in estuaries used for aquaculture. This paper will describe methods used to further develop the application of this technique for sanitary surveys and outline its usefulness as an indicator of human sourced contamination.

Keywords: contamination, fluorescent whitening compounds, source tracking, sanitary survey

Introduction
There have been a number of highly publicised cases of contaminated estuarine waters used for oyster growing in NSW, Australia (Geary and Whitehead, 2011), and in each case, possible sources of faecal pollution have been identified from agricultural areas, waterway users, runoff from urbanised areas and unsewered small communities. In terms of human contaminant sources (apart from a direct sewerage treatment plant discharge to an estuary), it is difficult to separate the overall impact that OWMS have to estuarine water quality. In a number of these cases however, human viruses have been found in estuarine filter feeders such as oysters, so a human source of contamination has to be acknowledged.

There are a variety of methods which have been used to distinguish wastewater contaminant sources in the environment. Of the source tracking methods available, a substantial amount of work has been undertaken examining the fate of human derived effluent using the fluorescing property of optical brighteners. These FWCs which are added to washing powders to adsorb to fabrics and brighten clothing by fluorescing when exposed to ultraviolet light, are also regarded as good markers or indicators of human wastewater contamination in environmental waters, and where present with faecal bacteria, may suggest that failing OWMS are present (Table 1).

Table 1. Likely cause of faecal contamination when certain numbers of faecal bacteria and levels of FWCs are observed (after Hartel *et al.,* 2007).

Faecal bacteria numbers	FWC concentration	Likely cause
High	High	Failing OWMS or leaking sewer pipe
High	Low	Human or other warm-blooded animals
Low	High	Grey water in storm water runoff
Low	Low	No evidence of faecal contamination

The optical brighteners in these laundry products emit light in the blue range (415-445 nm) to compensate for undesirable yellowing in clothes (Hartel *et al.,* 2007). FWCs as indicators of human sourced contamination have been used in New Zealand, while groups in the USA have also used this effluent property in sanitary surveys to identify wastewater from failing on-site systems in sensitive oyster growing areas. Devane *et al.* (2006), Gilpin *et al.* (2002) and Hartel *et al.* (2007) have reported the usefulness of this technique when combined with other indicators including human faecal sterols, although natural organic matter can also contribute to some of the observed fluorescence in estuarine water samples. A method using UV exposure to differentiate fluorescence from natural organic matter has also been reported (Cao *et al.,* 2009).

Another technique which has been used to distinguish between contributions from various sources of faecal contamination in estuarine waters is faecal sterol analysis (FSA). Distinguishable sterol profiles for humans, herbivores and birds have been found to be sufficiently distinctive to be of diagnostic value in determining whether faecal pollution is of human or animal origin (Leeming *et al.*, 1996). FSA has been successfully used to trace faecal pollution in similar estuarine environments and has also been used in part of this study.

Methods

In this study the capabilities of two Turner Designs® fluorometers (Trilogy and Aquafluor) were investigated in the laboratory to detect FWCs in a variety of samples from known and unknown sources. In addition, field work was undertaken in 2011/12 at an unsewered development at North Arm Cove, Port Stephens (NSW) to examine the usefulness of the FWCs as a human signal indicator in stormwater runoff entering the estuary and within the estuary itself. Stormwater runoff samples were collected following a number of rainfall events and estuarine waters were also sampled and analysed. The FWC work has been combined with FSA work and the traditional bacterial indicators to determine whether the OWMS were likely to be contributing to the faecal contamination currently being experienced within the estuary following rainfall.

Within this estuary there are two small unsewered communities on rocky and steep foreshores. Many of the OWMS are close to the water, visible from a survey boat and directly adjacent to the harvest area and leases. Inspections of the OWMS by Great Lakes Council which are conducted at intervals of 12 or 24 months have been primarily aimed at identifying system failure and/or illegal greywater disposal. In North Arm Cove, which is the larger of the two communities, the number of OWMS totalled 307 with the predominant systems being pump-out (101), aerated (94) and soil absorption systems (88). The remaining 24 systems comprised biological, chemical, composting, soil mound, sand and wet composting systems. All pump-out systems with respect to pump truck volumes are periodically monitored to assess whether effluent is being discharged from the holding tanks.

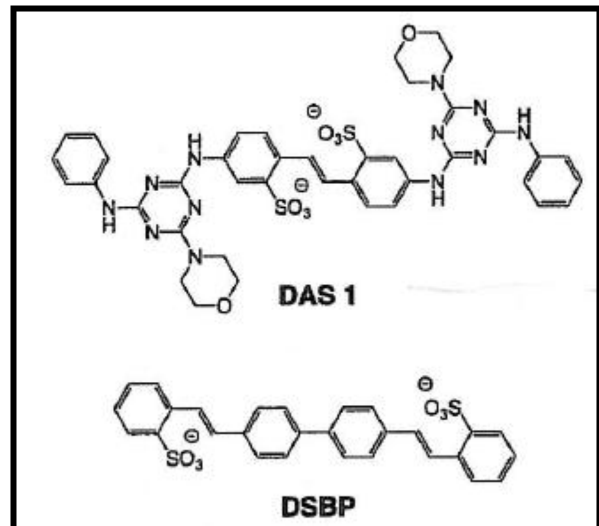

Fig. 1. Chemical structures of the two types of FWCs used in Australian laundry powders (Stoll & Giger 1998 pp. 2042).

There are two primary types of FWCs that are added to laundry powders which are: disodium distyrylbiphenyl disulfonate (DSBP or FWA5) and disodium anilinomorpholinotriazinyl aminostilbene sulfonate (DAS1) (Fig. 1). Laundry detergents contain approximately 0.10-0.20% (w/w) of FWCs (with DAS1 used in greater weight proportions), of which between 20-95% binds to the fabrics during washing, with the remainder being discharged (Devane *et al.,* 2006; Stoll *et al.,* 1997). There is no regulatory limitation on the dosing of FWCs in laundry applications. The dosage essentially comes

down to cost versus performance; there is a peak (or plateau) in performance when using these compounds, thus rendering an overdose both expensive in terms of formulation cost and useless in relation to performance enhancement.

Results

Work undertaken in the laboratory by Kable (2012) has determined which commercially available laundry products contained none, one or both of these identified FWCs. Work also undertaken on the fluorometers found that there is a difference in their capability with respect to their ability to determine each of the fluorescent response signals for the two compounds. The Trilogy fluorometer performed better than the Aquafluor for a range of standard concentrations of each compound. While not all commercially available laundry washing powders contained both FWCs, the response signal was clearly stronger for the DSBP compound, so this was typically used with the Trilogy instrument in analysing unknown samples collected as part of the field work.

With regard to the field work at North Arm Cove, six water samples were collected during four runoff events in 2011/12 and the results are summarised in Table 2. The four stormwater collection sites (apart from the two creeks which enter the estuary) represent stormwater runoff from the unsewered community. The results presented in Table 2 are mean concentrations for the rainfall events sampled (Kable, 2012). There was an FWC signal at each of the stormwater runoff sampling sites as well as a significant faecal bacterial component which may have an impact on the oyster growing waters of the estuary. Faecal sterol analyses were not undertaken for these samples.

In addition, eight water samples were also collected by the NSW Food Authority from sample sites on the foreshore and within the estuary at North Arm Cove during one rainfall event on 24/11/11. The rainfall event which occurred between 22 and 27 November was sufficient to result in the closure of the harvest area (where the rainfall event exceeded 40mm in 48hrs). The rainfall between 22 and 24 November (when sampling occurred) was 53.5 mm according to records obtained from near the estuary. The water quality data in Table 3 relates to electrical conductivity, faecal bacteria, faecal sterol (coprostanol) and FWC concentrations. Sample sites 1, 2, 3 & 4 are sites within the estuary adjacent to the oyster harvest areas where there is regular testing for faecal coliforms. Site 2 is at the head of the harvest area and within the estuary, but adjacent to a smaller tributary stream which was not sampled on this occasion. Sample site 7 is Bundabah Creek which enters the estuary, while samples 9 and 10 are from the main stormwater drains from the urban development around the estuary. Sample site 11 is a control site away from urban development in the Port Stephens estuary.

Table 2. Mean stormwater quality runoff quality for rainfall events sampled at North Arm Cove (Kable, 2012)

Site No.	EC (mS/cm)	E.coli (cfu/100 mL)	FWC (µg/L)
1U	0.204	18442	22.2
1D	0.168	10017	22.5
2	0.150	3797	24.8
3	0.225	3008	23.5
Bulga Creek	0.787	6875	22.2
Bundabah Creek	17	4661	23.5

Table 3. Summary of measured key parameters in water samples collected 24/11/11

Site No.	EC (mS/cm)	FC (cfu/100 mL)	FWC (µg/L)	Coprostanol (ng/L)
1	36	36	2.11	0
2	21.5	110	12.08	20.2
3	36	24	1.44	0
4	36	2	1.05	0
7	17	240	14.15	21.3
9	stormwater	128	19.85	3.2
10	stormwater	1900	21.89	22.2
11	30	3	0.8	0

The bacterial analyses revealed high faecal coliform (FC) counts (>85 cfu/100mL) at four sites – the two stormwater sites (sites 9 and 10), the main creek entering the estuary (site 7) and site 2

in the harvest area. At each of these sites, salinity was reduced due to the freshwater inflows to the estuary during and following the rainfall event. The four water samples with the highest faecal coliform counts also returned the highest concentrations of fluorescent whitening agents. While there are no standards for FWCs in environmental waters and little data available locally, these concentrations are elevated relative to the control site (site 11) and sites 1, 3 and 4. Studies which have been undertaken overseas and using different analytical instrumentation to that used here have suggested that FWC concentrations similar to those at sample sites 2, 7, 9 and 10 are also indicative of the presence of human sourced contamination. These four samples were also the only samples in which coprostanol, the key sterol indicator of potential human faecal contamination, was detected.

With regard to the interpretation of the FSA results, faecal contamination of human and/or bovine origin is generally indicated by a coprostanol/cholestanol ratio $\geq$ 0.4 which was observed only in the case of sites 2 (0.46) and 7 (0.42), although a value close to this threshold (0.36) was also recorded for site 10. In these cases the low epicoprostanol/coprostanol ratios indicated that the contamination involved relatively fresh faecal material while the non-detection of 24-ethylcoprostanol suggested that the contamination was more likely human than bovine in origin. While the ratio values calculated for these samples (not shown in Table 3) are indicative of likely human faecal contamination, it should be recognised that the interpretive reliability of the ratio analysis is far less robust at relatively low coprostanol concentrations such as those detected here. Notwithstanding, detection of coprostanol and high FC counts in these samples in conjunction with the highest FWA concentrations, may indicate that these sites had been contaminated with a detectable level of human wastewater. These results are preliminary and certainly suggest that further investigation and sampling as part of the program are warranted.

On the basis of these limited results, it is not possible to clearly state that the faecal contamination present in the estuary is directly derived from a human source. One of the reasons for this is the presence of naturally occurring organic matter in stormwater runoff which contributed to some of the fluorescence signals recorded. Further investigation into the use of FWCs as an alternative marker or indicator to detect the presence of a human source of contamination is still required. In summary, FWCs were able to be detected in laboratory samples, in effluents and in environmental samples using the instruments mentioned above. The test does therefore appear to have the potential to be used as a rapid initial field screening method to identify human derived contamination in shoreline sanitary surveys, however, the contribution from naturally occurring organic matter does need to be differentiated in stormwater runoff samples.

Conclusions

Demonstrating direct linkages between the wastewater management practices of small communities and estuarine water quality is difficult at the catchment scale and may not be possible using standard monitoring techniques and microbiological indicators. There is a close relationship between the wastewater disposal practices in small communities, increasing development, and the need to maintain estuarine water quality so that aquaculture such as oyster growing can be undertaken without compromising human health. Determining the fate of surface and particularly subsurface contaminants from the land application of wastes is difficult and often expensive. Whilst traditional faecal indicator bacteria have been used for a variety of reasons (ease of analysis, cheaper cost, easier interpretation), other techniques can assist in determining whether the contaminant sources in estuaries are likely to be human or animal.

Acknowledgements

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Integrated water quality forecasting system for shellfish harvesting areas

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Abstract
The Annapolis Basin is prone to contamination by fecal coliform bacteria as a result of precipitation events. Contamination levels are sufficient to force the closure of aquaculture lease areas for several weeks each year negatively impacting the approximately 170 soft-shell clam harvesters in the area. We have developed a system to track contamination and predict areas of poor water quality (72 hours). Each forecast predicts the concentration and spatial extent of contamination and is tailored to provide water quality reports and statistics for shellfish harvesting zones within the area. Forecasts are determined by linking dynamic environmental variables to a robust database of contamination sources, estuarine hydrodynamics, watershed hydrology, and fecal coliform loading scenarios. Watershed loading concentrations and decay rates of fecal coliform are calculated using hydrological, hydrodynamic, and advection dispersion models which incorporate land cover attributes and flow dynamics. At each watershed loading confluence, estuarine hydrodynamics of the basin are modeled to simulate the transport and dispersion of suspended fecal coliform in response to dynamic tidal elevations. Model data can assist decision makers in identifying and mitigating the contamination problems while minimizing resource investments. Direct and indirect model results can serve to minimize risk of contamination and reduce closure periods.

Keywords: Hydrodynamics, particle tracking, watershed, environmental model

Introduction

The Annapolis Basin in Nova Scotia, Canada supports a rich population of soft-shell clam (*Mya arenaria)*. At present, approximately 170 clam harvesters in the area produce an annual $2.3 million (CAD) in revenue farming the resource. The basin is prone to contamination by fecal coliform bacteria as a result of precipitation events. The Canadian government implements controls through the Canadian Shellfish Sanitation Program (CSSP) to ensure only products that achieve strict safety and quality standards reach domestic and international markets. These controls require Environment Canada to monitor water quality around shellfish harvest areas and confirm fecal coliform levels do not exceed minimum threshold (14 MPN/100 ml). Regulatory sampling within the basin after rainfall events often reveals fecal coliform levels exceeding the minimum threshold, resulting in an indiscriminate closure of all Annapolis Basin harvesting activities for several weeks each year. At present, regulatory water quality monitoring is limited to the basin itself. Wastewater treatment plants are considered the primary source of fecal contamination, while other point and non-point sources identified by regulators have been limited to the immediate basin area (Roberts *et al*., 2009). As a result, contributions of fecal bacteria from watersheds draining to the basin are not well understood. The current state of industry regulation ensures the safety of consumers, but does not address sources of contamination. As a potential solution, we set a framework for investigating and tracking these sources within the Annapolis Basin, Nova Scotia.

The integrated forecast system in this study was developed to help improve the understanding of how contamination is transported within the Annapolis Basin estuary and surrounding watersheds. Our approach incorporates contaminant source location data, meteorological models, watershed models, and estuarine models to monitor fecal coliform contamination in real-time. Further, contamination levels and their sources are monitored over shellfish beds. These data are

stored in a long-term database for further analysis. Model data can assist decision makers in identifying key sources of environmental contamination, thus aiding in both mitigating contamination problems, and minimizing resource investment. Long-term analysis of model results will help improve the understanding of how contamination is transported throughout the basin, and will serve to help regulators reduce closure periods while ensuring there is no risk to the consumer.

Methods

A crucial aspect of hydrodynamic modeling is the accurate definition of geomorphology within the study area. Bathymetric geomorphology was modeled using high resolution multibeam echosounder survey data provided by the Department of Fisheries and Oceans (DFO). Surveys were supplemented with Canadian Hydrographic Services (CHS) chart sounding data, where required. Terrestrial geomorphology was modeled using high resolution light detection and ranging (lidar) survey data acquired and processed by the Applied Geomatics Research Group (AGRG). Inland terrestrial elevations that fell outside the 1 m resolution lidar survey extent were obtained from the Nova Scotia Topographic Database (NSTDB) 5 m resolution contour data. The surface was gridded as a 1 m cell resolution terrestrial digital elevation model (DEM) for watershed modeling, and a 50 m cell resolution bathymetric DEM for the purpose of estuarine modeling (Fig. 1). Accurate meteorological data were also a crucial model input, and were obtained from project partner Scotia Weather Services Inc. (SWS). Precipitation, evapotranspiration and ultraviolet radiation predictions were provided as a 72 hour forecast every 6 hours for each watershed in the study area.

Watershed drainage extents and river networks were delineated using the high resolution terrestrial DEM and validated using coarser NSTDB provincial datasets. Watershed hydrological, hydrodynamic and particle tracking models were created using the Mike 11 software suite produced by DHI. A total of 60 models were developed for watersheds around the Annapolis Basin estuary. Watersheds with known contamination problems, large drainage areas, or desirable distribution were selected as calibration areas (n = 6) and were gauged to monitor discharge (Fig. 1). The hydrological model component was used to generate lateral inflows to river networks from catchment areas in response to precipitation events using a rainfall dependent inflow and infiltration (RDII) framework. Differing physical characteristics of each catchment impacted the capacity of surface storage, lower zone storage, and ground water storage areas. Specific model parameters, which managed the routing characteristics between stores, were adjusted to match the gauging station records in response to precipitation events provided by the SWS meteorological model. Each study watershed was characterized based on the proportion of land-cover and surface geology using data from the NSTDB and terrestrial DEM. The relationship between RDII model parameters and watershed characteristics was assessed and used to develop RDII models for watersheds without gauging stations. The hydrodynamic watershed component was used to route the lateral inflow produced by the RDII model through river networks and produce output discharge volumes and current magnitude results at the confluence point between watershed and estuary. River branch dimensions were calculated using high resolution DEM and field surveys at several culvert and bridge locations. The particle tracking component of the watershed model used the hydrodynamic data to attenuate and disperse contamination entering the system. Loading concentrations were calculated by assessing the number of homes without town sanitation and the land-use with specific attention to agricultural areas. Point source impacts were also assessed by identifying the direct input points, such as wastewater treatment plant effluent pipes and town grey-water pipes. Simpler models were developed for these point sources to simulate contaminant releases in response to precipitation thresholds. The integration system was designed to run all watershed models in unison with the SWS weather forecasts every six hours.

Estuarine hydrodynamics and particle tracking were simulated using the Mike21 software suite produced by DHI. All hydrodynamic and particle tracking results were stored in a database due to

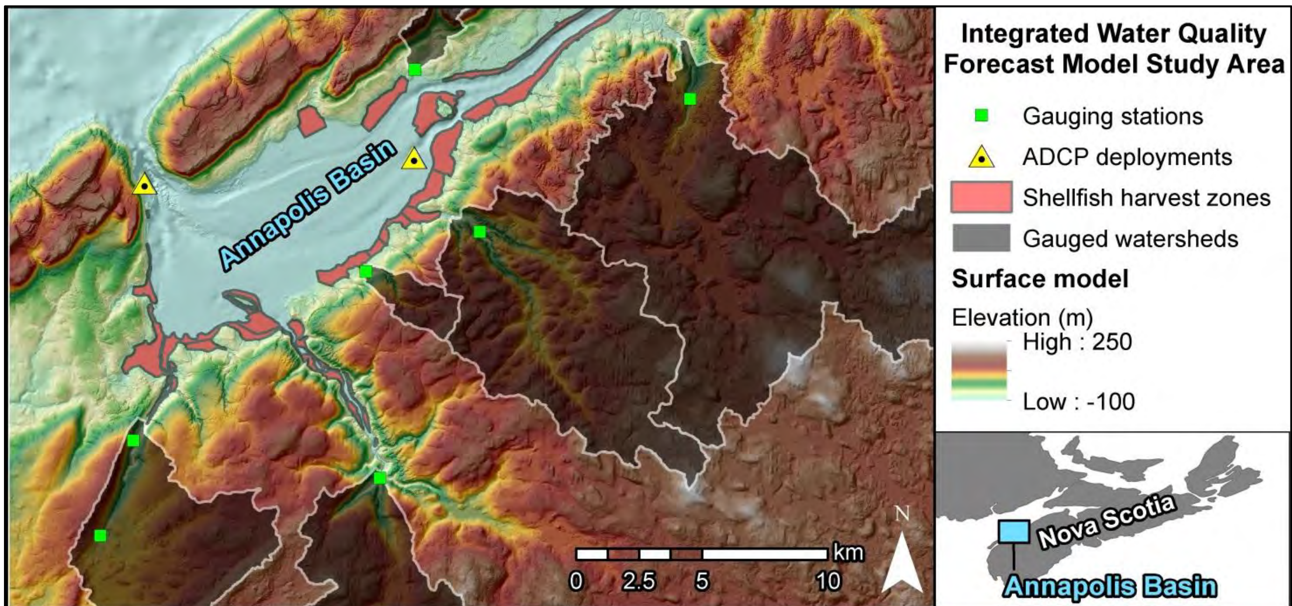

Fig. 1. Digital elevation model of the Annapolis Basin surface geomorphology highlighting gauged drainage areas and ADCP locations

the forecasting nature of the system and the length of time required to run these models. Tidal predictions were obtained for the Annapolis Basin at a five minute resolution for an 18.6 year tidal cycle using the Department of Fisheries and Oceans WebTide Tidal Prediction Model (v0.7.1). Estuarine hydrodynamics were simulating by calculating water level variations and flows over the bathymetric surface in response to these tidal predictions. Two acoustic Doppler current profilers (ADCPs) were deployed to validate water surface elevation, current direction, and speed (Fig. 1). The estuarine particle tracking component simulated contaminant attenuation and dispersion in response to the modeled hydrodynamics. Nominal particle tracking release concentrations were modeled at each confluence between a modeled watershed and the estuary and stored within the database.

Results and Discussion

Estuarine hydrodynamic simulations and were found to be comparable to data collected using ADCPs (surface elevation: Pearson's correlation = 0.99, r^2 = 0.99; current magnitude: Pearson's correlation = 0.89, r^2 = 0.78; current direction: Pearson's correlation = 0.79, r^2 = 0.61; n = 5064). Correlation coefficients of both current magnitude and direction were low due to model result averaging over the 50 m grid cell. Using a finer grid resolution may have improved model results, but computing time and space restraints made this unrealistic, especially given the level of accuracy already achieved. Early results of nominal particle tracking runs in the estuary are comparable between modeled contamination extents and field samples collected after rainfall events (Fig. 2).By storing estuarine model results in a database for future retrieval, the system was able to successfully predict the extent and magnitude of contamination within the estuary by linking real-time watershed and point source contaminant results with pre-run estuarine particle tracking models.

Watershed model results are numerous and ongoing, but results will be presented and discussed for the gauged Moose River watershed. The coefficient of determination (R^2) and the Nash-Sutcliffe Efficiency Index (E) (Nash and Sutcliffe 1970, 282-90) were used to compare model results to observed values. The hydrological model was determined to be appropriate for calculating inflow to the hydrodynamic model, with similar simulated and observed discharge (R^2 = 0.93, E = 0.91). The hydrodynamic model was deemed suitable for particle tracking, yielding simulated flows comparable to observed flows both during the calibration period (R^2 = 0 .91, E = 0.84), and validation period (April 1 to July 31, 2010, R^2 = 0.88, E = 0.85). Validation of the particle tracking model is ongoing, as limited sampling prevents both calibration and validation. Preliminary calibration of the model was

completed using data from the 2010 field season, and resulted in relatively low values of R^2 and E between measured and predicted concentrations of contamination (R^2 = 0.51 and E = 0.38). Obtaining and validating data regarding potential contamination sources has been difficult, agricultural livestock counts, manure spreading and storage practices are not made public in Nova Scotia. As well, there is a lack of available information on average animal counts for unique habitats such as wetlands or forested areas in Nova Scotia. Further sampling and field assessments have been performed to increase the accuracy of the potential loadings database, and are predicted to have a positive impact on model results.

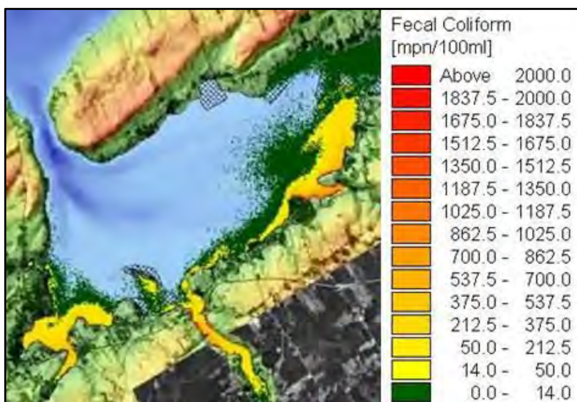

Fig. 2. Estuarine particle tracking results showing the extent and concentration of fecal coliform contamination originating from gauged watersheds after a rainfall event.

Conclusion

The integrated system design was successful. The integrated model receives 72 hour meteorological forecasts every six hours. Watershed simulations are run in unison with the provided meteorological forecasts. The 72 hour forecast period is used to retrieve the relevant pre-run estuarine particle tracking contamination extent for each watershed. Estuarine concentrations are then adjusted to reflect forecasted watershed model results. Finally, estuarine forecast results are recorded over shellfish beds. Currently, work is being done to preserve the data and make it available to the end-user via tailored reports and a dedicated web user interface. Model data can assist decision makers in identifying and mitigating contamination problems while minimizing resource investments, and can also serve to minimize risk of contamination during harvesting periods.

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Adding value throughout the supply chain

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Introduction

Raising molluscan shellfish values can be achieved, both directly by industry operators and indirectly by regulators and scientific researchers, at all stages along the supply chain, from production through transportation to the marketplace. This is not simply 'added value' in the sense of processing and raising prices, but a broad portfolio of operations to improve truly product quality as well as responding to consumer demands for greater ease, convenience and partial (if not total) preparation.

Production or harvesting sector: Industry actions can include cleaning the product (a very basic concept, but frequently ignored), selection through the grading process for desirable traits such as growth rate and flesh content, depuration when there are relatively low levels of potential bacterial contamination, identification of optimal growing sites away from anthropogenic inputs, biotoxin hotspots, etc., and participation in structured management arrangements with fellow stakeholders and regulators.

Regulators can strive to improve the management of biotoxin events, the efficiency of sanitary surveys and improve rapid response to events such as sewage treatment plant (STP) overflows (on a risk assessment basis). They can also 'work' the bureaucratic chain, pressing for increased investment in STPs and more effective enforcement of environmental legislation. Routine monitoring of viruses in parallel to statutory *E.coli* monitoring, to assess the background level of perceived contamination using current indeterminate methods (RT-PCR) would also be a positive, proactive value adding activity.

Added value also results from regulatory monitoring of other contaminants (heavy metals, PAH, etc), based on a rigorous risk assessment.

Inshore locations for cultivation increase risk and lower the value of our product, with examples of mussels in Vigo, Spain, oysters near Sete and Concale, France, Sangoo Bay in China – but not in South Australia (no rivers, no runoff, low population) – the exception to the rule!

But there will be additional major challenges for the industry in the future, revolving around the move to offshore cultivation, to escape from:

- Space restrictions;
- Visual pollution complaints;
- Anthropogenic inputs.

However, there will be no escape from algal blooms e.g. Western approaches of UK, Baltic Sea. And monitoring of offshore conditions will most likely involve 'remote' monitoring (satellite based), which in turn will need increased cooperation, more structured collaboration and improved communication between all parties.

Transportation: In this link of the supply chain, the first essential is to identify the optimal mode of transport for the product, which will reflect both the format of the product (fresh, live, chilled or frozen), the available infrastructure (road, rail, air, sea) and time/distance constraints.

Packaging: The optimal blend of the various parameters (strength, thermal efficiency, insulation materials, use of cool packs, cost) requires detailed assessment.

Temperature: Probably the most important parameter for transport, and while it is largely out of the producer's control, it can be monitored using modern credit card sized data loggers which enable/empower the producer (evidence of failure to perform cool chain commitments). Not all shellfish require the same environmental constraints – *Vibrio parahaemolyticus* does not grow in Sydney Rock Oysters between 4 and 25 C,

unlike in *Crassostreagigas*, so careful assessment is necessary.

Percussion: There are also different levels of fragility between species, and although they all look pretty 'rock-like', they are sensitive to shock. Again, percussion loggers are available to enable producers to monitor the handling of their shipments and thereby to enhance quality and add value to the product.

Processing: This can range from simple preparation of 'raw' shellfish for direct sale through to making the product 'consumer friendly' by taking them partly or virtually all the way through the process of preparation of a dish! In view of the well documented aversion of many consumers in certain markets to preparing shellfish, this can prove a very valuable activity, creating and developing novel outlets.

Marketplace: Presentation is important; however priorities vary across the world and between markets, with pristine, chilled stands epitomising the western' view, while live presentation is a priority in Asian markets. 'Live' and 'Fresh' can also characterise western markets, when they are close to the producer! In addition, value can be added to the product through promotion and advertising, ranging from education about health benefits to highlighting the 'style' of the product (taste, texture, coolness!).

Conclusions

In the process of adding value to molluscan shellfish, which must be a priority for producers, I believe it is essential for the industry to be proactive and collaborate vigorously with scientists and regulators – producers must be participating 'at the table', not retreating to a silo or developing a bunker mentality! We have many allies within the scientific, regulatory, environmental and political arenas, and it should be a primary objective to build on these relationships and thereby add value throughout the supply chain.

It may be difficult, but producers must refocus from 'production' to 'full chain assessment', collaborating in assessment of environmental factors, analysing all aspects of transportation, including monitoring of the parameters affecting product quality (temperature, percussion, etc), while developing innovative consumer-friendly products and becoming actively involved in the marketing and promotion of their products, recognising that different markets require different approaches.

Accepting a role in the provision of future supplies of protein for the expanding global population and stimulating demand for molluscan products generates an ethical added value for the sector as well as creating additional economic returns – a combination that will support future expansion of this sustainable and natural industry, which contributes positively to human health.

Microbial source tracking to identify and manage sources of fecal contamination in shellfish growing waters

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Abstract
Shellfish sanitary quality is affected by a variety of different pollutants and sources in populated coastal areas. Sewage and other sources of fecal-borne microbial contaminants can cause shellfish harvesting closures due to potential public health hazards. Traditional bacterial indicators used to classify shellfish and overlying water quality cannot be used to identify pollution sources. A variety of microbial source tracking (MST) methods and approaches have been developed to help identify sources of fecal pollution to enable management action to reduce pollution below harmful levels. MST is frequently most useful in areas where conditions associated with pollution are well defined based on preliminary studies and sanitary surveys. MST methods have varying capacity for accurate, quantitative and sensitive detection of one or more source types in shellfish harvest areas, as well as specific limitations that suggest use of multiple methods as the best strategy for identifying pollution sources. Ongoing research suggests new approaches that may help provide more in depth understanding of pollution source identification strategies.

Keywords: sanitary survey, targeted sampling, library dependent method, genotyping, fecal bacterial indicators, fecal pollution

Problem Description
The management of shellfish beds to a significant extent requires minimizing exposure of shellfish to fecal contamination to prevent human exposure to microbial pathogens and potential disease incidence. To re-classify shellfish areas that have harvest-limiting classifications, resource and public health managers need to identify and eliminate sources of fecal pollution. Sanitary surveys are a critical step in this process, but as point and other obvious sources of pollution are identified and eliminated, further progress in upgrading classifications can be limited by traditional approaches.

In the US where most point sources of fecal microbial pollution have been eliminated or are effectively disinfected, shellfish areas affected by nonpoint source (NPS) pollution can have harvest limitations and the elevated levels of fecal indicator (fecal coliforms, *E. coli)* bacteria are often of unknown public health significance. New approaches are needed under these conditions to identify NPS pollution sources.

Regulatory strategies such as the Total Maximum Daily Load (TMDL) approach for eliminating use limitations for surface waters in the US are often addressed by what is viewed as less costly modeling efforts based on assumed sources and mechanisms of transport and persistence. Some models are excellent surrogates for direct analysis of water quality, but many are based on simple assumptions that can only provide general source identification, The outcome for many modeling efforts is useful forecasting capacity for likely contamination conditions that define when harvesting can or cannot occur, i.e., the development of conditional classifications. Is it enough to define conditions under which fecal pollution allows for or prohibits shellfish harvesting, or is it best to identify and eliminate pollution source to eventually enable harvesting without limitations? Obviously there will always be extreme events that will cause fecal pollution problems, like Hurricane Sandy along the New Jersey coast in October 2012 where full re-opening of harvest areas did not occur until April 2013 (http://www.state.nj.us/dep/bmw/sandy.html).
Application of MST, however, is the best available

approach for identifying the actual and most significant pollution sources affecting water quality that can also enable expenditure of limited resources to eliminate sources in a way the results in actual improvements in water quality.

Microbial Source Tracking Approach and Methods

A variety of source identification methods, including microbial source tracking (MST) methods and approaches, have been developed to help identify sources of fecal pollution. For this study, only MST, and not chemical, methods will be described. MST is an approach that uses one or a variety of target microorganisms or their genetic markers, detection methods and data analysis approaches to identify the actual sources of fecal pollution that contaminate shellfish waters. MST is often not applied because of misconceptions about the accuracy or the expense of studies, however, by applying MST, resources are directed at actual problems so money is saved in the long term.

There are numerous review papers that have been written to compare and provide updated assessments of MST methods and their applications (Harwood *et al.*, 2014). Several key points have emerged from these overviews: (1) MST techniques are 'experimental science' and should be used with clear understanding of inherent limitations; (2) No MST technique can accurately identify all fecal sources, all have some degree of inherent inaccuracy, and no MST method can be applied to all objectives.

These caveats to the use of MST suggest the best overall approach is to use a variety of 'tools' to identify pollution sources (US EPA, 2005). This may involve multiple MST methods, or a combination of assessment strategies that include GIA analysis, modeling and targeted sampling and analysis. Polyphasic or multi-tiered approaches can help to increase the predictive power of a MST 'tool box' (Santo Domingo *et al*., 2007).

A carefully executed decision making process is another key component of MST applications, and is best undertaken when accompanied by thorough research of all related background information that defines the nature of the pollution problem (Stoeckel, 2005). The basic steps are as follows:

-Existing data should be reviewed and efforts to conduct shoreline surveys, targeted sampling and surveys of local knowledge should be conducted to provide information to inform and focus MST studies.

-Identify the pollution problem. Is it a seasonal problem, or one associated with changes in human population and activities or environmental conditions?

-The spatial extent, temporal occurrence and environmental conditions that define the problem should be identified as the basis for the experimental design and sampling strategy for the MST study.

-Formulate objectives relative to categorizing sources, quantification (presence/absence, source loading), and application, i.e., linkage to public health risk or regulatory indicators.

appropriate MST method(s). This should be informed by nature of the pollution problem and the conditions under which it occurs. Technical demands, cost and availability of expertise are also significant considerations.

-Data collection and analysis considerations.

-Interpretation and application of results.

The critical aspects of different MST methods to consider have also been summarized in many review papers. The main differences in MST methods are whether the method is a library-dependent or independent, dependent on microbial culture or is culture independent, the targeted species or source identifier, indicator or genetic marker, the scope of source identification, the cost, availability and study area relevance, and the specificity, sensitivity, accuracy, reproducibility, and quantification potential. Target organism(s) may be indicators that relate to harvest area regulations, other non-regulatory indicator species of fecal contamination, or direct detection of pathogens. Targeted organisms or genetic markers can vary in persistence, becoming indicators of short or longer-term pollution. The range of source identification is often limited to identifying human sources because these are of most concern for risk management of public health, even though many studies indicate that non-human sources can be significant. All MST methods have limitations; and

the degree to which these are well defined an important consideration in their application.

Application of MST for Shellfish Harvest Areas
There have been many studies with a wide variety of MST methods and study designs to address fecal pollution problems in shellfish harvesting areas. These studies vary in spatial scale, the nature of the problem, study design, and objectives. Many papers in the primary literature report on studies that evaluate new detection and MST methods with no testing of environmental samples of shellfish or overlying waters. Others include some environmental samples in method evaluation papers (Mauffret *et al.,* 2013), while some are actual application studies for MST procedures that expand the conceptual understanding of MST application. For example, Shanks *et al.* (2006) found bovine sources were more likely sources than human sources in fecally polluted Tillamook Basin in Oregon, and that levels of bacterial indicator (*E. coli*) and the Bacteroidales genetic markers used for source tracking were affected differently by environmental factors.

Many recent MST studies suggest a combination of different markers and analytical approaches can provide effective pollution source identification. Mauffret *et al.* (2013) found procedures that were useful for tracking pollution sources in French oysters were not effective for cockle and clam samples. McQuaig *et al.* (2012) detected markers for human sewage in California coastal beach areas affected by NPS pollution using a combination of fecal indicator bacteria and source-specific genetic markers. Ahmed *et al.* (2007) found library dependent and independent methods were both useful and complimented each other in identifying sources of human-borne fecal pollution in coastal Australia. Jones (2008) summarized several studies in the Northeast US that used *E. coli* ribotyping capable of identifying a wide array of source species (35 different human and non-human source species) to show how fecal pollution sources could be made up of quite different source species profiles despite relatively short geographical distances between sites. Another study on the coast of Maine exhibited a shifting profile of source species with season, where human sources were not detected and wild animals and birds were the most significant sources in summertime, but human sources became the major source in winter (Jones, 2009). Both the Jones studies and that by Kelsey *et al.* (2008) showed locally and temporally specific source species libraries enhanced the effectiveness of ribotyping for identifying sources in specific locations.

Many MST studies with capacity for identifying a wider range of potential source species show non-human sources to be more significant pollution sources in shellfish areas (Jones, 2009, Kelsey *et al.,* 2008). MST results can be surprising, as in finding otters, backyard chickens, or other wild animals as the most prevalent sources in small catchments (Jones, unpublished). The public health significance of non-human sources is less than for human sources, yet there is known potential for disease risk associated with the feces of many animal species.

Norovirus and hepatitis A are common causes of shellfish-borne disease associated with fecal pollution. Even where wastewaster is treated, different viruses have different susceptibility to disinfection agents (Sigstam *et al.,* 2013). The common focus of MST studies on bacteria because of their use for regulatory standards may be a limitation for assessing viral contamination. Some studies have focused on the use of F+-specific RNA coliphages as indicators of different sources of viral contamination (Kirs and Smith, 2007) while others have used direct detection of viral pathogens (Ueki *et al.,* 2005). Ongoing research is defining differences in viral that may lead to improved use of viral indicators for source detection (Rajko-Nenow *et al.,* 2013).

Expected Outcomes and Challenges.
What can be reasonably expected from application of MST for eliminating fecal pollution from shellfish areas? MST study objectives relative to source identification accuracy are important for follow-up management actions. Many MST studies focus on the question of whether human sources are present and/or significant (McQuaig *et al.,* 2012). There are source identifiers used in many MST methods for human sources, and their application can help focus management actions to eliminate human pollution sources. MST methods

Table 1. MST (*E. coli* ribotyping) pollution source identification in two studies where cats were significant pollution sources.

Study/Source	Pet	Human	Bird	Livestock	Wild animal	Unknown sources
% Identification						
Seabrook storm drain	77 (94% cats)	3	10	0	0	10
Southern Maine watershed	22 (95% cats)	3	11	11	15	35

that use a wider array of markers for non-human source types or species can also define the significance of human and other sources as pollution sources. This broader approach is essential for enabling reduction of indicator bacteria for re-classifying areas, and can help frame the public health significance of the overall fecal pollution.

Non-human pollution sources of less of a public health threat, and their management can often be more difficult relative to human source reductions. MST studies that use methods that can identify multiple source species and types often produce findings where an array of different non-human sources are significant. In some cases, one source species can dominate, like Canada geese in the salt ponds of Martha's Vineyard (Jones, 2009). With a clearly identified source, management action can be taken to eliminate animal sources by lethal and non-lethal methods (Nugent *et al.,* 2008). Fecal pollution from pets can be significant in some areas, and their management can vary in complexity. A storm drain in Seabrook NH was significantly contaminated with *E. coli* from cats (Table 1). An investigation determined that a woman was dumping cat feces from a litter box directly into the storm drain, and direct communication with her eliminated this source. Cats, both domestic and feral, can be significant sources of feces to the landscape (Dabritz *et al.,* 2006). Feral cats were the most significant fecal pollution source in a southern Maine watershed (Whiting-Grant *et al.,* 2004), where a multi-tiered management program was initiated

Each technique can be expected to have limitations; false positives are associated with library dependent methods, and false negatives are associated with library independent methods. For methods that do not target the indicator bacteria used to classify shellfish harvesting areas, the relationship of findings to water quality and shellfish classification regulations is an important consideration.

Proliferation of indicator species under warm conditions can complicate classification status and potentially complicate some MST methods. Jones (2009) found that the most significant fecal pollution sources in a watershed affecting shellfish harvesting in Maine exhibited significant seasonal changes; this may in part be due to varying persistence and/or proliferation of the target organism (*E. coli*) as water temperature changed. Development of new indicators that do not have this limitation should be a focus of regulatory agencies. Overall, the best result that can be accomplished with MST is an estimate of the relative contributions of pollution sources in the study area at the time and conditions of sampling, and use of multiple methods can enhance source identification.

Future Directions

Recent advances in MST-related research suggest major advances in our understanding of fecal pollution sources and their public health significance. Almost all of the most promising methods currently in development are library and culture independent methods, and many target 16S rRNA genes in bacteria of the Order Bacterioidales. The advantages of these new methods is that they are abundant in many fecal sources, the genetic markers have been found to be highly source specific, and the bacteria do not proliferate in the environment. They have undergone extensive lab and field-testing, and there are markers for a wide range of source species, including humans, birds, cows, and deer.

Other newer methods that also hold promise are microbial community sequencing analysis and mitochondrial DNA analysis. Community sequencing can provide a broader context and deeper understanding to the persistence and fate of both indicators and pathogens under different and varying community and environmental conditions (Shanks *et al.,* 2013). This type of research could also lead to new and more reliable fecal pollution source indicators. Mitochondrial DNA analysis has been found to be source specific, rapid and sensitive (Baker-Austin *et al.,* 2010) although conditions for the determination of human sources are difficult to achieve because of the high probability of contamination from lab workers and other human sources.

Summary

In summary, fecal pollution in shellfish waters often results from a variety of sources that may be categorized into types that inform management approaches: humans, pets, livestock, wild animals and birds. MST methods also vary in their capacity to provide accurate identification of different sources; some are human specific, others are capable of identifying a wide variety of sources. Many methods, however, are not adequately field-tested, so their limitations have yet to be full defined (Layton *et al.,* 2009). New methods are mostly ‘library’ and culture independent, and hold promise for being accurate, rapid and inexpensive tools. These new genetic marker based methods also need to determine the persistence of the targeted nucleic acids/species in the environment and relate findings to regulatory frameworks. In general, MST is useful for focusing efforts to eliminate the most significant pollution sources, saving resources in the long run. Regulatory agencies may be reluctant to use the results of MST based on what are perceived to be limitations and lack of accuracy. These perceptions need to be overcome to enable use of all possible strategies to improve water quality and gain access to closed shellfish beds.

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Section II – Viruses and Microbial Contaminants

Epizoic barnacles act as a reservoir for pathogenic bacteria on commercial shellfish beds: Implications for the shellfish industry.

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Abstract
Routine monitoring of commercial shellfish beds in the European Union currently focuses on quantifying the bacterial content within shellfish flesh as an indicator of faecal contamination. Previous studies have documented the presence of other significant bacterial reservoirs within commercial shellfish beds e.g. sediments. This study examined the importance of epizoic barnacles as a potential bacterial reservoir across three intertidal mussel (*Mytilus edulis*) beds in North Wales, UK. Results demonstrated that over 80% of the total coliform reservoir was held within the epizoic barnacles in comparison to the mussel flesh, concluding that epizoic barnacles represent a significant bacterial reservoir within commercial shellfisheries. The implications for the shellfish industry are discussed.

Keywords: Indicator organisms, Faecal Coliforms, Bacteria, Reservoirs, Human Pathogens

Introduction

The consumption of bivalve shellfish has been cited as the causative agent in several cases of foodborne illness (Potasman *et al.,* 2002) primarily due to their ability to bio-accumulate pathogenic micro-organisms (Roslev *et al.,* 2009). To protect consumers, and to preserve the quality of the shellfish products, the industry is closely regulated. In the European Union (EU), by law, all commercial shellfish beds must be routinely monitored for potential microbial contamination (EU 2004, a,b).

Indicator species are often used as a proxy for pathogenic species (Field and Samadpour, 2007). EU legislation (EU, 2004a) uses *Escherichia coli* as a generic indicator organism for both pathogenic bacteria and viruses, assigning each commercial shellfish bed a classification based on routinely monitored *E. coli* concentrations within the shellfish flesh (Table 1). The assigned classification minimises the risk to consumers and also helps to promote the economy of the shellfish industry by providing reassurance to consumers that the product they are purchasing is considered safe for consumption.

Table 1. Summary of the European Microbial standards based on *E. coli* per 100g by 5 tube, 3 dilution MPN method (EU, 2004a).

Classification	*E. coli*	Information
A	< 230	Live shellfish may be collected and sold directly for human consumption
B	< 4,600	Live shellfish may be collected and placed on the market for human consumption only after purification treatment or relaying as to meet the standards for class A.
C	< 46,000	Live shellfish may be collected but placed on the market for human consumption only after relaying to meet the standards for class A.

However, current legislation focuses only on the bacterial levels contained within the shellfish flesh itself potentially ignoring other sources of contamination. Recent shifts in consumer preference for food stuffs viewed as more "natural" (Acebron and Dopico, 1999) combined with an increasing global pressure to supply the human population with cheap protein (Naylor *et al.,* 2000) have led to the increase in sales of

shellfish sold complete with their epizoic (associated) organisms e.g. barnacles.
Whilst other potential bacterial reservoirs on commercial shellfish beds have been identified e.g. sediments (Martinez-Manzanares *et al.,* 1992), epizoic organisms have yet to be fully assessed as a reservoir for potentially pathogenic micro-organisms.

Research on the bacterial content of shellfish flesh is abundant (Oliveira *et al.,* 2011), however research on other potential pathogenic reservoirs found *in situ* on commercial mussel beds is currently lacking. Therefore the primary aim of this work was to determine if epizoic barnacles associated with the common mussel *Mytilus edulis* represented a significant *in situ* bacterial reservoir for pathogenic bacteria and the potential implications this may have for the global shellfish industry.

Materials and Methods

Sampling Location

Three intertidal, commercial mussel (*Mytilus edulis* L.) beds were sampled between 1st April and 10th April 2011. Located in the Conwy region (North Wales, UK), Conwy Bridge (53.280279N, -3.838767W), Llanfairfechan (53.259132N, -3.980289W) and Conwy Morfa (53.298015N -3.854535W) represented three commercially harvested shellfish beds that are routinely monitored for bacterial contamination. Mussels and their associated barnacles were collected by hand from 15 random sample points per mussel bed and subsequently pooled prior to laboratory analysis for bacterial determination. All samples were transported and stored at 4°C and processed within 6 h of collection.

Determination of Bacterial Load

Only live shellfish were chosen for evaluation. Shellfish samples were washed with sterile seawater to remove any residual sediment and debris before surface swabbing with 100% methanol to eliminate the surface biofilm. Samples were left to dry for 30 min at room temperature to allow the methanol to fully evaporate before aseptically removing 50 g of the encrusting barnacles and adding them to 50 mL of 25% strength Ringer's solution. The associated mussels were then opened aseptically and 50 g of flesh and extra cellular fluid was obtained. Barnacle and mussel samples were homogenised for 60 sec at 10,000 rev min^{-1} using a Bamix® blender (Seal Rock Enterprises Ltd., Bishop's Stortford, UK). From the resulting homogenate, 200 µL was plated onto Brilliance® selective agar (#CM1046; Oxoid Ltd, Basingstoke, UK) to determine total coliform counts. All plates were inverted and incubated at 37°C and bacterial colony forming units (CFU) enumerated after 24 h.

Statistical Analysis

Data was analysed using PASW statistics v18 (IBM Corp., Armonk, NY). Normality was assessed using a one sample Kolmogorov-Smirnov test ($P \geq 0.05$). Bacterial count data was analysed using the Independent Samples Kruskal-Wallis test and any significant differences ($P \leq 0.05$) were investigated further using the independent samples Mann-Whitney U test, with fixed factors of either site or bacterial species and three replicate units per analysis.

Results

Total coliform concentrations were significantly higher in barnacles compared to mussels across all three sample sites ($P < 0.05$; Fig. 1). There was also a significant difference in coliform concentration observed in both barnacles ($P = 0.001$) and mussels ($P = 0.033$) between the three sampling sites. Total coliform levels in mussel tissues were all below the upper threshold for European Union "Class B" classification (4,600 *E. coli* CFU 100 g^{-1}). In contrast, barnacles at all sites showed total coliform concentrations in exceedance of the upper threshold for this critical classification.

Coliform concentrations corrected by weight, per unit area, of mussel bed (data not shown) and expressed as a percentage of the total (Table 2), show that across all sites the epizoic barnacles are a much larger reservoir for coliform bacteria than their associated mussels. The bacterial reservoir contained within the mussel tissues is
less than 20% of the reservoir contained within the barnacles attached to the shell of the mussel. Llanfairfechan showed the lowest coliform concentrations (Fig. 1) and also the lowest relative

coliform reservoir present within the mussel tissues.

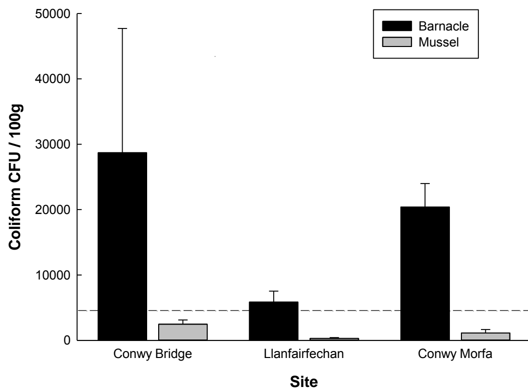

Fig. 1. Total coliform population observed in mussels and barnacles across three commercial shellfish beds. For reference the dashed line represents the upper EU threshold for 'class B' grading of mussels (4,600 *E. coli* CFU 100 g^{-1}) (EU 2004a). In all cases n = 15 where data points represent the mean ± SEM.

Table 2. Proportion of the coliform reservoir associated with mussels and their epizoic barnacles in three commercial shellfisheries.

	Coliform reservoir (% of total)	
Shellfishery	**Mussel**	**Barnacle**
Conwy Bridge	13.6	86.4
Llanfairfechan	3.0	97.0
Conwy Morfa	16.6	83.4

Discussion

The results of this study demonstrate that epizoic barnacles are a significant bacterial reservoir in commercial shellfish beds, containing over 80% of the total coliform concentration per unit area. Little data exists on the *in situ* bacterial flux between shellfish and their epizoic organisms. This study highlights the need for further research in this area.

The ability of sediments to act as a reservoir for bacteria has been well documented (Martinez-Manzanares *et al.,* 1992) as a direct result of this, shellfish collection protocols for routine monitoring state that shellfish samples must be rinsed to remove sediment and debris to avoid recontamination during transit (CEFAS 2008). Epizoic barnacles have been shown to be a significant bacterial reservoir (Clements *et al.,* 2013a). The capability of epizoic barnacles to recontaminate harvested shellfish was first documented in 1947 (Clegg and Sherwood, 1947) however, the limited research and documentation in this area (Sagoo *et al.,* 2007) has failed to quantify the bacterial flux between organisms *ex situ*. Further study is needed to quantify this flux and to determine accurate protocols to minimise the impact of secondary contamination of commercially harvested shellfish during transit and storage.

It has also been inferred that different barnacle species may show differential accumulation of bacteria (Clements *et al.,* 2013a). Further research is also required to quantify the accumulation of bacteria between different barnacle species and assess the impact of this to the shellfish industry.

Recent research on commercial shellfish entering depuration or purification facilities complete with epizoic barnacles have shown that the barnacles eliminate bacteria at a slower rate than the corresponding shellfish (Clements *et al.,* 2013b). Bacterial indicator concentrations within the epizoic barnacles indicated that a longer depuration time may be necessary for shellfish entering depuration complete with epizoic barnacles. The same research also tentatively suggested that depuration times could be halved for shellfish entering the system without epizoic organisms. This would have huge economic implications to the shellfish industry.

The shellfish industry has a responsibility to provide consumers with a product that is of good quality and is safe to eat. Compliance with current EU legislation (based on indicator organisms) minimises the risk to the consumer. However, current legislation and protocols should 'factor in' new research to not only protect the consumer, but to safeguard the shellfish industry.

Acknowledgements

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Convergence program administered by the Welsh Government in association with Bangor Mussel Producers and Deepdock Ltd.

This work represents a short synopsis, for a full account of the work undertaken please refer to Clements *et al*. (2013a).

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Evaluation and application of viral source tracking in New Zealand

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Abstract
Faecal pollution of shellfish growing waters presents a risk to human health because of the potential presence of pathogenic bacteria and viruses. Rapid, accurate identification of human and animal faecal pollution sources is important for effective water quality management. We have developed a viral source tracking system (Viral ToolBox) for identifying human and animal faecal pollution sources in New Zealand. Specific real-time quantitative PCR (qPCR) assays for viruses excreted in animal and human faeces or urine were developed. The assays were evaluated for host specificity and virus prevalence in environmental samples including wastewater, shellfish, biosolids, environmental waters and animal faeces. Assays detecting human polyomavirus, human adenovirus species F, and norovirus GI and GII were confirmed to be human specific and assays detecting bovine polyomavirus (cows), porcine adenovirus type 3 (pigs), 'ovine/bovine' adenovirus (sheep, cows) and norovirus GIII (sheep, cows) were animal specific. Norovirus GIII and bovine polyomavirus assays successfully identified animal faecal sources. The Viral ToolBox can identify human and animal faecal pollution sources and will assist resource managers, shellfish farmers and public health officers to determine sources of pollution for effective management of water quality, particularly shellfish growing waters.

Keywords: norovirus, adenovirus, polyomavirus, real-time qPCR, faecal source tracking, shellfish, environmental waters

Introduction

Contamination of shellfish growing waters and recreational waters by faecal pollution can present a risk to human health because of the presence of pathogenic bacteria and viruses. It is therefore important that faecal pollution sources are identified rapidly and accurately so that strategies to eliminate the pollution source can be introduced. Indicator bacteria (eg. *E coli*, enterococci) are used by regulators to assess water quality but do not discriminate between animal, avian or human sources. Microbial source tracking (MST) is well established for identifying and distinguishing between human and animal pollution sources and therefore can aid in management and mitigation of faecal contamination. Methods used for MST include molecular fingerprinting, bacteriophage typing, and identification of bacterial PCR markers, antibiotic resistance markers, mitochondrial DNA, faecal sterols and fluorescent whiteners (Santa Domingo *et al*., 2007; Stoeckel & Harwood, 2007).

Over several years we have been developing a source tracking system using human and animal viruses. Initially a F-RNA phage genotyping system was developed using a multiplex RT-quantitative PCR (qPCR) assay for genogroups I, II, III and IV (Wolf *et al*., 2008). This was used in environmental studies for comparison with norovirus presence, but correlation with norovirus was only observed following sewage pollution events and not following rainfall events. In addition, phage genotyping was not 100% discriminatory for animal and human sources. The viral source tracking approach was therefore extended to include detection of human and animal viruses by RT-qPCR and qPCR assays. These assays were then evaluated as viral source tracking tools for identification of faecal contamination sources in shellfish and waters.

The rationale for developing a virus based source tracking system was that enteric viruses are excreted in large numbers in faeces, viruses are host specific

for animals or humans and techniques for enteric virus recovery and analysis from environmental samples are now well established. The viruses selected for our "Viral ToolBox" (VTB) included noroviruses (RNA genome), adenoviruses (DNA genome) and polyomaviruses (DNA genome), each of which contain types or species that infect either humans or animals.

Several specific RT-qPCR / qPCR assays were developed for detection of viruses known to occur in animal or human faeces or urine and which were potential candidates for source tracking purposes. Assay sensitivity and specificity were evaluated for a range of sample types including shellfish, wastewater, biosolids, environmental waters, and animal and human faeces. The methods used were as described in previously published reports (Kageyama *et al*., 2003; Hewitt *et al*., 2007; Greening & Hewitt 2008; Wolf *et al*., 2008; Wolf *et al*., 2010).

The relative prevalence of the different enteric viruses in the New Zealand environment was determined and the appropriate assays selected for inclusion in the VTB.

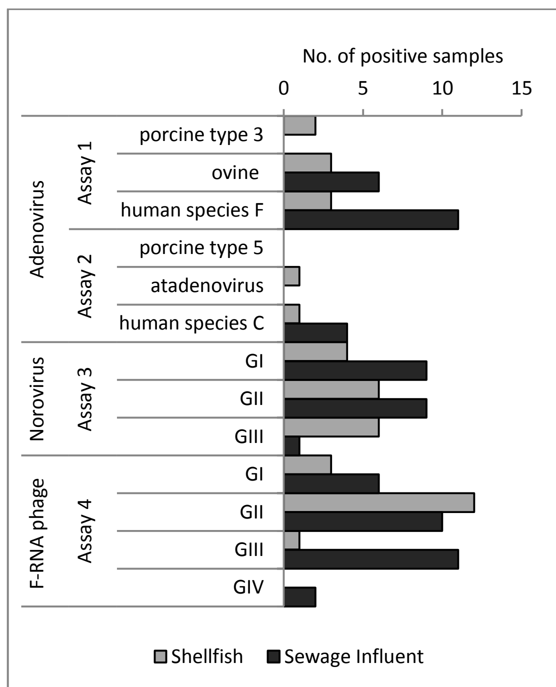

Fig 1. Range of viruses detected by initial 'viral toolbox' assays in raw sewage (n = 11) and shellfish (n = 16) samples.

The initial VTB comprised four multiplex qPCR assays. These included human adenovirus species F (types 40, 41), human adenovirus species C (types 1, 2, 5, 6) and norovirus GI and GII for human pollution and for animal pollution, porcine adenovirus types 3 and 5 (pigs), ovine/bovine adenovirus (ovine adenovirus types 2,3,4,5, bovine adenovirus type 2), atadenovirus (sheep, cattle, deer and goats) and norovirus GIII (sheep, cows) (Wolf *et al*., 2010). These assays were tested for specificity and sensitivity on animal faeces (pigs, sheep, cows, and wildfowl) (Wolf *et al*., 2010) and then on shellfish and sewage samples (Fig 1).

Atadenovirus and human adenovirus species C were each detected in only one sample and porcine adenovirus 5 was not detected. The original combination of assays was subsequently modified. Assay 2, comprised of one human and two animal adenovirus assays, was removed because of low prevalence and the F-RNA phage assay, Assay 4, was removed due to its non-specificity. These assays were replaced with human and bovine polyomavirus assays because polyomavirus is extremely species specific and is excreted in high concentrations in urine. Although the prevalence and concentration of polyomavirus in the NZ environment was previously unknown, other researchers overseas have reported this virus as useful for source tracking studies (Hundesa *et al*, 2006; Albinana Giminez *et al*., 2009; McQuaig *et al*., 2009).

The modified VTB is comprised of four separate assay groups. All are monoplex assays except VTB 3, which continues to be a multiplex qPCR assay (Table 1).

Table 1. Attributes of Viral ToolBox (VTB)

Assay	Target virus	Matrices	Source
VTB 1	Norovirus GI	waters & shellfish	human
	Norovirus GII		
VTB 2	Human polyomavirus	waters	human
VTB 3			Multiple:
	Human adenovirus F	waters	human
	Porcine adenovirus 3		pig
	Ovine/bovine adenovirus		sheep & cattle
VTB 4	Norovirus GIII	waters & shellfish	sheep & cattle
	Bovine polyomavirus	waters	cattle

Samples analysed with the modified VTB included influent wastewater (31), biosolids (11), shellfish (27), estuarine water (11), urban stream water (7) and river water (22). The results are shown in Figs. 2 and 3.

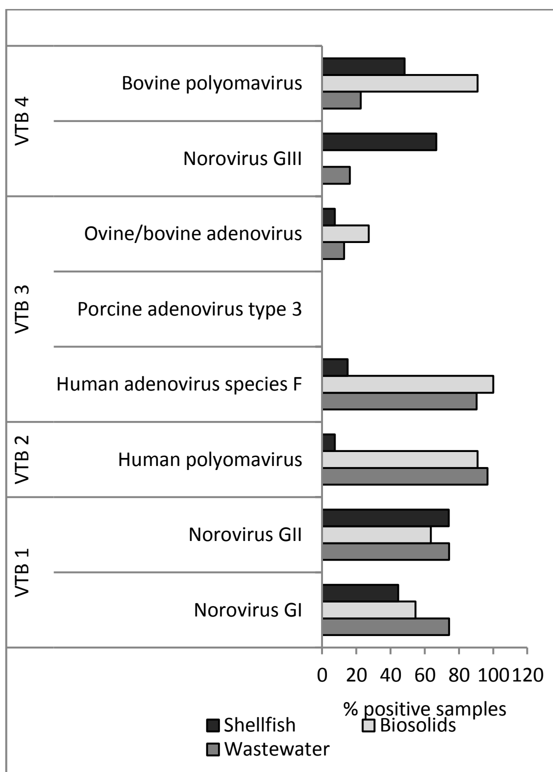

Fig. 2. Prevalence of viruses in NZ shellfish, biosolids and influent wastewater using the Viral ToolBox (VTB).

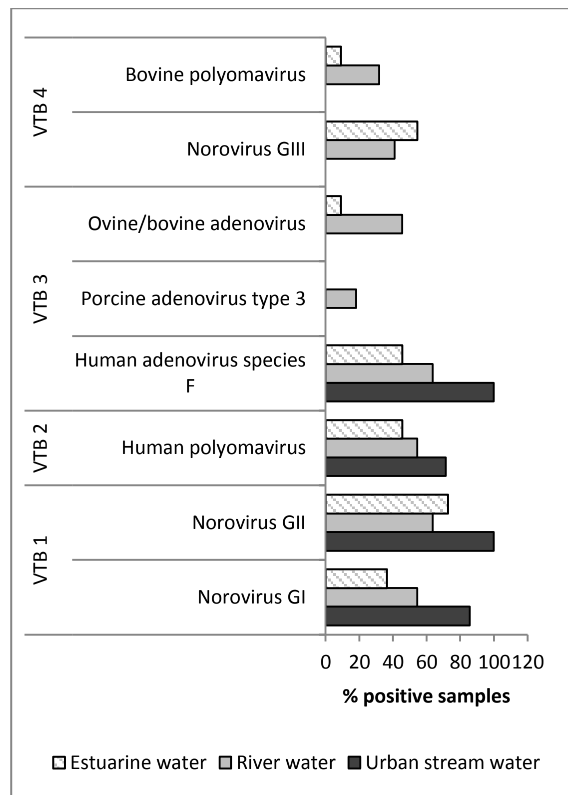

Fig. 3. Prevalence of viruses in NZ estuarine, river and urban stream waters using the Viral ToolBox (VTB).

Human polyomavirus was prevalent in the environment and was detected in 68% (39/57) of water samples. No animal viruses were detected in urban stream water. Porcine adenovirus type 3 was only detected in river water and all viruses (except porcine adenovirus type 3) were detected in shellfish. Norovirus GIII and bovine polyomavirus used together identified animal pollution. Human norovirus GI (44%), GII (74%) and animal norovirus GIII (68%) were frequently detected in shellfish. However, there was a marked lower prevalence of human adenovirus and polyomavirus in shellfish compared with norovirus. This may be due to low recovery rates for adenovirus in shellfish observed using the protease digestion method.

Concentrations of human polyomavirus, adenovirus species F and norovirus GII were generally similar in water samples, ranging from 2-3 log_{10}/litre of water. Human polyomavirus, human adenovirus species F, and

norovirus GI and GII were confirmed to be human specific and were prevalent in NZ. For determination of animal specific sources, assays for bovine polyomavirus (cows), porcine adenovirus type 3 (pigs), 'ovine/bovine' adenovirus (sheep, cows) and norovirus GIII (sheep, cows) were found to be useful in New Zealand.

Summary

Four VTB assays were evaluated as tools for tracking sources of faecal pollution in samples of New Zealand wastewater, biosolids, environmental water and shellfish. All assays were specific for human or animal faecal contamination. A major advantage of the VTB assays is that they can be customised to fit the situation under investigation and the sample types available for analysis. They can also be used with other MST methods. Optimal information on pollution sources can be obtained when a combination of source tracking tools are used. These methods can assist in management of water and shellfish
quality by identifying sources of faecal pollution.

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Improving the management of the risk of human enteric viruses in shellfish at harvest

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Abstract

A series of eight retrospective case studies from five commercial oyster growing areas in New Zealand and New South Wales, Australia, was undertaken to identify the fundamental reasons why the current bivalve shellfish classification and management systems can fail to protect consumers from human enteric viral contamination in shellfish at harvest. The case studies were based on norovirus illness events since 1990 in growing areas with Shellfish Quality Assurance Programmes. The study showed that faecal coliform/*E. coli* indicators failed to predict the risk of enteric virus contamination in shellfish, placing a high level of reliance on the sanitary survey component of the programme. On-site sewage management systems failure, resulting from design, installation and/or maintenance issues, was the most commonly identified viral contamination source. Common factors contributing to the failure of the sanitary survey component to protect consumers highlight the need for on-going access to reliable, high-quality information on pollution sources. Environmental policy and its implementation were identified as key factors in maintaining water quality in shellfish growing areas.

Keywords: Norovirus, enteric viruses, shellfish, oysters, sanitary survey, environmental policy

Introduction

Bivalve molluscan shellfish are filter feeders and can accumulate human pathogens, and chemical contaminants (such as heavy metals and marine biotoxins) from their growing waters. Consequently bivalve shellfish pose a high food safety risk and therefore receive special consideration in food safety laws throughout the world. Internationally there have been a number of approaches to classification of shellfish harvest areas and management programmes to reduce the food safety risks at the time of harvest. These approaches appear largely effective in preventing food-borne illness associated with chemical contamination and bacterial pathogens. However, there is a large amount of documented epidemiological evidence indicating that shellfish consumers are not always protected from illness arising from human enteric viruses such as Hepatitis A and norovirus. (Butt *et al.*, 2004; Guillois-Becel *et al.*, 2009; Richards, 2002). This is a significant food safety problem for the shellfish industry internationally, and will pose on-going human health and market access issues unless the problem is remedied. The cost of viral illness associated with shellfish consumption is not just that associated with personal morbidity and mortality issues, but also potentially with loss of public confidence in the shellfish industry to supply safe food product. The loss of this reputation in today's global food market is economically significant.

Australia and New Zealand both have Shellfish Quality Assurance Programmes (SQAP) that incorporate: a) a public health sanitary survey of shellfish growing area catchments; b) water and shellfish sampling; and c) controlled harvest times. The shellfish industries and regulatory authorities of both countries share an interest in ensuring their shellfish programmes reliably manage the risk of viral contamination of shellfish. Based on the premise that analysis of previous events could improve the management of the risk of virus contamination of shellfish in the future, this study analyzed eight retrospective case studies drawn from New Zealand and New South Wales, each based on a norovirus (NoV) illness event

associated with the consumption of oysters that were identified as having been contaminated with NoV before harvest. The aim was to identify the key factors resulting in failure to prevent human enteric virus contamination of shellfish in growing areas and resultant viral illness outbreaks in consumers.

Methodology

The broad framework for case study-based research used in this project was adapted from methodologies set out in Eisenhardt (1989), Kohn (1997) and Baxter & Jack (2008). A detailed description of the methodology used is provided in Hay *et al*. (2013). Briefly, a multi-case study using cross-sectional analysis based on a sample of events from two countries (New Zealand and New South Wales, Australia) was undertaken. In order to maintain relevance to current conditions, the sample of cases was temporally bounded to events since 1990, and bounded in scope to shellfish growing areas with an operative SQAP at the time of the illness outbreak. This limited the study to NoV illness outbreaks only. Eight case studies, representing 89% of eligible cases, were selected.

Based on the findings of McCoubrey (2007), two points of control were identified as potentially representing fundamental issues of shellfish quality at harvest: i) The SQAP that ensures that shellfish that are harvested for consumption are safe to eat; and ii) The legislative/regulatory environment that influences the quality of water in shellfish growing areas. Consideration of these two control points provided the skeleton for a conceptual framework to structure the data collection and case study analysis.

Data collection and data analysis were interlinked iterative processes. The primary sources of information in data collection included: Growing Area Sanitary Survey reports; Growing Area Annual Review reports; epidemiological reports of illness outbreaks; reports of growing area investigations following illness outbreaks; and Council reports and records. This was supplemented by direct communication with participants. A systematic process was used to collect, analyze and review data to ensure that there was consistency amongst the three researchers.

Data analysis consisted of two phases: analysis of within-case data (a detailed case study description of each site) and cross-case analysis (which examined cases in groups to identify fundamental issues common to all sites). In addition to analysis across all cases for some variables, cross-case analysis included: a) analysis of cases grouped based on common causes of viral contamination; b) within-growing area analysis where there were several cases within one growing area; and c) analysis across the growing areas that had several cases each. Other groupings were analyzed as themes or issues emerged.

Results

All growing areas in the study were either classified as “Conditionally Approved”, or (in one case) “Conditionally Restricted”. Compliance with standards within the SQAP was analysed for each case, with the following results:

a) In every case, at the time of harvest of the oysters implicated in the NoV outbreak, routine water and/or shellfish monitoring results indicated that the growing area was compliant with its classification.

b) In all cases, oysters implicated in the NoV outbreak had been harvested in compliance with the growing area harvest criteria. In 3 of 5 growing areas the harvest criteria had been revised within the 3 years prior to the NoV outbreaks.

c) In the one case in which an oyster depuration process was required after harvest (i.e. the case from the “Conditionally Restricted” growing area), the post-depuration oyster sample from the batch implicated in the NoV outbreak contained no detectable *E.coli*.

In addition, in all cases in which data were available from cases from “Conditionally Approved” growing areas, water and oyster samples taken from the growing area either when NoV contamination was known to be present in the oysters, or when the source of NoV contamination was subsequently known to have been impacting on the growing area, had faecal coliforms/*E. coli* levels below the regulatory level of 14 MPN/100 ml (faecal coliforms in water samples), 300 MPN/100g (faecal coliforms in oyster samples) or 230 MPN/100g (*E. coli* in oyster samples).

The range of implicated sources of viral contamination across the case studies included discharges from on-site sewage systems (implicated in 6 of 8 cases), effluent from wastewater treatment plants (3 of 8 cases), spillages from sewerage reticulation systems (2 cases) and discharges from boats (considered a possible source in 3 cases, but not able to be definitively proven). NoV outbreaks occurred in the months from June to December (i.e. winter to early summer), but those in which on-site sewage systems were implicated as sources occurred in the wetter months from June-September.
There were sufficient data in four cases to investigate the reasons why viral contamination of the growing area from on-site systems within the catchment occurred. These included:

- Inadequate maintenance, which is the responsibility of the system operator, but in some cases, should have been monitored by the Council;
- The operation of systems of a design inadequate to prevent contamination of watercourses and not approved by Council;
- The modification of approved systems, or the installation of systems in a manner that was not compliant with the approved design; and
- Failure of systems (particularly effluent disposal fields) designed and installed in accordance with Council requirements and standards.

Similar types of management issues were identified with respect to other contamination sources (e.g. poor implementation of maintenance programmes, design inadequacies, and operation of WWTP and sewerage reticulation systems outside their design specifications; policy with respect to effluent discharge from boats not designed to protect against virus contamination of shellfish).

In all cases, the presence of the actual or potential sources of NoV contamination that were identified in the outbreak investigations had previously been acknowledged in a broad sense in the Sanitary Survey reports as potential risks. However, in all except one case the growing area investigations following a NoV event, which involved an unusually high level of cooperation and communication between councils and the agencies responsible for shellfish management, revealed much more detailed information. Had this information been available earlier, it could have significantly impacted on the assessment and management of the risk of virus contamination.

Failure to continue to manage the risk of sources of viral contamination that had previously been implicated in NoV illness events was identified as an issue in the growing areas in which several NoV cases occurred. In some case studies this was evident in inadequately detailed documentation of investigations and in the failure to institute and sustain management plans to ensure that issues that caused virus contamination were not repeated.

Discussion and Conclusions

Although illness outbreaks arising from shellfish contaminated with enteric viruses at harvest are rare in New Zealand and Australia, the study highlighted a number of problems with growing area management, technical issues and environmental policy. As in many other countries, SQAP in Australia and New Zealand use bacterial indicators, i.e. faecal coliforms and *E. coli*, to assist in the classification of shellfish growing areas, to provide the basis for setting harvest criteria, to confirm the growing area classification on an on-going basis, and to confirm the effectiveness of depuration processes. The results of this study suggest that the manner in which *E.coli*/faecal coliform indicators are used in the current shellfish programmes fail to consistently predict the risk of enteric virus contamination in shellfish harvested for market. This reflects the poor between faecal coliform/*E. coli* and enteric virus levels in shellfish and growing waters on a sample-by-sample basis observed by many other researchers (e.g. Gerba, 1979; Lowther, 2011). This places a very high reliance on the sanitary survey component of the SQAP in managing the risk of enteric viruses.

The case studies suggest that the implementation of the sanitary survey components of the programme failed to adequately protect consumers from illness arising from NoV contamination because there was insufficient reliable information gathered during the sanitary survey process.

Reliance on other agencies such as Councils to provide sufficient, reliable, high quality information about growing areas and their catchments is problematic and there can be barriers to Food Authority officers accessing private properties to inspect potential contamination sources themselves.

The design of the SQAP, which incorporates infrequent detailed sanitary surveys, assumes little change will occur in the risk of viral contamination in the growing area through time. The case studies suggest that this assumption is not correct. Change can be impacted by increasing pressures from competing resources, including urbanization of coastal areas. Particularly in New Zealand, there is little cross-agency planning, and environmental policy is not well designed to protect water quality in shellfish growing areas, especially with respect to the cumulative impact of many small changes. The implementation of existing environmental policies by Councils can be very poor, driven by lack of resources, poor management and lack of technical expertise. These issues need to be addressed to ensure the on-going protection of shellfish consumers from enteric virus illness.

In the absence of a reliable universal indicator, alternative methods of confirming the validity of observations made in sanitary surveys are needed. These might include the application of microbial source tracking techniques close to pollution sources, and on occasion, direct virus testing.

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Evaluation of the sanitary status of the Dutch shellfish production waters over a 7 year period

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Abstract
In the Netherlands, a monitoring program is set up according to EU regulation, in which production areas are monitored year round for *E.coli*, toxic phytoplankton and marine biotoxins. The sanitary status of the Dutch production waters is evaluated using results achieved over a 7-year monitoring period (2006-2012). Based on European legislation, most Dutch production waters are classified as A. Occasional violation of 230 *Escherichia coli* colony forming units (cfu)/100 g was always followed by a compliant sample a few days later. Diarrhoeic shellfish poison (DSP) producing algae are the predominant phytoplankton in Dutch production waters. DSP blooms occur yearly. Occasionally a bloom of Amnesic Shellfish Poison (ASP) producing algae occurs, with violation of the criteria for algae counts in 2006, 2008 and 2010. However, these blooms did not lead to the presence of marine biotoxins in shellfish. In addition to the routine monitoring plan, presence of viruses in one of the production areas was investigated in 2007-2008. Norovirus genogroup II was detected in 5% of the bivalves studied and hepatitis E virus in 4%. Human adenoviruses were detected in 1% of the oysters and human parechoviruses in 1% of the mussels studied. Norovirus genogroup I and enterovirus were not detected.

Introduction

The European Union (EU) has prescribed specific rules for the control of shellfish production areas (Regulation (EC) No 854/2004) in order to ensure that shellfish do not contain micro-organisms and toxic substances in quantities considered dangerous to human health. Practical applications of the EU legislation for management of shellfish production areas is described in a series of manuals published by the European Reference Laboratory (EURL) for bacteriological and viral contamination of bivalve molluscs (CEFAS, UK) and for marine biotoxins (AESAN, Spain). In the Netherlands, a monitoring plan to control the sanitary quality of the shellfish production waters is set up according to this EU legislation. The production waters are classified based on the level of the fecal indicator organism *Escherichia coli*. Areas with contamination levels < 230 colony forming units (cfu)/100g are classified as A, areas with a contamination level between 230 and 4600 cfu/100g (with a tolerance level of 10% of samples < 46000 cfu/100g) are classified as B. Areas with contamination levels between 4600 and 46000 cfu/100g are classified as C. In the Netherlands there are 12 production areas for which, according to the sanitary survey, all but one have a class A status. Lake Veere has a class B status. The main reason for this B classification is the large amount of recreational activity in that area. A monitoring plan is set up according to the outcome of the sanitary survey of the production areas. Parameters included in the monitoring plan are *E.coli* counts, toxin producing phytoplankton counts and marine biotoxin analyses (ASP, PSP and DSP).

The stipulated method for *E.coli* enumeration in shellfish is the MPN method (ISO 16649-3). However, recently an alternative method based on the TBX method (ISO 16649-2) has been validated against the MPN method and is now used as a valid alternative for monitoring samples (Pol-Hofstad *et al*., 2013). Figure 1 shows an

overview of the violations of *E.coli* criteria for class A areas from 2006 to 2012. Figure 1a shows violation throughout the years for production areas in the north of the Netherlands, figure 1b shows violations for areas in the south of the Netherlands (note, Lake Veere is shown here although it has a B-classification). All areas experience occasional violations, from 1.5 to 2.3% of the yearly samples exceed the *E.coli* criteria for the class A classification. In 2011 and 2012, this percentage decreased to 0.5% of the total amount of analysed samples. Evaluation of yearly data led to declassification of two areas in the North (Frisian Wad and Groninger Wad) in 2006 and 2007 as a result of more frequent violation of the *E.coli* criteria. In 2008 these two areas were upgraded to class A again based on their *E.coli* results from 2007.
No specific pattern or differences can be distinguished between areas in the north or the south. Violations occur throughout the year with a higher frequency in summer periods. A non-compliant result is always followed by a compliant result the week after (except for one occasion in 2007 and one in 2008, where there were two successive minor violations), making it difficult to track the source of contamination.

Enumeration of potential harmful phytoplankton and analysing marine biotoxins are also part of the monitoring plan in all production areas. Marine biotoxins are determined using chemical methods. For DSP toxin analyses, the stipulated method was the mouse-bioassay. In the Netherlands the rat-bioassay was used instead, because of national animal testing legislation. However in April 2011, legislation allowed for replacement of the rat-bioassay with an LC-MS method (EC 15/2011). Phytoplankton is monitored using a light microscopy method to distinguish between different species based on their visual appearance.

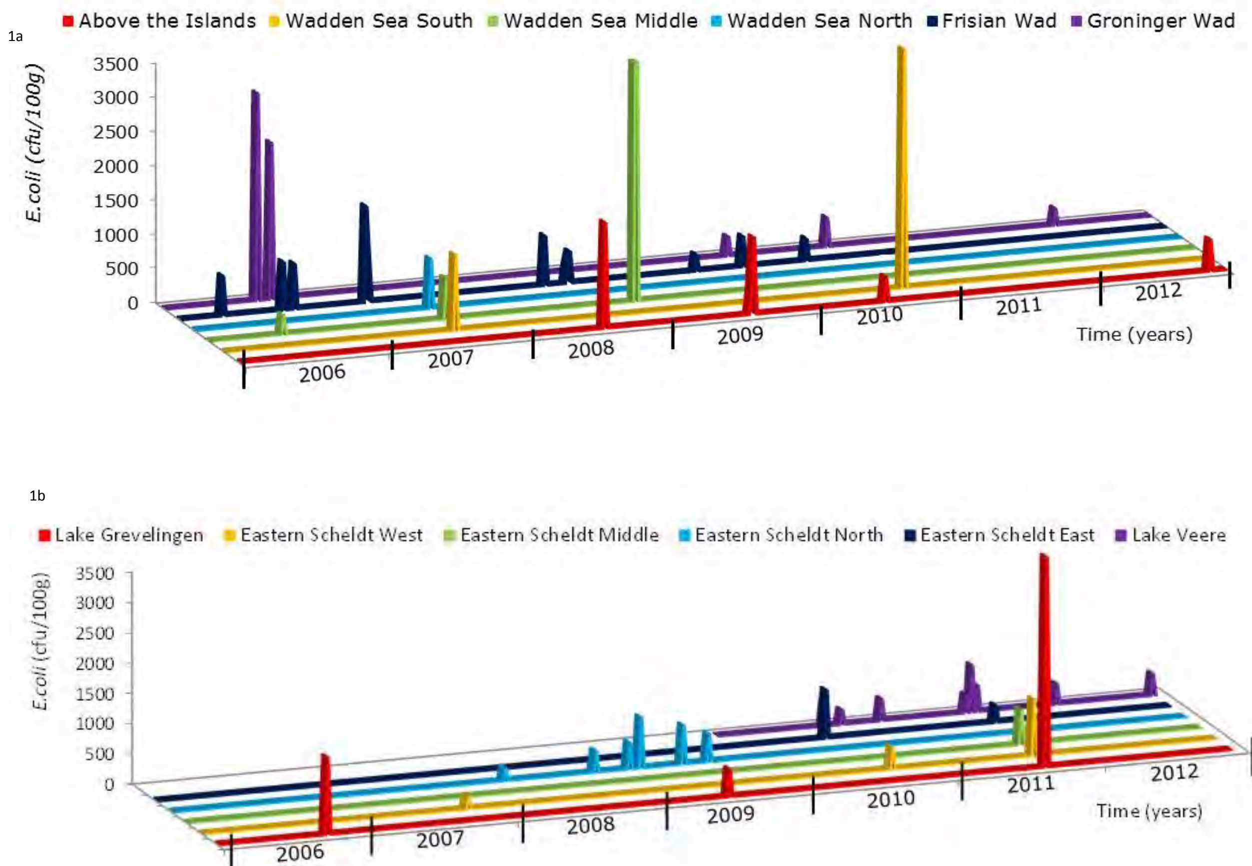

Fig. 1. *E.coli* levels above 230 cfu/100g in northern (1a) and southern (1b) production areas in the Netherlands from 2006 till 2012.

Results are used as an early warning system. If violation of phytoplankton criteria occur, the area is placed under a more frequent and extended monitoring programme, both for phytoplankton and marine biotoxins. In the Netherlands the criteria for the 3 phytoplankton groups are as follows: ASP producing algae: 500,000 cells/l , PSP producing algae: 1000 cells/l and DSP 100 cells/l. Evaluation of results obtained in the years 2006 up to 2012, showed that PSP producing algae were not observed in the Dutch production waters in numbers exceeding the criteria. ASP producing algae were found occasionally in some production areas, but ASP toxin was not detected in the shellfish flesh. In 2007 the criteria for ASP algae were evaluated. Since ASP toxin had never been detected in shellfish, even when higher numbers of ASP producing algae were found, it was decided to increase the criteria for ASP producing algae from 100,000 cells/l to 500,000 cells/l. Violations of these new criteria in 2008 and 2010 did not lead to detection of ASP toxin in the shellfish flesh from those areas confirming the safety of the new limit for ASP producing algae. DSP producing phytoplankton are the predominant algae in Dutch production waters. Violations of the criteria have occurred in all of the years evaluated to date and in most production areas. DSP has been detected more frequently in some of the northern areas then others and some southern production areas are not implicated at all. Notably, the conditions in the Eastern Scheldt do not seem to allow substantial growth of DSP producing algae. Most positive samples were found in the summer period and the beginning of August. DSP algal blooms are found to last from just 1 week up to 8 weeks. In contaminated areas, DSP toxin is detected only in low amounts (LC-MS method). These findings are in agreement with earlier monitoring results described in Fels-Klerx *et al.,* (2011), where results between 1999-2009 were evaluated. For DSP producing algae, the authors tried to evaluate the relationship between the start of DSP producing algae growth and the onset of DSP toxin accumulation in shellfish. For 2007 and 2008 LC-MS data was available, when combined with rat-bioassay data it was shown that DSP toxins were still present five weeks after the peak in DSP producing algae. This relationship will be studied in further detail in the future to improve the early warning system.
Recently EFSA has published an opinion concerning contamination of shellfish with norovirus and the possibilities of establishing limits for norovirus in shellfish. In addition, CEN initiated the development of a standardized method for the detection of viruses in foods, including bivalve molluscs, published as ISO/TS 15216:2013 (Microbiology of food and animal feed -- Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR) . In 2007 and 2008, the presence of several enteric viruses was determined in oysters and mussels obtained from a class A area (Eastern Scheldt) in the Netherlands.

Norovirus genogroup II was detected in 5% of the oysters and mussels studied, hepatitis E virus was detected in 4% of the samples. Human adenovirus was detected in 1% of the studied oysters and parechovirus was detected in 1% of the mussels. Hepatitis A virus and enterovirus were not detected. In all cases, the levels of *E. coli* complied with the limit used for category A harvesting areas, indicating that less than 230 *E. coli* CFU were present in 100 g of shellfish. This confirms the current opinion that *E. coli* does not predict the presence of pathogenic viruses. However, virus detection was undertaken using molecular methods, these indicate the presence of viral RNA/DNA but do not provide information on the infectivity of the virus and thus on potential health risks.

Research is currently ongoing to determine the prevalence of Norovirus in other Class A areas in the Netherlands.

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Table 1. Prevalence of Norovirus genogroup I (NoV I) and II (NoV II), hepatitis A virus (HAV), hepatitis E virus (HEV), human adenovirus (hAdV), enterovirus (EV) and parechovirus (PeV) in oysters (Crassostrea gigas) and mussels (Mytilus edulis) from the Eastern Scheldt.

	Oysters (n=84)	Mussels(n=84)
NoV I	0	0
NoV II	4 (5%)	4 (5%)
HAV	0	0
HEV	3 (4%)	3 (4%)
hAdV	1 (1%)	0
EV	0	0
PeV	0	1 (1%)

Detection and genotyping of norovirus from clinical and shellfish samples: epidemiological implications

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Abstract
Noroviruses (NoV) are the main cause of gastroenteritis worldwide and outbreaks associated with shellfish consumption periodically emerge. In this work, a total of 2643 stool samples from patients with acute gastroenteritis from Galicia (NW Spain) and 81 mussel samples collected from the same geographical area were analysed by qRT-PCR for NoV, using specific GI and GII primers and probe sets. In addition, genotyping was carried out by partial sequencing of the capsid gene. All the strains detected from clinical samples were classified as NoV GII, we observed 8 different genotypes with a clear predominance of GII.4 and GII.14. NoV strains from shellfish samples belonged to genotypes GI.4, GII.4, and GII.6. Only NoV GII.4 and GII.6 were detected in both mussel and clinical samples. Among the different GII.4 variants observed, it is interesting to point out that the same sequences of variant 2006b were detected in clinical and shellfish samples, although with some delay in the latter, which demonstrated a clear epidemiological linkage.

Introduction
Norovirus (NoV), genogroups I and II, have emerged as the most common causes of outbreaks and sporadic cases of acute nonbacterial gastroenteritis in children and adults. These viruses are easily transmitted by the fecal-oral route either directly, from person to person, or indirectly, via contaminated surfaces, water or food (Koopmans and Duizer, 2004). Shellfish play an important role in the transmission of NoV, as filtering of large volumes of seawater contaminated by faecal waste may result in the accumulation of these pathogens to considerable levels in shellfish tissue (Burkhardt and Calci 2000). Thus, consumption of raw or improperly cooked shellfish is a major risk factor for food-borne outbreaks (Koopmans and Duizer, 2004; Le Guyader *et al*., 2008).
The aims of this work were to compare the NoV genogroups and genotypes found in patients affected with gastroenteritis who sought medical care in A Coruña, Galicia (NW Spain) with those detected in mussel samples collected in the same geographical area in order to establish phylogenetic and epidemiologial relationships among clinical and shellfish strains.

Material and Methods
Sample collection and processing
A total of 2643 stool samples from patients of all ages affected with gastroenteritis were obtained from Complejo Hospitalario Universitario de A Coruña (CHUAC), Galicia (NW Spain) during a one-year period (July 2010-June 2011). Also, 81 mussels samples were obtained over an 18-month period (October 2010-March 2012) from seven different harvesting areas in Ría do Burgo, A Coruña, Galicia (NW Spain).
Each stool sample was diluted (10% wt/vol) in PBS supplemented with NaNO3 2M, 1% of BSA and 0.1% Triton X-100. On the other hand, digestive tissues of 10 mussels per sample were dissected, pooled, and homogenized with a volume of 0.1% peptone water pH 7.5. Samples were centrifuged at 1000 x g for 5 min to recover the supernatant.
RNA extraction of each sample was carried out in duplicate with a commercial NucleoSpin® RNA Virus kit (Macherey-Nagel, Germany) following the manufacturer's specifications.

Norovirus detection and quantification
Extracted RNA was used for NoV GI and GII detection by real-time RT-PCR (qRT-PCR) with TaqMan probes (da Silva *et al*., 2007). NoV qRT-

PCR and quantification of the virus were performed as previously described (Manso and Romalde, 2013).

Norovirus Typing
Genotyping of the detected NoV strains was carried out on the basis of partial capsid gene sequences. For this purpose a semi-nested RT-PCR protocol with specific primers for NoV GI and NoV GII was performed. RT was carried out with Expand Reverse transcriptase (Roche, Germany) and 5 µl of purified RNA and 100µM of random hexamer stock as reverse primer following the manufacturer's specifications. The first round PCR was conducted using primers GOG1F and G1SKR for NoV GI and GOG2F and G2SKR for NoV GII (Kageyama *et al*., 2003; Kojima *et al*., 2002). The second PCR for NoV GI was conducted with primers G1SKF and G1SKR. For NoV GII, the second round of PCR was carried out with primers G2SKF and G2SKR. All the PCRs were conducted using Expand High Fidelity PCR System (Roche, Germany) following the manufacturer's specifications. RT-PCR amplicons for NoV GI and GII were 330 bp and 344 bp, respectively. Amplification products were analyzed by electrophoresis using a 1.5% agarose gel and visualized with ethidium bromide. Amplicons were purified from the gel with the QIAQuick Gel Extraction Kit (Qiagen) and sequenced.

Phylogenetic analysis of NoV Strains
Sequence analysis was performed with the DNASTAR Lasergene SEQMAN program. Phylogenetic reconstructions were performed based on sequences of the NoV partial capsid gene. Sequences of NoV reference strains were retrieved from Genbank. Neighbour Joining (NJ) phylogenetic trees were constructed with MEGA5 software using the Kimura-2-parameter and a bootstrap of 1000 replicates (Balboa *et al*., 2011).
Sequences were also analysed with the Norovirus Genotyping Tool Version 1.0 (Kroneman *et al*., 2011), for comparison.

Results
In clinical samples from patients affected by gastroenteritis, NoV's were detected in 28.26% of the cases, without significant differences among the different age groups. Suitable sequences for subsequent phylogenetic characterization of the strains were only obtained in 41% of the positive samples. All the strains were classified as NoV GII. Within this genogroup, the detected strains belonged to 8 different genotypes: GII.1 (0.67%), GII.3 (2.65%), GII.4 (36.42%), GII.7 (8.6%), GII.6 (2.65%), GII.12 (2.65%), GII.13 (13.24%), GII.14 (33.11%). Within GII.4, 10 strains were classified as the 2006 variant, 70 as the 2010 variant and 59 could not be assigned to any specific variant employing the Norovirus Genotyping Tool.

In the mussel samples, NoV's were detected in 61.7%, of the samples. Only 39% of the detected NoV strains gave suitable sequences for phylogenetic characterization. The NoV strains were classified as GI.4 (37.5%), GII.4 (50%) and GII.6 (12.5%). Only NoV GII.4 and GII.6 were detected in both mussels and clinical samples. A phylogenetic tree based on the NJ method was constructed with the sequences of the 80 GII.4 strains detected in clinical samples and characterized as 2006 and 2010 variants and the 8 strains detected in mussels (Fig. 1). Visual inspection of the tree revealed that 7 strains from mussel samples and 10 strains from clinical samples constitute a monophyletic group together with the reference strains belonging to the NoV GII.4 2006b variant, with a bootstrap value of 74%. Sequence similarities among these strains ranged from 99.4% to 100%, and 7 mussel strains and 7 clinical strains were identical. Those sequences were detected in clinical samples during August 2010 and in mussels from March 2011. The other GII.4 sequence from mussels is located together with the GII.4 2010 variant strains from the clinical samples, forming a cluster with a bootstrap value of 70%. However, this strain is rearranged in a separate branch, grouping together with the reference strain GII.4 2009 (JN595867).

The phylogenetic tree constructed for the GII.6 sequences, including 2 strains detected from mussels and 4 strains from clinical samples (Fig. 2), revealed a higher diversity of the strains in the clinical samples. In addition, it was also observed that mussel strains constitute a robust cluster together with 1 clinical strain, with bootstrap values of 97%. The similarity between the clinical strain and the mussel strains was 97.6%.

Fig. 1. Phylogenetic reconstruction based on the partial capsid gene sequences of NoV GII.4 2006b and 2010 variant strains (according to the Norovirus Genotyping Tool Version 1.0) and reference strains using the NJ method. Strain code: B, strains detected in mussel samples; H, strains detected in clinical samples. Bar, nucleotide substitutions per site. Only bootstrap values above 70% are shown (1000 re-samplings) at each branch point

Discussion

The NoV strains detected in this study were characterized within 8 different genotypes, indicating the high diversity of NoV, even in a limited geographic region. Such variability is similar to that observed in other studies (McAllister *et al*., 2012).

NoV GII.4 was the dominant genotype, in general and taking into account the origin (clinical or shellfish) of the samples. This finding is consistent with other recent molecular epidemiological and surveillance studies that found GII.4 to be responsible for the majority of NoV infections and outbreaks worldwide (Siebenga *et al*., 2009; Bull *et al*., 2010). In addition, this genotype was the only one detected in shellfish in a study performed in Portugal, which is geographically close to Galicia (Mesquita *et al*., 2011).

The high incidence of NoV GII.4 in the population could facilitate the transmission of the virus (Victoria *et al*., 2009). However, is important to note that it has been reported that NoV GI is more

often implicated in outbreaks because of its higher resistance to breakdown during wastewater treatment (da Silva *et al*., 2007). In fact, the genotype GI.4 was also predominant when analysing the shellfish samples separately.

In only 41% of the clinical samples and in 39% of mussel samples, suitable sequences were obtained. Similar results were obtained in previous reports (Mans *et al*., 2013). This is probably related to the variety of single nucleotide polymorphisms (SNP) found even in the relatively conserved capsid region, which makes the design of suitable primers and probes difficult. On the other hand, it also means that it is extremely important to undertake appropriate RT-PCR optimization and verification assays (Kageyama *et al*., 2003; Loisy *et al*., 2005).

Phylogenetic analysis of NoV GII.4 strains detected in mussel and clinical samples revealed that the 2006b variant mussel strains are 100% homologous with most of the 2006b variant strains detected in the population. However, a delay of approximately 6 to 8 months was observed between their detection in clinical samples and their first reported detection in mussels. Although in the literature there are many examples of relationships among NoV sequences found in shellfish and clinical samples during gastroenteritis outbreaks (Koopmans and Duizer, 2004; Le Guyader *et al*., 2008, 2010; Nenonen *et al*., 2009; Iizuka *et al*., 2010), to our knowledge this finding is one of the first pieces of scientific evidence on how strains circulating in the population can reach the shellfish harvesting areas. Further studies are needed in order to understand the fecal-oral cycle for NoV, which can be useful to establish appropriate preventive measures

Acknowledgements

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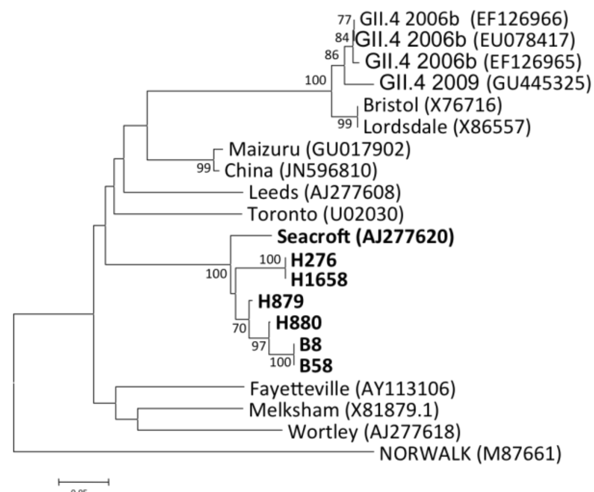

Fig. 2. Phylogenetic reconstruction based on the partial capsid gene sequences of NoV GII.6 strains by NJ method. Strains code: B, strains detected in mussel samples; H, strains detected in clinical samples. Bar, nucleotide substitutions per site. Only bootstrap values above 70% are shown (1000 resamplings) at each branch point.

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Quality programmes as a commercial opportunity

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Abstract
This presentation summarises food safety consumer trends and experience in the industry to discuss whether a major opportunity for creating value from shellfish safety programmes is available. Shellfish safety programmes have four principal stakeholders. The first three – industry, science and regulatory authorities are at the forefront of decision making. The fourth though, the consumers of shellfish products are relatively invisible even though they are the *Raison d'être*, the reason for existence, the ultimate purpose of molluscan shellfish safety.
The presentation will examine how industry and science both have a duty to be responsive to consumer concerns and trends and will illustrate how awareness of growing consumer concerns over animal welfare and ethics was, alongside the need for more quantitative science, the major driver of the development of the chemical based biotoxin test methodologies in New Zealand. That awareness can then be converted into market opportunity, leveraging commercial returns from the existing investment in quality assurance programmes.
Consumers' perceptions of value are multi-layered. Food product safety is clearly, and increasingly one of those layers. Alongside olfactory qualities, there is an increasing demand for ethical and safe foods. That is, shellfish safety therefore represents a potentially significant value creating opportunity. The now widely adopted chemical based test methodologies and programmes provide an ethical product assurance that should have consumer appeal. However that opportunity is largely neglected by industry. Quality assurance is viewed as a "cost". However it also represents an opportunity to create and harvest value that at the present time is largely lost. The presentation will consider the opportunities and the pros and cons of utilising shellfish safety as a value adding tool.

Keywords: Consumer, Asset Management, Compliance, Productivity, Profit.

Introduction

Alongside the immense changes in science and technology over the past fifty years, and to a large degree driven by it, we find ourselves surrounded by the most massive social and economic upheaval since the industrial revolution. According to some commentators – the biggest upheaval in the history of civilisation. It would be most surprising if that upheaval was passing our industry by. Yet – perhaps because we have become so accustomed to change, we've come to take that change for granted – or maybe we're just so focused on the science that we barely notice what's going on around us – especially in the two areas discussed in this presentation.

Perhaps we're a bit like the boiled. If you drop a frog in a pot of boiling water, it will of course frantically try to clamber out. But if you place it gently in a pot of tepid water and turn the heat on low, it will float there quite placidly. As the water gradually heats up, provided you turn the heat up slowly enough, the frog will sink into a tranquil stupor and before long, with a smile on its face, it will unresistingly allow itself to be cooked. This illustrates the point that things change around us without us noticing, or more importantly, without us changing our strategies accordingly. While the frog story is usually used to illustrate negative things sneaking up on us unnoticed, it also serves to illustrate how opportunities can emerge unnoticed as well.

In a quote incorrectly attributed to Charles Darwin it is said that it is the most adaptable, not the smartest or the most powerful that survives. The

quote actually comes from Leon Megginson, Professor of Management and Marketing at Louisiana State University when using Darwin's conclusions to illustrate business responsiveness and adaptation. The full quote actually says ***"It is not the most intellectual of the species that survives; it is not the strongest that survives; but the species that survives is the one that is able best to perceive and adapt to the changing environment in which it finds itself."*** Then clearly the precursor to adaptability is the ability to sense the changing environment. Our survival as an industry and our relevance as assurers of food safety is dependent upon our ability to recognise and respond to change.

Molluscan shellfish safety has made immense strides over the last two decades but within the quite narrow scope of science and regulation. It seems just possible then that some of the changes in the world in which we find ourselves have eluded our attention.

We think of molluscan shellfish safety programmes as a tool to meet our regulatory requirements. Compliance is a cost of production. A grudge purchase in marketing terms. Something from which we receive no perceived benefit – we just have to do it to satisfy the regulators. As such we are product and process oriented, not consumer oriented.

But what if those same programmes represented an unexploited opportunity to create value for industry? We have perhaps missed the significance of the growing consumer concerns about food safety and the tools that our work provides to help allay those concerns. For consumers, food safety has emerged from the mists of the subconscious into being a conscious part of the purchase decision criteria. We have not spotted that consumers are willing and constantly do pay a premium for quality including for assurances just like those we provide. We have also missed the change from reliance on tangible assets – plant and machinery - to intangible or intellectual assets that have accompanied the emergence of the so-called "knowledge economy". Molluscan shellfish safety is almost entirely dependent on intellectual assets and those assets therefore warrant closer scrutiny and management than they currently receive.

This presentation therefore summarises food safety consumer trends and experience in the industry to discuss whether two major but unexploited opportunities for creating value from shellfish safety programmes are available. First the opportunity to monetise the consumer benefits delivered by our programmes. Then second – the opportunities on offer from the shift from reliance on tangible to intangible assets.

Shellfish Safety Programmes

Shellfish safety programmes have four principal stakeholders. The first three – industry, science and regulatory authorities are at the forefront of decision making. The fourth though, the consumers of shellfish products are relatively invisible even though they are the *Raison d'être*, the reason for existence, the ultimate purpose of molluscan shellfish safety.

Industry and science both have a duty to be responsive to consumer concerns and trends and I will illustrate how awareness of growing consumer concerns over animal welfare and ethics was, alongside the need for more quantitative science, the major driver of the adoption of the chemical based biotoxin test methodologies and associated protocols in New Zealand. Although we did not appreciate it at the time, the adoption of those methods provides us with the tools to respond to a significant consumer trend, mitigate against global trends in communication and potentially create new value opportunities by more effectively managing our "intellectual assets".

The tension between industry's profit motive and the regulators public safety motive is often represented as an irreconcilable tug of war. The West in particular has been caught up in a productivity model involving driving out cost – the opportunity to create value has been largely forgotten. Search "productivity" on Google and you are swamped with references to efficiency and you have to search very hard to find the words "creating value" – but remember we increase productivity and profitability by both driving down the unit cost of production or driving up the unit

value of output. In our constant struggle to balance quality and cost we instinctively look towards lowering cost rather than increasing value – assuming that value is outside of our control. It is not. We need to reconcile this tug-of-war and we will achieve that when we begin to recognise molluscan shellfish safety not as a cost to be tolerated but rather as a marketing opportunity to be embraced.

As scientists and regulators we focus on objective quality criteria and standards. Our processes are increasingly quantitative. That's the very foundation of what we do but as we will see, consumers purchase decisions are a lot more complex and highly subjective. From farm to fork consumers are demanding much more than food that tastes good or even meets nutritional needs. Consumers are demanding greater accountabilities, greater transparency and greater assurances of safety. Their perceptions play a critical role and are influenced by much more than just the olfactory qualities of the food they consume. Although the confidence that people have in the food supply chain varies from region to region, consumers are no longer content to assume that regulatory authorities have adequately assured the safety of the products that they purchase and consume. They want and need to receive overt assurances. Interestingly in terms of physical product safety we are all familiar with safety marks, arguably the best know being the ubiquitous CE and car safety ratings that are promoted with powerful emotive messages.

Two majors changes that we must be aware of:

1. **Communication Technologies**
2. **Consumer Behaviour**

Changes that suggest that we quality people need to be having much more engaging conversations with the marketing people. The monetisable consumer quality assurances that our molluscan shellfish safety could, and I suggest, should be part of our shellfish Inc brand that can be leveraged off by individual company brands. In this regard we can, I suggest, derive a great deal of benefit from our continuing collaborations.

The evolution and global adoption of internet based and mobile communication technologies has democratised consumers' access to information. Un-managed this represents an immense threat to us - exploited properly an unparalleled opportunity. What were once local news stories are now global stories. A food poisoning or biotoxin event anywhere on the planet can and does immediately become a global story. If we want to see a topical example of that consider the recent horse meat scandal in the UK and Europe. It has little more than vicarious interest to those of us in the Southern hemisphere. Under the reign of traditional media it would have represented one or two brief news stories. Instead – at the time of writing - searching Google for "Horse Meat Scandal" produced 263,000,000 results just one month after the first news release. That means that what happens in shellfish safety is of universal interest – and concern. Combine that with the fact that we know that consumers do not distinguish between one event and another and we have a potent cocktail. That is, one failure in the food supply chain has implications right across the chain. One molluscan shellfish safety failure anywhere in the world has flow on effects for all of us.

Not only is news dissemination broad it is almost instantaneous. Once we had time to attempt to manage news release – news spread in time frames measured in days or even weeks. Now we don't have that luxury. A personal example to illustrate: When the second major Christchurch earthquake struck – two years ago on February 22 - even though enough of the telecommunications infrastructure survived to keep the system up, the dramatic surge of people using cellphones collapsed the system within seconds. Thankfully our family in Christchurch were able to get a message out to us before the system went down. Starved of information we turned to the internet. There were no New Zealand posts for quite some time but within twelve minutes we were able to read details and see photos on the Huffington Post which is based in New York. When something happens in one place, the world knows about it – immediately. Furthermore, news reporting is no longer the domain of professionals who theoretically have a duty of care around accuracy, but members of the public who through the various social media channels are communicating their

feelings as well as the facts. That creates an immediate vulnerability when something does or even just appears to go wrong.

The second of these two changes is the rise in concern about food integrity. I use integrity as a much broader description than just quality as we traditionally consider it. The horse meat scandal is a timely example! The meat was likely processed in accordance with the appropriate safety standards. It was its passing off as beef that led to the scandal. Quality assurance as we provide is an important part of that overall integrity assurance.

Safety concerns growing

In Europe for example, consumers are remarkably cynical about food safety. In a report commissioned by the Directorate-General Health and Consumer Protection and the European Food Safety Authority and published in Eurobarometer, food poisoning was the highest food risk perception amongst consumers, albeit that only 16% identified it unprompted. However when prompted *"concerns appear quite wide spread"*. Most concern was about external factors over which consumers perceive that they have no control including residues, viruses, bacterial contamination and unhygienic conditions in the production and supply chain. That's very close to our space. Although more than 50% expressed confidence in public authorities 47% of citizens think that when deciding on priorities, authorities would favour the economic interests of producers over the health of consumers and only 46% believe that enforcement of the strict rules in place is done properly. This is a great example of "perception" because I doubt very much if you were to survey industry that we would say we believed our economic interests were being favoured over the consumers' wellbeing. Eurobarometer went on to say that the results are not necessarily a reflection of the actual situation but rather are *"indicative of respondents' propensity to worry and to admit being worried about food safety risks."* That is immensely meaningful – consumers' perception of risk colours their interpretation of objective facts. In the absence of evidence to the contrary, consumers are inclined to worry about food safety. It is within our control to reduce that level of worry. And when we reduce that level of worry we should anticipate increased demand for our products and everything that implies in terms of profitability. In other words, the output of our quality programmes is monetisable.

In science and regulatory terms we endeavour to make our decisions as objective as we can – we want the facts to "speak for themselves". But in consumer decision terms perceptions play a prominent role. That is the whole basis of branding and business positioning. In consumer decision making, we know that subjective elements play a major role in the decision to purchase and in the price paid.

Perception

Perception has a major impact on the price realised:

- In the UK, blind tastings of baked beans ranked Heinz beans as sixth best. When the tastings were conducted with the brand known to the tasters, Heinz was ranked number 1!
- In the USA, consumer panels ranked identical wines higher when they thought that the price was higher.
- In Denmark, free range eggs have gained a major market share and a 20% price premium even though scientific testing reveals no nutritional differences between free range and battery eggs.

The message here is that perceptions are all powerful and we have the tools to help counter those negative perceptions.

Consumers are demanding more and more information. Trendwatching.com calls it INFOLUST.

Research published in the USA by PR firm Context Marketing had these major findings:

- Consumers are paying closer attention to food quality claims, they are becoming more savvy about their evaluations and the more educated and affluent customers have a surprisingly detailed understanding of the issues;
- Safety concerns lead the list – especially the assurance that the things that they <u>do not want</u> in their foods are not there. Food safety is important to the majority of shoppers and especially women who bring somewhat greater concerns to food choices. Women pay more attention to food safety information.

They control as much as 85% of global consumer spending and from 80% of UK spending on food (*UK Office of National Statistics 1997*) to 93% according to She-conomy. We can reasonably anticipate that this figure or something similar is universal. Further, according to the World Bank, women's spending power continues to grow at a significant rate.

- Most consumers will pay a 10% or more premium for "quality" and 12% said they would pay more than a 10% premium.
- Notably, the language that the food industry uses to describe product attributes, quality etc is different to what consumers understand and 59% of women feel misunderstood by food marketers (She-conomy).

Add to that the emergence of the millenials or generation Y and their even greater demands for sustainability and transparency, especially when it comes to food. You can't buy millenials loyalty – you can only earn it through transparency and integrity.
This is a potent mix of risk and opportunity.
Consumers' perceptions of value are multi-layered. Food product safety is clearly, and increasingly one of those layers. Alongside olfactory qualities, there is an increasing demand for ethical and safe foods. That is, shellfish safety therefore represents a potentially significant value creating opportunity. The now widely adopted chemical based test methodologies and programmes provide an ethical product assurance that should have consumer appeal.

Yet despite our investments and collective efforts in assuring that very safety that consumers seek and are prepared to pay a premium for, we make little effort to leverage value from it. Once we avoided drawing attention to our work because of concerns about the mouse bioassay and the implications of testing on animals. As we have moved to chemical based methodologies for biotoxins and as our protocols and methodologies to control bacterial and viral contamination continue to become more robust the opportunities to create additional value for industry and for the science organisations that support us grows ever greater.

Kano
According to Dr Noriaki Kano consumer satisfaction can be defined in three tiers – "Expected" where consumers take for granted that things like product safely will be taken care of. The second is specified quality and while the first rarely receives conscious consideration, this tier is upper most in the consumers mind when choosing and experiencing a product or service. The third tier is that part of the product experience that exceeds the customers' expectations and it is this that thrills or delights.

When I first encountered the Kano model back in the early 1990s food safety was cited as an example of the first tier – it was assumed that it was taken care of. According to this model attributes in the lowest tier have little influence on choice and satisfaction. That is where our work has traditionally resided and as you can see from Kano's model, no matter how good we were it wasn't going to impact consumer perception and satisfaction. However things have, we know from the research, changed and consumers are now consciously factoring food product safety and integrity into their decision making. That means that our work can now, presented properly, play a meaningful role in achieving consumer satisfaction. Of course if we ignore this, our opportunity could equally become a threat.

Kiwi bioassay
In fact, in earlier addresses I identified awareness of the mouse bioassay as a threat to consumer choice. Now however with the advances in testing technologies we are in a position to translate the threat into a meaningful opportunity.

That means that we have unwittingly moved from being responsible for customer safety to customer satisfaction. Although that difference might seem like semantics, it is potentially a game changer. I suggest that our industries, wherever they are have an opportunity to look very carefully at what we do in the molluscan shellfish safety game, examine their own participation and look to how our processes, protocols and regulatory oversight can be used to provide consumer assurance of safety and hence enhanced consumer satisfaction.

I have already discussed the potential to enhance consumer confidence by leveraging our quality programmes and regulatory compliance. I now want to move on to intellectual asset management.

Assett Management

We are all familiar with tangible assets. In any organisation the tangible assets are comprised of plant and machinery and financial assets. We manage those with great care. We keep careful registers. Maintain them judiciously. We defend them against loss and insure them as part of our prudent risk management. We measure their contribution and report their value and performance in annual reports. Yet as we have moved further and further into knowledge economies, plant and machinery is no longer the principal asset upon which business success rests. There is a new asset class and it looks like this …

You cannot see them and that means that they have been neglected. A firm or industry's total assets are made up of physical assets, financial assets and intellectual assets

While in 1978, according to Standard and Poor's, 80% of the asset value of a company was in its tangible or physical assets, by 2006 a remarkable change had occurred. 80% of the value was now represented by the intangible or intellectual assets.

Intellectual (or intangible) assets are the drivers of business productivity, competitiveness and profitability. They exist as difficult to replicate combinations unique to each business. They are the "magic" ingredient that makes the difference between ordinary and exceptional – given similar plant and machinery etc.

We are most familiar with intellectual properties like patents and trademarks. Intellectual assets is a much broader classification including inventions, ideas, institutional knowledge & expertise, know-how, designs & design capability, plans, customer databases and relationships, production processes and quality systems, brands and reputation.

Although our intellectual assets are spread across a diverse range of organisations – industry, research, regulatory authorities, they none the less represent assets that our individual and collective industries rely upon. Therefore, I suggest that as industries we would benefit from a far more active engagement in their management – in fact they should receive the same level of attention to manage as would tangible assets of an equivalent value.

So that is about managing the assets more closely but there is a further potential too. Mature industries in successful economies not only embed their intellectual assets in their products but exploit the value in those assets directly.

To provide a useful analogy, Finland has transformed its economy from one of the poorest to one of the wealthiest developed nations, largely by exploiting the intellectual assets inherent in its wood fibre industry. It has leveraged the intellectual assets that underpinned its wood fibre production expertise – growing trees – in quite hostile conditions - to become the world leader in pulp and paper processing plant and machinery, pulp and paper chemicals and pulp and paper consulting. We need to be asking ourselves –

"Do similar opportunities exist for molluscan shellfish production and in this context, from the intellectual assets inherent in molluscan shellfish safety?"

Conclusion

I believe that leading the development of molluscan shellfish safety intellectual asset management, of assuring the security of those assets, understanding the implications of loss, succession planning for the holders of those assets where the knowledge is tacit – that is draws upon accumulated experience, should be an integral part of each industry's risk management and development strategies whether or not the assets are technically owned by industry, science institution or regulatory authorities.

We need to move beyond thinking of ourselves as national industries competing for our share of the "protein" market and instead think of ourselves as a global industry competing for our collective share of consumers' hearts and minds. Molluscan shellfish safety assurance needs to be thought of as

an integral part of the value chain. Then, in order to ensure that we fully satisfy our consumers, we need to prepare ourselves for an unprecedented level of exposure.

Every decision by every person in our production and value chain must be focussed on meeting consumer expectations – and those expectations now very clearly include utterly transparent assurances of food safety. As an industry we have an obligation to drive scientific and regulatory advancements to continuously progress molluscan shellfish safety.

Given modern consumers demands for integrity and transparency and their access to information through modern communication technologies, we have to move from having nothing to hide to pro-actively showing and proving that we have nothing to hide – not just transparent, but naked and proud of it!

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Application of risk analysis for ensuring food safety after the Fukushima nuclear accident: Focused on molluscan shellfish safety

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Abstract

Immediately after the accident at the Tokyo Electric Power Company's Fukushima Daiichi Nuclear Power Plant on 11 March 2011, the Japanese Government set provisional regulation values by adopting the "index relating to limits on food and drink ingestion" which had been determined by the Japanese Government in preparation for nuclear emergencies on a basis of an intervention level of 5 mSv/year.

On 1 April 2012, the Japanese Government adopted 1 mSv/year consistent with the current Codex Guidelines as an intervention exemption level, and established new limits. The limit of total radioactivity attributable to Cs-134 and Cs-137 is 100 Bq/kg for general foods, 50 Bq/kg for milk and infant foods, or 10 Bq/kg for drinking water.

Sampling of food and water for monitoring surveys began on 16 March 2011. Monitoring results of molluscan shellfish indicated the health risk associated with the consumption of mollucs is very low. In this paper, the way in which the risk analysis frame work was applied will be described.

1. Introduction

A magnitude 9.0 earthquake occurred off the northeastern coast of Japan on 11 March 2011, triggering a deadly tsunami with waves reaching as high as 40.5 m.

This disaster caused massive destruction across a wide area: there have been 15,883 confirmed fatalities with 2,676 people missing as of 10th May 2013 (NPA Japan, 2011).

The height of the tsunami which attacked the Fukushima Nuclear Power Plant (NPP) was higher than 14m. The tsunami swamped the reactors and the generators became inoperable. Elevation of temperature and pressure inside the reactors resulted in a partial meltdown and hydrogen explosions. Eventually, radionuclides were discharged from the stricken reactors.

A preliminary estimate made by the Nuclear and Industrial Safety Agency of Japan (NISA) indicated that 160 peta (P) Bq of 131I, 18 PBq of 134Cs and 15 PBq of 137Cs were spewed into the atmosphere between 11 and 16 March 2011 (Hamada *et al*., 2012).

The Tokyo Electric Power Company (TEPCO) estimated the outflow of contaminated water into the open sea as follows: 520 tons of water containing 4.7 PBq (a total level of ^{131}I, ^{134}Cs and ^{137}Cs) between 1 and 6 April 2011, 10 393 tons of

water containing 150 GBq between 4 and 10 April 2011 and 250 tons of water containing 20 Tera Bq on 11 May 2011 (NHERQ, 2011). On 12 April 2011, the Fukushima accident was provisionally rated as Level 7 on the International Nuclear and Radiological Event Scale (NISA, 2011), the same level as the Chernobyl nuclear accident in 1986.

The Fukushima NPP is located on the East coast of the island of Honshu, 200 km north-east of Tokyo. The coast runs north-south, facing the Pacific Ocean.

Even though the causes and nature of the radioactive contamination following these accidents are so different, it is possible to compare their radio-ecological consequences with respect to freshwater and marine aquatic biota.

Of the three radiation accidents, reactor failures that occurred at the Fukushima NPP, the Chernobyl NPP (1986), and the Kyshtym radiation accident (1957), the Fukushima NPP failures resulted in the lowest calculated radiation exposures to aquatic biota. (Fig. 1) (Kryshev *et al*., 2011).

Fig. 2 shows ^{137}Cs concentration and $^{131}I/^{137}Cs$ rations in seawater at less than 2km from Fukushima NPP. The high concentration recorded in the seawater in the immediate vicinity of the Fukushima NPP indicated that there were several sources of radioactive liquid effluents escaping directly from the NPP (Bailly du Bois *et al*., 2012). They consisted of the water used to cool the damaged reactors, part of which has washed over surfaces contaminated by radioactive deposits formed during the atmosphere release, and other water leaked out of the damaged reactors, and ran into the sea.

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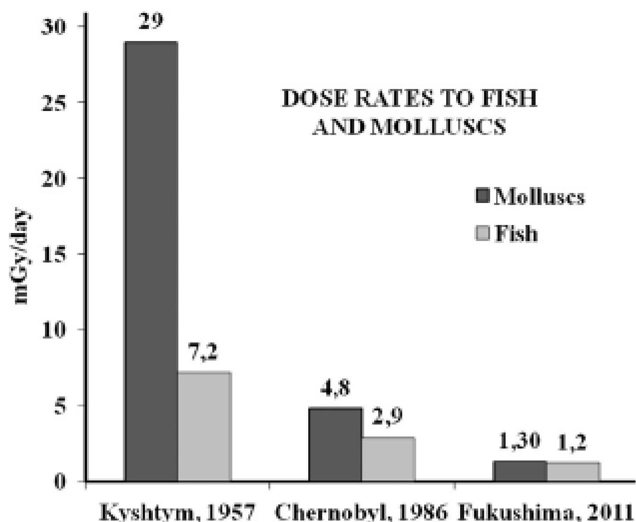


Fig. 1. Dose rates of internal irradiation calculated for aquatic biota in the exclusion zones of radiation accidents: Kyshtym accident at Lake Uruskul within the central part of the Eastern Urals Radioactive Trail (September and October 1957); the Chernobyl NPP cooling pond (April and May 1986); the coastal area of the sea near the Fukushima NPP (March and May 2011). Adapted from NERHQ (2011).

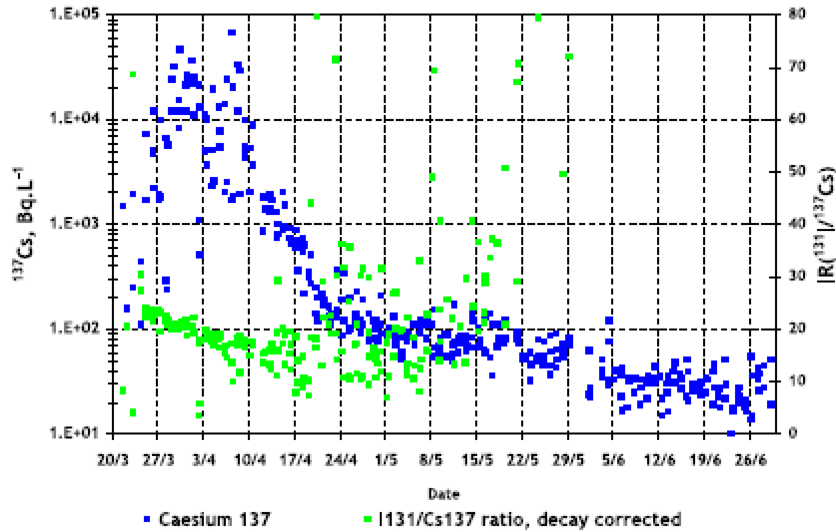


Fig. 2. Evolution of 137Cs concentrations and 131I/137Cs ratios in seawater at less than 2 km from the Fukushima NPP. Adapted from Ref 4.

Table 1. Provisional regulation value

Radionuclide group	**Category of food and water**	**Provisional Regulatory Value (Bq/Kg)**
Radioiodine	Drinking water and milk	300
		(100 for infant)
	Vegetable except corn, tubers and roots	2,000
Radiocesium	Drinking water and milk	200
	Vegetable, Cereals, Meat, Egg, Seafood and other foodstuffs	500
Uranium	Infant foods, Drinking water and Milk	20
	Vegetable, Cereals, Meat, Egg, Seafood and other foodstuffs	100
Plutonium and other transuranic alpha emitters	Infant foods, Drinking water and Milk	1
	Vegetable, Cereals, Meat, Egg, Seafood and other foodstuffs	10

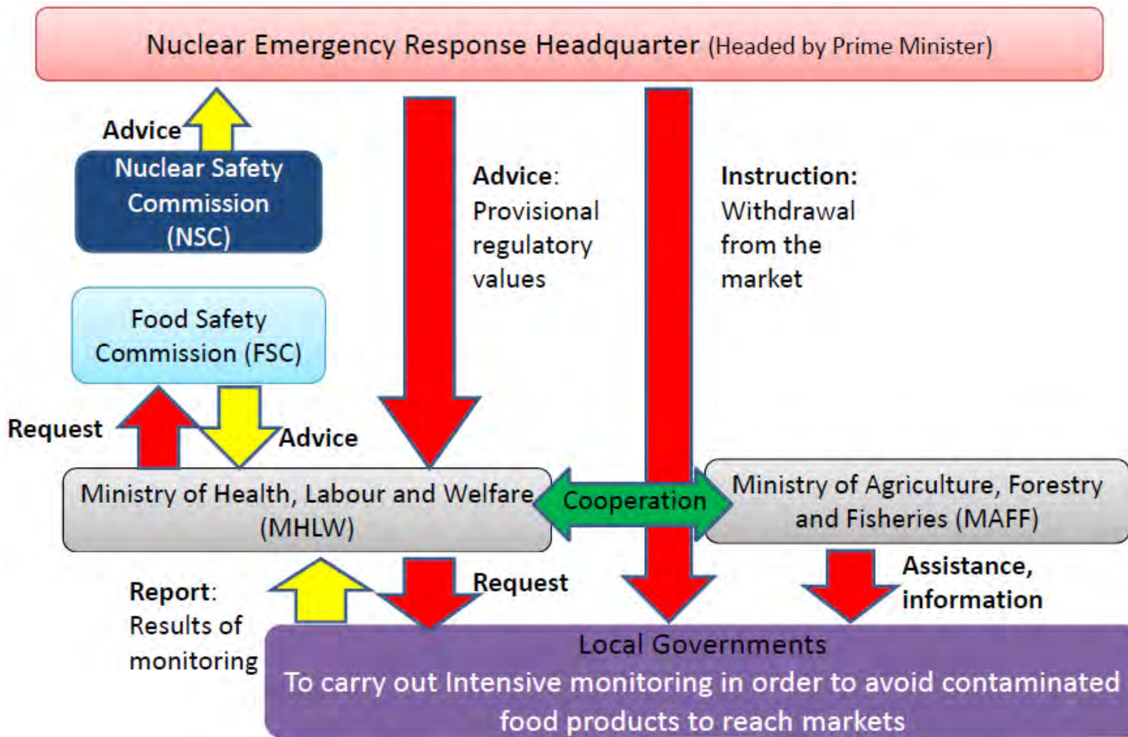
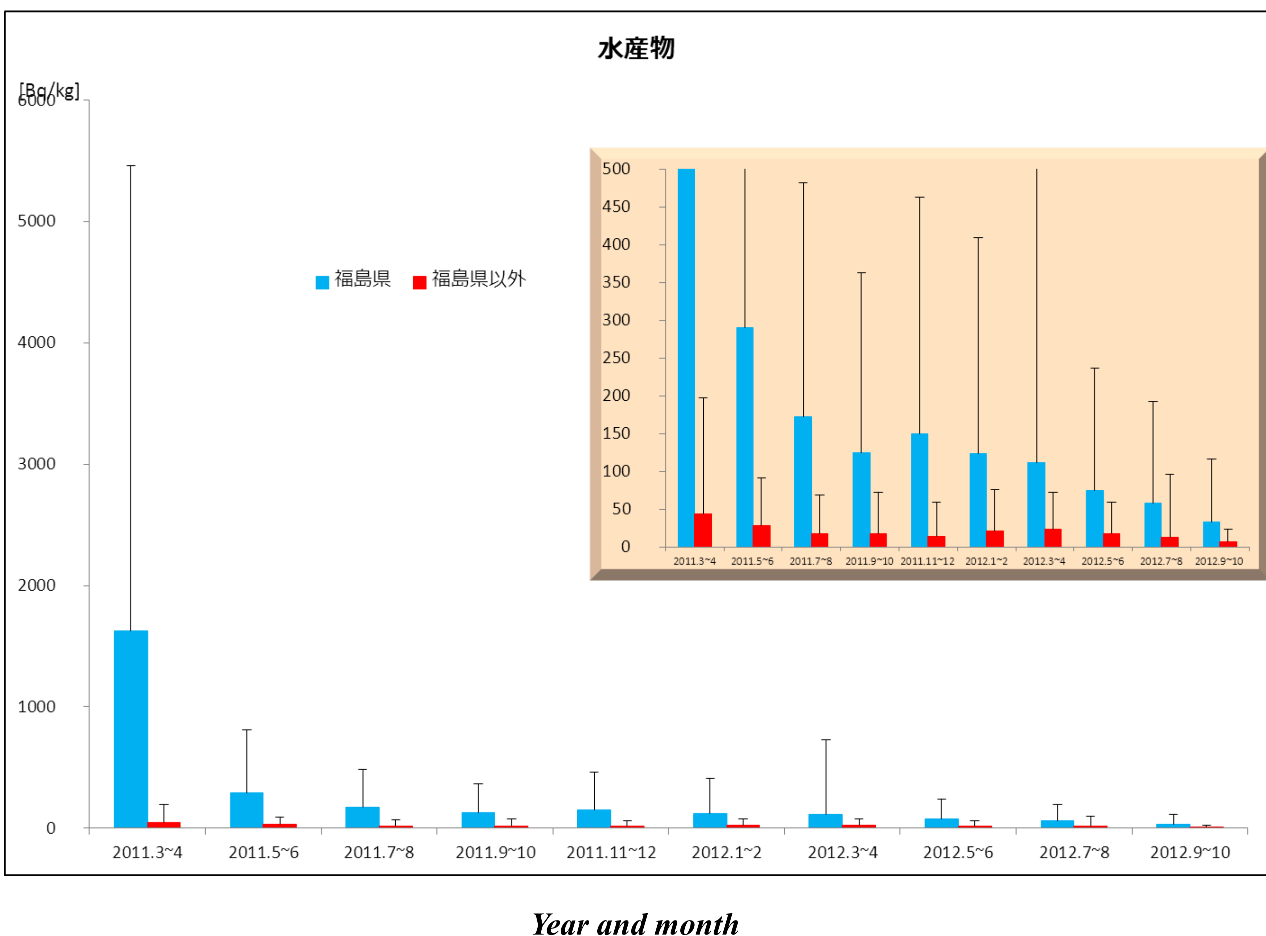


Fig. 3A. Administrative System for Food Safety in the Situation of Nuclear Emergency. Monthly average



Year and month

Fig. 3B. Administrative System for Food Safety in the Situation of Nuclear Emergency. Monthly average radioactive cesium levels in fish and fishery products Blue bars: products sampled in Fukushima prefecture. Red bars: products sampled in other prefectures

The influence of these liquid releases was particularly significant from March 26 to April 8 2011 in the vicinity of the nuclear facilities. The drop in the concentrations measured after April 10 2011 in the vicinity of the facilities showed that they were far smaller direct releases after this date.

2. Risk Management

In Japan, the Ministry of Health Labour and Welfare (MHLW) and the Ministry of Agriculture Fisheries and Forestry are food safety risk management agencies. The Food Safety Commission (FSC) of the Cabinet Office is a risk assessment body (see Fig. 3A).

Regarding nuclear emergencies, the Nuclear Safety Commission of Japan (NSC) advised the Nuclear Emergency Response Headquarter (NERHQ), which was established on 11 March 2011, that monitoring surveys of food and water should begin immediately because high levels of radioiodines and radiocesiums were detected in the topsoil and plants on 15 March 2011. On 16 March 2011, samples for such surveys started being collected.

2.1 Provisional Regulation values

On 17 March 2011, the MHLW set regulatory limits for radioactively contaminated food and water stipulated as 'provisional regulation values' (PRVs)(see Table 1), and directed that food and water contaminated above the PRVs should not be consumed (MHLW, 2011a). PRVs were initially exceeded in tap water, raw milk and leafy vegetables, and their distribution and consumption have been restricted since 21 March 2011.

Subsequently, the PRV for radioactive iodine in seafood was additionally set on 5th of April 2011 MHLW (2011b), because fish contaminated at levels of concern began to emerge. These PRV levels are based on protective action guides (PAGs) of a 50 mSv per year thyroid-equivalent dose for radioactive iodine and a 5 mSv/year effective dose for radioactive cesium in emergency situations. The NSC previously designated these levels in preparation for emergency situations. The PRVs were adopted from the preceding index values, except the value for radioiodines in drinking water and milk consumed by infants, and that in seafood. Although the PRV for radioiodine in water and milk ingested by infants was adopted from the 'guideline level' indicated by the Codex Alimentarius Commission (CAC) in 1995 (CAC, 1995), other values were adopted from the preset 'index values' that NSC had provided as evaluation criteria to launch discussion on whether NERHQ needed to restrict the consumption of food and water (NSC, 1980). Index values for radioiodine were defined in water and two food categories (i.e. milk, and vegetables except corn, tubers and edible roots), and those for other radionuclide groups (i.e. radiocesiums, uranium, plutonium and other transuranic α emitters) were defined in water and four food categories (i.e. all food). The intervention level (IL) considered in each category of food and water was a committed equivalent dose to the thyroid of 11.1 mSv/year

Table 2. Comparison of provisional regulation values and the new standard enforced on 1st of April 2011

○Protective action guide levels for radioactive cesium in emergency situation[1]

Category	Limit
Drinking water	200
Milk, milk products	200
Vegetables	500
Grains	
Meat, eggs, fish, etc.	

○Radiological standards for radioactive cesium in the existing exposure situation[2]

Category	Limit
Drinking water	10
Milk	50
General Foods	100
Infant Foods	50

(Unit : Bq/kg)

for radioiodine, and a committed effective dose of 1 mSv/year for other radionuclide groups (e.g radiocesiums). Index values (i.e. derived IL) were set not to exceed IL in any age groups of infants (0–1 year of age), children (1–6 years) and adults (≥17 years), and the same index values were thus given to these three age groups. Index values were calculated as radioactive concentrations of indicator radionuclides (^{131}I for radioiodine, ^{134}Cs and ^{137}Cs for radiocesiums) by postulating the relative radioactive concentration of coexisting radionuclides (i.e. ^{132}I, ^{133}I, ^{134}I, ^{135}I and ^{132}Te for ^{131}I; ^{89}Sr and ^{90}Sr for ^{134}Cs and ^{137}Cs) (Hamada *et al.*, 2012).

These PRVs were enforced until 31st of March 2012.

2.2 The New Radiological Standards for Foodstuffs

The aim of new radiological standards for foodstuffs is to reduce the maximum dose due to food consumption from 5 mSv per year to 1 mSv per year. To achieve this, standards were derived for four categories: (1) “drinking water,” “infant foods” and “milk,” which are deemed to need special consideration from the viewpoint of public concern; and (2) “general foods” for other foods were established (Yamaguchi, 2012).

The targeted radionuclides are all the radioactive radionuclides on the NISA list emitted by the Fukushima NPP accident, and whose half-life is over 1 year. Standard limits have not been established for radioactive iodine and uranium, because the levels of 129I and uranium are too low to consider in terms of radiation safety. Radionuclides other than ^{134}Cs and ^{137}Cs require a longer time for measurement. Therefore, the new values were expressed as radioactive concentrations of ^{134}Cs and ^{137}Cs considering the contribution of ^{90}Sr, ^{238}Pu, ^{239}Pu, ^{240}Pu, ^{241}Pu and ^{106}Ru, set not to exceed a committed effective dose of 1 mSv/year, and given to water and beverages, infant food, cow’s milk and other foods.

To derive standards, the emission ratio of each radionuclide from the NPP and the transfer factors of each radionuclide to each food are considered. For fish and fishery products, there is

insufficient data and scientific knowledge, so radiation dose due to radioactive cesium was assumed to be 50% when the new standards were developed. Regarding these conditions, the maximum concentration of each food category was derived by taking into consideration food consumption and the dose conversion coeffcient according to age category.

The limit of total radioactivity attributable to ^{134}Cs and ^{137}Cs is 100 Bq/kg for general foods, 50 Bq/kg for milk and infant foods, or 10 Bq/kg for drinking water, and they were 1/4th to 1/20th of the PRVs (for comparison, see Table 2) (MHLW, 2011c). To establish these limits, the following assumptions were made (CAC, 2013):

- The ratio of radio-contaminated foods is 50% of all the foods that are distributed on the Japanese marketplace.
- To consider radionuclides other than radioactive cesium, the total of cesium-134 and cesium-137 was calculated and a factor of 1.2 was applied to cover other radionuclides.

New radiological standards were enforced on April 1, 2012.

2.3 Monitoring of radionuclides in food

Monitoring has been performed in 17 prefectures based on monitoring plans.

According to the MHLW, from 18 March 2011 to 31st March 2012, 137,037 samples were tested and 1204 samples were found to exceed the PRVs. After the new standard was enforced, from 1 Apr 2012 to 25 Jan 2013, a total of 225,004 samples were tested, and 2,097 samples were were found to exceed the Standard Limit enforced after 1st April 2012.

In fish and fishery products sampled in the Fukushima prefecture in March to April 2011, the monthly average concentration exceeded 1000 Bq/kg, however, the level dropped sharply in the next month, and decreased gradually afterword. After one year, the average level was below 100 Bq/Kg. (Fig. 3B)

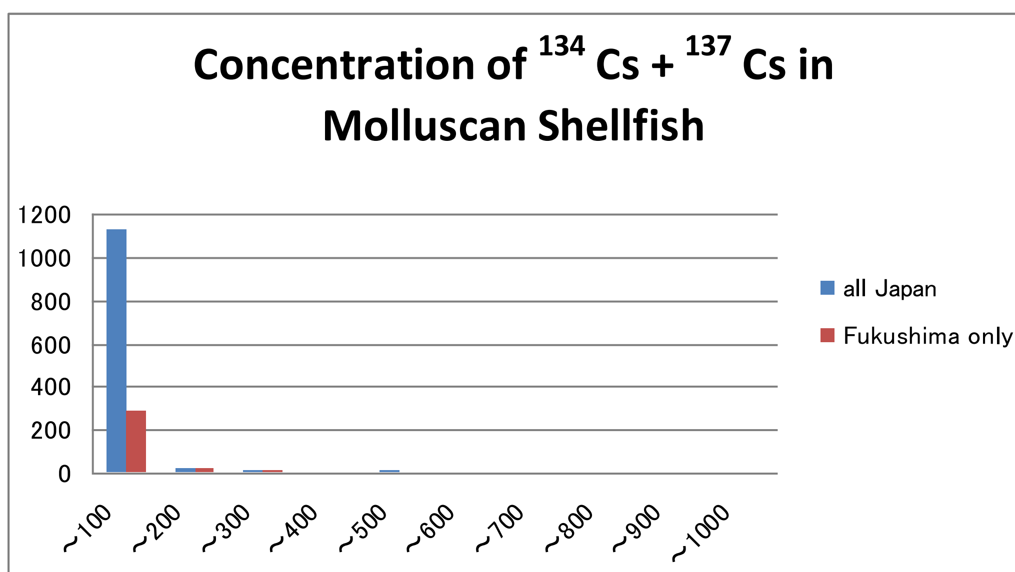


Fig. 4. Concentration of 134Cs and 137 Cs in Molluscan Shellfish

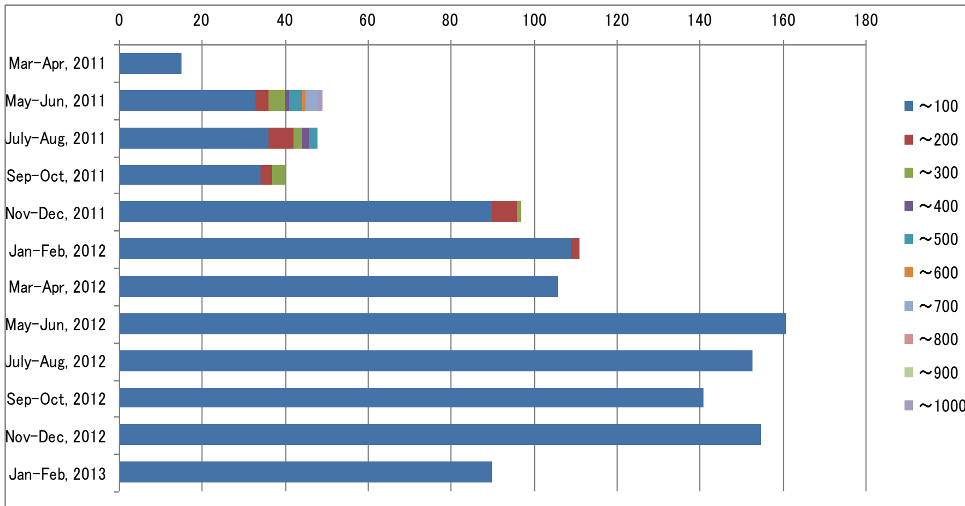


Fig. 5. $^{134}Cs + ^{137}Cs$ in Molluscan Shellfish per month

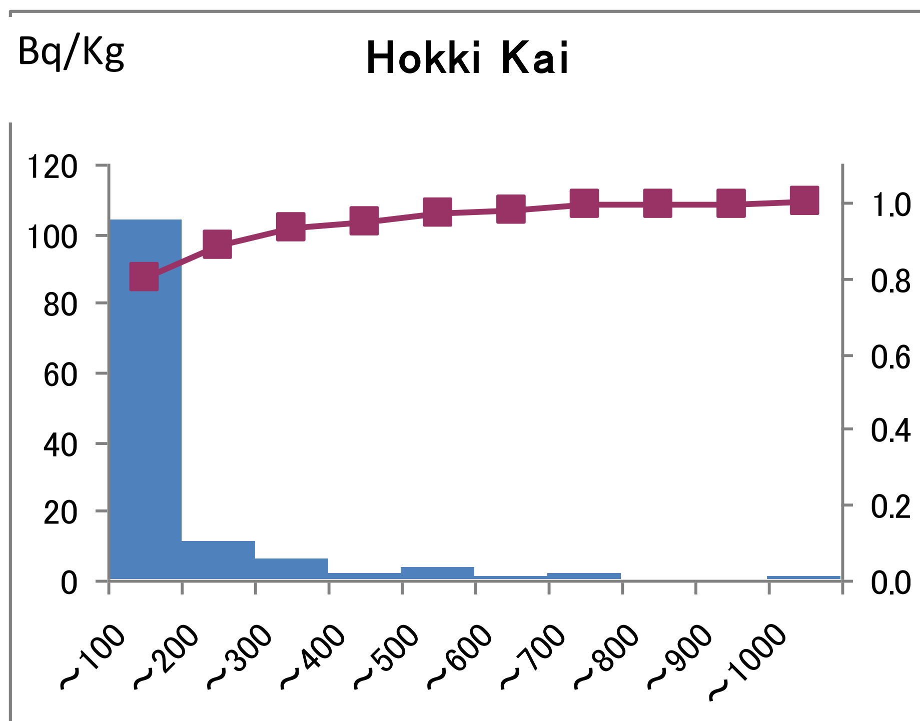


Fig. 6. Monitoring results of radiocesium in molluscan shellfish and Hokki gai (*Pseudocardium sachalinense*)

Fig. 4 shows the monitoring results of molluscan shellfish from March 2011 to March 2013. A total of 1,166 samples were tested and 1,123 samples were below 100 Bq/Kg.

In May-June 2011, even though most of the samples were below 100 Bq/kg, some samples contained around 1,000 Bq/Kg of $^{134}Cs + ^{137}Cs$. After March 2012, all the samples contained less than 100 Bq/Kg (Fig. 5).

Among molluscan shellfish, Hokki gai (*Pseudocardium sachalinense*) contained relatively high levels of radiocesium imimdiately after the accident. A total of 130 samples were tested, and 104 samples were below 100 Bq/Kg. (**Fig.** 6)

2.4 Recall and destroy food exceeding standard limits

Marine fish samples were taken once a week in Fukushima, Miyagi and Ibaragi prefectures, while sampling frequency was decided based on the previous detection in Iwate and Chiba prefecture.

Tests are based on the Food Sanitation Law. When the level in the food exceeds the limit, the lot is considered as a violation of the Food Sanitation Law, and the monitoring programme is enhanced. When the high level contamination was considered to be regionally spread, shipments were restricted based on the Act on Special Measures Concerning Nuclear Emergency Preparedness. Further, if extremely high levels are detected in the food, consumption restriction is enforced based on the Act on Special Measures Concerning Nuclear Emergency Preparedness.

3. Risk Assessment

In Japan, all new food safety requirements shall be based on risk assessment performed by the FSC, except for emergency situations. MHLW set PRVs on 17 March 2011 without being assessed by the FSC. On 20 March 2011, MHLW requested FSC to evaluate health risks associated with the consumption of contaminated food and water. FSC drew up an emergency report (FSC, 2011a) on 29th March 2011, which stated that;

1) Radioactive Iodine

   The thyroid gland equivalent dose of 50 mSv per year and the corresponding effective dose of 2 mSv per year is sufficiently safe to prevent radiation exposure from food (calculated by applying a tissue weighting factor of 0.04 for thyroid according to ICPR 2007).

2) Cesium

   There is no challenging evidence on the adequacy of emergency risk management based on the effective dose of 10 mSv per year proposed by ICPR Publication 63. Thus, for radioactive cesium, the annual effective dose of 5 mSv per /year was considered as highly conservative in terms of preventing radiation exposure caused by food and securing human health.

3) Comments on both radioactive Iodine and radioactive cesium

   The emergency report did not discuss the adequacy of the PRVs that had been already adopted by the MHLW, but rather emphasized the need for appropriate future discussion by risk managers (MHLW and MAFF).

The FSC further published the final risk assessment in October 2011 (FSC, 2011b). The conclusions were drawn mainly from the following three papers.

(i) A cohort study of cancer incidence among residents of the high natural background radiation area in Kerala, India indicates that exposure to a cumulative dose of >500 mGy does not increase cancer risks (Nair *et al*., 2009).

(ii) A cohort study of mortality among atomic bomb survivors in Hiroshima and Nagasaki indicates that the excess solid cancer risks linearly increase in the dose range of 0–125 mSv, which was statistically significant, but

were not significantly related to the dose at 0–100 mSv (Preston *et al*., 2003).

(iii) A cohort study of cancer mortality among atomic bomb survivors in Hiroshima and Nagasaki indicates that compared with the control (0 Gy) group, the excess relative leukemia risks significantly increase at an organ-absorbed dose of ≥0.2 Gy=200 mSv, but not at <0.2 Gy (Shimizu *et al*., 1990). The draft also pointed out the possibility that infants and children are more radiosensitive than adults especially for thyroid cancer and leukemia. Taken together, it was documented in the draft that the risk management authority (i.e. MHLW) should determine how to assign the lifespan effective dose of 100 mSv to each year.

After a call for public comments and minor amendments, the FSC submitted the final report to MHLW (FSC, 2011c) along with partial disclosure of the public comments (FSC, 2011d), but the above-described conclusion was unchanged.

For risk assessments, several models were demonstrated by international organizations, in which data derived from high dose levels were applied to low dose exposure e.g. 100mSv (or 50 – 200 mSV). However, model validation was very difficult, so the FSC determined risk based on epidemiological data obtained from those people actually exposure to radioactive materials.

With regards to cancer (excluding leukemia) for doses in the range of 0 to 125mSV, if the dose increases, the risk of solid cancer increases, and this increase was statistically significant. However, for a dose range of 0-100mSV, the increased risk is not statically significant.

Regarding epidemiological data related to *in utero* and children:

- Children (<5 year) were at higher risk of leukemia (Noshchenko *et al*., 2010)]
- Those younger at the time of exposure tended to have higher thyroid cancer risks per unit of dose (Zablotska *et al*., 2011)

When exposed in utero, exposure to 1 Gy within the most vulnerable period (8-15 weeks following conception) increases the frequency of mental retardation. For the gestation period 16 -25 weeks, no cases of severe retardation were observed at exposures of less than 0.5 Gy (UNSCEAR, 1993).

Based on the findings above, the FSC concluded on 27th October 2011 that (FSC, 2011b):

- More than "around 100 mSV" cumulative effective dose of radiation during a lifetime could increase health risk.
- Exposures to young children increase vulnerability of thyroid cancer and leukemia.
- "around 100 mSV during a lifetime" is not a threshold value. Instead, this is the value which risk managers have to consider when taking appropriate risk management actions.
- Health effects from the exposure below 100 mSV are difficult to verify based on the currently available knowledge. Even if people are exposed to more than "around 100 mSv", it will not necessarily mean they will have an elevated level of cancer risk.

4. Risk Communication

To enhance understanding of risk associated with food contaminated with radioactive materials, Japanese Governments organized more than 140 town hall meetings throughout Japan. The FSC, MHLW and MAFF co-hosted a total of 34 meetings in FY2011 and 2012. In these meetings, representatives from the FSC, MHLW, MAFF, consumer organizations and industries etc presented their views, followed by panel discussions.

The key issues raised in most of the risk communication events were:

- Zero risk is not achievable
- What is risk?
- The scientific basis of the standard limits enforced on 1st April 2012
- Risk to risk comparison (e.g. risk derived from exposure to radioactive nuclides in food vs. tobacco, alcohol etc). If the current cancer risk is assumed to be 1, exposure to 1000 - 2000 mSV of radiation could be considered to be 1.8, Tobacco to be 1.6, exposure to 100-200 mSv of radiation to be 1.08, and passive tobacco effects for a non-smoking female to be 1.06.
- Food contained radioactive materials even before the Fukushima Nuclear Plant accident.
- The amount of radioactive exposure from other sources than food in daily life.

Two of the key messages for risk communication are: 1) the annual dose from food containing ^{134}Cs and ^{137} Cs is extremely small compared to the dose from naturally occurring ^{40}K (Fig. 7); and ii) compared with a naturally occurring dose, the dose derived from radioactive cesium in food is relatively small (only 28%) (Fig. 8).

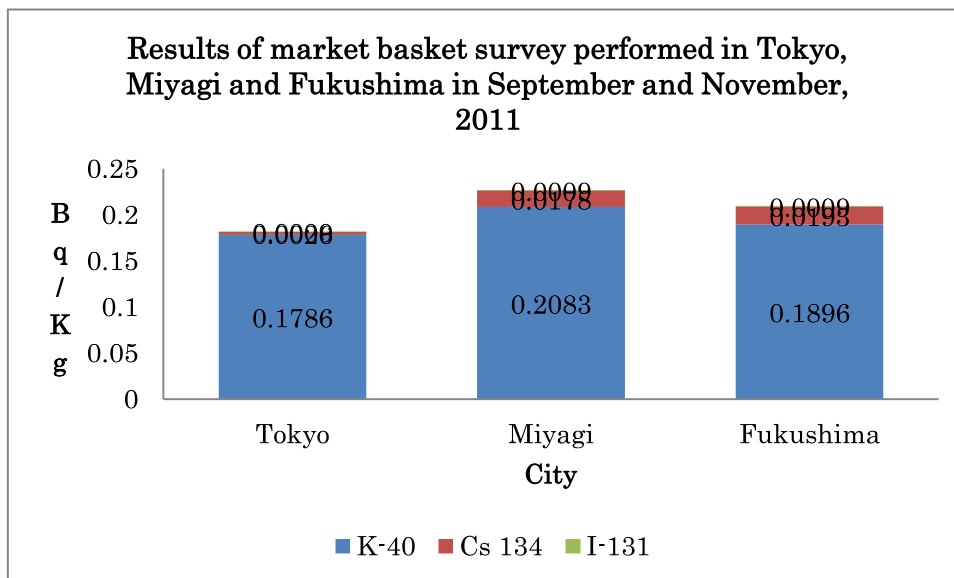


Fig. 7. Results of market basket survey performed in Tokyo, Miyagi and Fukushima in September and November, 2011.

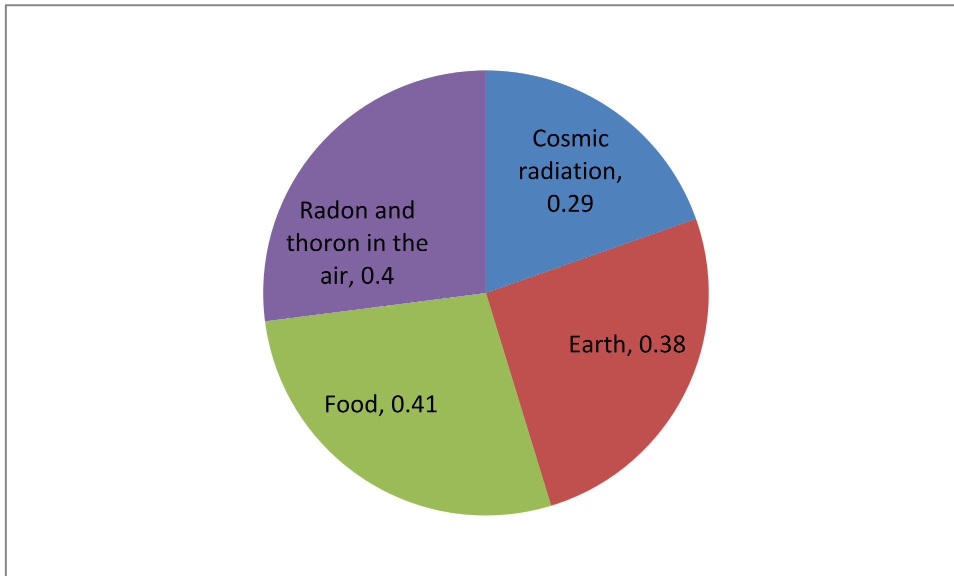


Fig. 8: Comparison of naturally occurring doses of radiation, the annual dose per average Japanese person totals 1.5 mSv

Conclusion

Currently it is safe to say that molluscan seafood safety is a well controlled situation, but long-term monitoring will be needed. In order to effectively manage emergency food safety events, multi agency collaboration, interaction, and co-ordination is critical.

Science based, transparent decision making is an essential element of the risk management activities. In an emergency situation time is limited, however, the risk analysis framework should still be applied. In unusual situations like this case, risk communication is important, when dealing with probabilistic scenarios.

For food safety risk management, we tend to heavily rely on testing, however once testing starts, it is very difficult to stop it. Currently monitoring is still an essential part of the risk management activities, but the monitoring strategy should be reviewed and updated based on the results of previous years until it is thought that monitoring is no longer needed.

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Incidence of *Vibrio parahaemolyticus* in oysters along Northern Manila Bay, Philippines

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Abstract
Codex guidance recommends all shellfish-producing countries assess the total and pathogenic *Vibrio* levels in commercial shellfish production areas. As no data existed on local *Vibrio* populations in the Philippines, we conducted a study to assess the prevalence and densities of *V. parahaemolyticus* populations in oysters from the major producing areas of Northern Manila Bay. Oysters were collected from seven stations during the wet (Nov 2011) and dry (April 2012) season. A most-probable number (MPN) enrichment followed by colony isolation on TCBS agar was used during both collection seasons. Presumptive *V. parahaemolyticus* colonies were identified by the presence of typical (sucrose negative) green colonies on TCBS. Additionally, for samples collected during the dry season, the BAX® Vibrio Real-Time (RTi) PCR Assay was used for total *V. parahaemolyticus*, while pathogenic *V. parahaemolyticus* was determined using an Rti-PCR assay that targets the thermostable direct hemolysin (*tdh*) and the *tdh*-related hemolysin (*trh*) genes. Lower densities of total *V. parahaemolyticus* were detected during the wet season (3-16 MPN/g) compared with the dry season (290–15,000 MPN/g). Densities of pathogenic *tdh*+ (0.23-93 MPN/g) and *trh*+ (0.93-240 MPN/g) comprised ~1% of the total *V. parahaemolyticus* recorded. These levels are consistent with levels found in shellfish from countries that routinely report *V. parahaemolyticus* illness. Seafood-borne *V. parahaemolyticus* illness has not been reported in the Philippines, but this may be attributed to the absence of an established reporting system. The data presented here indicates the potential for *V. parahaemolyticus* illness from consumption of oysters harvested from Northern Manila Bay, Philippines.

Keywords: Vibrio parahaemolyticus, pathogenic, *tdh*, *trh,* wet season, dry season.

Introduction

Vibrio parahaemolyticus is a gram-negative halophilic marine microorganism native to estuarine waters throughout the world. This bacterium is the leading cause of gastroenteritis due to consumption of raw or inadequately cooked seafood, especially oysters (Scallan *et al.,* 2011, Iwamoto *et al.,* 2010, Joseph *et al.,*1982, McCarter, 1999, Deepanjali *et al.,* 2005, Vongzay *et al.,* 2008). Infection by *V. parahaemolyticus* most commonly presents as acute diarrhea (McLaughlin *et al.,* 2005, Khouadja *et al.,* 2013); however, abdominal cramps, nausea, vomiting, headache, fever and chills have also been reported in *V. parahaemolyticus* infections (Takeda, 1998). Clinical manifestation of infection after ingestion of seafood containing *V. parahaemolyticus* varies depending on the magnitude of infection. It can occur as early as 4 hours or as late as 96 hours after ingestion (Lee *et al.,* 2003; McLaughlin *et al.,* 2005; Takeda, 1998). The pathogenicity of *V. parahaemolyticus* is primarily associated with the presence of the thermostable direct hemolysin (TDH) and TDH-related hemolysins (TRH) that are encoded by the *tdh* and *trh* genes, respectively (Nishibuchi *et al.,* 1986, 1989, 1992, Nordstrom *et al.,* 2007, Johnson *et al.,* 2009, Khouadja *et al.,* 2013). Not all *V. parahaemolyticus* are

pathogenic (FAO/WHO, 2001), thus, we only consider the pathogenic (*tdh*$^{+}$ and/or *trh*$^{+}$) strains as a potential health hazard, which typically comprise 0.3 to 3% of the total *V. parahaemolyticus* population in environmental and food samples (Cook *et al.,* 2002, Kaysner *et al.,* 1990, Khouadja *et al.,* 2013).

Oyster production in the Philippines started in 1979 and is considered one of the major aquaculture industries in the country. Because of the ready availability and relatively low market value of oysters, the oyster industry has continuously provided an inexpensive source of protein available to a large array of consumers. The oyster consumption per capita of Filipinos is 0.24 kgs/year (BAS, 2012); however, since not all Filipinos eat oysters, this index value could be higher if only oyster consumers were considered. Most local oyster consumers prefer to eat oysters raw or half-cooked, thus making them susceptible to any microbiological hazard that maybe present in the animal.

The oyster production areas in Northern Manila Bay in Central Luzon, particularly in the province of Bulacan, are among the most productive in the Philippines. This area is ranked third among 23 oyster producing provinces and accounts for 17% (4,045MT) of the total oyster production (22,523MT) that was reported in 2010 (BAS, 2011). Ninety eight percent (98%) of these products were consumed locally, and 0.15% were used for oyster sauce, which was distributed mainly to local markets. The remaining product was used as feeds in aquaculture. To date, there are no reports of *V. parahaemolyticus* outbreaks, nor do any extensive studies on the incidence of these pathogenic bacteria in seafood products from the Philippines exist. However, 1,334 cases of acute watery diarrhea (DOH, 2009) and 51 cases of acute bloody diarrhea (DOH, 2012) have been documented in Central Luzon, some of which we suspect may be caused by *V. parahaemolyticus*.

This survey was a proactive measure to identify a potential public health hazard due to *V. parahaemolyticus* in oysters that are eaten raw and are regarded as a delicacy in the Philippines. This study was aimed at determining the temporal presence and abundance of *V. parahaemolyticus* in Northern Manila Bay and identifying the relative proportions of pathogenic strains, those carrying the *tdh* and/or *trh* genes, from the total *V. parahaemolyticus* detected. Densities of total *V. parahaemolyticus* was also correlated with water temperature and salinity conditions for the two distinct seasons, wet and dry, in the Philippines.

Materials and Methods

Sample collection and preparation

Seven sampling stations were established in the oyster farming areas along Northern Manila Bay (Fig. 1). These cover the municipal waters of Malolos, Paombong and Hagonoy, Bulacan in Central Luzon, Philippines. The sampling sites were selected to be representative of the major oyster farming production areas in Northern Manila Bay that account for the majority of reported production in Central Luzon. Oysters were collected from these stations during the wet (Nov 2011) and dry (April 2012) seasons. Water temperature and salinity data were recorded and were correlated with the density of total and pathogenic *V. parahaemolyticus*.

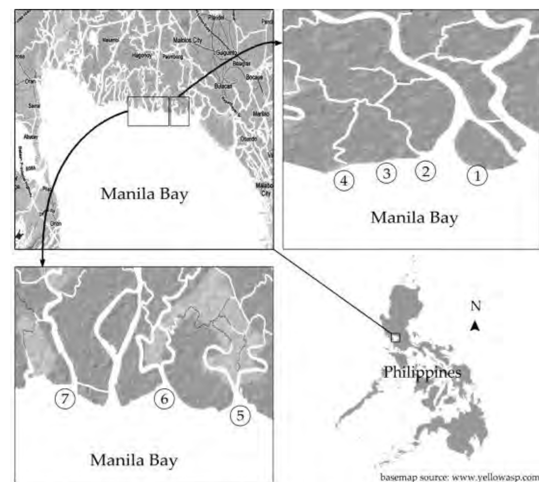

Fig 1. Location of the oyster sample collection sites

Twenty five (25) oysters were collected from each of the seven stations. Dirt and other loose materials from oysters were scraped and scrubbed with a clean stiff brush at the site of collection. Cleaned shell stock were then placed in sealed plastic bags and stored in a plastic cooler with crushed ice and covered with paper to avoid direct contact with the live oysters. The samples were

then transported to the National Fisheries Research and Development Institute (NFRDI) laboratory for microbiological analysis. At the laboratory, the oysters were aseptically cleaned with distilled water prior to shucking. The hands of the technician were scrubbed thoroughly with soap and potable water and latex or nitrile gloves were worn while cleaning the oysters. Clean oysters were shucked using a sterile shucking knife. The whole animal meat was transferred to sterile blender without adding diluent and was homogenized for 60-120 sec at 14,000rpm.

Bacteriological analyses

A most-probable number (MPN) enrichment followed by colony isolation on TCBS agar (Kaysner and DePaola, 2004) was used during both collection seasons. A 1:10 dilution was prepared by transferring 1g of the homogenate to 9ml of phosphate buffered saline solution (0.01M phosphate buffered saline; NaCl-0.138M; KCl-0.0027M; pH 7.4). Three 1g aliquots of homogenate were added to 9ml APW and labeled as 10^{1}. Serial dilutions were prepared from 10^{-1} up to 10^{-6} from the PBS solution and 1ml of each dilution was used to inoculate triplicate 9ml APW tubes. The APW tubes were then incubated overnight at 35+/-2^{0}C.

After 18-24 hrs of incubation, a 3mm (1µl) loopful from the top 1cm of all APW tubes showing growth was streaked onto TCBS Agar. Presumptive *V. parahaemolyticus* colonies were then identified by the presence of typical (sucrose negative) green colonies on TCBS. At the NFRDI laboratory, suspect *V. parahaemolyticus* isolates were confirmed using the API 20E (bioMerieux, Inc., Hazelwood, MO) according to the manufacturer's instruction.

Extraction and preparation of DNA

One (1) ml aliquots of samples were extracted from each positive MPN enrichment tube and boiled for 10 minutes. The heated samples were then immediately plunged into ice until cold and stored frozen until shipped to the FDA Gulf Coast Seafood Laboratory in Alabama, USA for confirmatory and pathogenicity analysis using Real-Time PCR (Rti-PCR).

Real-time PCR amplification

MPN-Rti-PCR was employed for identification of total and pathogenic *V. parahaemolyticus*: the BAX® Vibrio Assay (DuPont Qualicon, Wilmington, DE, USA) was used for total *V. parahaemolyticus*, while pathogenic *V. parahaemolyticus* was determined using an assay targeting the *tdh* and *trh* genes (Jones and Lüdeke, 2012).

Statistical analysis

Pearson's correlation coefficient analysis (Bluman, 2012) was used to describe the linear relationships of total and pathogenic *V. parahaemolyticus*, salinity and temperature data. Two tailed *t*-test was used to test the significance of the correlation coefficient (*r*). Mann-Whitney rank sum test was used to test significance of differences of *V. parahaemolyticus* levels across seasons, sites, and methods. The significance level for all tests was a 95% confidence interval (α=0.05).

Results and Discussion

For comparison of the total *V. parahaemolyticus* levels during the wet and dry seasons, conventional MPN enumeration was employed with suspect isolate confirmation by API 20E (Table 1). No significant difference in levels across sites was observed. However, the difference in total *V. parahaemolyticus* levels between seasons was statistically significant (P=0.016). The significantly higher concentration of total *V. parahaemolyticus* in the dry season indicates the environmental conditions during that time of year favor vibrio persistence and/or proliferation.

Results of this study revealed a high degree of positive correlation between increase in temperature (r=0.51) and salinity (r=0.62) with the density of total *V. parahaemolyticus*. This could be due to the biology of the bacteria as influenced by their adaptation to variations in temperature and salinity conditions (Kaneko and Colwell, 1974).

Table 1. Density of total *Vibrio parahaemolyticus* (Vp) collected during wet (November 2011) and dry (April 2012) seasons as estimated using conventional methods. Values are presented as means (range) for each parameter.

	Wet Season	Dry Season
Total Vp (MPN/g)	7.9 (3.0-16)	4,200 (2,300-9,300)
Salinity (ppt)	19 (17-20)	31 (27-35)
Temperature (°C)	26 (25-28)	31 (30-32)

Notably, there is a lower and wider range of temperature and salinity during wet seasons (Table 1). The lower water temperature and salinity conditions were influenced by the influx of heavy rains from May to December, which are the typhoon months in the Philippines.

The correlation between total *V. parahaemolyticus* MPN/g and temperature agrees with previous observations from Italy, Brazil, and the US Gulf of Mexico (DePaola *et al.,* 1990, DePaola *et al.,* 2003, Sobrinho *et al.,* 2009, Vezzulli *et al.,* 2010). A similar relationship was also observed in Singapore oysters where Huang *et al*. (2012) hypothesized that the warm marine environment would support growth of *Vibrio spp*. These data indicate that the higher incidence of total *V. parahaemolyticus* would be expected during the dry season in the Central Luzon when the temperature is relatively warmer.

Increasing salinity in this study also demonstrated a positive correlation (r=0.62) with the density of total *V. parahaemolyticus*. This observation is consistent with the finding of Zimmerman *et al.* (2007) in oysters of Mississippi from the US Gulf of Mexico. Our data reveal that at lower salinity (<20 ppt), densities of total *V. parahaemolyticus* were relatively low. As cited (Zimmerman *et al.,* 2007, Martinez *et al.,* 2008), the optimal salinity for *V. parahaemolyticus* growth is ~23 ppt which is supported with our observations. The oyster farming areas in Northern Manila Bay (Fig. 1) were mostly located near the mouth of a river, or channels which serve as waterways for run-off from the terrestrial environment during the rainy season. It is interesting to note, however, that a completely opposite finding was observed in the oyster and clam culturing environment in Taiwan (Yu *et al*., 2012) and Spain (Martinez-Urtaza *et al*., 2008) where the high density of *V. parahaemolyticus* was characteristic of the decreasing salinity level.

During the dry season sampling, total and pathogenic (*tdh*$^+$ and/or *trh*$^+$) *V. parahaemolyticus* were also enumerated using MPN-Rti-PCR (Table 2). Although differences in levels were observed for total *V. parahaemolyticus* from the conventional MPN, this was not statistically significant (P=0.8). On average, pathogenic *V. parahaemolyticus* comprised less than 3% of the total *V. parahaemolyticus* population, with a significantly higher proportion of *trh*$^+$ strains than *tdh*$^+$ strains (P=0.02).

Table 2. Density and relative abundance of total and pathogenic (*tdh*$^+$/*trh*$^+$) *Vibrio parahaemolyticus* collected during the dry (April 2012) season as estimated by MPN-Rti-PCR.

	Density	Proportion of total population
Total Vp (MPN/g)	5,400 (290-15,000)	---
tdh$^+$Vp	14 (0.23-93)	0.4% (0.01%-2.2%)
trh$^+$ Vp	110 (0.93-240)	2.8% (0.04%-8.3%)

The thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) genes have been closely associated with the severity of diarrheal illness and they have various biological activities including hemolytic activity, cardiotoxicity, and enterotoxicity (Shimohata and Takahashi 2010). The proportion of pathogenic *V. parahaemolyticus* strains that we observed is similar (<3%) to those cited by others (Rojas *et al.,* 2011; DePaola *et al.,* 2003).

While relatively high levels of virulent tdh^{+}/trh^{+} genes were detected in some samples during this study, it should be noted that trh^{+} *V. parahaemolyticus* was detected more frequently which, according to Vongxay *et al*. (2008), is a less virulent strain of pathogenic *V. parahaemolyticus*. Trh^{+}*V. parahaemolyticus* yielded a positive correlation with total *V. parahaemolyticus* (r=0.73) while tdh^{+} *V. parahaemolyticus* did not show significant correlation (r=-0.09), this could theoretically explain the relatively higher concentrations of trh^{+} genes and lower concentrations of tdh^{+} genes that were detected as the total *V. parahaemolyticus* density increases. Nonetheless, these levels are consistent with levels found in shellfish from countries that routinely report *V. parahaemolyticus* illness (Kaysner *et al.,* 1990, Daniels, 2000, McLaughlin *et al.,* 2005) and thus may be considered as a potential health hazard. Correlation analysis also revealed a negative correlation between the density of pathogenic *V. parahaemolyticus* with temperature (r= -0.52) while a weak positive correlation with salinity (r=0.13) was observed, suggesting that the pathogenic populations of *V. parahaemolyticus* are dependent on temperature, but not salinity.

This type of survey in Northern Manila Bay was the first ever initiated and it is interesting to note that the baseline data collected was comparatively higher than the concentrations that have been reported in some regions (Kirs *et al.,* 2011, Yu *et al.,* 2012), but similar to other regions (Deepanjali *et al.,* 2005). It should be noted, however, that only two representative collection months were considered in this study. A thorough investigation through analyzing a substantial sample size and seasonal data is highly recommended to generate a more conclusive finding. In spite of the relatively high concentrations detected, seafood-borne *V. parahaemolyticus* illnesses or outbreaks have not been reported in Northern Manila Bay or in the Philippines in general. This may be attributed to the absence of an established reporting system. This could also be due to higher tolerance of locals who are accustomed to eating raw and undercooked oysters in the community, although a dose-response study is necessary to validate this assumption.

Summary

This work is the first report on the prevalence of total and pathogenic *V.parahaemolyticus* in the oyster farming areas of Northern Manila Bay and, to the best of our knowledge, in the Philippines. In this study, levels of total *V. parahaemolyticus* were correlated with high temperature and salinity conditions in the environment. Pathogenic (tdh^{+} and trh^{+}) *V. parahaemolyticus* comprised 0.4 - 2.8% of the total *V. parahaemolyticus* detected. These levels are consistent with the levels in shellfish from countries that routinely report *V. parahaemolyticus* illness, which indicates the potential hazard of these pathogenic bacteria to human health. Close monitoring of the densities of total and pathogenic *V. parahaemolyticus* is, therefore, necessary during seasons when these pathogens are most likely to occur. Health advisories on proper handling and cooking practices may then be disseminated to prevent illness from the consumption of oysters with high levels of pathogenic *V. parahaemolyticus*. The strong correlation between total and pathogenic *V. parahaemolyticus* densities with temperature suggest that this scientific knowledge can be incorporated into management strategies focused on time- temperature controls that aim to minimize growth of *V. parahaemolyticus* after harvesting the oysters.

At present, there are no regulatory limits or handling practices imposed by the Philippine authorities for *V. parahaemolyticus*. Our data have established baseline information on the prevalence of total and pathogenic *V. parahaemolyticus* in oysters. This information can be a reference in developing management and regulatory guidelines following further validation studies.

Recommendation

From the food safety and management perspective, the strong correlation between *V. parahaemolyticus* density and temperature can be equated into a scientific tool to safeguard the general public on the potential hazards of *V. parahaemolyticus* infection. The data suggest that temperature control can serve as an effective post-harvest handling practice to prevent *V. parahaemolyticus* proliferation in oysters after

removing them from the marine environment. Temperatures of <18 °C (Vezzulli *et al.,* 2010) and ≤ 10 °C (Kaneko *et al.,* 1974, DePaola, 1990) have been suggested as levels that can prevent *V. parahaemolyticus* growth. Foo *et al*. (1974), on the other hand, suggested that the spread of any food-borne disease can be halted with the strict adherence to personal hygiene and through avoiding consumption of under-cooked food.

Additional sample collection during the rainy and dry months is proposed to arrive at a better understanding on the seasonal abundance of total and pathogenic *V. parahaemolyticus* in Northern Manila Bay. It is also suggested to study the strain characteristics of the pathogenic *V. parahaemolyticus* that have been detected in this study to determine if they belong to the pandemic strains that have been identified and reported in other countries that occasionally report *Vibrio parahaemolyticus* related illnesses and outbreaks.

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Evaluation of spatial contamination patterns for norovirus and faecal indicator bacteria near to a coastal sewage discharge using *Mytilus edulis* as biosentinels

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Abstract
Bivalve shellfish have the capacity to accumulate norovirus (NoV) from waters contaminated with human sewage. Consequently, shellfish represent a major vector for NoV entry into the human food chain, leading to gastrointestinal illness. Identification of areas suitable for the safe cultivation of shellfish requires an understanding of NoV behaviour upon discharge of sewage into coastal waters. This study exploited the potential of *Mytilus edulis* to accumulate NoV and employed the proposed international standard method for quantification of NoV within mussel digestive tissues. To evaluate the spatial and temporal spread of NoV from an offshore sewage discharge pipe, cages of mussels were suspended from moorings (n=13) deployed in a 1km grid array around the outfall. Caged mussels were retrieved after 30 days and NoV (GI and GII), coliforms and *E. coli* enumerated. The experimentally derived levels of NoV GI and GII in mussels were similar, with NoV spread from the outfall showing a distinct plume which matched very closely to a tidally-driven effluent dispersal model. A contrasting spatial pattern was observed for coliforms. These data demonstrate that coliform / *E. coli* concentrations do not accurately reflect viral dispersal in marine waters and contamination of shellfish by sewage-derived viral pathogens.

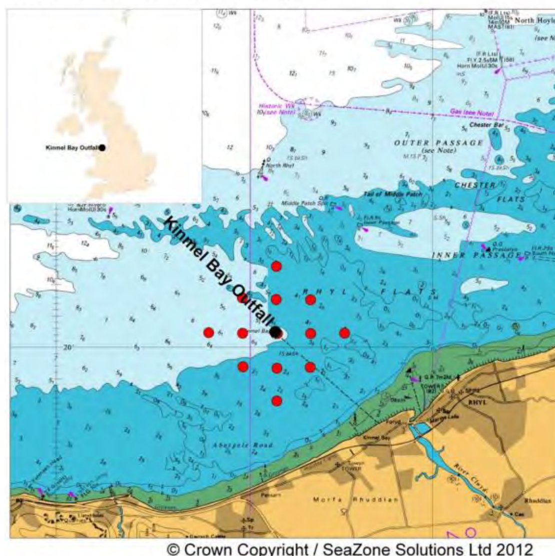
Keywords: food safety, mussels, human sewage, shellfish harvesting, viral gastroenteritis

Introduction
Contamination of bivalve shellfish with norovirus (NoV) from human faecal sources represents a well-established human health risk (Lees, 2000). Bacteria including coliforms and enterococci have been used to estimate the level of faecal contamination of water and / or shellfish and may be referred to collectively as Faecal Indicator Bacteria (FIB). In Europe, *Escherichia coli* (*E. coli*), a coliform species commonly found in the lower intestine of warm-blooded organisms, is adopted as the traditional indicator of faecal (sewage) contamination in shellfish and used for risk assessment and management (Anon., 2004). However, studies have indicated that *E. coli* provides a poor indicator of the risk of NoV contamination. Reasons for this poor correlation include the different environmental persistence of viruses and bacteria in marine water and differences in their seasonal discharge pattern (Fong and Lipp, 2005).
Direct recovery and concentration of viral pathogens from coastal waters is problematic, often requiring large sample volumes and providing only a time-specific measure of contamination. However, bivalve shellfish have been shown to efficiently accumulate viruses (Asahina *et al.,* 2009; De Donno *et al.,* 2012; Nenonen *et al.,* 2008) and sensitive quantitative methods which detect NoV genomes in molluscan shellfish using molecular techniques (PCR) now exist (Lees and CEN WG6 TAG4 2010; Anon 2013).

Materials and Methods
Site selection
The offshore sewage outfall pipe at Kinmel Bay, North Wales (53.336901N, 3.569200W (WGS84);

Fig. 1), which serves a total population equivalent of 77,953, was selected for this study. The discharge is consented for up to 38,860 m^3 d^{-1} with a dry weather flow not exceeding 15,941 m^3 d^{-1}. Sewage released from the outfall receives only secondary treatment (activated sludge). No tertiary treatment is applied. The outfall discharges into the coastal waters of Liverpool Bay at 4 km offshore, in 6.9 m of water at Lowest Astronomical Tide, to achieve compliance with EU bathing water quality standards. We hypothesized that these conditions could result in a significant release and persistence of potential human pathogens in marine waters.

Fig. 1. Outfall Location and sample sites



Sampling Regime and Shellfish Biosentinels

A diamond-shaped array of 13 independent sampling points was selected (Fig. 1) based on model simulations of sewage plume behaviour. The individual sample points were separated by 1 km in *x* and *y* dimensions. To minimise variability associated with growing conditions, *Mytilus edulis* were collected via a short trawl of broadcast-cultivated animals, from a commercial bed with an EU Class B classification. The animals were washed, size graded and 200 animals randomly selected for baseline enumeration of NoV and *E. coli* at time zero (T_0). Ten replicate samples of 10 animals were analysed for NoV and 10 replicate samples of 50 g shellfish flesh for coliforms and *E. coli.* Groups of 35 live animals of the same batch were then placed in net bags (300 x 300 mm). The net bags were placed in plastic cages and suspended at a sea depth of 1 m by attaching to a plough anchored Polyform A3 buoy. The cages were deployed on 12/03/12 and after 30 d the mesh bags containing shellfish were recovered. The samples were stored on ice before return to the laboratory for processing within 6 h.

Quantification of Norovirus in Mussels

NoV quantification in mussel digestive tissue was determined using quantitative reverse-transcription PCR (qRT-PCR) as described by (Lowther *et al.,* 2012a). Modification was made to the formation of the positive extraction control, to the quencher used for the GII probe (TAMRA) and in addition, aliquots of chopped glands were frozen (-20°C) and thawed once prior to Proteinase K digestion rather than being digested fresh or after short-term (24hrs) refrigerated storage (4°C). The positive extraction controls consisted of homogenates prepared as per samples after the addition of 1 lenticule® disc of NoV Reference Material for each genogroup (HPA) to ten digestive glands. Average quantities enumerated from three aliquots of extracted RNA/sample give overall quantities in detectable genome copies/g digestive gland. For T_0 n=10. For *in situ* samples n=1 per site/month.

Quantification of *E. coli* and coliforms

Bacterial colony forming units (CFU) were enumerated from shellfish flesh by direct plating onto selective agar as described in Clements *et al.* (2013). For T_0 n=10. For *in situ* samples n=3 per site/month.

Statistical and geostatistical analysis

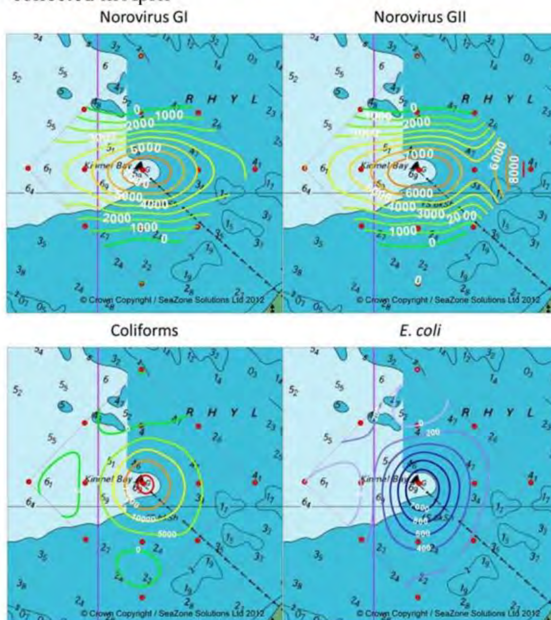
To ensure our data are comparable with UK survey data generated by the National Reference Laboratory (Lowther *et al*., 2012a), samples returning "not detected" results for a particular NoV genogroup were assigned a score of 20 copies/g for that genogroup (half the limit of detection (LOD)). Samples giving positive results below the limit of quantification (LOQ; 100 copies/g) were assigned a score of 50 copies/g. Statistical analysis was carried out using IBM SPSS Statistics 20 and Geostatistical analysis and presentation was carried out in ArcMap 9.3.1 using the Spatial Analyst Extension.

Results

Norovirus and Bacteria in Mussels

After 30 d, GI NoV levels had increased from a T_0 baseline value of 52.2 copies/g at all sites except two at which it was not detected and two at which levels remained <LOQ. For GII NoV, levels increased from a T_0 value of 3312 copies/g at four sites and decreased at all other sites. Similarly, *E. coli* contamination increased in mussels directly over the outfall from the T_0 value of 400 ± 163 to 1167 ±166 CFU/100g. The spatial patterns of NoV and coliforms / *E. coli* around the discharge point were however, very different with NoV showing much greater dispersion and symmetry about the outfall (Fig. 2).

Fig. 2. Norovirus and indicator organisms in mussels collected in April



For NoV GI and GII, contours represent scored data as detectable genome copies / g digestive gland. Not detectecd scores 20 gc / g. 1-100 (<LOQ) scores 50 gc / g. n= 1. For Coliforms and *E. coli* contours represent mean CFU / 100g shellfish flesh and intravalvular fluid. N=3.

Both GI and GII NoV results showed a pattern of contamination elongated to the East and West of the outfall. For NoV GI, levels decreased with distance in all directions from the outfall. But for NoV GII, highest levels (9958 c/g) were observed at the most Easterly sample point, 2 km to the East of the outfall. *E. coli* was detected at highest levels over the outfall but was not detected to the West of the outfall, being skewed East and towards the shore. Total coliforms were detected at highest levels over the outfall, were also skewed East and slightly towards shore, but were detected at all sites. On a site-by-site basis, there was a strong correlation between NoV GI and GII concentrations (r_s=.905; P<0.001). Total coliforms and *E. coli* also correlated (r_s=.747; P=.003). Correlation between total coliform and NoV GI concentrations was weakly significant (r_s=.601 P=.030), but correlation with GII was non-significant (r_s =.543 P=.055). *E. coli* did not correlate with either NoV GI (r_s=.296 P=.326) or GII (r_s=.220 P=.470).

Discussion

The relatively high T_0 value for GII NoV allowed for clear differentiation between sites where levels in resituated mussels increased (up to 3-fold) and sites where they decreased to levels below the LOQ (approx. 66-fold decrease; 3311 to half LOQ), suggesting that the pattern observed is representative of contamination *in situ.* Furthermore, spatial contamination patterns for GI and GII NoV were correlated, except for a disparately high GII result at the easternmost site. Further work seeks to integrate model data for the nearby Clwyd River (Fig. 1), into which sewage is also discharged, possibly resulting in an additional impact of greater magnitude at Eastern sites and containing different GI/GII composition.

The most contaminated sites by either NoV genogroup all occupy the East-West transect through the centre point of the array, over the outfall, and concentrations declined steeply with distance both to the North and South. This is in visual agreement with hydrodynamic model predictions for the sewage discharge plume (data not presented). However, agreement between model predictions and measured *E. coli* and coliform concentrations was less apparent. Furthermore, whilst *E. coli* correlated with total coliforms and NoV GI correlated strongly with GII, the only statistically significant correlation between the FIB selected for enumeration and NoV was coliforms with NoV GI and this association was not strong.

Indeed NoV GI and GII were detected in mussels at very high levels at sites at which *E. coli* was not detected, notably to the West of the outfall. We are aware that the tidal current was flowing to the East at the time of sampling and therefore animals to

the West are likely to have been less recently exposed to the effluent plume. This is consistent with evidence that FIB are an indicator of recent faecal contamination but norovirus can persist longer in shellfish tissue. The water is deeper to the West of the outfall and a differential effect of water depth upon NoV/FIB behaviour is also plausible given potential association with particles and related sedimentation / resuspension phenomena. But importantly, all sentinels were suspended at 1 m below the surface rather than on the seabed.

Given that current regulations in Europe are based on concentrations of *E. coli* in shellfish flesh, mussels containing these levels of NoV could legitimately be sold for consumption following minimal treatment - potentially exposing consumers to an unacceptable risk of illness. It is possible that the method applied detected some inactivated NoV and may overestimate the amount of infectious virus present. However there is recent evidence that amount of genome detected is generally proportional to risk (Lowther *et al.,* 2012b).

Conversely, FIB were detected at sites at which NoV was not detected, with the distribution of FIB being somewhat more skewed towards the shore. We hypothesise that secondary non-point sources, which may be of animal origin, affect this pattern. Therefore, this study suggests that FIB indicate the presence of faecal contamination but may not accurately reflect persistent contamination by viral pathogens associated with human-sewage effluent.

Much of the research concerning accumulation / elimination dynamics in shellfish has focussed upon oysters which are associated with more outbreaks than other species, possibly as a result of traditional raw consumption. However, with potential in Europe for virological standards applicable to all bivalve molluscan shellfish, similar data relating to *Mytilus edulis* (and other bivalves sold for consumption) is urgently required.

Acknowledgements

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Treatment time and environmental factors affect the reduction of *Vibrio parahaemolyticus* concentrations in relayed oysters (*Crassostrea virginica*)

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Abstract

Vibrio species are significant causes of shellfish-borne disease. The US shellfish industry has experienced increasingly more frequent *Vibrio*-associated disease outbreaks linked to shellfish consumption. In the Northeast US where infections have been rare in the past, recent illnesses caused by *Vibrio parahaemolyticus* (Vp) associated with changing climatic conditions have caused public health and economic impacts, and required new management control measures. The objective for this study was to determine time requirements for shellfish relaying, where shellfish are trans-located from contaminated, lower salinity areas to higher salinity waters with low *Vibrio* contaminant levels, to reduce Vp levels in oysters under multiple environmental conditions. Oysters were relayed from the harvest area in New Hampshire to a nearby site in Maine with higher salinity water during June-November in 2011 and 2012. Conventional culture methods and real time PCR were used to quantify Vp levels in triplicate oyster samples collected at days 0, 2, 7, 10 and 14. Vp levels were variable with inconsistent reductions from days 2 to 7, but Vp levels were reduced to relatively safe levels after 10 days. The minimal shellfish relay duration for effective Vp reduction appeared to be between 7-10 days under a range of water temperatures and salinity.

Keywords: shellfish relay duration, salinity, water temperature, real time PCR

Introduction

A variety of bacterial pathogens may reside in bivalve shellfish, yet only some species pose significant threats to consumers of raw or undercooked shellfish (Drake *et al.,* 2007). *Vibrio parahaemolyticus* is at the top of bacterial safety concerns in seafood for public health officials and the shellfish industry, as it is the leading cause of bacterial gastroenteritis from consuming raw or improperly cooked oysters in the United States. The US shellfish industry has experienced increasingly more frequent *Vibrio*-associated disease outbreaks linked to shellfish consumption in northern, colder water areas, where infections have until recently been rare. In the Northeast US, recent outbreaks of illness caused by *Vibrio parahaemolyticus* are emerging with changing climate conditions, resulting in economic and public health impacts, as well as instigating management control measures.

The National Shellfish Sanitation Program and the Interstate Shellfish Safety Conference have created guidelines to reduce microbial pathogen threats in shellfish to protect consumers from *Vibrio* infections. Different types of post-harvest processing methods (ultra-low temperature quick-freeze, high hydrostatic pressure, hot water pasteurization) are effective in reducing *Vibrio* populations to acceptable concentrations, i.e., <30 MPN Index/g prior to being sold to market. These processes are, however, capital-intensive and the treatment processes kill the shellfish and/or render them less desirable to consumers and thus less valuable for the shellfish industry. Effective post-harvest treatments that do not kill shellfish and that do not involve high capital expenditures are more desirable.

Depuration and relaying are accepted strategies for reducing fecal-borne bacteria from shellfish, allowing shellfish harvesting from mildly contaminated areas. Relaying is a process of trans-locating shellfish from non-approved growing waters to approved shellfish growing waters. This natural process reduces fecal coliform levels while

maintaining healthy, live shellfish. Previous studies have explored use of both depuration and relaying for *Vibrio* reduction and found that depuration is generally ineffective in reducing *Vibrios* (Jones *et al*., 1991; Tamplin *et al*., 1992) yet several different relay approaches have significantly reduced levels of *V. parahaemolyticus* and *V. vulnificus* (Jones, 2009; Motes *et al.*, 1996; Yu *et al.*, 2010; Audemard *et al*., 2011).

The Great Bay estuary of New Hampshire and Maine, USA (Fig. 1) was the study area, where shellfish-borne *Vibrio* disease incidence has been extremely rare. Environmental conditions in the GBE are spatially variable and seasonal climatic conditions (i.e., water temperature, rainfall) vary seasonally with temperature extremes of <0 °C to ~30 °C, allowing rigorous testing of post-harvest shellfish treatment strategies. Pathogenic *Vibrio* species are not present in near shore Atlantic Ocean/Gulf of Maine waters, but are found in the estuary, with consistent detection during June to September when water temperatures are warmer. The exception is Spinney Creek, a small tidal pond that has very little freshwater inflow and a tidal dam at the mouth that allows high tide salt water to enter, which creates elevated salinity conditions where *Vibrio* levels are extremely low or absent. The objective for this study was to identify the treatment duration and environmental conditions required for reductions of *V. parahaemolyticus* in oysters relayed from a tidal river in the estuary to Spinney Creek.

Oysters (*Crassostrea virginica*) were harvested by diving or tonging from an oyster bed in the Piscataqua River (43°10’08.49”/70°49’42.54”), Dover, New Hampshire (Fig. 1) during June to November in 2011 and 2012 and relayed to Spinney Creek, Eliot, Maine. Water column conditions were measured at the harvest and relay sites using a YSI 85 meter. Enough oysters were harvested and transported to allow for analysis of *V. parahaemolyticus* levels in three separate sub-samples of 12 animals at each sample time. During 2011, samples were analyzed after 0, 2, 7 and 14 days of relaying, and in 2012 after 0, 7, 10 and 14 days.

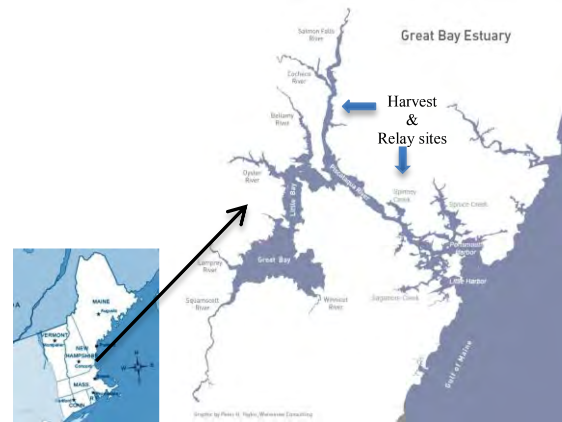


Fig. 1. The Great Bay estuary of Maine and New Hampshire, USA, with harvest and relay site locations.

V. parahaemolyticus concentrations in oyster homogenates were determined by the FDA Bacteriological Analytical Manual (Kaysner & DePaola, 2004) 3 tube MPN method in combination with real time PCR. Turbid tubes were streaked onto CHROMagar *Vibrio* (DRG International) for selection of Vp isolates. Vp confirmation was carried out using qPCR in a Cephied Smart Cycler based on Nordstrom *et al.* (2007). Omnimix Mastermix (Cephied, Sunnyvale, CA) in conjunction with tlh marker primers and probe, and an IAC internal control (BioGx, Birmingham, AL) were the PCR reagents used. The qPCR parameters were as follows: Stage 1- initial denaturation at 95 °C for 1.5 min (Hot Start), Stage 2- 45 cycles of denaturation at 95 °C for 5 s then primer annealing at 59 °C for 45 s.

The salinity at Spinney Creek (SC) was always higher than the salinity at the harvest site in the Piscataqua River (PR) from June through September in both 2011 and 2012 (Fig. 2). The salinity differences between the two sites ranged from 1.1 to 12.1 ppt for individual sample dates. Water temperatures at the two sites were more similar. The water temperature in Spinney Creek was slightly higher (<2.3 °C) than in the Piscataqua River during June and July, temperatures were the same in August and Spinney Creek was cooler (by 1.7-7.4 °C) during September as cooler ocean water flowed into the pond at high tides.

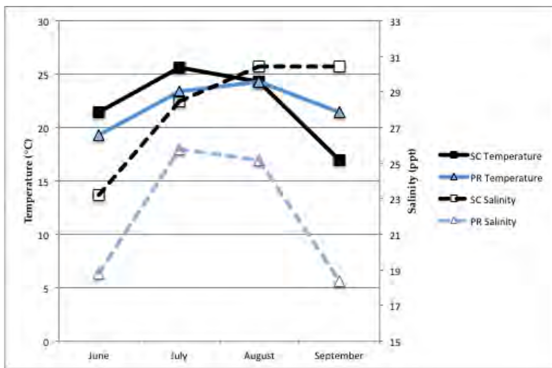


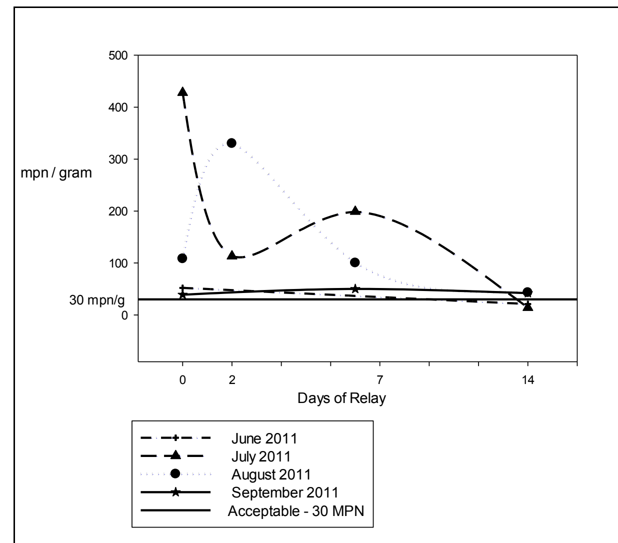
Fig. 2. Water temperature and salinity in Spinney Creek (SC) and the Piscataqua River (PR): average monthly values for 2011-12.

Target reduction levels (<30 MPN/g) for *V. parahaemolyticus* were achieved during the 14-day relay process in five of the eight monthly trials, while *V. parahaemolyticus* concentrations were reduced to relatively safe levels (<43 MPN/g,) after 14 days of relaying to Spinney Creek in the other three trials (Fig. 3). The greatest reduction occurred in July 2012 when concentrations were reduced from 4100 to 40 MPN Vp/g.

V. parahaemolyticus concentrations were more variable on days 2 and 7 than after 14 days in 2011, especially in August when salinity differences between the harvest and relay sites was minimal (1.1 ppt). This lead us to change our sampling scheme in 2012 to focus on the time between days 7 and 14 to enable a better understanding of the minimal time required to achieve consistent reductions. It appears that reductions to relatively safe levels do occur in a consistent fashion by days 10 and 14.

V. parahaemolyticus concentrations in 2012 harvested Piscataqua River oysters were always higher than in 2011 during June to August, and the corresponding 14-day reductions during 2012 were greater than those in 2011. Water temperatures in Spinney Creek were lowest and the lowest degree of reduction occurred in September both years, although the initial Vp and 14-day relay levels were ≤42 MPN/g and thus close to the target reduction level.

Relaying oysters from the Piscataqua River to Spinney Creek for 14 days always resulted in final *V. parahaemolyticus* concentrations that were below or near to safe levels. *V. parahaemolyticus* concentrations varied and were not consistently reduced after 2 and 7 days, perhaps due to changes in oyster condition caused by relaying to different conditions. The minimal time for consistent reductions to occur appears to be between 7-10 days.



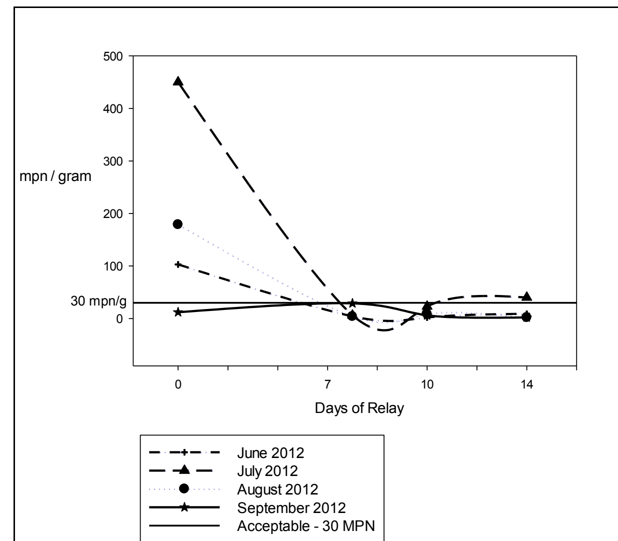


Fig. 3. *V. parahaemolyticus* concentrations in oysters relayed to Spinney Creek after 2, 7 and 14 days (2011) and 7, 10 and 14 days (2012). The Day 0 concentration in July 2012 was 4115 Vp/g wet weight tissue.

Relaying contaminated shellfish to areas with low *Vibrio* concentrations is an effective strategy for maintaining shellfish quality and reducing *Vibrio* concentrations to safe levels. The results of this study are consistent with previous studies of relay to high salinity conditions, although the range of

environmental conditions in this study exceeded previous studies.

The variability in Vp response to high salinity over the first week could indicate uncertain dynamics of the oyster microbial community, in which different oysters vary in the number of preexisting salt-tolerant bacterial colonists, or in the host response itself. However, the consistent reduction in Vp following this period likely reflects their eventual competitive inferiority. Ongoing work is focused on understanding the shifts in microbial community populations within relayed oysters. We are presently analyzing bacterial community members using 16S rRNA sequences identified from the relayed oysters discussed in this study. We expect to identify changes in the bacterial community prior, during, and after relay, both as a function of *Vibrio* abundance and external environmental conditions.

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Section III – Algae and Biotoxins

Harmful algal blooms in the Australian region: changes between the 1980s and 2010s

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Abstract
While microalgal blooms in a strict sense are completely natural phenomena (e.g. *Trichodesmium* cyanobacterial blooms reported in the Coral Sea by Captain Cook in 1770), since the 1980s their impacts on Australian public health, tourism, and fisheries have increased in frequency, intensity and geographic distribution. To a major extent this reflects increased scientific awareness (pinnatoxins in SA oysters). In other cases, algal bloom problems reflect increased utilisation of coastal waters for aquaculture and fisheries (fish-killing *Chattonella marina* raphidophytes in Port Lincoln, dinoflagellate *Dinophysis* Diarrhetic Shellfish Toxins in NSW pipis, diatom *Rhizosolenia amaralis* causing bitter mussels in Port Philip Bay). Eutrophication has only rarely been rarely invoked as a causative factor (dinoflagellate *Karlodinium veneficum* in Swan River; haptophyte *Prymnesium parvum* in NT barramundi ponds). Other harmful species have been newly introduced via ship ballast water discharge (PST dinoflagellate *Gymnodinium catenatum* into Tasmania) or exhibit significant range expansions in relationship to climate change (*Noctiluca scintillans* red tides into the Southern Ocean). Algal blooms may also pose unexpected problems for desalination plants. Heightened scientific and regulatory attention has triggered the development of many new technologies (molecular probes, remote sensing) and approaches for monitoring (continuous plankton recorder) and management of algal bloom phenomena.

Keywords: Harmful Algal Blooms; Fish Kills; Shellfish Toxins; Australia

Introduction

The microscopic plankton algae of Australian coastal waters provide critical ecosystem services in terms of food for filter-feeding bivalve shellfish and larvae of crustaceans and finfish. In most cases, the proliferation of so-called 'algal blooms' (millions of cells per litre) therefore is beneficial to human society. However, in other situations algal blooms can have a negative effect, causing severe economic losses to aquaculture, fisheries and tourism and major environmental and human health impacts. The Harmful Algal Bloom (HAB) designation is a societal concept rather than scientific definition—blooms are considered to be harmful if they cause injury to human health or socioeconomic interests. Three different types of algal blooms can be distinguished (Table 1) (Hallegraeff 1993):

1. Species that produce basically harmless water discolorations

Some nontoxic HABs still can cause damage to ecosystems, fishery resources and recreational facilities, simply due to the high biomass of accumulated algae, which can create noxious scums and foam, and cause indiscriminate marine faunal mortalities via decay and oxygen depletion. Whitelegge (1891) reported on massive marine mortalities in Port Jackson, NSW, associated with water discolorations by a dinoflagellate *Glenodinium rubrum* sp.nov. (closely resembling the nontoxic *Scrippsiella trochoidea*). A recent Australian example is the *Noctiluca scintillans* dinoflagellate red tides (Nov. 2012; Fig.1A) responsible for temporary closure of 10 Sydney beaches including iconic Bondi Beach.
However beyond the inconvenience to beachgoers lies the threat of a more serious ecological disturbance. *Noctiluca* exhibits voracious phagotrophic feeding behaviour and consumes a

variety of prey such as diatoms, dinoflagellates, zooplankton and fish eggs. This large (200-1000 µm diameter), bioluminescent dinoflagellate was first documented from Sydney Harbour in 1860, but visible "red tides" were not reported from the region until the 1990s reputedly in response to eutrophication stimulating seed populations in estuaries (DelaCruz *et al.,* 2002).

Table 1. Different types of harmful algal bloom in Australian waters (after Hallegraeff 1993).

1. Species that produce basically harmless water discolorations; however, under exceptional conditions in sheltered bays, blooms can grow so dense that they cause indiscriminate kills of fish and invertebrates through oxygen depletion. Examples: dinoflagellates *Akashiwo sanguinea*, *Gonyaulax polygramma*, *Noctiluca scintillans*, *Scrippsiella trochoidea*; cyanobacterium *Trichodesmium erythraeum*
2. Species that are non-toxic to humans but harmful to fish and invertebrates (especially in intensive aquaculture systems) by damaging or clogging their gills. Examples: diatoms *Chaetoceros criophilum*; dinoflagellates *Karenia mikimotoi*, *Karlodinium veneficum*; haptophyte *Prymnesium parvum*, raphidophytes *Heterosigma akashiwo*, *Chattonella marina*
3. Species that produce potent toxins that can find their way through the foodchain to humans, causing a variety of gastrointestinal and neurological illnesses, such as: • Paralytic Shellfish Poisoning (PSP) (dinoflagellates *Alexandrium catenella*, *A. minutum*, *A. tamarense*, *Gymnodinium catenatum*) • Diarrhetic Shellfish Poisoning (DSP) (dinoflagellates *Dinophysis acuminata, D. fortii, D. tripos)* • Amnesic Shellfish Poisoning (ASP) (diatoms *Pseudo-nitzschia australis, P.multistriata, P. cuspidata)* • Ciguatera Fish Poisoning (CFP) (dinoflagellates *Gambierdiscus carpenteri, G. belizeanus, G. yasumotoi)*

Major bloom events in NSW are associated with natural nutrient upwellings creating abundant longshore diatom prey biomass. *Noctiluca* was first observed in Tasmania in 1994, carried south by the East Australian Current, but has since become firmly established there with overwintering populations. In 2008, the species was found for the first time in Qld, WA, SA, and most dramatically in 2010 it appeared to have newly moved into the Southern Ocean (240km south of Tasmania) (Fig.1) raising concerns about grazing impacts on iconic krill-based foodwebs. (McLeod *et al.,* 2012). The arrival of *Noctiluca* into Tasmania also heralded a new threat to the expanding salmonid fish farm industry which responded by creating air bubble screens to disperse up to 4m thick suffocating surface slicks.

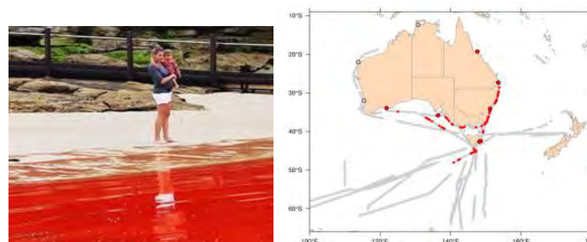

Fig. 1. Left. *Noctiluca* dinoflagellate red tide (Clovelly Beach, Nov 2012, Daily Telegraph); Right. Continuous Plankton Recorder survey of *Noctiluca* (red) 2009-12 (courtesy Anthony Richardson, CSIRO).

Other common water discolorations in Australian coastal waters are caused by the cyanobacterium *Trichodesmium erythraeum* (already reported by Captain Cook in 1770, occasionally leading to beach closures in Sydney and Perth), dinoflagellates *Akashiwo sanguinea, Gonyaulax polygramma, Scrippsiella trochoidea* and the ciliate *Mesodinium rubrum* (all found in NSW estuaries) (Hallegraeff 2002).

2. Species that are non-toxic to humans but harmful to fish

Other HAB species can release compounds that are, strictly speaking, not toxins (e.g., reactive oxygen species (ROS), polyunsaturated fatty acids (PUFAs), mucilage (Dorantes *et al.,* 2012; Marshall *et al.,* 2003; Mooney *et al.,* 2008). Critically, that means that these species are not of human health significance, but still can be injurious and even lethal to finfish, especially when held captive in aquaculture operations. An example is the *Chattonella marina* raphidophyte blooms associated with $45M loss of aquacultured tuna in Port Lincoln in 1996; (Hallegraeff *et al.,* 1998; Fig.2). Smaller aquaculture fish kills have been caused by a mixed *Karenia* dinoflagellate bloom (including K. *mikimotoi, K. umbrella*; $4M loss in Tasmania in 2003; de Salas *et al.,* 2004),

the raphidophyte *Heterosigma akashiwo* (e.g. Huon River, Tas) and haptophyte *Prymnesium parvum* (in Darwin barramundi ponds; Seger *et al.,* 2014). Recurrent wild fish kills by the dinoflagellate *Karlodinium veneficum (=micrum)* have occurred in the Swan River, WA, since 2003.

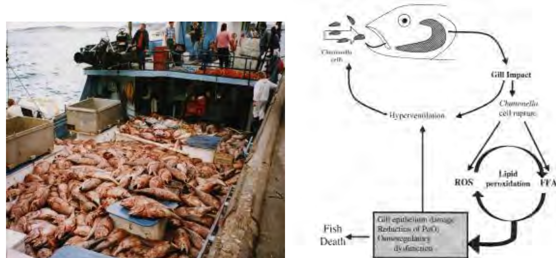

Fig. 2. Left. $45M tuna aquaculture mortality in Port Lincoln 1996, associated with a *Chattonella marina* raphidophyte bloom; Right. Elucidation of the fish killing mechanism to be the result of algal cell rupture releasing a reactive cocktail of ROS and free fatty acids (Marshall *et al.,* 2004).

3a. Species that produce toxins of human health significance: Toxic Shellfish

Of greatest concern to human society are HAB species that are toxigenic. Human consumers of contaminated seafood may be poisoned, suffering acute toxic symptoms and even fatalities. Paralytic Shellfish Toxin producing dinoflagellates were reported in Port Hacking, NSW, as early as 1945 (*Alexandrium catenella* reported as *Gonyaulax conjuncta* sp.nov. by Wood 1954). This species produced limited toxicity in wild mussels from Port Phillip Bay in 1988 (up to 4.8 mg/kg), and in wild oysters from Sydney Harbour in 1993 (3 mg/kg). *Alexandrium minutum* was first reported from the Port River, SA, in 1986-87 (up to 27 mg /kg in wild mussels). In Oct/Nov 2012 an *Alexandrium tamarense* dinoflagellate bloom along the east coast of Tasmania led to harvesting closures of mussels, oysters, scallops, abalone, and rock lobster causing an estimated $12M loss. Embarrassingly, the 2012 Tasmanian PSP outbreak was only picked up when Tasmanian mussels exported to Japan were found to contain 15x allowable toxin levels (up to 10 mg/kg). In response, a global recall of all Australian shellfish occurred, the economic cost of which and associated loss of reputation of Australian seafood product are still being felt. While the causative dinoflagellate taxon had been known from Tasmania in low concentrations for 15-20 years (Hallegraeff *et al.,* 1991; Bolch & de Salas, 2007), previous *Alexandrium tamarense* bloom events in SE Australian waters (e.g. Cape Jaffa SA, Bell Bay Tas) all belonged to the mostly non-toxic "Tasmanian group V ribotype" (Scholin *et al.,* 1995) even though a toxin-producing strain is also known (Murray *et al.,* 2012) .

Fig.3. Australia-wide distribution of toxigenic *Alexandrium* species.

Unexpectedly the 2012 blooms were made up of the toxic Group I and IV genotypes (Bolch & Murray, pers. comm). This represents a new bloom phenomenon for Tasmania and a link with climate-driven ecosystem change in the area is being pursued (Hallegraeff *et al.,* 2010). Contamination of rock-lobster and abalone are newly recognized major threats to export trade (Homan *et al.,* 2011). The difficulty for routine monitoring of mixtures of toxin- and non-toxin producing genotypes of *A. tamarense* is emphasized. Until 2012 the only biotoxin problem for the Tasmanian shellfish industry was caused by the more easily recognizable, large chain-forming, dinoflagellate *Gymnodinium catenatum*, which exhibits predictable seasonal blooms confined to the Derwent and Huon estuaries (up to 100 mg/kg; Hallegraeff *et al.,* 2012). The Huon River ultimately was classified as unsuitable for shellfish farm leases.

While PSP undoubtedly poses the greatest risk for Australian shellfish safety, minor problems have been caused by Diarrhetic Shellfish Poisoning in 1997-8 from human consumption of NSW pipis containing *Dinophysis acuminata, D. tripos*

(Burgess & Shaw 2001; 56 hospitalisations) and precautionary closures in 2010 of NSW oyster farms contaminated with Amnesic Shellfish Toxins (from *Pseudo-nitzschia cuspidata*; up to 34 mg DA/kg; Ajani *et al.,* 2013). Problems in 2007 with pinnatoxin contamination of SA oysters (Rhodes *et al.,* 2011) were resolved by a decision to deregulate this compound. Problems with bitter tasting shellfish associated with the diatom *Rhizosolenia amaralis* (as *R. chunii*) occurred in Port Phillip Bay in 1987, 1993-4 but also in South Australia 2004 (Parry *et al.,* 1989).

3b. Species that produce toxins of human health significance: Ciguatoxic Fish

Human ciguatera poisonings are known from Queensland (>1000 cases, 2 fatalities; Gillespie *et al.,* 1986), the Northern Territory and occasionally from consumption of pelagic fish from northern NSW, but more seriously >500,000 Pacific Islanders suffer ciguatera during their life time. This debilitating human illness is caused by eating seafood contaminated by ciguatoxins and maitotoxins, bioaccumulated from benthic *Gambierdiscus* dinoflagellates. Ciguatera causes fish markets to ban product sales from entire estuaries (eg. Platypus Bay, Qld), but impacts penetrate far beyond the tropics through seafood trade. There is no immunity, toxins are cumulative, persistent, and the debilitating symptoms often recur. Ciguatera poses a major hurdle to fisheries and seafood market development throughout the Pacific and Caribbean (Fig.4; >$20M loss p.a.). Port and tourism developments, coral bleaching and global warming are all believed to increase ciguatera. We newly recognised widespread *Gambierdiscus* populations along the southern NSW coast (Bermagui-Merimbula, 2006-2012; (Kohli *et al.,* this conference**)**, a major shellfish aquaculture, recreational fisheries and tourism region, unprepared for this problem.

What was once thought to be a single causative dinoflagellate species, *G. toxicus*, even though a >100-fold variation in toxicity had long been recognised, is now considered to be a species complex of 12 different morphotaxa (Litaker *et al.,* 2009), of which *G. carpenteri*, *G. yasumotoi*, *G. belizeanus* have now been identified in Australia. NSW populations have been shown to produce a novel maitotoxin analogue, which potentially could accumulate in fish digestive organs, liver and flesh (Kohli *et al.,* 2014).

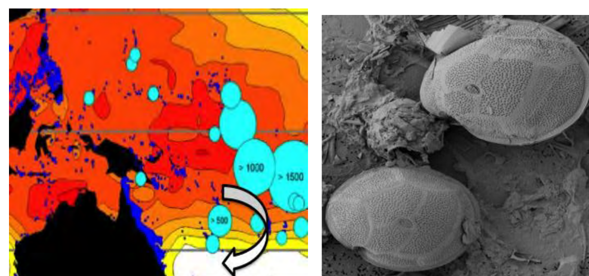

Fig. 4. Left More than 500,000 Pacific Islanders suffer from ciguatera, the causative organisms of which appear expanding their distributions worldwide (arrow); Right SEM of *Gambierdiscus carpenteri* dominated benthic algal community from Merimbula, southern NSW 2012.

3c. Algal toxins of human health significance in desalinated drinking water?

Recent concerns have also been raised that residues of marine algal toxins can end up in desalinated drinking water (Caron *et al.,* 2010). In 2009 an Arabian Gulf *Cochlodinium polykrikoides* algal bloom temporarily closed 18 desalination plants for up to 55 days. A combination of increased water usage and extended years of drought has triggered significant $12B Australian investment in building desalination plants using reverse osmosis technology (Perth 2006, Gold Coast 2009, Sydney 2010, Adelaide 2012, Melbourne 2012, Western Australia 2012). Existing knowledge of marine biotoxins has largely focused on algal toxins which bioaccumulate many orders of magnitude in commercial shellfish or fish. Considering the average human daily water consumption and the proposed 100-300 Dalton molecular weight cut-off by reverse osmosis, none of the currently known algal biotoxins is expected to cause acute human health impacts when present in desalinated drinking water . However early evidence shows that 1% of toxins pass reverse osmosis membranes (Laycock *et al.,* 2012). Worldwide experience with chronic human health problems caused by initially unrecognised cyanobacterial toxins in freshwater drinking reservoirs (Falconer, this conference), suggest that this is an area that calls for internationally coordinated research. Microalgal blooms may also impart harmless odour problems

to drinking water.(eg. from dimethyl sulfoxide, geosmin, ammonia) for which a response protocol is needed.

Table 2. Environmental and Human Societal Drivers of an apparent increase in Australian Harmful Algal Blooms

1. Increased scientific awareness of toxic species. Example: Pinnatoxins in South Australian oysters in 2007, newly associated with dinoflagellate *Vulcanodinium rugosum* in 2011 (Rhodes *et al.,* 2011).
2. Increased utilization of coastal waters for aquaculture. Example: Diarrhetic Shellfish Toxins from *Dinophysis acuminata/fortii* in Ballina NSW pipis in 1997/8 (100+ poisonings; 56 hospitalisations) (Burgess & Shaw, 2001)
3. Stimulation of plankton blooms by cultural eutrophication. Example: Fish-kills by dinoflagellate *Karlodinium veneficum* in Swan River since 2003 (Mooney *et al.,* 2008).
4. Transport in ships' ballast water or associated with translocation of shellfish stocks. Example: PSP dinoflagellate *Gymnodinium catenatum* into Tasmania since 1971 (McMinn *et al.,* 1997).
5. Stimulation by unusual climate conditions/ climate change. Example: Range extension of red-tide dinoflagellate *Noctiluca* from NSW (since 1860) to Tasmania (1994) into Southern Ocean (2010) (McLeod *et al.,* 2012).

While harmful algal blooms, in a strict sense, are completely natural phenomena that have occurred throughout recorded history, in the past three decades the public health and economic impacts appear to have increased in frequency, intensity and geographic distribution. Four explanations for this increase have been proposed (Table 2) (Hallegraeff, 1993). Aquaculture operations act as sensitive 'bioassay systems' for harmful algal species and can bring to light the presence in water bodies of problem organisms not previously known to exist there. The increase in shellfish farming worldwide is thus leading to more reports of Paralytic (since 1793), Diarrhetic (1976), Neurotoxic (1840), Amnesic (1987) and Azaspiracid (1998) Shellfish Poisoning. Similarly, increased finfish culture is drawing attention to algal species which can cause damage to the fishes' delicate gill tissues. People responsible for deciding quotas for pollutant loadings of coastal waters, or managing agriculture and deforestation, should be made aware that one probable outcome will be an increase in HABs.

In countries that pride themselves on having disease- and pollution-free aquaculture, every effort should be made to quarantine aquaculture areas against unintentional introduction of nonindigenous harmful algal species (eg. the IMO *Convention for the Control and Management of Ships' Ballast Water and Sediments*). Nor can any aquaculture industry afford not to monitor for an increasing number of harmful algal species in water samples and increasing number of algal toxins in seafood products using increasingly sophisticated analytical techniques. Last but not least, global climate change is now adding a new level of uncertainty to many seafood safety and HAB monitoring programs (Hallegraeff, 2010). Increasing temperature, enhanced surface stratification, alteration of ocean currents, intensification or weakening of local nutrient upwelling, stimulation of photosynthesis by elevated CO_2, and heavy precipitation and storm events causing changes in land runoff and micronutrient availability may all produce contradictory species- or even strain-specific responses. We can expect: (i) range expansion of warm-water species at the expense of cold-water species; (ii) species- specific changes in abundance and seasonal window of growth of HAB taxa; (iii) earlier timing of peak production of some phytoplankton; and (iv) secondary effects for marine food webs, when individual zooplankton and fish grazers are differentially impacted by climate change. Some species (e.g. toxic *Alexandrium* dinoflagellates) will benefit from land runoff and/or water column stratification, tropical benthic *Gambierdiscus* dinoflagellates may respond to increased water temperatures and coral reef disturbance and may become more successful, while others may diminish in areas currently impacted. Evidence is also accumulating that toxin content of harmful algal cells may increase under ocean acidification scenarios (Fu *et al.,* 2012). The greatest problems for human society will be caused by being unprepared for significant range expansions or the increase of algal biotoxin problems in currently poorly

monitored areas. While Australia in the past may have been less affected by HABs than Japan, Europe or North America, we are now faced with serious problems that call for more vigilance in monitoring, more sophisticated technological approaches (Anderson *et al.,* 2012; Murray *et al.,* 2011) and more coordinated management than currently practiced in Australia.

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Identification and seasonal occurrence of potentially toxic *Pseudo-nitzschia* diatoms in oyster growing estuaries of New South Wales, Australia

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Abstract: Species belonging to the potentially toxic diatom genus *Pseudo-nitzschia* are a significant component of the phytoplankton community in south-east Australian waters. Toxigenic representatives of this genus produce domoic acid and are responsible for the majority of regulatory exceedances in New South Wales (NSW) oyster-growing estuaries. This toxic genus has been implicated in 6 toxic events during the sampling period 2005-2009 (max. concentration of 34 mg DA kg^{-1} oyster tissue). However, identification to species level is difficult and requires both electron microscopy and molecular techniques for unambiguous identification. Detailed analyses revealed 10 different species in NSW coastal waters, *Pseudo-nitzschia americana*, *P. arenysensis, P. calliantha*, *P. cuspidata, P. fraudulenta, P. hasleana, P. micropora, P. multiseries, P. multistriata* and *P. pungens,* including two confirmed domoic acid producers, *P. cuspidata* (25.4 pg DA $cell^{-1}$) and *P. multistriata* (11 pg DA $cell^{-1}$). Species diversity and the seasonal occurrence of regulatory *Pseudo-nitzschia* groupings have important implications for monitoring and management of shellfish harvest areas in NSW. The ubiquitous species, *Pseudo-nitzschia cuspidata,* represents the greatest challenge for coastal shellfish culture in NSW.

Introduction

Phytoplankton (microalgae) are a diverse group of primary producers, which provide nutrition to filter feeding organisms including shellfish. A number of species produce natural toxins that can bioaccumulate in shellfish to concentrations considered harmful to humans (Hallegraeff *et al.,* 2003). Amnesic Shellfish Poisoning (ASP) is one such illness, whereby the neurotoxin domoic acid (DA) is produced by members of the cosmopolitan, diatom genus *Pseudo-nitzschia* (Bates *et al.,* 1989). The New South Wales (NSW) Shellfish Program requires local shellfish programs to collect samples for the detection and enumeration of *Pseudo-nitzschia* with the aim of minimising the economic and health effects related to this toxigenic genus. The identification of these organisms to species level, however, is a difficult task. Furthermore, the regulatory action limits are currently based on the '*Pseudo-nitzschia delicatissima* group' (500,000 cells L^{-1}), '*P. multiseries* and *P. australis* group' (50,000 cells L^{-1}) or 'Total *Pseudo-nitzschia'* (500,000 cells L^{-1}), each of which contain toxic and non-toxic representatives. In view of recent advances in the taxonomy of *Pseudo-nitzschia* worldwide, the aim of this study was to re-examine the diversity and seasonal occurrence of *Pseudo-nitzschia* in NSW in light of shellfish sanitation requirements.

Methods and Materials

Water samples (500 ml to 1 L) for light microscopy cell counts and comparison to NSW Shellfish Program's Phytoplankton Action Limits (PALs (http://www.foodauthority.nsw.gov.au/industry/industry-sector-requirements/shellfish/)) were collected fortnightly from a depth of ~50 cm from 76 harvest areas (31 oyster growing estuaries) for a period of five years (June 2005 to December 2009) (Fig. 1). Lugols's Iodine was immediately added to samples to preserve phytoplankton cells for

later enumeration using a Zeiss Axiolab or Zeiss Standard microscope equipped with phase-contrast (maximum magnification x1000). Samples for transmission electron microscopy (TEM) and molecular sequencing were also also collected from NSW oyster-growing estuaries using a 20-µm mesh phytoplankton net haul (245 mm diameter, 1.2 m length). Clonal cultures were established using single cell isolation techniques and subsampled for TEM analysis, molecular sequencing and toxin production.

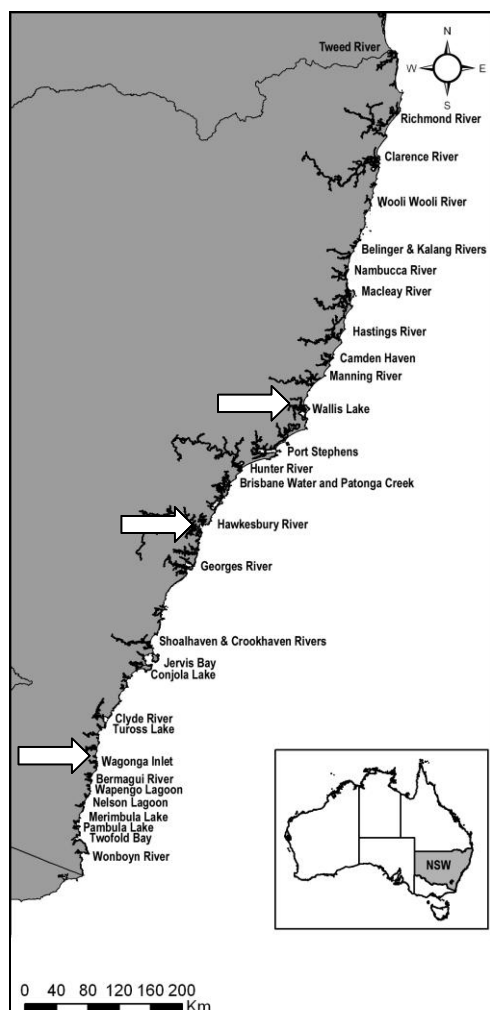


Fig. 1. Map of oyster-growing estuaries in New South Wales, Australia, showing three high-risk estuaries Wallis Lake, Hawkesbury River and Wagonga Inlet (indicated by arrows).

Subsamples for microscope examination were acid cleaned (Hasle and Fryxell, 1970), mounted on to 200 mesh formvar-coated grids and examined using a Philips CM10 TEM equipped with an Olympus SIS Megaview G2 digital camera. Sample preparation for molecular sequencing of the nuclear-encoded rDNA regions followed the CTAB method (Doyle and Doyle, 1987) with modifications by Lundholm *et al.*, (2002). Ten ml subsamples were freeze dried prior to dispatch to Cawthron Institute (New Zealand) for the detection of domoic acid using LC-MS/MS (limit of detection 0.5 ng sample^{-1}).

Results and Discussion

The majority of regulatory exceedances for phytoplankton across the sampling period were for the "Total *Pseudo-nitzschia* group" (Table 1) with high cell densities of *Pseudo-nitzschia* resulting in 6 ASP events – one each for Wallis Lake, Hawkesbury River, Shoalhaven/ Crookhaven River, Wagonga Inlet, Merimbula Lake and Twofold Bay (Fig. 1).

Table 1. Potentially toxic phytoplankton monitored in the oyster-growing estuaries of NSW and the number of events for each taxa where they exceeded the Phytoplankton Action Limits for the sampling period 2005 to 2009.

Toxic Phytoplankton	No. of Events over PALs
Alexandrium catenella/fundyense	47
Alexandrium fraterculus	1
Alexandrium margalefi	8
Alexandrium minutum	8
Alexandrium peruvianum/ostenfeldi	5
Alexandrium pseudogonyaulax	52
Alexandrium sp.	3
Alexandrium tamarense	4
Dinophysis acuminata	58
Dinophysis caudata	8
Dinophysis tripos	3
Gymnodinium catenatum	4
Karenia papillionacea	7
Phalachroma rotundata	1
Prorocentrum lima	7
Pseudo-nitzschia delicatissima group	54
Pseudo-nitzschia fraudulenta/australis	34
Pseudo-nitzschia heimii/subpacifica	4
Pseudo-nitzschia multiseries/australis	53
Pseudo-nitzschia pungens/multiseries	18
Total *Alexandrium* spp.	126
Total *Dinophysis/Phalachroma* spp.	136
Total *Pseudo-nitzschia* spp.	310

Ten *Pseudo-nitzschia* species in New South Wales coastal waters were confirmed using electron microscopy and molecular sequencing: *Pseudo-*

nitzschia americana, P. arenysensis, P. calliantha, P. cuspidata, P. fraudulenta, P. hasleana, P. micropora, P. multiseries, P. multistriata and P. pungens (Ajani et al., 2013a), three being new reports for the Southern Hemisphere (P. arenysensis, P. hasleana and P. micropora) (Ajani et al., 2013b) and two producing domoic acid, P. multistriata (11 pg DA $cell^{-1}$) and P. cuspidata (25.4 pg DA $cell^{-1}$). The latter species was responsible for the largest ever toxic event in NSW, closing the Sydney rock oyster (Saccostrea glomerata) harvest areas in Wagonga Inlet for 16 weeks in 2010 (max. concentration of 34 mg DA kg^{-1} oyster tissue).

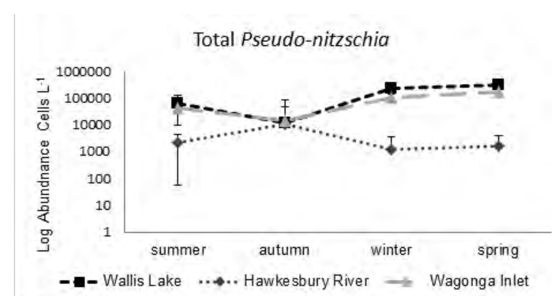
Table 2. *Pseudo-nitzschia* species found in the current study and their current toxicity status. Bold taxa have been found to produce domoic acid in other locations around the world (see review Lelong *et al.,* 2012); *found to produce domoic acid in the current study

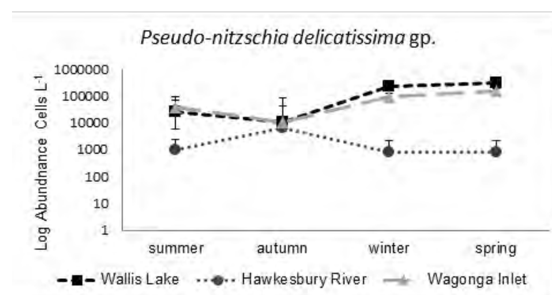
'P. delicatissima group.' (mean valve width <3μm)	'P. seriata group.' (mean valve width >3μm)
P. arenysensis	*P. americana*
P. calliantha	***P. fraudulenta***
P. cuspidata*	***P. multiseries***
P. hasleana	***P. pungens***
P. micropora	
P. multistriata*	

Other species previously found in SE Australian coastal waters include *P. australis, P. dolorosa, P. delicatissima, P. galaxiae, P. lineola* and *P. heimii* (Ajani *et al.,* 2013a and references therein).

Seasonal occurrence of 'Total *Pseudo-nitzschia*' and '*P. delicatissima* group' for the high-risk estuaries Wallis Lake and Wagonga Inlet revealed highest mean cell densities in the austral winter and spring, with a minimum abundance in autumn (Fig. 3a-b). Conversely, the peak mean abundances of 'Total *Pseudo-nitzschia*' and '*P. delicatissima* group' in the Hawkesbury River, were observed in the autumn, with minimum cell densities in the winter and spring (Fig. 3a-b). Other NSW estuaries (data not shown) revealed a high level of temporal variability for both these regulatory groups with no clear latitudinal pattern emerging. The regulatory group '*P. multiseries* and *P. australis*' remained low and relatively constant across all seasons and all estuaries (Fig. 3c – Wallis Lake, Hawkesbury River and Wagonga Inlet presented).

Morphologically, *P. arenysensis, P. calliantha, P. cuspidata, P. multistriata, P. micropora* and *P. hasleana* have loosely been placed in the '*Pseudo-nitzschia delicatissima* group' (Hasle and Syvertsen, 1997, Lelong *et al.,* 2012) with mean cell width <3 μm wide (Table 2). This '*Pseudo-nitzschia delicatissima* group' was historically considered less harmful than the '*Pseudo-nitzschia seriata* group' (>3 μm wide), with fewer members being confirmed producers of domoic acid. In a recent review of *Pseudo-nitzschia* however, (Lelong *et al.,* 2012 and references therein), many of these species, have now been confirmed as toxic candidates. On the other hand, species isolated in this study and belonging to the '*P. seriata* group' included *P. americana*, *P. fraudulenta, P. multiseries* and *P. pungens* (Hasle and Syvertsen, 1997, Lelong *et al.,* 2012) (Table 2).





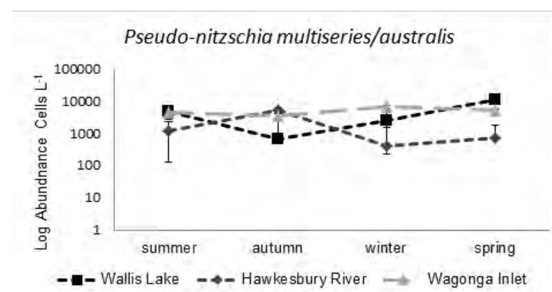


Fig. 3a-c. Seasonal mean abundance of regulatory *Pseudo-nitzschia* groupings for the high-risk

estuaries Wallis Lake, Hawkesbury River and Wagonga Inlet.

All species belonging to the '*P. seriata* group', with the exception of *P. americana*, have been found to produce domoic acid in other locations throughout the world (Lelong *et al.,* 2012). Interestingly, while *P. multiseries* has been a consistent producer of domoic acid in all strains and in all studies throughout the world, local strains in NSW were found to be non-toxic. The regulatory limit, nonetheless, is based on the total enumeration of *P. multiseries* and *P. australis*, the latter species having been found to produce domoic acid in Australian waters (Lapworth *et al.,* 2001).

Considering the species diversity, the temporal and latitudinal variability and the relatively 'short-term' phytoplankton dataset collected to date, we suggest that there is no current scope for alteration to the fortnightly sampling frequency used by the NSW Shellfish Program. Furthermore, this study shows that the general acceptance of the '*P. delicatissima group*' as the more non-toxic group is no longer deemed accurate in NSW waters and as such, regulatory authorities need to place a greater emphasis on this group. To this end, it is an imperative to identify *Pseudo-nitzschia* to species level during bloom events. With this knowledge the regulatory authority can proceed with the most appropriate bloom management procedures.

In conclusion, species of *Pseudo-nitzschia* commonly occur in the coastal waters of NSW, blooms of which account for a significant number of shellfish aquaculture harvest closures. With species diversity now established, more focused smaller-scale studies are required to gain a deeper understanding into the factors that regulate seasonal occurrence, domoic acid production and bloom dynamics of this genus. Finally, we suggest a future spotlight on *Pseudo-nitzschia cuspidata*, a highly abundant and challenging species for coastal shellfish culture in NSW.

Acknowledgements

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First reported closure of a classified mussel production area in Northern Ireland due to Domoic Acid

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Abstract

Northern Ireland production areas have experienced closures due to biotoxin levels above the regulatory limit in most years however the frequency is low, ranging from 2% of samples in 2008 to 4% of samples tested in 2011. Whilst levels of domoic acid above the regulatory limit have frequently been detected in whole scallops, July 2012 was the first occasion that mussel production was affected. From mid-June 2012 *Pseudo-nitzschia* counts increased at a number of sampling points in Belfast Lough. By mid-July 2012 counts were above 150,000 cells L^{-1} initiating additional water and tissue sampling. Domoic Acid levels peaked at 28 µg/g, breaching the regulatory limit and resulting in the closure of the affected harvesting area. This report illustrates the key role that phytoplankton and tissue monitoring plays in protecting public health and supporting the aquaculture industry, even when the assessed risk of a toxic event is low.

Keywords: Domoic Acid, ASP, *Pseudo-nitzschia*, mussels

Introduction

There are approximately 40 classified shellfish production areas in Northern Ireland, located in seven marine sea loughs. Classification of the production areas, monitoring for harmful algae and marine biotoxins is the responsibility of the Food Standards Agency in Northern Ireland (FSA in NI). The Agri-Food and Biosciences Institute undertakes the monitoring on behalf of FSA in NI to agreed sampling plans, based on risk assessments undertaken on each production area. Water sampling is fortnightly from designated sampling points and tissue sampling is undertaken from designated points on a monthly or twice monthly cycle, depending on the risk assessment. The sampling plan is designed to ensure that each production area is sampled at approximately twice monthly intervals. Northern Ireland production areas have experienced closures due to biotoxin levels above the regulatory limit in most years however the frequency is low, ranging from 2% of samples in 2008 to 4% of samples tested in 2011 (Fig. 1). Closure of production areas has resulted from the detection of lipophilic toxins in mussels and oysters and the detection of domoic acid in King Scallops. The affected site, Belfast Lough, is a marine bay situated at the mouth of the River Lagan, on the eastern coast of Northern Ireland. The Inner area of the Lough has been identified as being hypernutrified (as a result of anthropogenic impacts), and is subject to eutrophication (Service, 2008). Phytoplankton growth in Belfast Harbour and the Inner Lough is rarely limited by nutrients. Diatoms dominate the phytoplankton community and may occasionally bloom when the water column is sufficiently stable.

Year	Samples Received	PSP	ASP	Lipophilic Toxin
2008	318	0	8	0
2009	385	0	12	5
2010	419	0	11	2
2011	415	0	3	13
2012	369	0	1	0

Fig. 1. Monitoring Samples above the Regulatory Limit in Northern Ireland: 2008-2012

The shellfish industry has redeveloped in the last 20 years using bottom culture of the common mussel *Mytilus edulis.* Currently, Belfast Lough has 14 classified production areas with 6 representative monitoring points (RMPs) designated with associated harvesting points assigned to each representative monitoring point. Sampling is extended to include associated

harvesting areas which are in production if an RMP returns a result which breaches the regulatory limit.

Domoic Acid Incident 2012

In July 2012 a large bloom of *Pseudo-nitzschia* sp. was recorded in water samples from a number of monitoring sites in Belfast Lough. During the event a maximum cell abundance of 258,520 cells L^{-1} was recorded (Fig. 2). Initial observations using light microscopy showed the species to be of the *Pseudo-nitzschia* '*seriata*' complex.

Elevated levels of domoic acid can pose a significant risk to human health. EC Regulation 853/2004 establishes a limit of 20 mg/kg of domoic acid in whole tissue or any part edible separately. The method of analysis consists of a methanol/water extraction followed by strong anion exchange cleanup and analysis by HPLC with UV detection (Quilliam *et al.,* 1995). In 2012, Domoic Acid levels remained below the level of quantification (LOQ) of 0.3 mg/kg at all monitored sites within Belfast Lough up to the 11th June when levels at the sampling site, Urey, rose to 1.01 mg/kg. By the 9th July levels above the LOQ were detectable at a further two sites within Belfast Lough (Middle Bank and Dougold). Domoic Acid levels at all three sites peaked between the 16th and 30th July 2012 and returned to levels below the LOQ by the 6th August 2012 (Fig. 3). Associated harvesting points for the beds were not in production and therefore additional monitoring of these sites was not required.

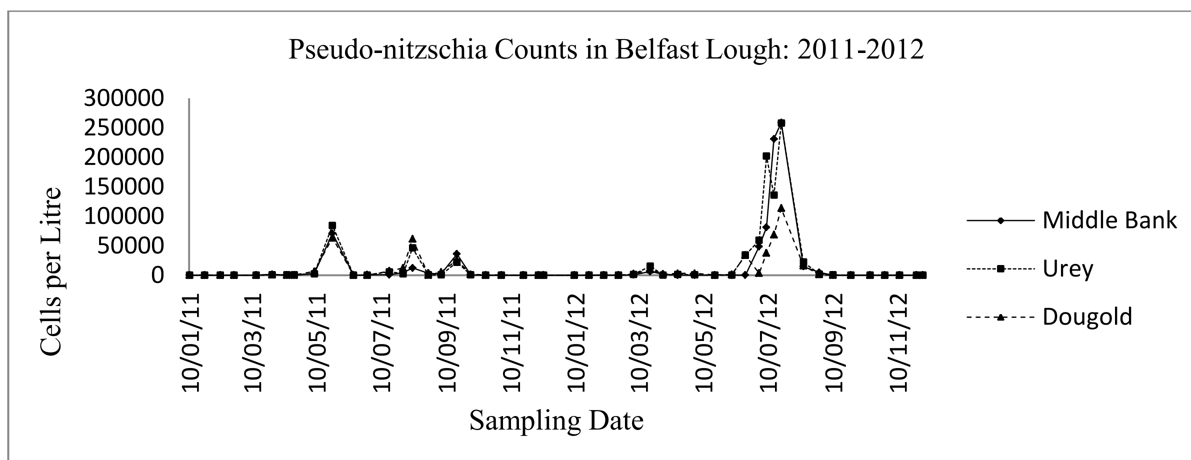

Fig. 2 *Pseudo-nitzschia* Counts in Belfast Lough: 2011-2012

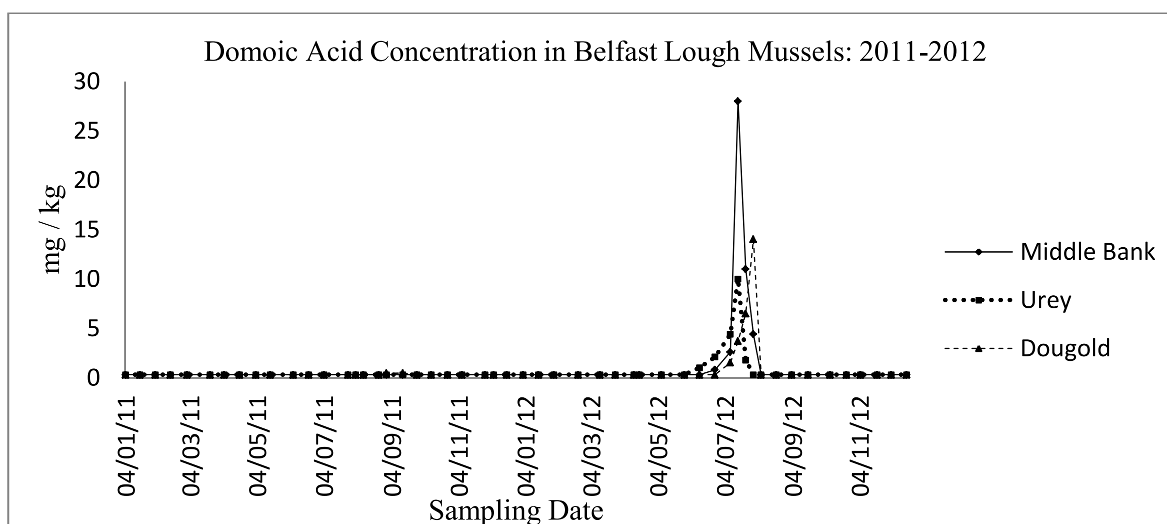

Fig. 3. Domoic Acid Levels in mussels *(Mytilus edulis)* from Belfast Lough: 2011-2012

Discussion

Species of *Pseudo-nitzschia* are the most abundant toxin producing algae in coastal waters of Northern Ireland. Species of this genus with maximum abundances of up to 424,000 cells l^{-1} recorded in Carlingford Lough in 2002 and counts of approximately 250,000 recorded in Belfast Lough in 2009. Previous blooms of *Pseudo-nitzschia* in the waters of Northern Ireland have not resulted in associated toxicity in mussels. Further investigation is required to establish whether this reflects blooms of less potent domoic acid producers or simply highlights the disparity between cell counts and associated toxicity in shellfish. In bivalves, domoic acid depuration time has been shown to be species-specific and have a wide-ranging variability. Most mytilids and other bivalves depurate domoic acid very quickly, while *Pecten* spp can retain the toxin for months (Blanco *et al.*, 2002). The incident in Belfast Lough confirms rapid depuration in mussels with toxicity falling to levels below the level of quantification within 2 weeks.

Conclusions

The first closure of classified *Mytilus edulis* production areas in Northern Ireland due to Domoic Acid highlights the importance of regular biotoxin monitoring. However tissue monitoring alone is not sufficient. The rapid depuration rate for Domoic Acid in mussels observed in this incident suggests that in the case of Domoic Acid, tissue sampling frequency of greater than two week intervals during higher risk periods, could potentially result in the harvesting of produce which breaches the regulatory limits. The data confirms the vital role played by phytoplankton and biotoxin monitoring in the early identification of biotoxin events and protecting the consumer and the aquaculture industry.

Acknowledgements

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Alexandrium species in New South Wales (NSW) coastal waters: historical distributions and identification of high-risk zones.

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Abstract
Accumulation of paralytic shellfish poisoning (PSP) toxins, produced by marine planktonic dinoflagellates, can occur in all major commercial shellfish species. Aside from the potential risk to human health, aquaculture industries have reported severe economic losses due to regulatory closures. Members of the dinoflagellate genus *Alexandrium* are known PSP producers. Since 2005, there has been an apparent increase in reports of *Alexandrium* blooms in New South Wales (NSW), with species causing over 50% of algal related shellfish harvest zone closures. Our current knowledge of the distribution of the species in NSW is examined with an emphasis on high-risk zones.

Keywords: Harmful algal blooms, *Alexandrium*, Paralytic shellfish toxins.

Background

Aquaculture is one of Australia oldest industries. Its significance to indigenous Aboriginal coastal communities has been established through archaeological findings (Attenbrow, 2010), while the beginnings of the modern industry in NSW evolved after the arrival of European settlers. One early development was the commercial availability of Sydney rock oysters (*Saccostrea glomerata*), which began in 1872 (Love and Langenkamp, 2003). Today, there are 71 commercial oyster harvest zones in NSW, spanning across more than 2,000 km of coastline (Fig. 1). Oyster production estimates from the 2011/12 harvest season exceeded $AUD30 million (Livingstone, 2012). The majority of production ($AUD28.5 million) was focused on the cultivation of Sydney rock oysters. The remaining harvest comprised both diploid and triploid Pacific oysters (*Crassostrea gigas*), flat oysters (*Ostrea angasi*) and sale of oyster spat.

A major threat to this immensely valuable industry is the contamination of shellfish product with microalgal toxins. During toxic events the harvest zones undergo mandatory closures.

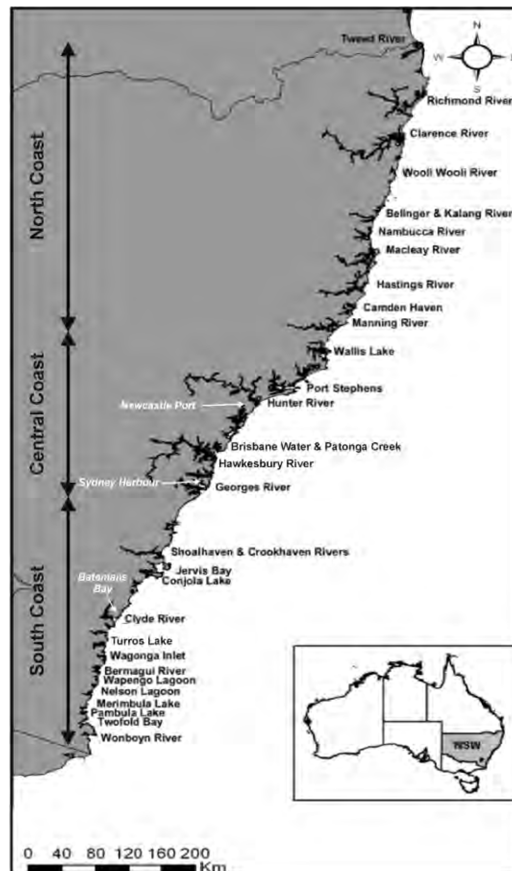

Fig. 1. Location of oyster producing estuaries along the coastline of New South Wales, Australia.

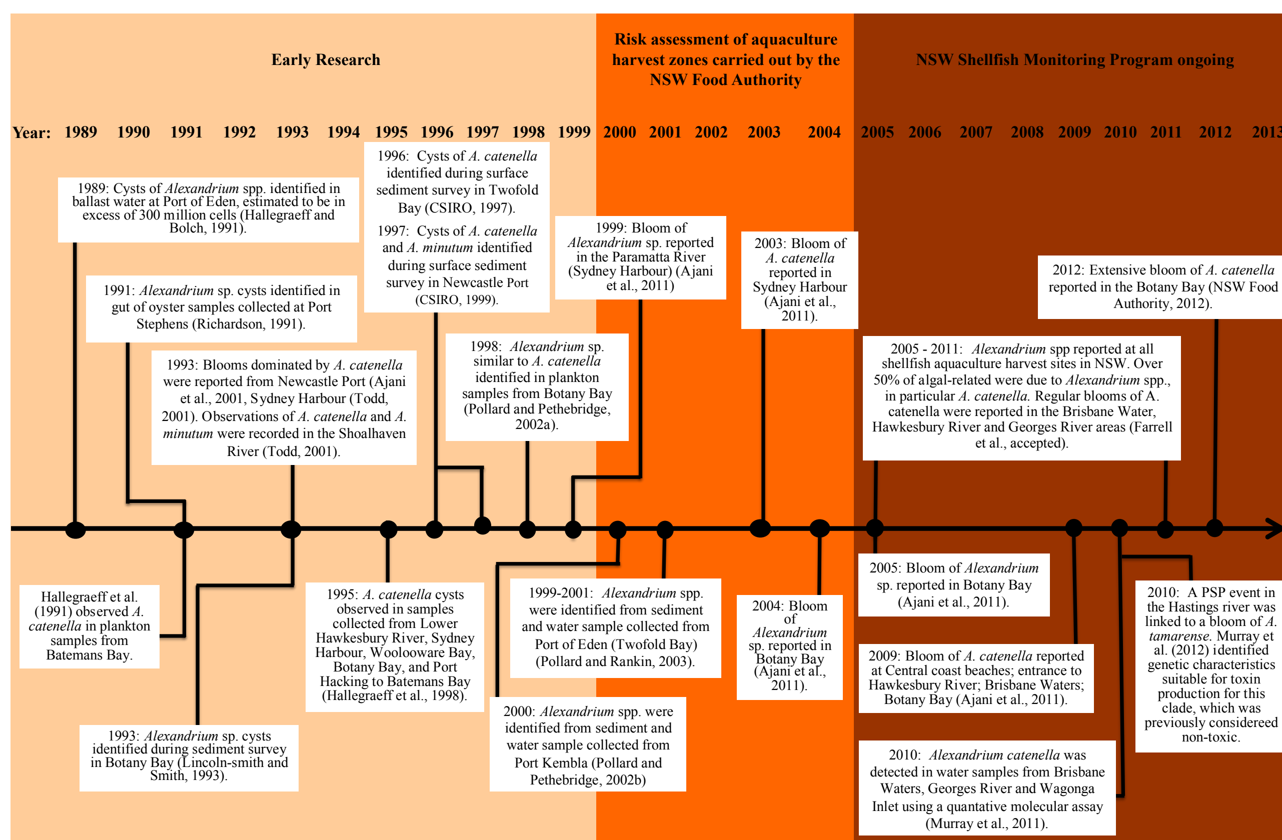

Fig. 2. Timeline of early research and current developments in the understanding of distribution and dynamics of *Alexandrium* spp. in NSW.

This is necessary for the protection of consumers. However, it also represents serious economic losses for shellfish farmers. In recent years, *Alexandrium* spp. and associated PSP events have emerged as an increasing threat to aquaculture zones in NSW (NSW Food Authority, 2011a).

Alexandrium spp. and PSP toxins in NSW

In NSW, species of *Alexandrium* (*Alexandrium minutum, A. catenella* Group IV ribotype, and possibly *A. tamarense* Group V) and *Gymnodinium catenatum* are recognised as the causative agents of PSP toxicity in shellfish (Hallegraeff *et al.*, 1988; Hallegraeff *et al.*, 1991; Negri *et al.*, 2003; Murray *et al.*, 2012).

In Australia, the earliest report of PSP symptoms in shellfish was from Batemans Bay, NSW (See Fig. 1) and the first observation of *A. catenella* in NSW was in 1945 at Port Hacking (Le Messurier, 1935; Wood, 1954; Hallegraeff *et al.*, 1991). Modern research on *Alexandrium* in the Australian region was initially in response to large bloom events in South Australia and Victoria during the 1980s (Hallegraeff *et al.*, 1988; Hallegraeff, 1992).

A review of the available literature on the occurrence of the genus in NSW is provided in Fig. 2. Early accounts of *Alexandrium* spp. in the region during the 1980s and 1990s were sporadic and based on water column and bottom sediment surveys in select areas, along with occasional reports of large blooms (e.g. Hallegraeff *et al.*, 1998; Todd, 2001; Ajani *et al.*, 2011).

The NSW Food Authority is the government body responsible for the NSW Shellfish Safety Program. The development of the program began in 2000, when risk assessments were carried out for all harvest areas. From 2005, regular monitoring of phytoplankton and biotoxins was instigated in all harvest regions. From each zone, phytoplankton samples are collected fortnightly, while biotoxin analysis on shellfish flesh is carried out on a monthly basis (NSW Food Authority, 2011b). The main aim of the program is to ensure that seafood is safe for consumption. However, the information collected has a secondary purpose in that it has provided a valuable baseline from which the risk of *Alexandrium* spp. in harvest zones can be assessed.

Analysis of NSW Food Authority Data 2005 - 2011

Since 2005, there has been an increasing trend in the number of annual closures and positive PSP shellfish tests triggered by the genus (Farrell *et al*., 201 3). In a recent assessment of the program data, *A. catenella* was identified as being the predominant cause of the closures shown in Farrell *et al*. (2013). It should be noted that *G. catenatum* was not observed in significant cell concentrations to be considered as the source of the corresponding PSP events. In the same study, Farrell *et al*. (2013) have recognised the Hawkesbury River, Brisbane Waters and the Georges River as high-risk zones for *Alexandrium* events. Seasonal blooms of *A. catenella* occur on a near annual frequency in these shellfish producing estuaries, and the underlying dynamics need to be clearly defined.

Discussion and Conclusion

Although the information compiled for the distribution of *Alexandrium* spp. up to the year 2000 was infrequent, the observations of the genus across the state represented a serious threat to the development of a sustainable aquaculture industry. The implementation of the Shellfish Safety Program has been successful in managing this risk, with no reports to date of human illness from contamination of commercial oysters during phytoplankton events (A. Zammit, pers comm.). However, with the observed increase in toxic *Alexandrium* events in NSW, the threat is escalating. Globally, PSP events have been reported with increasing frequency and intensity (Glibert *et al*. 2005). On a local level this has been highlighted by a recent (Nov. 2012), extensive bloom of *Alexandrium* along the east coast of Tasmania (DHHS Tasmania, 2012).

As part of an evolving strategy, to increase our understanding of the dynamics triggering the onset of seasonal *Alexandrium* blooms in NSW, intensive investigations are necessary in high-risk zones. Such insights are vital to the development of predictive tools, which would augment the current monitoring program and allow producers to alter harvest management plans, to reduce the impact of harmful events.

Acknowledgements

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Comparison of phycotoxin composition and distribution in toxigenic plankton from the north and south Atlantic

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Abstract
Two oceanographic surveys for toxigenic phytoplankton in the South and North Atlantic Ocean, including the adjacent Irminger Sea and the Arctic coasts of Greenland and Iceland, were conducted for analysis of putative toxic microalgal species and their respective toxins. During both expeditions, plankton was sampled by phytoplankton net (20 µm mesh) vertical hauls with subsequent size-fractionation, and by filtration of Niskin bottle water samples from discrete depths. In addition, sediment samples at selected stations were taken for identification and analysis of organic-walled dinoflagellate cysts (dinocysts). Among the toxins detected in both areas were domoic acid (DA), pectenotoxins (PTXs), yessotoxin (YTX), and paralytic shellfish toxins (PSTs). In addition, in the northern hemisphere, dinophysistoxins (DTXs) and spirolides were present, but these toxins were not found in Argentinean waters. In the sediments of San Jorge Gulf of Argentina, cysts of the dinoflagellate species *Alexandrium tamarense* and *Protoceratium reticulatum* were found, and their respective toxins (PSTs and YTX) were associated with the planktonic samples from the same stations.

Keywords: algal toxins, phytoplankton, dinocysts, PSTs, DSTs

Introduction

The concept of latitudinal cosmopolitanism, e.g. that species occurring at high latitudes in both the Northern and Southern hemispheres may be similar if not identical, has rarely been applied to consideration of toxic marine microalgae and their toxins. The objective of this work was to compare the occurrence of toxic microalgae and associated toxins in the South and North Atlantic, Irminger Sea and the Arctic coasts of Greenland and Iceland. During field expeditions plankton samples were collected and size-fractionated for toxin analysis. Species composition information was supplemented with additionally sediment samples for dinocyst analysis from the southern Atlantic.

Methods

Two ship expeditions were carried out in the northern and southern hemisphere: along transects from Ushuaia (Tierra del Fuego) to Mar del Plata (Argentina) and from Uummannaq Fjord (Greenland) to Reykjavík (Iceland). The first expedition was carried out in the southern hemisphere in Argentinean shelf waters between latitudes 38°S and 56°S in March/April 2012, and the second expedition in the northern hemisphere in coastal waters of western Greenland and Iceland between 60°N and 71°N in July/August 2012.

Vertical net tows were conducted at each station through the upper 30 m (northern hemisphere) and the upper 20 m (southern hemisphere) of the water column with a 20 µm mesh Nitex plankton net. Net tow concentrates were filtered sequentially through Nitex mesh of 200, 55 and 20 µm by gravity filtration and split into size-fraction aliquots for extraction of lipophilic and hydrophilic toxins.

The cell pellets from the plankton net tows were harvested and extracted as described in Krock *et al.* (2008). In short, cells were harvested by

centrifugation and extracted by reciprocal shaking with methanol for lipophilic components and with 0.03 M acetic acid for hydrophilic toxins.

After filtration, hydrophilic extracts were analyzed for PSP toxins by separation of target analytes in reverse-phase mode by high-performance liquid chromatography with post-column derivatization and fluorescence detection (LC-FD), according to Krock *et al.* (2007). Analysis of multiple lipophilic toxins was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), as described in Krock *et al.* (2008).

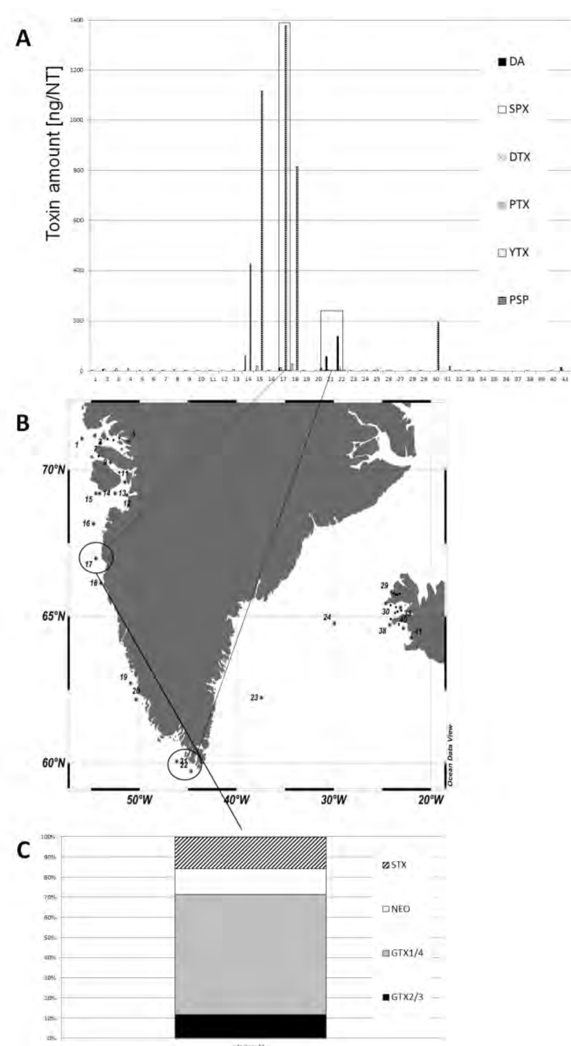

Fig. 1 A) toxin groups detected in the 20-50 µm size-fractions of plankton net tows collected on the Greenland coast; B) geographical location of sampling stations in the Arctic and Irminger Sea along the Greenland and Iceland coast; C) PSP toxin profile (% composition) at Station 17.

Surface sediments were collected with either a Van Veen or a Shipek grab sampler. The top 0-1 cm sediment and the flocculent layer samples were kept in dark cool (4 °C) conditions until analysis.

A 5 cm^3 wet sample from each station was sieved sequentially through 150 µm and 10 µm Nitex screens and the fraction retained on 10 µm was analyzed for the presence of dinocysts. One calibrated tablet of the spore-bearing plant *Lycopodium clavatum* spores was added as exotic markers before treatment to allow calculations of concentrations of cysts/g of dry sediment. Samples were treated by cold 10% hydrochloric acid and heavy-liquid separation with $ZnCl_2$. The residues were finally sieved and collected on a 10 µm mesh and mounted in glycerin jelly. Dinocysts were counted by light microscopy (Nikon Eclipse 600) under 600 x and 1000 x magnification. Slides containing the illustrated specimens are stored at the Laboratorio de Palinología (INGEOSUR-UNS), Bahía Blanca, Argentina.

Results and Discussion

The Arctic transect

Toxins found at stations along the northern transect were dominated by PSP toxins, with values up to 1400 ng per net tow (ng/NT), followed by domoic acid (DA) at the southern tip of Greenland (Stations 21, 22; Fig. 1B) and spirolides (SPX) in the Disko Bay area. These toxin results for the coastal Arctic imply a prevalence of *Alexandrium* and *Pseudo-nitzschia* spp. among toxigenic microalgae, especially in the Disko Bay region of western Greenland. In fact, the presence of toxic *Pseudo-nitzschia* from this coastal region has been described recently (Hansen *et al.,* 2011).

In contrast, dinophysistoxins (DTXs) and pectenotoxins (PTXs) produced typically by *Dinophysis* spp., and yessotoxin (YTX), usually associated with the dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum* or *Gonyaulax spinifera*, were only detected sporadically and at low concentrations <50 ng/NT (Fig. 1A).

The PSP toxin profile in the 20 – 50 µm size-fraction of Station 17 (Fig. 1B) was characterized mainly by gonyautoxins (GTXs) 1-4, low percentages of neosaxitoxin (NEO) and saxitoxin (STX) and the absence of N-sulfocarbamoyl toxins (Fig. 1C). This toxin profile closely matches the composition of several *Alexandrium tamarense*, isolates from Attu and Maniitsoq on the Greenland west coast (Baggesen *et al.,* 2012).

The Argentinean transect

In general, toxin abundances (ng/NT) were higher in the southern than in the northern Atlantic and Arctic. The PTXs were the predominant toxin group found in Argentinean shelf waters, at concentrations up to 3500 ng/NT and dominated by PTX-2. These toxins were found in the entire Tierra del Fuego and Southern Patagonia coastal and shelf regions up to the San Jorge Gulf (Stations I1 to P45B; Fig 2A). The San Jorge Gulf, characterized by nutrient rich, cold waters from the south overlaid by warm, nutrient-depleted coastal waters is a region of high primary productivity (data not shown). In this region (Stations C43 to P45B), a massive bloom of the non-toxic dinoflagellate *Ceratium* spp. was observed, but this plankton assemblage also coincided with the highest toxins concentrations of the entire transect, with PSP toxins > 5000 ng/NT. From Station C43 two isolates of *A. tamarense* displayed the same toxin profiles as already described for Argentinean coastal strains (Montoya *et al.* 2010). In addition two isolates of *Protoceratium reticulatum* from the same Station C43 proved to be YTX-producing.

Dinocysts of potentially toxic species were also found in sediment samples of San Jorge Gulf, with the *Gonyaulax spinifera* species complex dominating in the samples analyzed from three stations. However, there was a significant difference between the cyst assemblage at Station C45, in the central area of San Jorge Gulf, and at Stations C43 and C43N in the mouth region of the Gulf. Cysts of *Alexandrium* spp. were constrained to the sediments of the inner gulf, and their abundance followed the abundance of the *G. spinifera* complex in this sample. *P. reticulatum* cysts reached the highest proportion (only ~13% of the total cyst population) at this inner station.

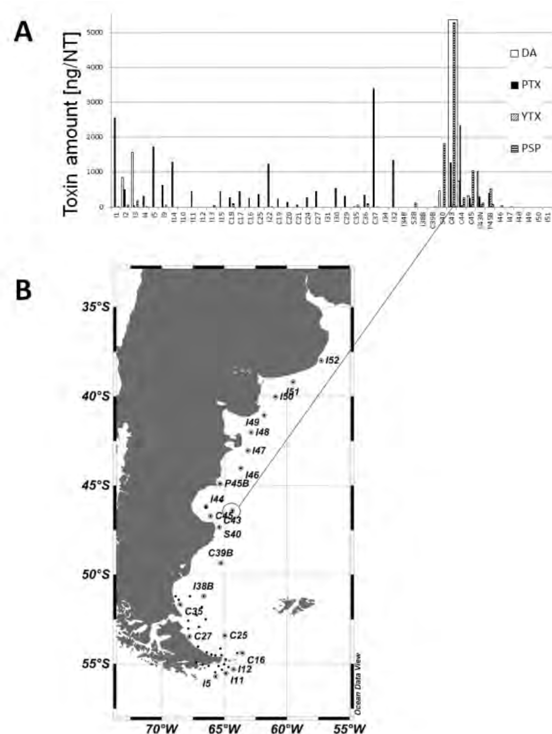

Fig. 2 A) Phycotoxin abundances and B) location of sampling stations along the Argentine coast and shelf region.

Significantly, vegetative cells of *A. tamarense* were isolated at Station C43 where the highest PSP toxin concentrations were also measured, but no *Alexandrium* cysts could be detected in the sediments. In contrast, *Alexandrium* cysts were found at station C45, where only moderate PSP toxin levels were measured. This uncoupling of the distribution of toxins from vegetative cells and of corresponding benthic cysts may reflect either small-scale temporal-spatial patchiness or the dominance of advective processes.

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Risk assessment from paralytic shellfish poisoning (PSP) due to bivalve consumption in Nha Trang city, Vietnam

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Abstract
This study is the first in Vietnam to evaluate the risk to consumers from PSP (Paralytic Shellfish Poisoning) due to bivalve consumption. To obtain shellfish contamination data, PST concentrations were determined by High Performance Liquid Chromatography (HPLC). The results showed that the PST concentrations in bivalves were lower than the limits set by Vietnamese and international using @Risk® software, the exposures of the studied human population to PST due to bivalve consumption were low. The comparisons of these levels with the ARfD (Acute Reference Dose) of PST toxins established by EFSA (European Food Safety Authority) allows us to conclude that there is no significant risk from the exposure to PST from bivalve consumption in Nha Trang city, Vietnam.

Keywords: risk assessment, bivalves, PSP, paralytic shellfish poisoning

Introduction

Bivalves have the capacity to accumulate PST (Paralytic Shellfish Toxins), a class of dangerous phycotoxins considered to be a global problem that requires better professional and public awareness (Martínez and Lawrence, 2003). On a global basis, almost 2,000 cases of human poisonings are reported per year, with a 15% mortality rate (Van Dolah, 2000; Chateau-Degat, 2003; Yan *et al.,* 2003). Human populations living in coastal regions are considered important bivalve consumers. Nha Trang city was chosen as a representative of a coastal community for shellfish consumption. This study was conducted to determine the PST concentration in bivalves to supply valuable information for PSP (Paralytic Shellfish Poisoning) risk assessment due to bivalve consumption by the population in Nha Trang city, Vietnam.

Materials and methods

PST determination

The PST concentrations were determined by the HPLC method of Oshima (1995) in five bivalve species (mussels (*Perna viridis*), scallops (*Comptopallium radula*), oysters (*Crassostrea belcheri*), cockles (*Anadara granosa*) and clams (*Meretrix meretrix*)). The sampling was performed in markets, temporary markets and restaurants in Nha Trang city (Vietnam) in 2009.

Calculation for PST dietary intake

The PST dietary intake by the population in Nha Trang city was calculated from consumption data (Nguyen *et al.,* 2010) and contamination data determined in this study. The probabilistic analyses were performed with @Risk international for Excel, version 4.5.6. The Monte Carlo method and Latin Hypercube sampling were used. The number of Monte Carlo iterations used for the calculations was 10.000.
The dietary intake of PSP by the population of Nha Trang was calculated by the following equation:

$$D=\sum_{i=1}^{n} Q_i C_i$$

D: Distribution of acute intake (µg/kg body weight) of PST by a particular population subgroup (6 population subgroups: men and women (18-29, 30-54 and 55 and over age)
Qi: Distribution of the consumption of the bivalves i (g/kg body weight/day)

Ci: Maximum PST concentration in the bivalves i (µg STXequivalent/g)

Risk characteristics

The acute intakes of PST (D) were compared with the Acute Reference Dose (ARfD) (0,5µgSTX eq./kg body weight) established by the European Food Safety Authority (EFSA 2009) and presented as % of ARfD: (E*100 / ARfD)(%).

Results and Discussions

The PST concentration in bivalves

The results of this study show that PST was not detected in mussels and scallops samples. The PST concentrations in cockles, clams and oysters samples were 0.11, 22.75 and 0.10 µg STXeq/100g, respectively. These results were within the maximum limit (80 µg STX eq/100g) of regulatory guidelines in Vietnam and other countries.

In general, mussels (*Perna* spp.) are known to accumulate PST toxins faster than other species of shellfish and also eliminate the toxin more quickly (Shumway *et al.,* 1990; Shumway *et al.,* 1995). Therefore, mussels (*e.g. Mytilus edulis*, *Perna viridis*) have been considered as indicator species for PST monitoring programmes by many countries. However, the interaction between algal toxins and shellfish is complex and unpredictable. It depends on species, subpopulations, environmental factors (*e.g.* season, water temperature) and shellfish diet (*e.g.* algal species, density) (Shumway *et al.*, 1995). Detoxification rates depend on the tissue of toxin storage within the animal. For example, toxins in the gastrointestinal tract (*e.g.* genus *Mytilus*) are eliminated more readily than toxins bound in tissues (*e.g.* in *Placopecten*, *Spisula*, *Saxidomus*) (Bricelj & Shumway, 1997). In this study, PST could not be detected in mussels. We therefore hypothesize that there was no PST in the environment. On the other hand, PST could have existed in the mussels for a short time and be quickly eliminated. In contrast, oysters do not accumulate PST toxin as readily as mussels. But, it takes considerably longer time to detoxify oysters, and hence they remain toxic for extended periods (up to 3 years) (Shumway *et al.*, 1990). Clams also can remain toxic for a long time (up to 2 years) (Shumway & Cembella, 1993).

Risk assessment

The maximum PST concentration was used for all calculations in risk assessment and the limit of detection (LD) replaced the results which were lower than the limit of detection (negative results). The acute intakes of PST for the different consumption rates and of these acute intakes to the ARfD are presented in Table 1.

Table 1. Acute intakes of PST (µg/kg body weight) due to bivalve consumption by the population in Nha Trang city, Vietnam and their % contribution to the ARfD according to different bivalve consumption rates

	Men						Women					
Percentile	18-29 years old		30-54 years old		>=55 years old		18-29 years old		30-54 years old		>=55 years old	
of exposition	intake	%ARfD	intake	%ARfD	intake	%ARfD	intake	%ARfD	intake	%ARfD	intake	%ARfD
5 th	0,008	1,59	0,008	1,57	0,006	1,26	0,010	2,09	0,008	1,51	0,007	1,33
10 th	0,010	1,94	0,010	1,97	0,008	1,59	0,014	2,70	0,010	1,97	0,008	1,69
15 th	0,011	2,23	0,012	2,34	0,009	1,89	0,016	3,21	0,012	2,36	0,010	2,04
20 th	0,012	2,50	0,014	2,71	0,011	2,18	0,018	3,66	0,014	2,73	0,012	2,40
25 th	0,014	2,77	0,015	3,04	0,012	2,43	0,021	4,14	0,016	3,11	0,014	2,75
30 th	0,015	3,06	0,017	3,44	0,013	2,70	0,023	4,67	0,017	3,49	0,016	3,10
35 th	0,017	3,35	0,019	3,79	0,015	2,96	0,026	5,20	0,019	3,90	0,017	3,50
40 th	0,018	3,69	0,021	4,18	0,016	3,23	0,029	5,72	0,021	4,28	0,019	3,88
45 th	0,020	4,03	0,023	4,58	0,017	3,50	0,031	6,28	0,024	4,71	0,022	4,30
50 th	0,022	4,38	0,025	4,98	0,019	3,78	0,034	6,86	0,026	5,16	0,024	4,74
55 th	0,024	4,73	0,027	5,40	0,021	4,11	0,037	7,46	0,028	5,63	0,026	5,19
60 th	0,026	5,10	0,029	5,87	0,022	4,43	0,040	8,05	0,031	6,11	0,028	5,66
65 th	0,028	5,51	0,032	6,34	0,024	4,77	0,044	8,74	0,033	6,66	0,031	6,18
70 th	0,030	5,94	0,034	6,85	0,026	5,13	0,047	9,44	0,036	7,16	0,034	6,72
75 th	0,032	6,43	0,037	7,43	0,028	5,52	0,051	10,26	0,039	7,77	0,037	7,32
80 th	0,035	6,95	0,040	8,04	0,030	5,97	0,056	11,11	0,042	8,43	0,040	7,98
85 th	0,038	7,51	0,044	8,77	0,032	6,48	0,061	12,10	0,046	9,13	0,044	8,73
90 th	0,041	8,26	0,048	9,63	0,035	7,10	0,067	13,30	0,050	10,05	0,048	9,66
95 th	0,046	9,20	0,054	10,75	0,039	7,89	0,074	14,84	0,056	11,28	0,054	10,83
Mean	***0,024***	***4,74***	***0,027***	***5,42***	***0,020***	***4,09***	***0,037***	***7,45***	***0,028***	***5,62***	***0,026***	***5,23***

The mean acute intakes of PST due to bivalve consumption of 6 groups of men (18-29, 30-54 and 55 years and over age) and women (18-29, 30-54 and 55 years and over age) were: 0.089; 0.105; 0.075; 0.146; 0.111 and 0.109 µg/kg body weight, respectively. The percentage of the mean acute intakes of PST to the ARfD due to bivalve consumption of these 6 groups were: 17,86%, 21,03%, 14,97%, 29,11%, 22,19% and 21,80%, respectively. In general, the percentages (%) of all acute intakes of PST to the ARfD according to different bivalve consumption rates were lower than 70%.

Conclusions

The acute intakes of PST due to bivalve consumption by the population in the Nha Trang city were lower than the ARfD of PST established by the European Food Safety Authority (2009), even though the maximum PST concentrations were used and the limit of detection (LD) replaced the negative results which were lower than the limit of detection used to calculate the levels of PST intake. The results achieved in this study permitted us to conclude that the degree of exposure to PST due to bivalve consumption was insignificant. However, we need more complementary studies to consider the PST concentrations in bivalves in different months.

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First report on the detection of yessotoxin from *Gonyaulax spinifera* in the Benguela current upwelling system

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Abstract

The Benguela current is one of the four major coastal upwelling currents on the eastern boundary of the ocean basins. This upwelling system provides nutrient-rich deep water to the euphotic zone, thus facilitating the proliferation of harmful algal blooms. One such bloom consisting predominantly of dinoflagellates was detected during late summer of 2011 in the coastal embayment of Walvis Bay, Namibia (23°S, 14°30'E). The phytoplankton species composition during this bloom included potentially toxic *Gonyaulax spinifera*. Environmental parameters as well as biological samples were taken during this event, and the toxin content for the oysters measured by LC/MS-MS and DSP MBA. Yessotoxin (YTX) and the analogues presumed 45-OH-YTX and homoYTX were the dominant toxins present in the mussel samples at concentrations of up to 5.4 mg YTX equivalents kg^{-1} shellfish tissue. The phytoplankton YTX profile was dominated by OH-YTX and homoYTX. The highest levels produced were 156.0 pg $cell^{-1}$ with OH-YTX and homo-YTX at 78.63 and 65.41 pg $cell^{-1}$ respectively. This report details the first detection of YTX and its analogues in shellfish samples as well as *G. spinifera* in the Benguela current upwelling system. It also details the first report of *G. spinifera* producing an OH-YTX as one of the dominant YTX analogues.

Keywords: Benguela Current, *Gonyaulax spinifera*, yessotoxin, homo-yessotoxin, hydroxyl-yessotoxin, shellfish.

Introduction

The cold Benguela Current extends from the southern tip of Southern Africa to the Angola-Benguela front of Angola. This current is one of the four major coastal upwelling currents on the eastern boundary of the ocean basins (Trainer *et al*., 2010). The principal upwelling cell is around Luderitz, where the area is divided into northern (encompassing Walvis Bay) and southern components (Trainer *et al*., 2010). As an upwelling system, it provides nutrient-rich deep water to the euphotic zone (Pitcher *et al*., 2010). This enhances the growth of phytoplankton and can thus facilitate the frequent proliferation of harmful algal blooms (Hallegraeff, 1995). These blooms can be caused by algal species that produce phycotoxins, which accumulate in filter feeding shellfish, thus affecting mariculture activities. The phycotoxins most commonly detected in the Northern Benguela are lipophilic toxins (Aquafact, 2012). Since these results were obtained using the mouse bioassay, there is no information on the types of lipophilic toxins present. During the week of 15th March 2011, a dinoflagellate bloom consisting of *Gonyaulax spinifera* was detected in the Walvis Bay area. This dinoflagellate has been known to produce yessotoxin (YTX) (Rhodes *et al.,* 2006; Riccardi *et al.,* 2009). YTX and its analogues are polyether toxins produced by the dinoflagellates *Protoceratium reticulatum, Lingulodinium polyedrum*, and *Gonyaulax spinifera* (Alvarez *et al*., 2011; Gerssen *et al*., 2010). YTX was first isolated in Japan from the scallop *Patinopecten yessoensis* (Murata *et al*., 1987). To date, 90 different analogues have been identified (Miles *et al*., 2005). Globally, shellfish from Norway, Italy, New Zealand, Russia and the USA have tested for YTX at varying concentrations from *P. reticulatum* and *L. polyedrum* (Howard *et al*.,

2008; Paz *et al*., 2008). YTX in shellfish from *G. spinifera* has only been detected in New Zealand and Italy (Rhodes *et al*., 2006; Riccardi *et al*., 2009). YTX was thought to be the predominant toxin until Riccardi *et al.* (2009) found homoYTX and minor concentrations of carboxy-YTX and 45-OH-YTX in algal samples. These analogues were believed to be shellfish metabolites of YTX and homoYTX (Miles *et al*., 2005; Roeder *et al*., 2011). In the Benguela Current system, YTX has only been detected in Southern Benguela from *P. reticulatum* cultures (Trainer *et al.,* 2010). This report details the first detection of YTX in mussel and phytoplankton samples from a bloom of *G. spinifera*.

Materials and methods

Study area

Four farm sites from the Walvis Bay area (Aquapark 1) with depths that range from 8-11m were sampled from Namaqua, Beira, Seatet and Shoreline. The deep-sea waves originate from SSE to SW and the waves progressively decrease in magnitude. A shadow zone is formed from the positioning of pelican point (where the farms are situated). This results in a southwards long shore current in the harbour (Voges and Morant, 2009). In the harbour, the water circulation is driven by predominantly southerly winds, which enable a clockwise water flow. The wind during autumn, winter and spring is predominantly in a SW direction, and NW during summer. Blooms are most likely to occur during late summer and early autumn when there are light, predominantly on-shore winds (Voges and Morant, 2009).

Environmental parameters

Seawater samples were taken from Aqaupark 1 from the surface, mid and bottom with a Niskin bottle. Temperature readings were taken at the corresponding depths. Samples for dissolved oxygen were collected in glass oxygen bottles and analysed using the Winkler method (Grasshoff *et al*., 1983). Samples for the analysis of inorganic nutrient concentrations (phosphate, silicate, nitrate and nitrite) for the corresponding depths were collected and filtered in 250 ml soft low-density dark polyethylene acid washed bottles. Samples were frozen until analysis using the standard colorimetric manual methods (Grasshoff *et al*., 1983). All samples were analysed in duplicate.

Phytoplankton sampling and toxin extraction

Phytoplankton samples were collected daily during the bloom from the first 5 m of the water column using a pipe to obtain an integrated sample of the photic zone. These samples were preserved and used for species identification and quantification using the Utermohl method (Hasle, 1978) with 20 ml setting chambers with an inverted microscope at 400x magnification. Vertical net haul samples were also taken at each station using a 20-µm-mesh phytoplankton net. Seawater from the bloom was filtered onto GF/F filters for toxin extraction. Lipophilic toxins were extracted from filters using 4 ml 80% methanol, sonicated in an ice-bath for 10 min and centrifuged at 14000 rpm for 10 min. This was repeated once more combining supernatants and adjusting the final volume to 10 ml.

Shellfish sampling and toxin extraction

Shellfish samples consisting of the mussel species *Perna perna* and *Mytilus galloprovincialis* were collected during the *G. spinifera* bloom (15th-18th March 2011) as well as on three other occasions after this bloom. Lipophilic toxins were extracted from 2 g homogenized mussel tissue using 9 ml 80% methanol, sonicated in an ice-bath for 5 min and centrifuged at 14000 rpm for 5 min. This was repeated once more combining supernatants and adjusting the final volume to 20 ml. This extracted all lipophilic toxins and was analysed using an LC-MS/MS. The individual YTX and its analogues were converted to YTX equivalents kg^{-1} (Roeder *et al*., 2011; European Food Safety Authority, 2008). As there is no TEF for carboxy-YTX it was omitted from the calculation.

LC-MS/MS analysis of lipophilic toxins

LC-MS/MS analyses were conducted using a Thermo Accela HPLC coupled to a Thermo TSQ Vantage quadrupole MS/MS. Chromatographic separation was done on a Kinetex 2.6 µm C18 column (50 mm x 2.1 mm) at 20°C with a flow rate of 0.2 ml min^{-1}. Both mobile phases, A-water and B-acetonitrile, contained 50 mM formic acid and 20 mM ammonium formate (all MS grade). The injection volume was 50 µl. A gradient

elution was applied, starting with 90% A reduced to 10 % A over 6 min, held for 8.5 min, then increased to 90 % A in 4.5 min and held for 2 min. Certified reference standard of YTX was used for fine tuning and calibration the MS, as it was the only available standard. It was purchased from the Canadian National Research Council and Cifga Laboratorio s.a. (Spain). The mass spectrometer was tuned for yessotoxin (-1141.5/-1061.6 m/z), homoYTX (-1155.5/-1075.5), 45-OH-YTX (-1157.5/1077.5) and carboxy-YTX (-1173.5/-1093.5) in selected reaction mode (SRM). YTX was quantified with an external calibration function (r^2: 0.999, limit of detection: 1ng YTX ml^{-1} respectively 10 µg kg^{-1} shellfish). The relative process standard deviation V_{xo} was ± 2,6 %. YTX analogue concentrations were roughly quantified by applying the calibration function of yessotoxin.

Results

Shellfish samples toxin analysis

YTX, homoYTX and presumed 45-OH-YTX, carboxy-YTX and homoYTX-OH were detected in mussel samples from the shellfish farms during the bloom period (Fig. 1). The sample from Namaqua on the 17th March had the highest concentration of YTX (5.4 mg YTX equivalents kg^{-1}). This coincided with the date at which the *G. spinifera* cells were at a maximum (Fig. 2). The second highest concentrations detected were from the 16th March from Beira followed by Seatet samples from the 30th March and 27th April (4.0, 2.4 and 3.0 mg YTX equivalents kg^{-1} respectively). The dominant toxin in all samples was presumed 45-OH-YTX (32-45%), followed by homoYTX (20-35%) in the samples from Namaqua and Seatet. These samples contained a mixture of both mussel species. Samples taken at Beira consisting of mature *Perna perna* mussels had YTX as the second most abundant analogue (25-30%).

Phytoplankton samples toxin analysis

The phytoplankton samples tested, from 17th March had the highest concentration of YTX equivalents at 59.18 ng ml^{-1} (Fig. 2). The vertical net haul sample taken on the 15th March contained 118.38 ng ml^{-1} of YTX eq. The YTX profiles of all samples were dominated by presumed 45-OH-YTX and homoYTX, which accounted for > 90% of the total cellular toxin content. This was observed in both net and composite samples. YTX was produced by the cells at 156.0 pg $cell^{-1}$ with the presumed 45-OH-YTX and homoYTX being produced at 78.63 and 65.41 pg $cell^{-1}$,respectively.

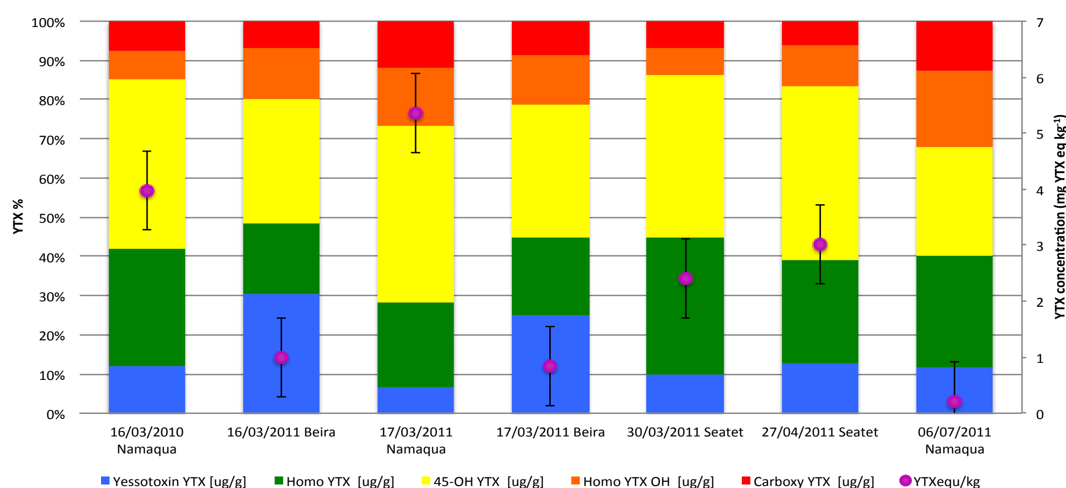

Fig. 1: Concentration of YTX in mussel samples from sampling sites in Aquapark 1and relative composition of analogues.

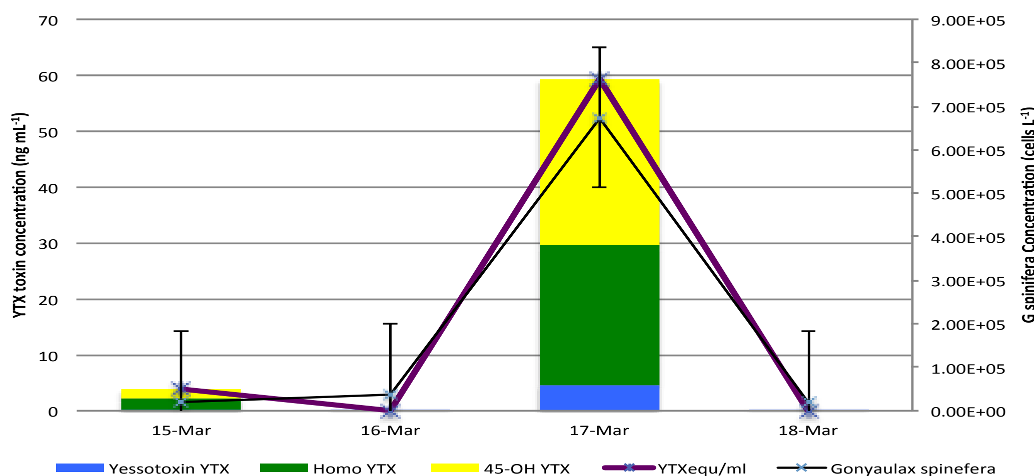

Fig. 2: Concentration of YTX analogues in phytoplankton samples and *G. spinifera* cell concentration during the bloom (15-18 March 2011).

Discussion

The YTX analogues profile in the mussel samples during the bloom correlated with the YTX profile observed in the phytoplankton samples. These values are within range of those detected in Italy (0.17- 9.0 mg YTX equivalents kg^{-1}) for the same species (Ricardi *et al*., 2009). The low level of carboxy-YTX (7-12%) is unusual as it is generally the second most abundant analogues found in shellfish. Feeding and depuration experiments in *Mytilus edulis,* and *Crassostrea gigas* found 45-OH-YTX and carboxy-YTX were the dominant toxins present (Roeder *et al*., 2011; Aasen *et al*., 2005). Thus the likely metabolic pathway of YTX in shellfish was that 45-OH-YTX and carboxy-YTX were produced from YTX. This was postulated since the *P. reticulatum* culture used for feeding had a dominant profile of YTX with traces of carboxy-YTX and keto YTX (<0.3 pg YTX eq $cell^{-1}$). In this work the distinct peak of presumed 45-OH-YTX could not be verified due to the lack of a standard, and it is possible that hydroxylation could have occurred in another area of the molecule. This will have to be examined in further work. The low levels of carboxy-YTX in the shellfish samples might have been due to the low YTX content in the *G. spinifera* cells. *G. spinifera* was first conclusively shown to produce YTX using an ELISA test on single cells from New Zealand (Rhodes *et al*., 2006). As this test uses antibody recognition, it was unable to provide any information on the analogues present. The toxin content of the cells detected was 176 and 200 pg YTX $cell^{-1}$. *G. spinifera* from the Adriatic Sea had homoYTX (8.6 pg $cell^{-1}$) present as a major component in its YTX profile (Riccardi *et al*., 2009). By contrast the YTX profile from the Benguela *G. spinifera* shows dominant production of OH-YTX and homoYTX. It is important that the structure of the OH-YTX compound identified is confirmed in future work as this is an important consideration with respect to the existing knowledge that 45-OH-YTX is produced by direct oxidative metabolism of YTX in shellfish (Miles *et al*., 2006). In conclusion, this is the first report of an OH-YTX being produced as the dominant YTX analogue in *G. spinifera*.

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Cyanobacterial toxin accumulation in shellfish, finfish and crustaceans: guidelines for consumer safety

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Abstract
Cyanobacterial toxicity has been a significant factor in livestock production in Australia since European settlement. The earliest report was of the deaths of cattle, sheep, horses, pigs and dogs in 1878 from *Nodularia* toxicity in an estuarine lake. Since then there have been cases of human toxicity from drinking water, and demonstrations of toxicity in mussels, fish and crustaceans. Guideline Values for cyanobacterial toxins have been developed for all three categories of aquatic food, using human consumption data and animal-derived No Observable Adverse Effect Levels (NOAEL) with appropriate safety factors. In the case of PSPs, existing Guideline Values were adopted. These guidelines are now in use during toxic waterbloom events in Australia during which aquatic species are harvested for human food.

Keywords: Cyanotoxins, Seafood, Guideline Values

Introduction
The common toxic cyanobacterial species *Nodularia spumigena,* forming a water bloom Lake Alexandrina, a brackish estuary in South Australia, killed cattle, sheep, horses, dogs and pigs drinking the water (Francis, 1878). Since this time there have been many livestock deaths due to cyanobacterial toxins in Australia, and elsewhere in the world. Thousands of sheep have been poisoned by *Microcystis* in South Africa, and by *Anabaena* in Australia.

The toxicity of *Nodularia* and chemistry of the toxin were researched in the 1980's in Australia and the USA (Runnegar *et al.*, 1988; Rinehart *et al.*. 1988). Eriksson in Finland showed uptake and concentration of a peptide toxin from *Oscillatoria* by freshwater mussels (*Anadonta cygnea*), demonstrating the possibility for shellfish poisoning of human consumers by cyanobacterial toxins (Eriksson *et al.,* 1989). Our laboratory demonstrated toxicity to mice of marine mussels (*Mytilus edulis*) collected during a naturally occurring water bloom of *Nodularia* in 1989/90 (Falconer *et al.,* 1992). Partly digested filaments of the cyanobacterium were visible in the gut contents of the mussels. Since this time many studies have confirmed the accumulation of cyanobacterial toxins in shellfish, fish and crustaceans used for human food, causing a potential health risk to consumers (Ibelings and Chorus, 2007; Stewart and McLeod, this volume).

Health significance and Guideline Values
Peptide toxins
Cyanobacterial toxins occur in a variety of chemical forms. The most studied are the cyclic peptide toxins nodularin (from *Nodularia*), which has a five-amino acid ring, and microcystin (from *Microcystis*) which has a ring of seven amino acids. These peptides are resistant to digestion as the majority of the acids are in D configuration, and are selectively toxic to organs as they require a transport mechanism to enter. Poisoning results in gastrointestinal and liver injury in consumers, as enterocytes and hepatocytes have the appropriate toxin transporters (Falconer, 2005). The biochemical mechanism of action is inhibition of specific protein phosphatase enzymes, causing cell disruption, together with tumour promotion through changes in control of growth of cells. Thus these peptide toxins cause acute toxicity through liver damage, which can cause death if consumed in sufficient quantity, and the potential to stimulate tumour growth through ongoing low-level exposure.

To derive safe guideline values for toxins in foodstuffs, a series of components have to be quantified (FAO;IOC;WHO, 2004). In particular the quantities consumed.

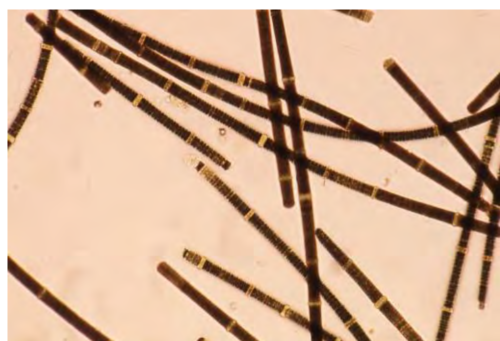
In Australia there have been recent surveys that provide data for high consumption (97.5th percentile) of mussels, prawns and finfish (Australian Bureau of Statistics, 1998; Department of Health and Ageing , 2007).

In the adult age group (17 years plus), this consumption is 178g/day mussels (2.4g/kg BW/day), 377g/day prawns (5.1 g/kg BW/day, 377g/day fish (5.1 g/kg BW/day).

For 2-16 year olds high level consumption is 148g/day molluscs (3.9 g/kg BW/daykg), 236g/day prawns
(6.2g/kg BW/dayBW), 319g/day fish (8.4 g/kg BW/day).

To derive the Tolerable (Acceptable) Daily Intake of a toxin, the highest No Observed Adverse Effect Level (NOAEL) must be obtained from human poisoning data or animal dose trials with toxin. These are standardised at 13 weeks of oral administration, usually with three dose rates, bracketing no effect, minimum effect and clear adverse effects of toxicity.

For microcystin-LR, the most extensively studied toxin, the NOAEL is 40µg/kg BW/day in mice (Fawell *et al*.,1994). The safety or uncertainty factors applied are 10x for interspecies sensitivity, 10x for intraspecies sensitivity (genetic, age etc), plus 2x for uncertainty over the data (possible carcinogensis, teratogenic and reproductive effects), giving an overall safety factor of 200, and hence a Tolerable Daily Intake of 0.2µg/kg/day for humans. The Acceptable Daily Intake is then 0.2 x Body Wt x allocation factor (proportion of the days intake from that source, assumed all from seafood in this study), hence 0.2 x 74 kg (Australian mean adult BW) x 1.0 =14.8µg/day/person.



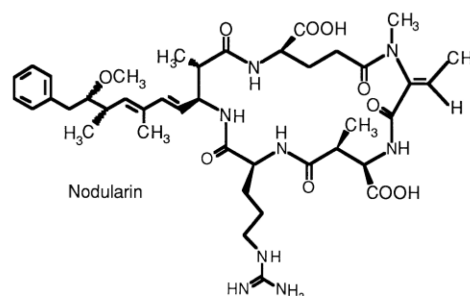


Fig. 1. *Nodularia* filaments and toxic cyclic peptide nodularin**.**

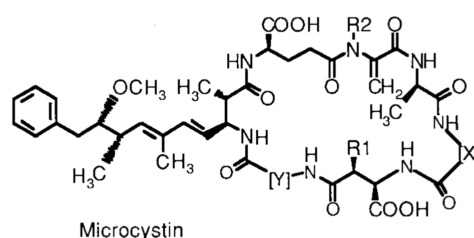


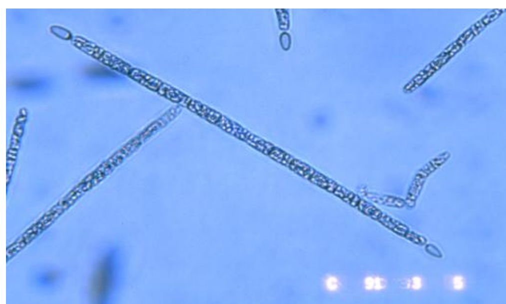
Fig. 2. *Microcystis* colony and microcystin

The Health Guideline Value for the seafood is the Acceptable Daily Intake devided by the consumption, which for mussels is 0.18 kg/day, hence giving a Guideline Value of 83µg microcystin/kg of shellfish for adults. The prawn GV is 39 µg/kg, and finfish GV 39 µg/kg.

Similar calculations are done for children of 2-16 years applying a mean bodyweight of 38kg, and the consumption data for that age group. This gives

a GV of 51 µg/kg of microcystin for molluscs, 32 µg/kg for prawns and 24 µg/kg for finfish.
Nodularin has the same toxicology as microcystin, and the same Guideline Values apply.

Cylindrospermopsin



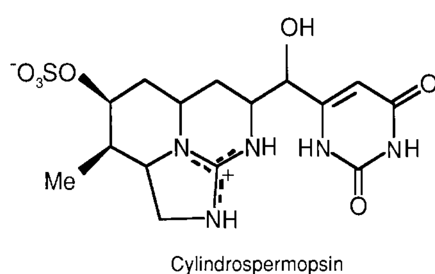


Fig. 3. Filaments of *Cylindrospermopsis* and structure of cylindrospermopsin

This alkaloid cyanotoxin is present in freshwater cyanobacterial blooms from which molluscs, crustaceans and finfish are harvested for human consumption. The toxin has been demonstrated to accumulate in crustaceans (Saker and Eaglesham, 1999) and in shellfish;(Saker *et al.,* 2004).
Subchronic 13 week oral animal toxicity trials in mice with cylindrospermopsin have given a NOAEL of 30µg/kg BW/day (Humpage and Falconer, 2003). The same safety factors, bodyweights and human consumption data have been applied to this cyanotoxin as to microcystin. While there is good evidence of mutagenic carcinogenicity for this toxin, no long-term carcinogenicity trials have been attempted. This is due to cost, including cost of production of the toxin by synthetic or natural harvesting and purification methods. In the absence of this key data for carcinogenic safety evaluation, the standard toxic threshold approach has been used, as applied to microcystin.

The adult Guideline Values for cylindrospermopsin are determined as 62µg/kg for shellfish, 29µg/kg for prawns and 29µg/day for finfish. For children of 2-16 years old Guideline Values are 39µg/kg shellfish, 24µg/kg for prawns and 18 µg/kg for finfish.

Fig. 4. *Anabaena circinalis* and saxitoxin

In Australia the cyanobacterial species *Anabaena circinalis* has caused extensive livestock deaths, notably when about 10,000 animals were killed by a 1,000km waterbloom along the Darling River in 1990. The toxins responsible were Paralytic Shellfish Poisons (PSPs) (Humpage et al., 1994). For the cyanobacterial PSPs, the accepted Guideline Value of 800µg/kg of 'saxitoxin equivalents' for human consumption of seafood is maintained. The saxitoxin derivatives in cyanobacteria include a preponderance of C toxins, which hydrolyse on boiling (and possibly on digestion) to the much more toxic gonyautoxins. These are highly toxic to ruminant species, as well as non-ruminant mammals.
A detailed consideration of cyanotoxin Guidelines Values and their calculation for seafood can be found in Mulvenna *et al*. (2012), from which the data for this paper has been drawn (Table 1).

Table 1. Summary from Mulvenna et al. (2012)

Health guideline values for cyanobacterial toxins in seafood, 12-16 age group (µg/kg of whole organism sample)

Toxin	**Fish**	**Prawns**	**Molluscs**
Cylindrospermopsin and deoxyCYN	18	24	39
Microcystin-LR or equivalent toxins, incl. Nodularin	24	32	51
Saxitoxins	800	800	800

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A new high resolution LC-MS method for identification, quantification and monitoring of palytoxin-like compounds in molluscs

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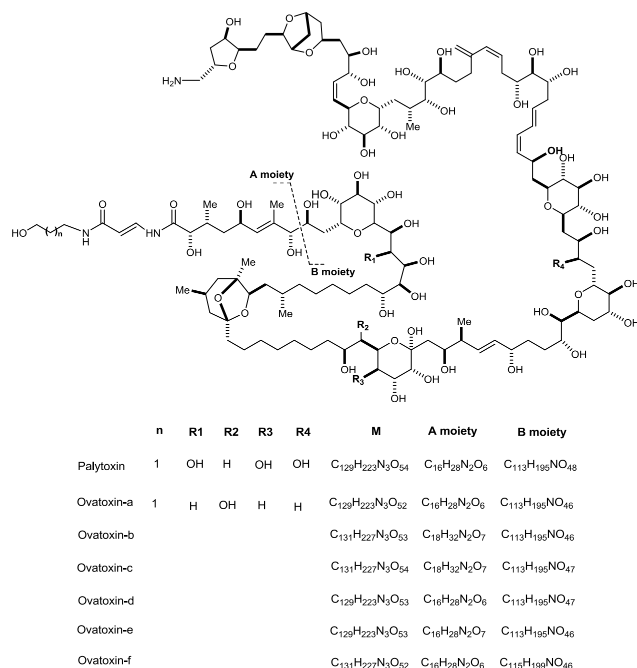
Abstract
Palytoxin and ovatoxins are posing a serious concern to human health in the Mediterranean area as they are the major toxins produced by *Ostreopsis* cf. *ovata*, a benthic dinoflagellate that since the late 90's repeatedly blooms along the Mediterranean coasts of Italy, France, Spain, and Greece. In order to prevent the potential sanitary risks associated with consumption of palytoxin contaminated seafood, the European Food Safety Authority (EFSA) indicated the need for efficient, rapid and sensitive methods to monitor the presence of palytoxin-like compounds in seafood. In the present study, the best conditions for extraction and clean-up of palytoxin from mussels and their determination using LC coupled to high resolution mass spectrometry (HR-MS) are presented.

Keywords: Ostreopsis cf. *ovata*, palytoxin, ovatoxins, High Resolution LC-MS.

Introduction
In the last decade, the benthic dinoflagellate *Ostreopsis* cf. *ovata* has been repeatedly blooming in the Mediterranean basin posing serious sanitary and economic problems to humans (Mangialajo *et al.,* 2011). Based on liquid chromatography-mass spectrometry (LC-MS) evidence our group identified a putative palytoxin and several much more abundant palytoxin congeners (ovatoxin-a, -b, -c, -d, -e, and -f), as the toxins produced by the alga (Ciminiello *et al.,* 2006, 2008, 2010, 2012) (Fig. 1). In most of the analyzed field and cultured samples of *O.* cf. *ovata*, ovatoxin-a dominated the toxin profiles, accounting for up to 89% of the total toxin content, followed by ovatoxin-b, -d+e, -c and putative palytoxin (listed in order of decreasing concentration) (Guerrini *et al.,* 2010; Honsell *et al.,* 2011). Ovatoxin-f was the major component of the unique toxin profile of an *O.* cf. *ovata* strain from the Central Adriatic Sea (Ciminiello *et al.,* 2012). Most of the health problems associated with *O.* cf. *ovata* blooms in the Mediterranean included a respiratory syndrome following exposure to marine aerosols (Durando *et al.,* 2006) and skin irritation (Tichadou *et al.,* 2010). However, palytoxin can also accumulate in some marine animals (crabs, filefish, triggerfish, mussels and mackerels) thus entering the human food chain and posing potential risks for seafood consumers (Aligizaki *et al.,* 2008; Amzil *et al.,* 2012).

A few fatal human poisonings following consumption of palytoxin contaminated seafood have been reported worldwide (Yasumoto *et al.,* 1986; Onuma *et al.,* 1999), although none in the European countries. Symptoms of the palytoxin-related intoxication include vasoconstriction, hemorrhage, ataxia, muscle weakness, ventricular fibrillation, pulmonary hypertension, ischemia, and death (Aligizaki *et al.,* 2011). Due to serious concern posed to human health by the presence of a palytoxin-producing algal species in the Mediterranean, the European Food Safety Authority (EFSA) in 2009 suggested a maximum limit of 30 μg kg^{-1} of palytoxin equivalents in shellfish, in order to avoid exceeding the acute reference dose for oral administration of palytoxin (0.2 μg kg^{-1} body weight). EFSA also indicated the need to develop efficient extraction procedures to be coupled to rapid and sensitive monitoring methods of palytoxin-like compounds in seafood.
In the present study, the best conditions for extraction and clean-up of palytoxin-like

compounds from mussels and their determination using LC coupled to high resolution mass spectrometry (HRMS) are presented.



	n	R1	R2	R3	R4	M	A moiety	B moiety
Palytoxin	1	OH	H	OH	OH	$C_{129}H_{223}N_3O_{54}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{48}$
Ovatoxin-a	1	H	OH	H	H	$C_{129}H_{223}N_3O_{52}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{46}$
Ovatoxin-b						$C_{131}H_{227}N_3O_{53}$	$C_{18}H_{32}N_2O_7$	$C_{113}H_{195}NO_{46}$
Ovatoxin-c						$C_{131}H_{227}N_3O_{54}$	$C_{18}H_{32}N_2O_7$	$C_{113}H_{195}NO_{47}$
Ovatoxin-d						$C_{129}H_{223}N_3O_{53}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{47}$
Ovatoxin-e						$C_{129}H_{223}N_3O_{53}$	$C_{16}H_{28}N_2O_7$	$C_{113}H_{195}NO_{46}$
Ovatoxin-f						$C_{131}H_{227}N_3O_{52}$	$C_{16}H_{28}N_2O_6$	$C_{115}H_{199}NO_{46}$

Fig. 1. Structure of palytoxin and ovatoxin-a and elemental formulae of all the ovatoxins so far known.

Extraction. Mussels were extracted following the best conditions for extraction of palytoxin from seafood proposed in our previous study (Ciminiello *et al.,* 2011): to a 1g-aliquot of homogenized tissue (epatopancreas and flesh) was added of 3mL of MeOH/H_2O (8:2), sonicated for 3 min in pulse mode, while cooling in ice bath, and successively centrifuged at 6500 rpm for 10 min. The extraction was repeated twice more obtaining a final extract of 10 mL. The extract was filtered through a 0.45 µm filter and directly analyzed by LC-HRMS. Spiking experiments before extraction were performed with palytoxin standard (Wako Chemicals GmbH) at 3 concentration levels (30, 60, and 100 µg kg^{-1}), thus including the limit proposed by EFSA for palytoxins. Recoveries of 90-94% after the 1st extraction step, of 4-7% after the 2nd step, and of 0-0.5% after the 3rd step were obtained. So we concluded that 1g of mussels could be efficiently extracted only once with 3mL MeOH/H_2O (8:2).

Matrix effect. Blank mussel extracts (1g tissue/3 mL) were spiked after extraction with palytoxin standard at 3 concentration levels (30, 60, and 100 µg kg^{-1}) and analyzed by LC-HRMS versus matrix free palytoxin standard. A strong ion suppression effect was observed (65-100%) which hampered detection of palytoxin in the matrix at 30 µg kg^{-1}. Thus, in order to detect palytoxin at the regulatory limit proposed by EFSA, a clean-up based on solid phase extraction (SPE) was considered.

SPE clean-up. Various reversed phase SPE cartridges were tested for clean-up of palytoxin in mussels, namely Sep-Pack C18 and Oasis HLB (Waters), Strata-X and Strata XL (Phenomenex).
A number of experiments were carried out to select the most efficient loading (MeOH/H_2O 2:8 and 5:95), washing (MeOH/H_2O 1:1, 4:6, 3:7, 1:9, H_2O 100%) and eluting (MeOH 100% and MeOH/H_2O 8:2,) conditions. Since palytoxin is strongly retained on C-18 sorbents, volumes of solvent necessary for quantitative palytoxin elution were also evaluated (1-15 mL).
In SPE method development, the matrix effect in SPE eluates was evaluated: a blank mussel extract was cleaned-up and the obtained SPE eluates were spiked with palytoxin. Comparison between LC-HRMS response of palytoxin pure standard and SPE spiked eluates provided information on matrix suppression/enhancement effect. Such SPE spiked eluates were then used as matrix-matched standards to evaluate the percentage recovery of each SPE procedure. Table 1 reports the best SPE conditions in terms of recovery and matrix effect that allowed palytoxin to be detected at EFSA limit of 30 µg kg^{-1}.

Table 1. Conditions of SPE clean-up

Cartridge	Strata X, 500mg/6mL
Load	MeOH/H_2O 5:95 (48 mL)[a]
Wash	H_2O 100% (3 mL)
	MeOH/H_2O 1:9 (3 mL)
Eluate 1	MeOH/H_2O 8:2 (3 mL)
Eluate 2	MeOH/H_2O 8:2 (4 mL)[b]
Eluate 3	MeOH/H_2O 8:2 (8 mL)

[a] In order to efficiently retain palytoxin on cartridge during loading operations, the crude extract (3 mL, MeOH/H_2O 8:2) was added of 45 mL of H_2O.
[b] Palytoxin was found in Eluate 2

LC-HRMS detection. LC-HRMS analyses were performed on a hybrid linear ion trap LTQ Orbitrap XL™ Fourier transform mass

spectrometer (FTMS) equipped with an ESI ION MAX™ source (Thermo-Fisher) and coupled to an Agilent 1100 LC binary system. LC conditions reported in table 2 were used. HR full MS experiments (positive ions) were acquired in the range *m/z* 800-1400 at resolution setting 30.000. The following source settings were used: spray voltage= 4kV, capillary temperature= 290°C, capillary voltage= 45V, sheath gas = 35 and auxiliary gas= 1 (arbitrary units), tube lens voltage= 165V.

Table 2. LC conditions used in LC-HRMS experiments

Column	3μm Gemini C18, 150×2.00 mm, (Phenomenex)
Eluent A	H_2O, 30 mM MeCOOH
Eluent B	95% MeCN/H_2O, 30 mM MeCOOH
Gradient	20–50% B in 20 min, 50–80% B in 10 min, 80–100% B in 1 min, hold 5 min
Flow rate	0.2 mL/min
Injection volume	5 μL

Extracted ion chromatograms (XIC) were obtained from the HR full MS spectra of palytoxin-spiked samples and real samples, by selecting the most abundant ion peaks of the $[M+2H-H_2O]^{2+}$ and $[M+H+Ca]^{3+}$ ion clusters of palytoxin and ovatoxins (Table 3). A mass tolerance of 5 ppm was used.

Table 3. Principal ions (*m/z*) of palytoxin and ovatoxins (mono-isotopic ion peaks)

Toxin	$\mathbf{[M+2H-H_2O]^{2+}}$	$\mathbf{[M+H+Ca]^{3+}}$
palytoxin	1331.2417	906.4851
ovatoxin-a	1315.2480	895.8255
ovatoxin-b	1337.2595	910.4976
ovatoxin-c	1345.2566	915.8286
ovatoxin-d	1323.2439	901.1533
ovatoxin-e	1323.2439	901.1533
ovatoxin-f	1329.2606	905.1616

The chromatographic peaks were identified by comparing their retention times and associated HR full MS to those of ovatoxins contained in a reference *O.* cf. *ovata* extract (OOAN0601) previously characterized (Ciminiello et al 2010) and analyzed under the same experimental conditions (Fig. 2). Calculation of elemental formulae in full MS spectra was performed by using the mono-isotopic ion peak of each ion cluster.

In quantitative analyses (triplicate injection), peak areas were measured and interpolated within the calibration curve of palytoxin standard at five levels of concentrations (50, 25, 12.5, 6.25, and 3.13 ng/mL). Linearity of the calibration curve was indicated by a correlation coefficient (R^2) of 0.9980. Measured limit of quantitation (LOQ) and limit of detection (LOD) for palytoxin standard under the LC-MS conditions used, were 3.13 and 1.9 ng/mL, respectively. LOD of palytoxin in mussel tissue was 30 μg kg^{-1}.

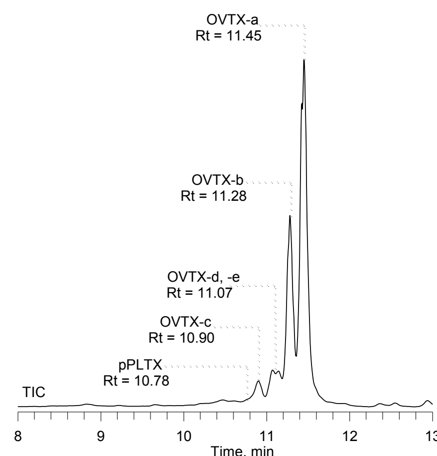


Fig. 2. Total ion chromatogram (TIC) of the *O.* cf. *ovata* culture extract containing putative palytoxin (pPLTX), ovatoxin (OVTX)-a, -b, -c, -d, and-e.

Application to real samples

The described method was applied to detection of palytoxin and ovatoxins in mussels collected in the period 2010-2012 within the Monitoring program of *Ostreopsis ovata* along the Campania coasts (Italy). It allowed to detect ovatoxin-a, -b, and -d, and -e in mussels up to 385 μg kg^{-1} (total toxin content). In most cases, the SPE clean-up step was not required.

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The patterns of hsp27 phosphorylation in cells exposed to mixtures of okadaic acid and palytoxin reveal synergistic effects

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Abstract
Living systems are currently exposed to mixtures of stressors, and mechanistic-based risk assessment demands characterization of toxicity pathways and molecular processes induced by mixtures of toxins possessing different mechanisms of action. We have studied the molecular effects of toxin mixtures by examining the phosphorylation of the stress response protein hsp27 in cultured human cells, following their exposure to okadaic acid (OA) and palytoxin (PlTX), either alone or in combination. We found that both OA and PlTX increase cellular levels of phoshorylated hsp27, but different patterns of phosphorylated aminoacids were found in hsp27 from cells exposed to either OA or PlTX. The analysis of enzymes involved in kinase cascades, including those phosphorylating hsp27, indicated that PlTX induces the activation of kinases, such as the p38 protein kinase, whereas OA stabilizes the cellular pool of the phosphorylated forms of the proteins, by inhibiting the dephosphorylation reaction. Our findings indicate that the molecular mechanisms responsible for phosphorylation of hsp27 in cells exposed to these toxins are not identical but the different pathways triggered by the two toxins cause a combined response. The molecular features we detected show that some combined effects of OA and PlTX are synergistic.

Keywords: toxicity pathways, synergism, stress response, p38 protein kinase, phosphoprotein phosphatases

Introduction
The extension and recurrence of blooms of *Ostreopsis* algae recorded in Mediterranean coastal waters in the last years (reviewed in Mangialajo *et al.*, 2011), their benthic co-distribution with *P. lima*, (Vila *et al.*, 2001; Simoni *et al.*, 2004), the recognition that those microalgae produce potent toxins belonging to palytoxin and okadaic acid groups, respectively (Rossini and Hess, 2010), justify the hypothesis that humans and other animals might be exposed to mixtures of these toxins. Palytoxin (PlTX) and okadaic acid (OA) represent the reference compounds of two toxin groups differing with regard to their chemistry and molecular mechanisms of action (Rossini and Hess, 2010). These considerations led us to investigate onto the molecular responses that mixtures of these toxins can induce in a biological system. Toxic responses ensue when functions are altered to an extent which escapes homeostasis. In mechanistic terms, toxic responses are perturbations of the molecular pathways carrying out normal functions, and toxicity pathways can be viewed as the trains of reactions found in living systems when severe alterations have led to adverse effects (NRC 2007). The actual functioning of biological systems, however, does not result from separate, independent processes, but consists of complex networks of interacting pathways.
In this study we have focused our attention onto responses triggered by mixtures of OA and PlTX, to gain insights onto major events participating to the cross-talks between molecular processes induced by these two toxins, involving different mechanisms of action (Rossini *et al.*, 2011). OA binds and inhibits ser/thr phosphoprotein phosphatases (Bialojan and Takai 1988), leading to stabilization of phosphorylated proteins in the cells (Haystead *et al.*, 1989). Protein phosphorylation is a key mechanism in the control of biological processes, and cell functioning is disrupted in cells exposed to OA, often leading to cell death (reviewed in Rossini 2000). The plasma membrane Na^+,K^+-ATPase is the recognized molecular target of PlTX, converting the pump into a non-specific cation channel (Habermann and Chhatwal, 1982). Membrane potential and ion

homeostasis collapse in cells exposed to PlTX, causing a plethora of perturbations (Rossini and Bigiani 2011), often culminating with cell death (Bellocci *et al*., 2011). In our past studies we have found that both OA and PlTX cause MCF-7 cell death, and the cytotoxic responses induced by these toxins are accompanied by changes in the cellular pool of the hsp 27 stress response protein (Sala *et al*., 2009 a and b). We have then analyzed the effects mixtures of OA and PlTX exert on the phosphorylation patterns of hsp27 in MCF-7 cells, to probe possible cross-talks between the toxicity pathways of these toxins. In particular we have ascertained whether OA and PlTX might induce different patterns of phosphorylation of functionally relevant serine residues of hsp27, such as Ser15, Ser78, Ser82 (Rogalla *et al*., 1999), whether a combination of effective doses of OA and PlTX might modify the responses which are induced by toxins individually, and evaluated possible pathways involved in the cellular responses induced by the two toxins and their interaction.

Materials and methods

The general structure of our experimental setting included exposure of MCF-7 cells to PlTX and OA at the concentrations and for the duration of treatments as specified under Results, followed by the preparation of cell extracts and analysis of molecular parameters of interest. The details of the methodology used in our study for the preparation and analysis of cytosoluble extracts can be found in Sala *et al*. (2009b). The only change inserted in experimental procedures regarded the handling of cells for the preparation of cell extracts. To avoid losses of cellular materials due to cell lysis in PlTX-treated samples (Prandi *et al*. 2011), cell cultures were washed with culture medium devoid of FCS, before being used for cell harvesting by scraping, and cell lysis, as detailed in Sala *et al*. (2009b).

Results and discussion

Initial experiments were carried out to identify proper experimental conditions of MCF-7 cell treatment with a combination of OA and PlTX. We treated MCF-7 cells with combinations of OA and PlTX at final concentrations in the 50-100 nM (OA) and 0.01-0.03 nM (PlTX) ranges. The DNA content of culture dishes was measured (Labarca and Paigen 1980) after 8 h incubations, and used to evaluate the cytotoxic effect of toxin treatments. The results we obtained showed that MCF-7 cell treatment for 8 h with a combination of OA up to 100 nM and of PlTX up to 0.03 nM determined a significant cytotoxic effect, without extensive loss of cells in culture dishes. These experimental conditions were then used in the following experiments.The phosphorylation of hsp27 protein was then analyzed after MCF-7 cells had been exposed to toxins added individually (either 100 nM OA, or 0.03 nM PlTX) or in combination. Proteins in cell extracts were fractionated by one-dimensional SDS-PAGE and were analyzed by immunoblotting (Sala *et al*. 2009b), using antibodies recognizing the total hsp27 protein pool (with affinity for both phosphorylated and non-phosphorylated proteins), as well as residue-specific phosphorylations on Ser15, Ser78, and Ser82 (Fig. 1).

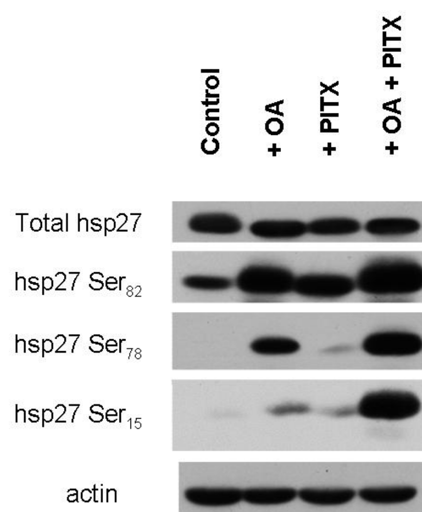

Fig. 1. Effect of okadaic acid, palytoxin and a mixture of the two toxins on the phosphorylation state and pattern of hsp 27 in MCF-7 cells. Cells were treated with 100 nM OA and 0.03 nM PlTX, either alone or in combination, or left untreated, for 8 h at 37°C, before being used for the preparation and analysis of cell extracts.

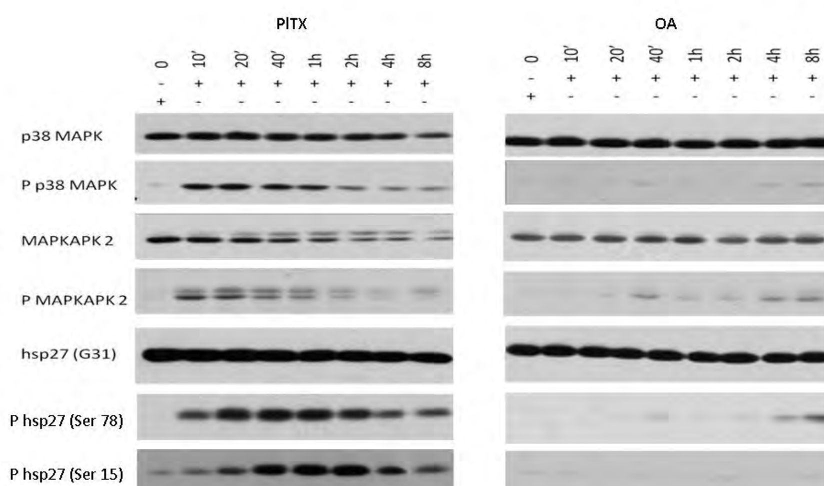

Fig. 2. Time-course of the effects of okadaic acid and palytoxin on the phosphorylation state of p38K, MAPKAPK 2 and hsp27 in MCF-7 cells. Cells were treated with either OA (top) or PlTX (bottom) at 37°C for the times indicated, before being used for the preparation and analysis of cell extracts. Immunoblotting was carried out using antibodies recognizing either the total pool of indicated proteins (p38 PK, MAPKAPK 2, hsp27) or their phosphorylated forms (p38 PK_P, MAPKAPK 2_P, P hsp27).

The results we obtained showed that the treatments did not cause relevant quantitative changes in the cellular levels of total hsp27, and confirmed our previous observations that both OA and PlTX induce increased phosphorylation of Ser_{82} in hsp27. The two toxins, however, differed with regard to their effect on the phosphorylation state of Ser15 and Ser78. OA caused a prominent increase in the phosphorylation of Ser78 and increased the phosphorylation of isoforms in Ser15. PlTX caused a marginal, if any, effect on the phosphorylation of both residues. The combined treatment of MCF-7 cells with OA and PlTX, caused increases in the levels of phosphorylated hsp27 in an isoform-related fashion, and the phosphorylation pattern induced by OA dominated the cellular response to the toxin mixture. Interestingly, the increase in the signal of phosphorylated Ser78 and Ser15 in cells exposed to the toxin mixture was more than the simple addition of those detected in cells treated with the two toxins individually. Overall, these results show that the phosphorylation patterns of hsp27 are toxin-related when cells are exposed to OA and PlTX, either individually or in combination, and are susceptible to combined effects of the two toxin classes. Those results indicated that the hsp27 protein represents a nodeof toxicity pathways leading to cell death induced by OA and PlTX. Furthermore, the patterns of hsp27 phosphorylation were toxin-related, suggesting that the molecular mechanisms responsible for the responses in cells exposed to these toxins, individually or in combination, might not be identical. It is known that the p38 protein kinase (p38K) pathway is responsible for the phosphorylation of hsp27 (reviewed in Kostenko and Moens 2009), and is a target of PlTX (reviewed in Wattenberg, 2011). More precisely, the phosphorylation of p38K by the protein kinases MKK3/MKK6 determines activation of p38K and the consequent phosphorylation of hsp 27, by either p38K itself or one of its downstream targets, the protein kinase MAPKAPK 2, following activation by p38K (Kostenko and Moens 2009). Furthermore, the phosphorylation state of many protein kinases is stabilized by OA, including the case of p38K (Rossini 2000). We then examined the effects of OA and PlTX on the phosphorylation state of p38K and MAPKAPK 2.

The results we obtained by immunoblotting analysis are reported in figure 2, and show that the time-courses of effects exerted by the two toxins markedly differ. The OA effect is slow, and modest increases in phosphorylated p38K and MAPKAPK 2 were detected over prolonged cell exposure to the toxin, in the absence of relative

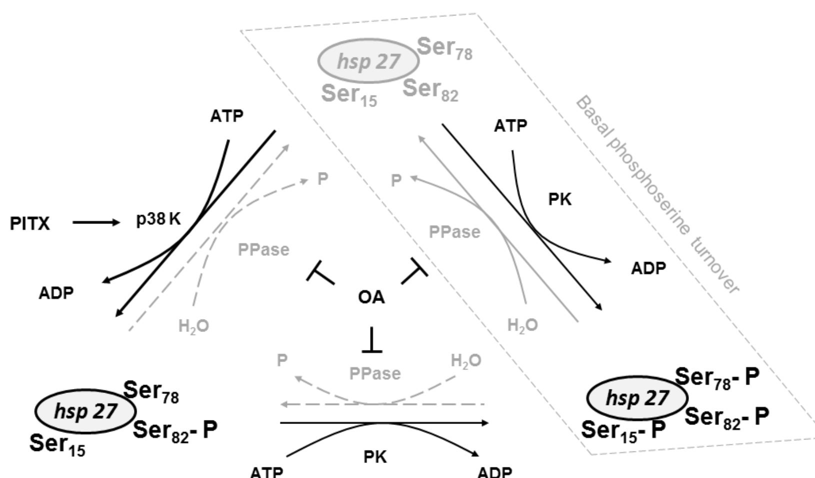

Fig. 3. Schematic representation of steps affected by palytoxin and okadaic acid in their combined effects on the phosphorylation state of hsp27 in MCF-7 cells. The reactions and protein isoforms indicated by light gray represent those which are not favored in toxin-treated cells. See text for explanations.

changes in the levels of total p38K and MAPKAPK 2. In turn, PlTX induced a very rapid phosphorylation of both p38K and MAPKAPK 2, showing maxima within the first h of cell incubation, followed by a slow decrease in the signal over the remaining portion of cell treatment. Interestingly, a progressive decrease in the levels of total p38K and MAPKAPK 2 was observed over prolonged cell exposure to PlTX under our experimental conditions. These results suggested that the increased phosphorylation of hsp27 found in cells exposed to OA resulted from a blockade of dephosphorylation reaction due to inhibition of PPases, rather than the stimulation of the protein kinase pathway of hsp27 phosphorylation. This hypothesis was probed by analyzing the phosphorylation state of ERK 1 and 2 isoforms, whose phosphorylation is unrelated to the p38K pathway but is enhanced by inhibition of PPases by OA (Rossini 2000), and the results we obtained confirmed that phosphorylation of ERK1 and 2 is stimulated by OA but not by PlTX treatment, and the response induced by OA is not affected by a combined treatment with PlTX (not shown). Overall, the data we obtained in this study support a model of increased phosphorylation of hsp27 in MCF-7 cells involving a decrease in the rate of dephosphorylation reactions induced by OA, and the activation of the p38K pathway by PlTX.

Thus, the inhibition of PPases by OA would favor accumulation of phosphorylated hsp27 through a slowing down of basal turnover of phosphorylated serine residues. The rapid activation of kinases responsible for the phosphorylation of hsp27 by PlTX, in turn, would cause a net increase in the rate of serine phosphorylation. The combined action of OA and PlTX, therefore, would be an overall increase of phosphorylated isoforms of hsp27, by the combined stimulation of the phosphorylation reactions and the decreased rate of phosphate removal from relevant residues in toxin-treated cells (Fig. 3). The major implication of our mechanistic study for risk assessment stems from the finding that more than additive (synergistic) responses can be found in cells exposed to a mixture of OA and PlTX. The exposure of animals and humans to low doses of these two classes of compounds in combination, therefore, might then cause adverse effects which are not expected when living systems are exposed to acceptable levels of only one toxin class.

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Risk and Mitigation of Food Safety Risks Associated with Abalone and Crustaceans

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Abstract
This presentation highlights the food safety risks associated with abalone and lobsters and suggests measures to reduce these risks. Abalone and lobsters are at risk of contamination by marine biotoxins. The PSP toxins in particular tend to concentrate in the epithelial layer and muscular part of the foot and to a lesser extent in the viscera. In lobsters, biotoxins tend to concentrate in the viscera, particularly the digestive gland. Depending on the degree of compartmentalization of PSP toxins in abalone, PSP can be mitigated by evisceration and by scrubbing the epithelium to ensure that it is properly removed. In a similar manner, PSP toxins in lobsters can be reduced by evisceration. Abalone and lobsters are at risk of heavy metal contamination, particularly in the viscera and to a lesser extent in the muscle. Once eviscerated, the heavy metal concentration tends to be substantially reduced. Abalone and lobsters are at risk of microbiological contamination, which can be reduced through depuration prior to marketing. Abalone and lobsters pose a potential risk in terms of food safety. These risks can, however, be mitigated through appropriate pre- and post-harvest actions.

Keywords: Risk; Mitigation; Abalone; Crustaceans; Biotoxin; Heavy metals; Microbiological

Introduction

Abalone and crustaceans are at risk of contamination from natural and anthropogenic hazardous substances, as well as infection by pathogenic microbiological organisms. This paper will discuss the associated risks and the mitigation of the risks to provide better food safety assurance of abalone and crustacean products for consumers.

The main risk to food safety relating to the consumption of abalone and crustaceans discussed in this paper, are biotoxins, microbiological contamination and heavy metals.

Biotoxin contamination

Biotoxins have been found to accumulate in abalone and crustaceans.

Paralytic Shellfish Poisoning toxin

Paralytic Shellfish Poisoning (PSP) toxins have been found in various gastropods (Chen & Chou, 1998) including, abalone (Deeds, Landsberg, Etheridge, Pitcher & Longan, 2008; Pitcher, Franco, Doucette, Powell & Mouton, 2001) as well as various crabs and lobsters (Deeds *et al*., 2008).

A study on the abalone, *Haliotis midae* with PSP toxin concentration exceeding 1 600 µg STX equiv./100g found that the toxin concentration was substantially reduced after removing the epipodial fringe (Pitcher *et al*., 2001). The viscera were also found to contain significant amounts of toxin and lesser amounts were found in the foot (Pitcher *et al*., 2001). Similarly a study on *Haliotis laevigata* that was fed on pellets containing PSP toxins found that the PSP toxin concentration in the abalone was reduced by 70% once the epipodial fringe was removed (Dowsett *et al*., 2011).

High concentrations of PSP toxins have been found in the gut and hepatopancreas of numerous crustaceans including spiny lobster (*Panulirus stimpsoni*), xanthid crab (*Zosimus aeneus), Telmessus acutidens,* Dungeness crab *(Cancer magister)*, reaching concentrations ranging from 1 500 to 2 700 µg STX eq/100 g viscera (Deeds *et al*., 2008). PSP toxins have also been found in the

muscle tissue, though generally at a substantially lower concentration than the viscera (Deeds *et al*., 2008).

Diarrhetic Shellfish Poisoning toxin

The lipophilic Diarrhetic Shellfish Poisoning (DSP) toxins okadaic acid (OA) and yessotoxin (YTX) have been found in the digestive glands of *Haliotis discus hannai* in Korea at concentrations of 4.7 ng/g and 1.3 ng/g respectively. No toxins were found in the foot of the abalone (Kim *et al*., 2012). OA has also been found in the abalone *Haliotis tuberculata* from Galicia (Gago-Martínez, Comesaña-Losada, Leao-Martins & Rodríguez-Vázquez, 1996).

Okadaic acid has also been found in the digestive glands and the muscle tissue of the brown crab (*Cancer pagurus*) in Norway, after cooking (Torgersen, Aasen & Aune, 2005). It is, however, not clear whether the toxin was absorbed by the muscle tissue during cooking or if the toxin was present in the muscle when harvested. It is assumed that the toxin accumulated in the crabs from the consumption of toxic mussels (Torgersen *et al*., 2005).

Amnesic Shellfish Poisoning toxin

There do not appear to be reports of Amnesic Shellfish Poisoning (ASP) toxins in abalone. ASP toxins have, however, been found in crustaceans such as the sand crab (*Emerita analoga*), a suspension feeder (Powell, Ferdin, Busman, Kvitek & Doucette, 2002). ASP toxins have furthermore been found in the dungeness crab (*Metacarcinus magister*), the rock crab (*Cancer pagurus*), stone crab (*Menippi adina*) and the spiny lobster (*Palinurus elephas*) (Altwein *et al*., 1995), which are known to scavenge and catch live prey. The toxins, though concentrated primarily in the viscera, have also been found in the muscle in low concentrations (Altwein *et al*., 1995).

Mitigation of biotoxin poisoning

Appropriate pre- and post-harvest monitoring programmes could be implemented which include phytoplankton monitoring and testing of the flesh of shellfish species to be consumed. Where scientifically proven to be effective, the shellfish could be eviscerated and in the case of abalone the epipodial fringe removed.

Microbiological contamination

Live and fresh abalone and crustacean products pose a human health risk as a result of the consumption of shellfish contaminated with bacteria and viruses, particularly of sewerage origin (Butt, Aldridge & Sanders, 2004). Virus and bacteria related illnesses are associated more with raw products than cooked products (Butt *et al*., 2004).

Vibrionaceae, which includes *Vibrio parahaemolyticus*, *V. cholerae* and *V. vulnificus*, have been implicated in illnesses arising from the consumption of shrimps, crabs and lobster (Butt *et al*., 2004; Yano, Kaneniwa, Satomi, Oikawa & Chen, 2006). *V. parahaemolyticus* has also been isolated from abalone, though there was no human infection resulting from the abalone in these studies (Lee, Liu & Huang, 2003).

Aeromonas and *Plesiomonas* species have been associated with illnesses contracted from the consumption of shrimps (Butt *et al*., 2004). A study in Finland found that 16 % of the shrimp samples were contaminated with *Aeromonas* (Butt *et al*., 2004).

The abalone and crustaceans, however, do not appear to pose as high a risk as bivalves (Butt *et al*., 2004). A comparative study has shown that abalone accumulate bacteria and viruses substantially slower than bivalves and generally attain lower concentrations of bacteria and viruses (SUDEVAB, 2010).

Mitigation of food borne disease

Abalone and crustaceans should preferably be harvested from areas that have been approved for the harvesting of the shellfish. The end-of-line product should be tested at minimum on an *ad hoc* basis to at least verify the status of the harvesting area and ensure that the products are safe for human consumption.

Heavy metal contamination

Abalone (Bae, Yoon & Lim, 2011; Fabris, Turoczy & Stagnitti, 2006) and lobsters (Castro-González & Méndez-Armenta, 2008; Morais, Garcia & Pereira, 2012) have the propensity to

accumulate heavy metals as the metals are not easily oxidized or precipitated out of an ecosystem or organism (Raissy, Ansari & Rahimi, 2011). The accumulation and biomagnification of the metals increases up the trophic chain (Morales-Hernández, Soto-Jiménez & Páez-Osuna, 2004).

Studies in Australia, Korea and Japan showed the heavy metal concentrations of As, Pb, Hg and Cd in the edible portion of abalone to be safe for human consumption (Bae *et al*., 2011; Fabris *et al*., 2006). The As and Cd concentrations increased with size of the abalone whereas Hg concentration decreased with size (Fabris *et al*., 2006). The heavy metal concentrations tended to be higher in the viscera than in the foot muscle (Probyn & Samsukal, 2006; Walker, 1982).

A study on the accumulation of Pb, Hg and Cd in the edible portion of lobster in Australia showed that the concentration of the heavy metals were well below the regulatory limits. The As and Hg concentration was found to increase with size in lobster (Fabris *et al*., 2006). The lobster *Homarus americanus* was found to have metal binding proteins for Cd in its gut and was capable of accumulating high concentrations of the metal in the gut (Chou, Guy & Uthe, 1991), which may account for the higher levels of Cd in the viscera.

A study on Norway lobsters *Nephrops norvegicus* found that the highest concentration of Hg and MeHg was in the gills. The greatest burden of MeHg was in the tail muscle (41%) and gills (32%), whereas Hg occurred in the tissue (63%) and hepatopancreas (17%). Highest concentrations of Cd were in the hepatopancreas and gills, while the highest tissue burdens of Cd were in the hepatopancreas (91%). The highest concentrations of Pb were found in the gills and carapace, while most of the burden of Pb was in the carapace (42%) and hepatopancreas (25%) (Canli & Furness, 1993).

Mitigation of heavy metal contamination

The risk of heavy metal poisoning through the consumption of abalone and lobster could be significantly reduced by eviscerating the shellfish. As there may be leaching of heavy metals from the viscera into the muscle during cooking, the shellfish could be eviscerated prior to cooking, though this transfer of heavy metals should first be further investigated.

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Speaker Abstracts

(Grouped by session theme)

Conference Opening

Keynote
Long-Term Observations of Phytoplankton Will Underpin Our Marine Management

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Phytoplankton regulate the productivity and carrying capacity of ecosystems, provide the oxygen in every second breath we take, recycle nutrients and pollution, can contaminate shellfish and compromise human health, and regulate our climate through stimulating cloud formation and through carbon sequestration.This importance, together with their short life cycles, sensitivity to the environment, and their lack of exploitation, make them ideal ecosystem indicators of health and productivity. Here we highlight the importance of long-term observations through summarising the major changes in phytoplankton that have been observed globally and in Australia, describe monitoring programmes in Australia since the advent of the Integrated Marine Observing System (IMOS), detail how phytoplankton will increasingly be used as ecosystem indicators integrated into marine management, and challenge research and industry to work together to provide important baseline data for managing global change.An important opportunity exists for HAB monitoring by the shellfish industry to become an integral part of the Australian Phytoplankton Database, which already includes 22,500 records from the IMOS Australian Continuous Plankton Recorder and National Reference Stations data, and all available historical data from CSIRO and the published literature. The analysis of this database will improve our understanding of phytoplankton dynamics, including HABs, and improve our marine management.

Keynote
Engaging Consumers and Retailers: Evidence-Based Marketing of Food and Environment Safety

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Sustainability, toxins, microbial food safety – all issues that can alter the sales profile of any seafood provider. The best producers, processors and retailers will actively market their food safety and other actions to buyers and consumers, have the processes and data to validate such claims, and actively engage with various audiences to build trust.

Adding Value to the Industry

Quality Programmes as a Commercial Opportunity

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This presentation summarises food safety consumer trends and experience in the industry to discuss whether a major opportunity for creating value from shellfish safety programmes is available. Shellfish safety programmes have four principal stakeholders. The first three – industry, science and regulatory authorities are at the forefront of decision making. The fourth though, the consumers of shellfish products are relatively invisible even though they are the Raison d'être, the reason for existence, the ultimate purpose of molluscan shellfish safety. The presentation will examine how industry and science both have a duty to be responsive to consumer concerns and trends and will illustrate how awareness of growing consumer concerns over animal welfare and ethics was, alongside the need for more quantitative science, the major driver of the development of the chemical based biotoxin test methodologies in New Zealand. That awareness can then be converted into market opportunity, leveraging commercial returns from the existing investment in quality assurance programmes. Consumers' perceptions of value are multi-layered. Food product safety is clearly, and increasingly one of those layers. Alongside olfactory qualities, there is an increasing demand for ethical and safe foods. That is, shellfish safety therefore represents a potentially significant value creating opportunity. The now widely adopted chemical based test methodologies and programmes provide an ethical product assurance that should have consumer appeal. However that opportunity is largely neglected by industry. Quality assurance is viewed as a "cost". However it also represents an opportunity to create and harvest value that at the present time is largely lost. The presentation will consider the opportunities and the pros and cons of utilising shellfish safety as a value adding tool

Adding Value throughout the Supply Chain

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Industry operators can ‘add value’ to their molluscan shellfish products at all parts of the supply chain:

1. Production;
2. Transportation;
3. Marketplace.

At the production location, in addition to obvious actions such as cleaning the product and selecting for positive traits such as growth rate and flesh content, there are clear benefits from proactive collaboration between industry, scientists and regulators. Regular, formal, structured meetings allow for the more effective management of biotoxins, monitoring of viruses and assessment of heavy metals and other contaminants. Such structured systems may become more necessary as the cultivation industry moves further offshore and monitoring for toxic blooms becomes the responsibility of remote sensing. Areas for improvement and adding value in transportation include focussing on temperature control (quality of the product, shelf life and microbiological status), thermal properties of cartons, percussion issues and assessment of the mobile ‘environment’ (ambient temperature of truck, flight hold, etc).

In the marketplace (retail - shop or market stand -wholesale, food service) added value can range from presentation issues (use of ice, water spray, drainage, general appearance, etc) to product preparation ranging from oysters on the half shell through ‘vacpac’ mussels in sauces (white wine, Provencal, etc) to supply of sachets of oyster cooking sauces (Kilpatrick, Asian, etc). Comparison of a retail fish counter – mostly raw and unprepared product, either fresh or frozen – with a meat counter, with a multitude of prepared offerings (sausages, rissoles, burgers, kebabs, cuts of spiced meats BBQ-ready, pies, pasties, etc, etc, illustrates the stark difference offered to the time-constrained and cookery nervous consumer!

Value Adding Options for Australian Oysters

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Introduction

Freshly harvested oysters are savoured by consumers for their characteristic aromas of the sea. However, half-shell oysters, like most fresh seafood, possess a short shelf-life. Thus, any extension in shelf-life may allow access into new markets. Further, convenience and simplicity are important aspects for consumers and packaged ready-to-eat half shell oysters may offer an increased appeal to consumers in a retail environment. Processing techniques such as high pressure processing (HPP), modified atmosphere packaging (MAP) and freezing may be useful for extending the shelf-life of half shell product and may facilitate the entry of products into new markets and retail environments.

Aims

Review the technologies that are available to extend shelf-life, Assess the effect of packaging and various gas mixtures on shelf-life , Assess a standard commercial freezing process

Results

HPP is a technique that has been applied world-wide to oysters. An important benefit of this technique is the automatic shucking effect that occurs due to protein denaturation causing the adductor muscle to separate from the shell. Other potential benefits include increased product safety, extended shelf-life and enlarged shell meats. Shelf-life trials evaluating the use of MAP have indicated successful inhibition of microbial growth resulting in increased shelf-life. This indicates that spoilage organisms present in Pacific Oysters are highly susceptible to the bacteriostatic action of CO_2. However, informal tasting trials indicated that unusual flavours were present in oysters that had been processed with high concentrations of CO_2. Cooking may remove these flavors and if so, this technique could be suitable for readyto-cook products. Experiments involving a standard freezing process have also demonstrated that oysters can be frozen for extended periods thus allowing them to be available during times of peak demand.

Conclusion

Several processing techniques for oysters have been studied and the benefits and drawbacks of each have been identified.

The NSW Shellfish Safety Program – An Industry Perspective on the Current Issues and Ongoing Challenges

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The NSW oyster industry has a farm gate value of around $35m/yr and operates out of 70 harvest areas up and down the coast. The NSW shellfish program is a shellfish safety program that went through a major overhaul around 10 years ago, since then it has matured to become a stable platform under which both government regulators and industry can operate. The program is run by the NSW Food Authority, has 3-4 dedicated shellfish safety staff and costs around $1.6m per year to run. It is reviewed by a joint industry / Food Authority committee that meets around three times per year to ensure the program remains on track and is fit for purpose. This presentation will outline the key features of this program, current optimisation issues and future challenges that still need to be addressed. The key targets for the NSW shellfish industry are to ensure that all product in the marketplace is safe and that when practicable there is good availability of product in the marketplace. To achieve this the Shellfish Safety Program has some underpinning frameworks that are well accepted as a given by all parties. These namely being; sanitary surveys, harvest area classifications and the underpinning strong management framework that come with these processes. Areas for system optimisation / improvement lie around having strong, well understood closure and opening triggers such as salinity / rainfall data and lab results. The challenge is then working within these parameters to maximise the use of the good weather windows to get product to market as and when required. Issues around this that have been or are currently being addressed by the shellfish safety program are; ensuring farmers know when an area is open or closed and investigating whether pre-emptive harvesting is feasible when lab samples have been taken to trigger a harvest area opening. Another key optimisation area is around shellfish testing, which accounts for around ½ of the annual shellfish safety budget. The issues here are essentially; how much is enough, what should be tested for, what are the best methods / processes to give good turnaround time on results. A final area where much emphasis has been placed recently is improving incident management procedures when a system failure occurs. Future challenges mainly lie around issues to do with norovirus testing of shellfish and biotoxin testing of both water and shellfish.

Potential Microbiological Hazards

Keynote

Global Bivalve Production, Marketing and Safety Issues

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Global bivalve molluscs production (capture + aquaculture) has increased substantially in the last fifty years, going from nearly 1 million tonnes in 1950 to about 14.6 million tonnes in 2010. While production by capture has marginally declined from about 1.9 million tonnes to about 1.7 million tonnes in 2010, production by aquaculture increased from 8.3 million tonnes in 2000 to 12.9 million tonnes in 2010. The increase of bivalve mollusc production was driven by international demand since the early 1990s. Total bivalve trade has expanded continuously during the past three decades to reach US$ 2.1 billion in 2009. In terms of quantity, scallops accounted for 24% of export, while mussels contributed to 48%. Scallops are the most important species contributing to 46% of value, followed closely by mussels (26%). Most of the problems in international markets are related to hygiene, biotoxins, viruses, and other causes. FAO/WHO have been providing scientific advice to Codex Alimentarius Commission and during last biennium, two Expert Consultations were held in the area of bivalve safety. One of them was related to the question from the Codex Committee on Food Hygiene on public health risk associated with Salmonella in live bivalve molluscs and the value of having criterion for Salmonella in this commodity. Another Expert Consultation addressed issues related to performance characteristics of methodology used for detection and enumeration of Vibrio parahaemolyticus and collection of data required for development of risk management tools that have wide geographical applications. The outputs of these Expert Consultations will be discussed.

Salmonella spp. and Fecal Pollution Indicators Bacteria in Zones of Bivalve Mollusks along the Mediterranean Coast of Egypt

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This work has been done as a part of the Mediterranean coast monitoring program running in Egypt where bivalve mollusks are usually eaten raw. Bivalve mollusks samples and their water beds areas, including surface and bottom sea water samples, were microbiologically examined during four seasonal sampling cruises from March, 2010 to February, 2011 at eleven stations located along the whole Mediterranean coast of Egypt. All samples were analyzed for total bacterial plate count, levels of total coliforms, fecal coliform, and fecal streptococci as well as the prevalence of Salmonella spp. In addition, some environmental parameters of the coastal water samples including temperature, salinity, pH and dissolved oxygen were also measured Sampling techniques were done according to the standers methods and the membrane filtration technique was applied in all the microbiological analysis. The total plate count agar and the respective selective / enrichment media were used for the microbiological analysis. The isolated colonies were identified using the biochemical tests as stated in standard methods and the final counts were calculated as CFU/100ml water or CFU/100 gm mollusks meat. The incidence of Salmonella spp in the examined water samples varied between 2 - 5% depends on the investigated area and sampling season. Counts of the total viable counts as well as levels of total coliforms, fecal coliform, and fecal streptococci varied widely from <1 to 105cfu . There was an association between the fecal contamination indicators and the presence of the pathogen. The same trend was observed in the samples of mollusks meat. These results will help to understand the hygienic status of the coastal water along the Mediterranean coast of Egypt and may help to develop sanitary strategies for better mollusks shellfish safety.

Keywords: Bivalve mollusks; coastal water beds; Salmonella spp; fecal pollution.

Do Barnacles Act as a Potential Reservoir for Pathogenic Bacteria on Commercial Shellfish Beds?

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The monitoring of bacterial levels in commercial bivalve shellfish destined for human consumption is a common global practice designed to preserve both public health and the future economic prosperity of the shellfish industry. Recent research has focused primarily on identifying the sources of bacterial contamination affecting shellfish beds globally, aided by the development of new molecular tools. This study adopts a different perspective, by attempting to identify potential bacterial reservoirs among shellfish symbionts.

Previous studies have shown that on intertidal mussel (*Mytilus edulis*) beds sediments can act as a significant reservoir for bacteria with up to 1.5 billion *Escherichia coli* per kilogram of sediment. Anthropogenic activities as well as natural events such as storms, re-suspend the bacteria allowing the subsequent uptake by bivalve shellfish. This study examined the importance of encrusting barnacle species on three intertidal *M. edulis* beds in North Wales, UK. Results demonstrated that across all sites, encrusting barnacles had significantly higher coliform levels respective to their associated symbionts. Coliform levels ranged from 2 to 5 times higher in the barnacles suggesting that barnacles represent a significant bacterial reservoir on commercial mussel beds. This has important implications for the shellfish industry, particularly where commercial shellfish are marketed as “natural” and sold complete with their associated organisms.
This study illustrates the need for an extensive in situ analysis of global shellfish beds to identify and quantify bacterial fluxes on a localized scale to improve commercial shellfish quality and preserve public health.

Key Listeria monocytogenes Sequence Subtypes Isolated from Greenshell™ Mussels and from Human Cases both Contain Premature Stop Codons and a Low Invasriveness Profile

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Listeriosis is caused by the food-borne pathogen *Listeria* monocytogenes, which can be found in shellfish and processing plants. In New Zealand (NZ), the reported incidence of listeriosis was 0.5/100,000 people with 23 cases and seven deaths in 2010. To evaluate the risk to human health from *L. monocytogenes* associated with mussel production in NZ, multi-virulence-locus sequence typing (MVLST) was used to subtype 36 isolates collected from NZ seafood processing plants and six from human listeriosis cases. As a virulence measurement, selected isolates (22) from the four most common subtypes (ST1, ST2, ST3 and ST5) were also evaluated for their ability to invade the human intestinal epithelial Caco-2 cells. Results were compared with those from other studies. STs were clustered in different lineages: all NZ human isolates belonged to lineage II, while lineage I was composed mainly of seafood-related isolates. None of the nine NZ subtypes identified in this study matched previously described subtypes from other countries. Two subtypes (ST1 and ST3) found in mussels and their processing environment matched those of sporadic listeriosis cases, confirming a potential health risk associated with seafood. ST3 isolates (19 from mussel processing environments, two from human and one from mussel) contained an inlA Premature Stop Codon (PMSC) mutation type 3, reportedly causing low virulence. Invasion assays confirmed a low invasion phenotype for STs carrying inlA PMSCs. No relationship was found between the source of isolates and their invasion performance, with results varying from <10% to 70% invasiveness in comparison to a ScottA control. The close relatedness of some clinical and environmental strains, as revealed by identical MVLST profiles, suggests that local and persistent environmental strains are epidemiologically important. In contrast to other studies, our research revealed a third of clinical *L. monocytogenes* isolates carry inlA PMSCs.

Potential Risks from Shellfish other than Bivalves

Keynote

Risk and Mitigation of Food Safety Risks Associated withAbalone and Lobster

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The presentation highlights the food safety risks associated with abalone and lobsters and suggests measures to reduce the risks. Abalone and lobsters are at risk of contamination by marine biotoxins. The mode of contamination may, however, be different. The PSP toxins in particular tend to concentrate in the epithelial layer and muscular part of the foot and to a lesser extent in the viscera. In lobsters biotoxins tend to concentrate in the viscera particularly the digestive gland. It has been suggested that lobster and abalone accumulate toxins through the consumption of toxic organisms. Abalone toxicity has also been shown to be associated with PSP toxin producing bacteria. A case study has shown that once a causal toxic bloom dissipates the wild stocks are able to depurate the PSP toxins whereas the farmed abalone do not depurate as easily in certain areas suggesting a bacterial association. Depending on the degree of compartmentalization of PSP toxins in abalone, PSP can be mitigated by evisceration and by scrubbing the epithelium to ensure that it is properly removed. In a similar manner, PSP toxins in lobsters can be reduced by evisceration. Abalone and lobsters are at risk of heavy metal contamination, particularly in the viscera and to a lesser extent in the muscle. Once eviscerated the heavy metal concentration tends to be substantially reduced. Abalone and lobsters are at risk of microbiological contamination, which can be reduced through depuration prior to marketing. Abalone and lobsters pose a potential risk in terms of food safety. These risks can, however, be mitigated through a number of pre-harvest and postharvest actions.

A Study of the Potential for New Zealand Abalone *(Haliotis iris, Haliotis australis)* to Accumulate Paralytic and Diarrhoeic Shellfish Toxins

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Wild New Zealand abalone *(Haliotis iris and Haliotis australis)* were sampled at intervals throughout a bloom of *Alexandrium catenella* in the Marlborough Sounds, New Zealand, in March-May 2011 and tissues were analysed for Paralytic Shellfish Toxins (PSTs). Similarly
H. iris were sampled through a *Dinophysis acuta* bloom at Banks Peninsula in September-December 2011, and tested for Diarrhoeic Shellfish Poison (DSP). A minimum of 5 *H. iris* (and on some occasions in the Marlborough Sounds, H. australis), a pooled mussel sample (Perna canaliculus or *Mytilus edulis*) and a phytoplankton sample were collected on each sampling occasion. Abalone foot and viscera tissues were analysed separately. Toxin analysis for PSTs used the AOAC official method 2005.06, commonly known as the Lawrence method. An LCMS-MS multi-toxin method (McNabb, 2005) was used to detect DSP. PST levels (reported as saxitoxin equivalents) in mussel samples associated with the *A. catenella* bloom reached a maximum of 37.4 mg/kg. No PSTs were detected in any samples of abalone foot tissue. PSTs were detected at very low levels (around the limit of quantitation) in some viscera samples. Significant levels of DSP were found in mussel samples associated with the *D. acuta* bloom (maximum 0.39 mg/kg). No toxins from the regulated DSP group (dinophysistoxin, pectenotoxin or okadaic acid congeners) were detected in any samples of abalone foot. Low levels of pectenotoxin2 (<0.016 mg/kg) were found in some abalone viscera samples collected during the bloom, and okadaic acid was detected in two abalone viscera samples late in the bloom (maximum level 0.062 mg/kg). The results of this study suggest that the risk of contamination of New Zealand abalone by significant levels of PSTs or DSP is low during blooms of *Alexandrium catenella* and *Dinophysis acuta*. There have been no reported cases of toxic shellfish poisoning associated with commercially harvested abalone from New Zealand

Paralytic Shellfish Toxins in Tasmanian Abalone, *Haliotis rubra*: Toxin Chemistry

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Wild abalone, *Haliotis rubra*, were tested for the presence of paralytic shellfish toxins (PSTs) following a major HAB of the dinoflagellate *Gymnodinium catenatum* in Tasmania, Australia. This was conducted as part of a project investigating the risk of marine biotoxins in abalone. Testing was performed at the Cawthron Institute in New Zealand using AOAC official method 2005.06, commonly known as the Lawrence method. As expected, highly elevated PST levels were observed in bivalves (mussels; *Mytilus edulis*) collected from commercial abalone harvest areas located within the bloom-affected area. PSTs were also observed in wild Australian abalone, with the edible foot containing lower levels than the visceral portion. Their presence resulted in some harvest areas being subjected to harvesting restrictions. There have been no reported illnesses from the consumption of PST contaminated abalone in historical Australian reports or in association with this bloom event. Abalone toxin levels were typically lower than those found in mussels collected from the same site. A high degree of variability in toxin level was observed between individuals tested from the same site, although the toxin profile remained relatively consistent. In the foot, saxitoxin (STX) and decarbamoyl-STX were the dominant PSTs on both a toxicity and molar basis. In the viscera, the profile was more complex with additional less toxic PST congeners being observed. This included decarbamoyl-GTX2,3 and to a lesser extent low toxicity N-sulfocarbamoyl PSTs (C1,2; C3,4; GTX5; GTX6). In mussels, the C-toxins predominated on a molar basis although the majority of the toxicity was due to decarbamoyl-STX.

An important observation was that two substantial unassigned peaks were observed in chromatograms obtained from PST-contaminated abalone. These are thought to be oxidation products of the uncommon PST congener deoxydecarbamoyl-STX (doSTX). There is no reference material or robust toxicity data available for doSTX, which makes it difficult to quantify accurately and to include in any sample toxicity calculations. Several studies suggest doSTX is substantially less toxic than STX. This and other issues are being addressed in a follow up project.

Paralytic Shellfish Toxins in the Marine Gastropod *Zidona dufresnei:* Toxicity and Profiles determined by MBA and Two HPLC Methods

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The marine gastropod *Zidona dufresnei* is a commercially important species in Argentina. It inhabits the warm-temperate waters of the Southwestern Atlantic Ocean, in sandy bottoms up to 100 meters deep, near banks of mussels and scallops on which they feed. Consequently, *Z. dufresnei* can concentrate PSP toxins through feeding on contaminated bivalves. The muscular foot is the part of the animal intended for consumption, being obtained through thermal process followed by separation of shell and viscera, which are discarded. Since 1987 samples from each landing batch are analyzed for PSP toxicity using the mouse bioassay method (MBA, AOAC Method 959.08). The toxin concentration in foot muscle usually is below the maximum permissible limit of 80 µg eq STX/100g, but levels up to 5,600 µg eq STX/100g have been detected in the viscera. This implies a significant risk for people who get whole snails and consume the product with viscera attached or use viscera in cooking fluids. Several PSP outbreaks were recorded in Argentina following the consumption of whole snails (foot muscle and viscera), including cooking liquid. Samples of snails involved in two of these outbreaks were obtained for laboratory analysis. One of these outbreaks was recorded in October 1996, and another one in November 2009. PSP toxin concentrations were determined by MBA in samples of fresh snails and in leftovers of the cooked snails. PSP toxicity in fresh foot muscle was below the regulatory limit, but the values detected in viscera were above 1,500 µg eq STX/100g. Recently, PSP toxin concentrations were determined in foot muscle and viscera samples of *Z. dufresnei*, as well as cooked snails, using two LC-FLD methods. Results will be presented showing the PSP profiles in each of the samples analyzed as well as toxicity levels by MBA.

Virus and Vibrio Occurrence

Keynote

Detection and Genotyping of Norovirus from Clinical and Shellfish Samples. Epidemiological Implication

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A total of 2643 stool samples from gastroenteritis-suffering patients (July 2010-June 2011) and 81 mussels samples (October 2010-March 2012) from the same geographical area (A Coruña, Galicia, NW Spain) were analysed for NoV presence. The aims of this work were i) the comparison of Norovirus (NoV) prevalence and genogroups found in these two kind of samples and ii)the analysis of correlation between the numbers of NoV positive samples found in mussel and the water quality at the 7 collectionpoints. Presence of NoV of genogroups I and II was analysed by qRT-PCR with TaqMan probes. Positive samples were genotyped by seminested RT-PCR of a partial capsid gene sequence. In clinical samples from patients affected by gastroenteritis,NoV were detected in 28.26% of the cases. All the strains were classified as NoV GII. Within this genogroup, detected strains belong to different genotypes: GII.1 (0.67%), GII.3 (2.65%), GII.4 (36.42%), GII.7 (8.6%), GII.6 (2.65%), GII.12 (2.65%), GII.13 (13.24%), GII.14 (33.11%). In the mussel samples, NoV were detected in 61.7%, of the samples. NoV strains were classified as GI.4(37.5%), GII.4 (50%) and GII.6 (12.5%). Similar percentages of positive samples were found in harvesting areas catalogued as B and C. Despite the fact that both genogroups were detected in mussel samples, higher genotype diversity was observed within the clinical samples. The highest detection rate on NoV GII.4 in mussel samples may suggest a better affinity of this genotype to bivalve shellfish. Finally, and as previously reported, no clear association between the classification of harvesting areas and the presence of human enteric viruses was observed.

Pandemic Noroviruses – Where Do They Come From?

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Noroviruses (NoVs) cause over 90% of nonbacterial epidemic gastroenteritis and around 50% of all gastroenteritis cases worldwide. Progress made so far in the molecular biology of norovirus has revealed the presence of a particularly important genotype of the virus, known as NoV genogroup II, genotype 4 (GII.4), as the cause of global pandemics of gastroenteritis, accounting for 65-80% of all NoV infections. Over the last decade NoV epidemiology and transmission has mirrored that of influenza A virus with new antigenic epidemic GII.4 variants of NoV arising approximately every two to three years. The pandemic GII.4 NoVs and their associated period of activity include; the US-95/96 variant in 19968, Farmington Hills virus in 2002, Hunter virus in 2004, 2006b virus in 2007-8. Our continued NoV surveillance, as part of both the global NoV surveillance network (NoroNet) and the Australian and New Zealand NoV Surveillance Network, revealed that three large epidemics occurred in the late winters of 2009, 2010 and 2011 in Australia. The aetiological agent was a pandemic GII.4 variant known as New Orleans 2009 and this variant has also caused outbreaks across the globe including Europe, the USA and Japan in 2010. In March 2012, we detected a novel GII.4 variant, termed Sydney 2012. NoV surveillance demonstrates that it is currently responsible for 20%, 75% and 95% of NoV outbreaks in Sydney, Adelaide and New Zealand, respectively. Based on these early indications, it is possible that this variant could emerge to be the next GII.4 pandemic variant. Genome wide analysis of GII.4 sequences revealed that the two most recently identified GII.4 variants, New Orleans 2009 and Sydney 2012, are both ORF1/2 recombinants with breakpoints located at positions 4972 – 5016 nt and 4972 – 5100 nt, respectively. Therefore, intra-genotype recombination is a common feature of NoV GII.4 evolution and importantly, played a role in the emergence of the two most recent GII.4 variants in circulation, New Orleans 2009 and Sydney 2012.

Norovirus Contamination in Oysters Collected on French Markets.

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In France, between 1996 and 2010, 5% of food poisoning declared were attributed to shellfish consumption. Through their filtering activity shellfish can concentrate microorganisms and more specifically human enteric virus such as norovirus. Among the 561 outbreaks linked to shellfish, norovirus were implicated in 145 (31%), 68 being confirmed. The purpose of this study was to evaluate, the frequency and concentration level of norovirus in oysters marketed in one area (Vendée). Samples were collected over 18 months (February 2010 to May 2011) from supermarket, market or producer direct sales. Forty samples were collected monthly during the two winter periods, and 12 samples during other months leading to a total of 391 samples collected. Each sample, constituted at least of 12 oysters, was analysed after PEG precipitation and nucleic extraction (Nuclisen kit, bioMerieux). After extraction efficiency checking norovirus (genogroups I and II) were detected by real-time RT-PCR. Overall 345 samples (88.2%) were found negative for norovirus. The 46 positive samples (11.8%) were all detected during the winter period (November to April). Amounts of virus detected in oyster digestive tissues (DT) were low with 83% of the positive samples under the limit of quantification (70 RNA copies / g DT). A marked impact of the winter gastroenteritis outbreak was observed with a higher number of positive sample during winter 2010 compared to 2011 season. These results, both in terms of frequency and quantification, coupled with epidemiological and environmental conditions are important to determine risks periods. Such studies will help in the future to set up preventive measures to limit shellfish contamination.

Restrospective of Total and Toxigenic *Vibrio parahaemolyticus* Monitoring in Chile 2009-2012

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Chile has cold Pacific waters affected by Niño Current from Asia, which increases the sea water temperature (SWT) and, consequently, risk for presence of Vibrios and other pathogens in environmental water and seafood. This phenomena has obligated public health authorities and the seafood industry to promote cooked and frozen molluscs in recent years. In order to characterize the contamination levels of total and toxigenic *Vibrio parahaemolyticus* and its effect on human health, the reference Chilean laboratory has been conducting a study to evaluate the exposure to the pathogen over the past 4 summer seasons. Sample surveys consist of weekly collections of 5 samples of different bivalves in duplicate in a open market study from Región de los Lagos, a region where 1000 shellfish farms coexist with the Salmon industry. The species evaluated are *Protothaca taca, Mytilus chilensis, Choromytilus chorus* and *Aulacomya ater*. The methodology used for enumeration of *Vibrio* with tlh and tdh genes was the Most Probable Number method with Real Time PCR (qPCR-MPN) using dilutions of 100 to 0.0001g per MPN tube. At least for the summer of 2009, the estimates of abundance of *Vibrio* were able to be related to weekly clinical reports of illness in the area. This past summer, the estimates of abundance were correlated to oceanographic parameters like SWT and air temperature from specific regional locations. The number of samples collected each season were 80-90. The results indicate a good relationship between abundance of total and toxigenic vibrio and environmental conditions, suggesting that the SWT can be a good predictor of the presence of the pathogen. Abundances of 100 MPN/g in market samples were observed when the SWT were over 18,5°C. This information was communicated to epidemiologists and managers responsible for enforcement of the risk management policy of the country in that specific region.
Acknowledgment: Students from Universidad Andres Bello, Scientists from US FDA, Food Microbiology Unit at Food Microbiology Chilean Reference Laboratory ISP,Universidad de los Lagos, Universidad de Chile , Universidad de Santiago de Compostela. Project FAO USP PR 38361 and CORFO 09CN14-5951.

Long-Term Study on *Vibrio parahaemolyticus* Prevalence in New Zealand Shellfish

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The food-borne pathogen *Vibrio parahaemolyticus* (Vp) is present in New Zealand (NZ) seawaters, but there are no reported cases of food poisoning from NZ-grown seafood. Our study determined the current numbers of Vp in NZ oysters and Greenshell™ mussels and the prevalence of pathogenic strains of Vp. Only a few strains of Vp are pathogenic and little is known about their presence in NZ. Pacific oyster (233), Greenshell™ mussel (54) and dredge oyster (19) samples were obtained from commercial shellfish-growing areas between December 2009 and June 2012. Vp levels were determined using the FDA Most Probable Number method and the presence of pathogenic genes tdh and trh was assessed by conventional PCR. In 2012 samples were also analysed by Real Time PCR (RT-PCR). Pathogenic Vp levels were low, only detected in 10/215 North Island oyster samples (three tdh and seven trh) using the FDA methodology and a further two tdh and four trh Vp positive samples using RT-PCR. Pathogenic Vp reached maximum level of 42/g, well below the FDA recommendation (< 104/g). From the South Island, nonpathogenic Vp were detected in just 1/37 oyster and 2/16 mussel samples, all at 0.36/g. Non-pathogenic Vp was detected in 81% of Pacific oysters and 34% of mussel samples harvested from the North Island, reaching peak numbers of 2.4 x 104/g and 95/g, respectively. Numbers increased with increasing seawater temperatures, peaking in late summer when most shellfish-growing farmers do not harvest. Samples only exceeded 1,000/g when seawater temperatures exceeded 19°C so seawater temperature could be used as a warning of potential hazard. There were no major differences between methods for the enumeration of non-pathogenic Vp. RT-PCR had a higher detection power, particularly for pathogenic Vp strains, and was faster for routine analysis with a turnaround of 24 hours after sample receipt.

Risk Management of Current and Emerging Marine Toxins

Keynote

Use of LC-MS for the Management and Mitigation of the Effects of Harmful Algal Bloom toxins

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Toxins from marine and freshwater algae are chemically incredibly diverse and they exhibit a range of interesting effects in humans. The most startling of these effects are those toxins that are capable of causing death. Fortunately these toxins are found to occur only rarely but the severe consequence for consumers means that management and mitigation of these effects is important for many seafood industries. Shellfish accumulate toxins from the algae that they feed upon and the New Zealand shellfish industry tests its products for these toxins using a range of detection methods. Traditionally test methods based on mouse bioassay and intraperitoneal (I.P.) injection were used. However the use of mouse bioassay has resulted in the discovery of a range of toxins which are highly toxic to mice by I.P. injection but which are of questionable relevance to the wellbeing of shellfish consumers. This presentation will discuss the on-going process of developing and validating LC-MS methods to manage and mitigate against the risks from shellfish toxins. New Zealand adopted an LC-MS replacement for lipophilic mouse bioassay in 2001 and then adopted alternative methods for paralytic shellfish toxins (PSTs) in 2010. The process of developing these new methods and implementing them in the monitoring systems involved key stakeholders and has been complex for more than just technical reasons. As well as presenting a review of the impact of LC-MS methodology in current monitoring programmes the application of LC-MS to the emerging issues of pinnatoxins and palytoxin is also discussed.

Unambiguous Confirmation of Cyclic Imines as Emerging Toxins in Shellfish Harvesting Areas of Catalonia (Nw Mediterranean Sea)

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Cyclic imines (CI) are lipophilic marine toxins that bioaccumulate in seafood. They have a cyclic-imino group in their structure, responsible for their fast-acting neurotoxicity in mice. Among CI, only pinnatoxins have been suggested to cause human intoxications. Still, they are not regulated in Europe and the human risk assessment on CI performed by the European Food Safety Authority (EFSA) was not conclusive due to lack of information. This work presents the first detection of two CI in shellfish from Catalonia (Spain, NW Mediterranean Sea). 13desmethyl-Spirolide-C and Pinnatoxin-G were found at low concentrations in mussels and oysters from the Ebro Delta. Spirolides were first detected in the Atlantic Spanish coast in 2006 but pinnatoxins had never been reported in Spain. These toxins have been also detected in sea water using SPATTs in several samples collected along four years (2008-2012). The first detection and quantification by LC-MS/MS of spirolides and pinnatoxins was performed under alkaline chromatographic conditions using a 3200-QTrap triple-quadrupole (AB/Sciex) and the further identification was performed in an Orbitrap Discovery (Thermo Scientific). The identification of the 13-desmethyl-Spirolide-C and pinnatoxin G was confirmed by their retention times compared to certified standard solution, and by high mass accuracy analysis (<2 ppm) on the precursor ion and on three main characteristics fragments. Analogs were determined by the high accurate mass of their precursor ions. The complementary use of LCMS techniques permitted the quantification, characterization and unequivocally identification of emerging marine toxins in Catalonia. We also discussed the distribution of CI in different shellfish species, harvesting locations and seasons, the relationship between the concentrations in water and in shellfish, and the presence of CI in an European context. Our results support the requests of EFSA to include CI in the shellfish safety monitoring programs to perform accurate assessments of the risk posed by CI.

An Update on the situation of the control of Marine Biotoxins in the European Union.

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The contamination of seafood due to natural toxins present in the phytoplankton represents a worldwide concern of increased interest due to their frequent and widespread appearance. New and emerging toxins are also becoming more and more relevant in several places worldwide. The search of efficient and reliable analytical methodologies for the detection of all these toxins is still challenging, although an important progress has been made over the last few years, looking for a replacement of the long standing mouse bioassay. In particular in the European Union an important change in the Legislation has been made since July 2011, by replacing the mouse Bioassay for Lipophilic toxins (LPTs) by a chemical approach based on a Liquid Chromatography separation coupled to a tandem Mass Spectrometry. This change marked a new era in the detection of marine toxins. Alternative methods have been also developed for the control of other group of toxins such as Paralytic shellfish toxins (PSTs) and even for Emerging toxins. This work shows the situation in The European Union regarding the control of Marine Biotoxins. The efforts carried out in the EU to get adapted to the new methodologies will be discussed and the improvements carried out on the LC-MS/MS method for LPTs will be also presented. The presence of Emerging toxins such as Cyclic Imines as well as Ciguatoxins and Palytoxins in several places in Europe will be also discussed and the recent work carried out at the European Reference Laboratory for Marine Biotoxins for the development, optimization and application of LC-MS/MS for the detection of these Emerging toxins will be also presented and discussed.

Innovative Technologies

Keynote

Molecular Genetic Detection of Saxitoxin and *Alexandrium* Species in Marine Environmental Samples

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Saxitoxin and its analogues are toxins responsible for the syndrome paralytic shellfish poisoning. Several methods are used to monitor marine samples for potentially toxic species and to detect toxins in shellfish, however, most are comparatively time consuming, expensive, and require a high level of expertise. Saxitoxin has been recently discovered to be catalysed by a group of enzymes encoded by sxt genes. The unusual gene sxtA initiates the first step in the synthesis. We investigated the distribution and sequences of sxtA in dinoflagellates, in particular, in toxic species of *Alexandrium* and *Gymnodinium catenatum.* This gene exists in multiple genomic copies in investigated strains of *Alexandrium* species. We developed a qPCR assay targeting the sxtA gene to detect saxitoxin-producing dinoflagellates. We detected and quantified sxtA in bloom events of *Alexandrium* species in Australia and New Zealand that led to saxitoxin uptake in shellfish. The abundance of sxtA was significantly correlated with the abundance of the saxitoxin-producing species of *Alexandrium* species in each case. As molecular genetic methods continue to decrease in cost and become faster, this method is a very promising alternative to currently used detection tools.

A Receptor Binding Assay for Paralytic Shellfish Toxins: Results of anAOAC Collaborative Study and Progress inAssay Implementation

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Paralytic shellfish toxins (PST) represent one of the most globally important safety concerns for shellfish commerce. PST encompass over 21 different congeners of saxitoxin that occur in varying combinations in shellfish, making their analytical determination complex. The receptor binding assay (RBA) is a rapid, high throughput screening method that has been proposed as a suitable alternative to the mouse bioassay for providing a composite toxicity measurement. A recently completed collaborative study of the RBA for PST resulted in its acceptance as an AOAC Official Method of Analysis, OMA 2011.27. Nine laboratories from seven countries conducted the RBA, two laboratories analyzed the same samples by HPLC (OMA 2005.06) and three laboratories performed the AOAC mouse bioassay (OMA 959.08). A total of 21 shellfish homogenates were extracted in 0.1 M HCl and analyzed in three assays run on separate days. Samples included naturally contaminated shellfish samples of 7 different species collected from several geographic regions and represented a range of concentrations below and above the regulatory limit. Five samples were blind duplicates used for calculation of RSDr. The interlaboratory reproducibility (RSDR) for 21 samples run in 9 laboratories was 33.1%, yielding a HorRat value of 2.0. Removal of results for one laboratory that reported systematically low values resulted in an average RSDR of 28.7% and average Horrat value of 1.8. Intralaboratory repeatability (RSDr), based on 5 blind duplicates, was 25.1%. RSDr obtained by individual laboratories ranged from 11. 8% to 34.9%. Laboratories that are routine users of the assay performed better than non-routine users, with an average RSDr of 17.1%. Recovery of STX from spiked shellfish homogenates was 88.1 – 93.3%. Correlation with the mouse bioassay yielded a slope of 1.64 and r2 of 0.84, while correlation with the pre-column oxidation HPLC method yielded a slope of 1.20 and r2 of 0.92. When samples were sorted according to increasing toxin concentration (eg STX diHCl equiv./kg) as assessed by the mouse bioassay, the receptor binding assay returned no false negatives relative to the 800 eg STX diHCl equiv./kg regulatory limit for shellfish. The RBA is the only validated method other than the mouse bioassay that directly reports a composite toxic potency for PST in shellfish, integrating the contributions of multiple congeners bearing different inherent toxicities. Current progress towards implementing this assay for routine monitoring and regulatory testing will be presented.

Capillary Electrophoresis for Saxitoxins and Analogues: Comparison of Detection Methods

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The currently widely-used analytical method for the determination of saxitoxin and analogues (STX) is high performance liquid chromatography-fluorescence (HPLC-FLD). Although effective, the instrument setup can be tedious and time-consuming, especially when post-column oxidation is involved. We present the development of an alternative method for the analysis of STX based on capillary electrophoresis (CE). The current study presents the comparison of capacitively-coupled contactless conductivity (C4D), laser induced fluorescence (LIF), ultraviolet (UV) and mass spectrometry (MS) following their electrophoretic separation. The results indicate that all detection methods are capable of separating and analyzing all 11 STX with differences only in sensitivity and simplicity of the method. The CE-C4D method was 3 to 5 times more sensitive than the CE-UV and CE-MS methods (based on S/N ratio) and the optimization and validation of this method is presented. Capillary electrophoresis is a promising analytical method for STX as it can be down-scaled in dimension for development of portable detection instruments for rapid detection of STX in field and laboratory settings.

Development and Validation of an SPR Method for Tetrodotoxin in Gastropods and Fish

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Tetrodotoxin is a highly potent marine neurotoxin. In Europe this toxin is considered to be an emerging risk on the consumption of non-bivalve molluscs and migratory fish due to limitations in the legislation for the monitoring of this toxin and species types. In recent years, there have been episodes of tetrodotoxin poisoning in Europe but whether this is due to increasing occurrences of this toxin or better reporting is yet unclear. Nevertheless, changes in climatic conditions or increased pollution may be having an effect on the toxin production from bacterial species. In Europe, tetrodotoxin monitoring in seafood is currently performed using the internationally accredited AOAC mouse bioassay as a consequence for testing for paralytic shellfish poisoning (PSP) toxins though only in bivalve molluscs for regulatory purposes. Due to ethical and performance related issues associated with this bioassay, the European Commission has recently published directives extending procedures that may be used for official PSP control. An AOAC accredited HPLC method has now been accepted as a first action screening method to replace the MBA but as this method is not capable of detecting tetrodotoxin an alternative method is required. Surface plasmon resonance (SPR) optical biosensor technology has been proven as a potential alternative screening method to detect PSP toxins in seafood and the addition of tetrodotoxin would complement this assay in removing the mouse bioassay. Immobilisation of tetrodotoxin onto the biosensor chip surface was achieved via amino-coupling. Using a monoclonal antibody an inhibition assay format was achieved with sensitivity (IC50) of 5ng/ml. An SPR method was developed for the analysis of tetrodotoxin and validated following the guidelines contained in the European Commission Decision 2002/657/EC for chemical contaminant analysis and the performance of the assay demonstrated to be fit for purpose with a detection capability (CCβ) ≤ 200µg/kg.

Advanced Faecal Source tracking Techniques

Keynote

Microbial Source Tracking to Identify and Manage Sources of Fecal Contamination in Shellfish Growing Waters

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Shellfish sanitary quality is affected by a variety of different pollution sources in populated coastal areas. Sewage and other sources of fecal-borne microbial contaminants can cause shellfish harvesting closures due to potential public health hazards. Traditional bacterial indicators used to classify shellfish and overlying water quality cannot be used to identify pollution sources. A variety of methods have been developed to identify sources of fecal pollution to enable management action to reduce pollution to safe levels. This presentation is an overview of the presently useful microbial source tracking methods for identifying fecal pollution sources and their application for managing shellfish waters.

The Use of Fluorescent Whitening Compounds as Signals of Human Sourced Contamination in Estuaries

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In recent years, oyster harvesting in a number of rivers, estuaries and coastal lakes on the east coast of Australia has been adversely impacted by reduced water quality. Following periods of heavy rain a number of incidents of pathogen contamination of estuarine waters have occurred. Such incidents have significant adverse impacts and may result in long periods of closure to harvesting with consequent commercial implications for oyster supply and the livelihoods of those employed in the industry. On-site wastewater management systems (OWMS) and land application areas (LAAs) in coastal areas have often been considered possible sources of contamination of estuarine waters.

In sanitary surveys of estuaries, faecal contamination is typically assessed by microbial indicators which are ubiquitous in the environment and cannot be used to distinguish between sources of faecal contamination. The ability to distinguish between human and animal sources of contamination is particularly important where estuaries are used for commercial aquaculture and there is a need to know the fate or pathway of contaminants from LAAs. Chemicals associated with human metabolism and activity which are also present in faecal material (such as caffeine, faecal sterols, and pharmaceuticals) have been used to characterise and identify human faecal contamination with limited success. One such group of chemicals are fluorescent whitening compounds (FWCs) which are present in wastewaters containing laundry washing products. These compounds have been used to a limited extent to assist in identifying human contaminant sources in estuaries used for aquaculture. This paper will describe methods used to further develop the application of this technique for sanitary surveys and outline its usefulness as an indicator of human sourced contamination in estuaries.

Evaluation and Application of Viral Source Tracking in New Zealand

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Contamination of shellfish growing waters and recreational waters by faecal pollution can present a risk to human health because of the presence of pathogenic bacteria and viruses. Rapid, accurate identification of human and animal faecal pollution sources using microbial source tracking is valuable for effective water quality management. Our objective was to develop a viral source tracking system for detection of human and animal faecal pollution sources in the New Zealand (NZ) environment. Specific real-time quantitative RT-PCR assays for viruses known to occur in animal or human faeces or urine which were potential candidates for source tracking purposes were developed and evaluated for host specificity and virus prevalence in environmental samples. Samples analysed included shellfish, estuarine water, wastewater, river water and animal faeces (pigs, sheep, cows, wildfowl).

Of the assays evaluated, human polyomavirus (HPyV), human adenovirus species F (HAdV-F), and norovirus (NoV) genogroup I/II were confirmed to be human specific and were prevalent in NZ. For determination of animal specific sources, assays for bovine polyomavirus (BPyV) (cows), porcine adenovirus type 3 (pigs), 'ovine/ bovine' adenovirus (sheep, cows) and NoV genogroup III (sheep, cows) were found to be useful in New Zealand. The combination of NoV GIII and BPyV assays allowed the identification of animal faecal sources for tracking purposes. HPyV was prevalent in environmental waters (estuarine, wastewater, urban stream) being detected in approximately 39/57 (68%) of samples analysed. Similar levels of HPyV, HAdV-F and NoV were detected in samples.

This combination of quantitative RT-PCR assays (the Viral ToolBox) for the specific detection of human and animal enteric viruses in the environment will aid in microbial source tracking and so assist resource managers, shellfish farmers and public health officers to determine sources of pollution for effective management of water quality, particularly shellfish growing waters.

Dye Tracing Techniques Used in New South Wales Shellfish Growing Areas to Assess Pollution Sources

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The New South Wales (NSW) Food Authority – Shellfish Program has successfully employed a practical and cost effective dye tracing technique that has led to the positive identification of pollution sources impacting shellfish growing waters. Fluorescing dyes have long been utilised for hydrological and geological studies generally relying on a positive visual identification or water sample collection for in field fluorometer use to assess stream and groundwater flows and determine leaks from pipes and sewage systems. The technique utilised by the NSW Food Authority to assess pollution sources utilises activated carbon to adsorb dye which can be eluted in a laboratory for testing via spectrofluorophotometers. The advantage of this technique compared to either visual identification or a standard grab water sample is that it maximises the possible detection of tracer dyes and minimises the number of samples and overall sampling effort, in turn reducing overall cost. Activated carbon sample packets can be left unmonitored in the environment to ensure longer term exposure to possible dye detection, thus increasing the chance of recording a positive reading. This technique has successfully been utilised in NSW shellfish growing waters which has led to the remediation of known pollution sources impacting shellfish growing waters.

Emerging Risks from Cyanobacteria (I)

Keynote

Cyanobacterial Toxin Accumulation in Shellfish, Finfish and Crustaceans, Guidelines for Consumer Safety

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The risks of human poisoning from marine shellfish are well appreciated, and as a result safety guidelines have long been in place for the dinoflagellate and diatom toxins. By contrast the cyanobacterial toxins have been less studied as potentially toxic components of human food. Drinking water safety has received the most attention, since toxic cyanobacteria can proliferate in drinking water reservoirs, thereby causing a direct challenge to human health. They can also form dense water blooms in natural lakes and estuarine systems, from which shellfish, finfish and crustaceans are harvested for human consumption. The cyanobacterial toxins of most concern are water-soluble peptides that cause liver and organ injury and are possible human carcinogens. Alkaloid toxins also occur, with examples having cytotoxic or neurotoxic actions. Cyanobacterial toxins have been clearly identified in the gut contents and livers of aquatic food species, with lesser concentrations in muscle tissues. For the protection of the health of consumers, safe guideline concentrations have been determined for shellfish, finfish and crustaceans, both for young people and adults, based on the approaches of WHO for toxicants in food and water. The derivation of these guidelines and their application will be discussed in the context of consumer safety.

Use of Health Guideline Levels for Cyanobacterial Toxins in Seafood From The Gippsland Lakes

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The Gippsland Lakes are a system of coastal lagoons situated in south-east Victoria, approximately 200 kilometres east of Melbourne. Cyanobacterial blooms are a common occurrence within the Lakes with twelve blooms recorded since 1985. *Nodularia spumigena* bloomed in the Gippsland Lakes in 1999, 2001 and 2002 with seafood sampling confirming uptake of nodularin toxin in fin fish, mussels and prawns. Fish, eels and prawns are harvested commercially within the Gippsland Lakes alongside a significant recreational fishery for fish, mussels, prawns and crustaceans. An inshore prawn fishery also operates outside of Lakes Entrance where prawns are landed for commercial sale. The 2001 health guideline values for cyanobacterial toxin in seafood have recently been reviewed using a standard human health risk assessment approach. The latest nutritional survey information and seafood consumption rates were taken into consideration to inform the health guideline values. The updated health guideline values were applied in the *N. spumigena* bloom that occurred in the Gippsland Lakes from September 2011 till April 2012. During the bloom seafood samples were collected from fish, mussels and prawns. Nodularin toxins exceeded the health guideline levels in whole fish, mussels and prawns. Health advisories were issued based on seafood sampling results and health advisories were lifted when sampling detected nodularin below health guideline levels.

Management of Cyanobacterial Blooms in New South Wales: Case Study – Mitigating Food Safety RisksAssociated With Recreational and Commercial Fishing – Myall Lakes NSW 2012

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Following the largest recorded blue–green algal bloom in the Darling River in 1991 a task force was formed to develop an integrated Algal Management Strategy for New South Wales. After several reviews the Strategy is administered by the NSW State Algal Advisory Group (SAAG) and nine Regional Algal Coordinating Committees (RACC). An expert Technical Advisory Group advises the SAAG. In February 2012 a red alert for blue green algae was issued for the Myall Lakes, a 10,000 hectare waterway on the NSW mid north coast. The estuary supports important commercial and recreational fisheries and has a history of toxic algal blooms. Laboratory analysis confirmed the presence of *Anabaena circinalis* Rabenhorst ex BronetetFlahault 1888, a cyanobacteria that is known to produce paralytic shellfish poisons (PSPs).The contingency plan of Hunter RACC (a 'whole of government' response) was implemented. Actions included notification of the community, determination the nature and extent of the bloom and voluntary gutting and gilling of finfish prior to sale or diversion of low value finfish to bait. A mandatory closure to the harvesting of all molluscs and crustaceans by recreational and commercial fishers was also implemented whilst toxicity testing of flesh and water samples was undertaken. Industry and government worked together to rapidly identify and manage the risks, minimise publicity and effectively minimise contention. The fishing industry was supportive of the actions taken by the Hunter RACC. This reflects both the industry's concern for consumers and acknowledgement of the critical importance of the healthy image of seafood in the marketplace

Cyanotoxins in Fish and Shellfish – An Overview

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Cyanobacterial toxins are a recognised hazard to public health; cyanotoxins have long been known to be acutely toxic, causing mass fatalities of stock animals and wildlife. Some cyanotoxins are potent tumour promoters and suspect carcinogens, thus presenting risk management challenges relating to chronic, low-dose exposure. Australia's NHMRC has developed guideline concentrations for specific cyanotoxins in drinking water, and cyanobacteria and cyanotoxins in recreational waters are also subject to guideline management. Cyanotoxins in drinking water source supplies can be effectively detoxified by modern water treatment processes like chlorination and chloramination, but such safeguards are not available to consumers of various foods that may be contaminated by cyanotoxins, and cyanotoxins in fish and shellfish are an emerging public health issue. Advances in analytical capability, particularly over the past decade, have contributed to a rapidly growing body of literature on the detection and quantification of various cyanotoxins in seafood matrices. The microcystin group of cyclic peptides are the most studied, with reports from all inhabited continents of these toxins in seafoods. However, research into both the bioavailability and analytical quantification of protein-bound microcystins is required to gain a better understanding of the specific health risks to seafood consumers. Nodularin is a particular concern for researchers in Baltic Sea states, as that semi-enclosed brackish water system is regularly impacted by toxic Nodularia blooms; nodularin has also been found in wild-caught fish and farmed prawns elsewhere in the world. Saxitoxins of cyanobacterial origin have been detected in freshwater fish and shellfish; cylindrospermopsin has also been found in molluscs, fish and crayfish that are popular food items. The alkaloid neurotoxin anatoxin-a has been recovered from fish and mussel tissues following experimental exposures. The water-soluble cyanotoxins are clearly capable of bioaccumulating in tissues of seafood and shellfish, but they do not appreciably biomagnify through higher trophic levels. Therefore filter-feeding bivalves and planktivorous fish may present a relatively higher risk from dietary exposure to cyanotoxins, though trophic transfer of cyanotoxins into carnivorous fish can and does occur. The capacity for particular cyanotoxins to partition into specific edible tissue compartments needs to be better understood. Studies on the uptake and elimination kinetics of the main cyanotoxins in important commercially and recreationally-harvested seafood are needed to better assess the risks to seafood consumers.

Virus Risk Management Options

Keynote

Science-And Risk-Driven Approaches for Control of Norovirus In Molluscan Shellfish

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The sanitary quality of molluscan shellfish is dependent upon the quality of the water in which they grow. Potential pollution sources that affect these growing areas are categorized as point- and non-point sources. The impact of these pollution sources on shellfish and shellfish growing waters has traditionally been established by fecal coliform or *E. coli* levels. While these indicator microorganisms are used to determine overall sanitary quality, they are inadequate alone to index the risk of norovirus (NoV) contamination. This presentation will provide details on research approaches used to assess the contributions of NoV from wastewater treatment facilities, overboard discharges, transport and dilution of NoV in shellfish growing waters, and bioaccumulation and elimination of NoV by shellfish. Through these investigations we have established: (1) The frequency and levels of NoV present in treated and untreated wastewater; (2) The spatial distribution of NoV in shellfish in proximity to pollution sources; and (3) The selective, seasonal bioaccumulation and elimination of NoV by shellfish. Examples will be provided on the necessity to mitigate non-point land- and water-based pollution sources that may affect growing areas. Information from these investigations has proven invaluable for the joint U.S. /Canada quantitative norovirus risk assessment for molluscan shellfish.

Impact on Shellfish Quality in the Avon-Heathcote Estuary/Ihutai, Christchurch Following the 2011 Earthquakes

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On 22 February and 13 June 2011, 6.3 and 6.4 magnitude earthquakes in Christchurch caused substantial damage to the city's sewerage infrastructure. Over 60,000 m3/day of untreated raw sewage entered the city's rivers and estuary for one month after each event, with discharges of 10-20,000 m3/day prior to sewerage repairs in November 2011. Quarterly monitoring of *E. coli* and norovirus in cockles at five sites around the Avon-Heathcote Estuary/Ihutai was already in place to compare concentrations before and after the city's treated wastewater discharge into the estuary was decommissioned. Thus the consequence of unprecedented quantities of raw sewage on shellfish quality could also be investigated. Both E coli and norovirus concentrations at the two river mouth sites dramatically increased after the February earthquake. E coli concentrations increased to 16,000 MPN/100 g and norovirus was detected at extremely high concentrations (>10,000 genome copies/g shellfish digestive tissue). The June earthquake did not elicit a similar increase in *E. coli* and norovirus concentrations at the river mouth sites. This may indicate that the disturbance prevented the shellfish from feeding, as collection was within a few days of the earthquake. The earthquake on 4 September 2010 (magnitude 7.1) did not cause significant damage to the sewerage network, and only minor increases in *E. coli* and norovirus concentrations were recorded, similar to when treated wastewater was being discharged to the estuary via the City's wastewater treatment plant. Monthly monitoring was instigated after sewage overflows ceased and indicated that pre-earthquake contaminant levels returned within three months. Following both earthquakes signage was erected and public messages issued to ensure the public were aware of the public health risks of shellfish consumption. Quarterly monitoring is planned to continue until December 2013 and will provide two years of data after earthquake-related sewage discharges to the rivers and estuary have ceased.

Risk Management Strategies for Hepatitis A Contaminated Oyster Production Areas

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Monitoring HAV genomes in shellfish production areas could be useful for improving food safety. HAV contamination can be acute in coastal areas, such as Brittany, where outbreaks of hepatitis A have occurred in the past, and have been linked to consumption of raw shellfish. A quantitative probabilistic approach was carried out to estimate the mean annual risk of hepatitis A in a population of adult raw oyster consumers. Two hypothetical scenarios of contamination were considered, the first for a rare accidental event, and the second for regular and prolonged episodes of contamination. Different monitoring and management strategies were simulated, in particular for transferred products. Their effect was assessed by the relative risk reduction in mean annual risk. The duration of closure after detection of the shellfish area was also considered. Among the strategies tested, results show that monthly RT-PCR monitoring of HAV is more useful than bacterial surveys. In terms of management measures, early closure of the shellfish area without waiting for confirmatory analysis was shown to be the most efficient strategy. Monitoring every 15 days is clearly more efficient than every month. When contamination is not clearly identified or if contamination is heterogeneous, it can be better to wait for three negative results. Finally, any preventive measures — such as improving sewage treatment or producing shellfish in safer areas — that can reduce contamination by at least 2 log10 are more efficient and less costly. Finally we show that controlling and managing transferred shellfish is useful and can play an important role in preventing cases. Qualitative results from HAV monitoring can usefully supplement other measures that improve the safety of shellfish products in exposed areas.

Norovirus in Oysters: Methods, Limits and Control Options in the European Context

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Norovirus infection is a well recognised hazard associated with consumption of oysters harvested from human faecally polluted areas. Recent method advances utilising quantitative PCR permit, for the first time, the presence and levels of norovirus contamination in production areas to be investigated. Such initial studies suggest that norovirus, as detected by PCR, may be viewed as a relatively common contaminant of commercial shellfish beds. This presentation presents the findings of a European Food Safety Authority working group. The group reviewed progress on norovirus methodology and published information on norovirus levels in bivalve molluscs in production areas and in outbreaks. A particular focus was recent quantitative PCR data yielded by the same methodology from differently designed surveillance studies in production areas in UK, Ireland and France. Common observations in all countries were the relatively high prevalence of norovirus contamination (>30% sample positivity), the clear winter seasonality, and the overall range of contamination levels observed. A key data gap emerging was the relationship between norovirus PCR levels in bivalve molluscs and the associated infection risk upon consumption of those bivalves. The group considered evidence supporting a norovirus dose response relationship and thus the possibility for setting a control limit. Possible numerical limits were evaluated for their impact in a European setting. The findings of the working group are reported in an EFSA opinion on Norovirus in oysters: methods, limits and control options (EFSA Journal 2012;10(1):2500).

Emerging Risks from Cyanobacteria (II)

Keynote

The genetics of cyanobacterial toxin production

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In many aquatic ecosystems world-wide, including drinking water supplies, cyanobacteria (blue-green algae) can proliferate into so-called "harmful algal blooms". Members of this bacterial phylum have been evolving on Earth for around 3 billion years and can produce an unparalleled array of bioactive secondary metabolites, some of which are potent toxins. The past ten years has witnessed major advances in our understanding of the genetic basis for toxin production by a number of groups of cyanobacteria and marine algae. Understanding the role of these toxins in the producing microorganisms and the responses of their genes to a changing climate may suggest the means for controlling toxic bloom events in water supplies. The information gained from the discovery of these toxin biosynthetic pathways has enabled the genetic screening of various environments for drinking water quality management. In addition, the information gained from studying the toxins has also provided the information needed to screen for contaminated seafood. This seminar addresses the evolutionary history of one of the oldest life forms on Earth, the molecular genetics underlying bacterial toxin production, and the exploitation of this information for risk analysis.

Growth Promotion of the Toxic Cyanobacterium *Cylindrospermopsis raciborskii* By Herbivorous Zooplankton

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Cyanobacteria occupy diverse habitats and their ecological success is expected to increase under predicted future climate scenarios. Managing their abundance in freshwaters is therefore critical for reducing risks to human and animal health. To understand the mechanisms of toxic cyanobacterium *Cylindrospermopsis raciborskii* bloom formation in oligotrophic lakes, we tested the hypothesis that zooplankton nutrient recycling facilitates *C. raciborskii* growth. *C. raciborskii* can fix nitrogen, so we focussed on P as the growth-limiting nutrient. Dialysis experiments were designed to simultaneously test the direct (grazing) and indirect effects (nutrient regeneration) of zooplankton-algal interactions, enabling zooplankton to access food outside the dialysis tubing, and for zooplankton-derived nutrients to be accessible to algae inside the tubing. Controls with no zooplankton were also set up to account for nutrient contributions from algal prey. Zooplankton-derived nutrients alleviated P-limitation of *C. raciborskii* inside the dialysis tubes and stimulated growth. Furthermore, *C. raciborskii* growth was favoured above a green algal competitor when both algae were in dialysis tubes, indicating *C. raciborskii* is more efficient at taking up P recycled by zooplankton. Outside the dialysis bags, zooplankton grazed a green alga in preference to *C. raciborskii* and selectively consumed P-replete cells. *C. raciborskii* growth was therefore affected both directly and indirectly by zooplankton under these low nutrient conditions, suggesting that foodweb interactions can facilitate blooms of this cyanobacterium.

Cyanophage (Viral) Control of Toxic Cyanobacterial Abundance and Dispersal

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Globally, cyanobacterial blooms are increasing along with their adverse impacts on human heath. Cyanobacterial toxins are now more often appearing in a wide range of environments from drinking water supplies to the seafood industry. Yet factors that control their occurrence, bloom formation and collapse are still not clear. Could this be because natural cyanophage (viruses specific to cyanobacteria) are exerting an underlying natural control in addition to the physico-chemical factors? Here we test whether viruses from two Australian freshwater lakes (drinking water supplies) could control the abundance of two toxic species of cyanobacterium— *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa.* These cyanobacteria were selectively isolated from the two lakes. Microscopy confirmed the resulting culture were single cyanobacterial species. Filtration (through a 0.2 µm filter) was used to isolate viral communities from each lake sample, chloroform washed and stored in the dark at 4° C until their host populations were growing in culture. Then the natural lake viral cocktails were incubated with each cyanobacteria host growing under optimum conditions. *C. raciborskii* cells abundance decreased by 86% in 5 days, while the number of viruses in the culture increased stepwise. The cyanophage replication time was 21 h, with an average burst size of 64 viruses cell−1. TEM showed this cyanophage to be in the Siphoviridae family of viruses. *C. raciborskii* is a filamentous cyanobacterium. The cyanophage would often lyse only a single cell in a filament and so split it into smaller viable fragments. This process would help disperse the host in the wild. Hence this cyanohpage influences both the abundance and distribution of *C. raciborskii*.

For *Microcystis aeruginosa* it took 6 d for its abundance to decrease by 95%. The density of the cyanophage was positively correlated with the rate of *M. aeruginosa* cell lysis (r2 = 0.95). The cyanophage replication time was 11.2 h, with an average burst size of 28 viruses per host cell. TEM showed that two types of virus were controlling the host abundance and both belonged to the Podoviridiae group (short tails) of viruses. In the lake, the number of these cyanophage was 5.6 x 104 . mL-1, representing 0.23% of the natural viral population of 2.46 x 107 mL-1. Our results showed that this cyanophage could be part of a major natural control mechanism of *M. aeruginosa* in the source drinking water supply.

Occurrence of the Ciguatera Fish Poisoning Causing Dinoflagellate Gambierdiscus in Temperate Waters of New South Wales,Australia

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Gambierdiscus spp. are epiphytic, benthic dinoflagellates that are well known from tropical reef areas where water temperature range from 24 to 29°C. *Gambierdiscus* spp. can produce two major toxins i.e. Ciguatoxins (CTXs) and Maitotoxins (MTXs). Ingestion of tropical fish that have orally accumulated effective levels of CTXs and MTXs causes ciguatera fish poisoning (CFP) in humans. While the role of CTXs in CFP has been investigated, little is known about MTX and whether it plays a significant role in causing human illness. However, recently a feeding study carried out to probe the uptake of MTX has shown that it can accumulate in fish digestive organs, liver and mussels. CFP affects between 50,000 and 500,000 people per year with reports of a 60% increase in ciguatera fish poisoning in the Pacific islands over the last decade and suspected to be significantly underreported. Human poisonings are known from Queensland and the Northern Territory, Australia. Here, we report the occurrence of *Gambierdiscus* spp. in Merimbula and Wagonga inlets located in southern New South Wales, Australia. Scanning electron microscopy and molecular identification via 28s rDNA sequencing, newly identified the isolate from Merimbula as *Gambierdiscus carpenteri.* Various other potentially toxic genera such as *Prorocentrum, Ostreopsis* and *Amphidinium* were identified via light microscopy. Therefore, to study the dinoflagellate community structure in detail at both sites, a pyrosequencing approach based on 18s rRNA was applied. Phylogenetic analysis of the sequences obtained revealed the massive diversity of dinoflagellates at both sites. Toxin profile of *Gambierdiscus* and *Ostreopsis* isolates was analysed via LC-MS.

Marine Biotoxin Interactions

Keynote

Multiple Patterns of Phosphorylation of the Stress Response Protein HSP _7 in Cells Exposed to Mixtures of OkadaicAcid and Palytoxin

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The characterization of toxicity pathways of toxicants is a key step to proceed with mechanistic-based risk assessment. The complexity of this task is daunting when mixtures of different toxicants are considered. Still, living systems are currently exposed to mixtures of stressors. We have approached this complexity by proteomics, and our initial studies have shown that molecular responses induced by mixtures of toxins may not be simply predicted by combining those found when the system is exposed to anyone of the toxic agents. We have continued our studies with cultured human cells as a model system, and exposed them to okadaic acid (OA) and palytoxin (PlTX), either alone or in combination. This choice was based on the finding that toxins belonging to those chemical groups have been co-contaminating fishery products from some coastal waters in Italy. Hsp 27 is a major component of cellular responses to toxicants, which becomes phosphorylated in biological systems exposed to stressors. We found multiple forms of phosphorylated hsp 27 in cells exposed to OA, PlTX and a mix of these toxins. The analysis of enzymes involved in kinase cascades, including those phosphorylating hsp 27, indicated that PlTX would induce the activation of kinases, whereas OA would stabilize the cellular pool of the phosphorylated forms of the proteins, by inhibiting the dephosphorylation reaction. Our findings showed that the different pathways triggered by the two toxins contribute to a common response, represented by increased phosphorylation of hsp 27. Different patterns of phosphorylated hsp 27, however, are found in cells exposed to either OA or PlTX, both alone and in combination, providing further indications that the molecular mechanisms responsible for different phosphorylation patterns of hsp27 in cells exposed to these toxins might not be identical. This study is supported by the Italian MIUR (grant 2009JS5YX9).

Paralytic Shellfish Toxin Accumulation, Transformation and Tissue Localization in Philippines'Bivalve Mollusk

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This study was conducted in order to investigate several factors that influence toxin accumulation. Among these factors are the varying habitats of bivalve species, individual gut size and potential diet, the possible toxin transformations of the different PSP analogues and localization of PSP toxins in shellfish tissues. Shellfish specimens from various habitats namely: epifaunal, partially exposed benthic and infaunal were collected monthly from November 2007 until December 2008 in Sorsogon Bay, Philippines during bloom of *Pyrodinium bahamense* var. *compressum* (Pbc). Tissues were removed and hepato-pancreas was weighed. Stomach contents were analyzed for Pbc cells to compare the epifaunal from infaunal specimen. Extracts for toxin analysis from whole tissues and hepatopancreas were prepared and analyzed using HPLC. Toxin accumulation in *Perna viridis*, and *Papia undulata*, an epifaunal and infaunal species was compared both from wild and controlled laboratory conditions. Larger shellfish like *Atrina pectinata* was dissected to separate adductor muscle and other parts, and analyzed for PSP as described above. Specimen of *P. viridis* collected from the same area was used as control. Shellfish toxicity is highest in epifaunal followed by partially exposed benthic and the infaunal species. Results revealed individual toxicity variations among shellfish species ranging from non-detectable to >2000µgSTXeq/specimen. Verification experiment on a laboratory simulated habitat showed that burrowed *P. undulata* did not accumulate considerable toxin amounts unlike that of control, *P. viridis* after exposure to millions of cells. Pbc cells were observed in P. viridis diet but not in *P. undulata.* Seasonal toxin profiles revealed that initially, GTX5/B1 dominates over other analogues in shellfish tissues during peak of bloom and shift to STX or NeoSTX after certain period of time. Toxicity correlates well with shellfish gut size rather than body size. Adductor muscles of *A. pectinata* accumulate minimal toxin level in contrast with the results obtained from other tissues and indicator shellfish used.

Production of Paralytic Shellfish Toxins by Dinoflagellates May Be Controlled By Two Sets of Proteins on Two Levels

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Despite continuous efforts to elucidate mechanism of production of paralytic-shellfish-toxins (PSTs) in dinoflagellates, the exact mechanism is unclear. We had obtained a non-toxic *Alexandrium catenella* strain and a PST-secreting strain. These 2 strains have a 97% homology in their ITS DNA sequences. Difference in toxicities in these 2 strains may due to genetic differences, hence different gene products and different machineries. Protein expressions of these dinoflagellates were compared by 2-dimensional-gel-electrophoresis followed by MALDI-TOF mass spectrometry. About 60 odd proteins were found to be differentially expressed. To proceed further with this work, a transcriptome database was built in-house using the said dinoflagellates grew in mid-log phase in normal K medium. 70,000 genes out of a total of 100,000 were annotated. Searched against this transcriptome, several down-regulated proteins in the non-toxic *A. catenella* were identified and they are related to metabolism. Whether a low metabolic rate constitute low-PST-secretion rate is currently unknown. On the other hand, when the toxic A. catenella was grown in K medium with 1/10 amount of phosphate, the toxicity increased significantly and was in a disproportionate manner to the increase in cell size/volume. Using the same proteomic approach, protein expressions from samples with or without phosphate limitation was compared with 2DE. Another set of differentially expressed proteins (about 6) were found and some of them were identified. These proteins are different from the first set, highlighting that the increase in toxicity in these dinoflagellates in phosphate-limited condition may have turned on/off another set of proteins which bring about the difference in toxicity. To conclude, it is envisioned that the production of PSTs from *Alexandrium catenella* may be controlled by 2 sets of genes operating at the genomic and epigenomic levels.

Vibrios in the Environment

Vibrio parahaemolyticus Distribution in Southern Chilean Intertidal Shellfish (*Mytilus chilensis* and *Venus antiqua*)

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We evaluate the level of total and toxigenic *Vibrio parahaemolyticus* in intertidal *Mytilus chilensis* and *Venus antiqua*, around coastal lines of the Región de Los Lagos of Chile, five of them located around continental inlets and five located in the Chiloé's Island, by two sampling campaign performed between April to May of 2011 and January to March of 2012. This area has been recognized has a *V. parahaemolyticus* hot spot from several years. Samples were processed by NMP-qPCR by using probes directed to tlh and tdh genes. In each location, the shellfish were split for processing both directly and after a post thermal treatment (18 h at 28 °C). The values becomes saturated (>3500 NMP/g for +tlh) only in the incubated sampled for both shellfish, but mainly around continental inlets and not around Chiloé's Island. The detection for +tdh was always negative. These results indicate a very narrow distribution of *V. parahaemolyticus* populations in the Southern Chilean inlets. Additionally, the significative difference of values achieved for direct and post incubated samples confirmed the hypothesis that post harvesting temperature is a key factor for prevention of *V. parahaemolyticus* hazards in shellfish row consumption.

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Investigation of the Presence of Type Three Secretory Systems 2 (T3ss2) In *V. parahaemolyticus* Strains Isolated In Italy

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The presence of genes related to type three secretion systems 2 (T3SS2) of *V. parahaemolyticus* was investigated in 30 shellfish or environmental and 3 clinical strains of *V. parahaemolyticus* isolated in Italy between 2004 and 2011. These isolates were part of a larger collection of *V. parahaemolyticus* and possessed the genes coding for those consider the major virulence factors of *V. parahaemolyticus*, the thermostable direct haemolysin (TDH) or the TDH-related haemolysin (TRH). Four (vscC2, vscS2, vopB2, vopC) and five (vopP,vscS2, vscC2, vopB2, vopC) genes characteristic of the T3SS2βand T3SS2α were investigated, respectively. Of the clinical strains, the two O3:K6 tdh+/trh- isolates had all five genes of the T3SS2α and were negative for all four genes of the T3SS2β. The last clinical strain was a trh+/tdh-*V. parahaemolyticus*, which amplified by PCR all four T3SS2βgenes searched, while was negative for the presence of all T3SS2 αgenes. The trh+ isolates from shellfish or environmental samples confirmed the same distribution of T3SS2β and T3SS2α genes of the trh+ clinical strain. In conclusion, no differences in the distribution of T3SS2 genes were identified between the clinical and food or environmental *V. parahaemolyticus* isolates harbouring the trh gene. All trh+/tdh- isolates, independently of the origin, were confirmed to possess the T3SS2β, which is consistent with previous reports. The two tdh+/trh- clinical isolates possessed all the T3SS2α.

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Development of an Electrochemical Immunosensor for the Early Detection of *Vibrio* Species

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Current technology is insufficient for rapid on-site identification of the causative agents for waterborne diseases and existing time-consuming detection results in delayed management decisions. Vibrio bacteria are proliferate in disturbed aquatic environments, and can cause major disease outbreaks that affect a large range of organisms, including humans. They represent the perfect target for an early warning diagnostic tool to safeguard water resources and the aquaculture industry. This work describes the steps involved in the development an electrochemical biosensor for in situ detection of vibrio species in aquatic environments where *Vibrio parahaemolyticus* was used as the model organism. *V. parahaemolyticus* was captured onto the surface of a low-cost screen-printed electrode using an optimized sandwich-type capture format with anti-vibrio antibodies. Detection is based on the electrochemical monitoring of the activity of horseradish peroxidase (HRP), an enzyme label coupled with the secondary antibody, through its catalysis of hydrogen peroxide (H2O2) in the presence of the mediator hydroquinone (HQ). The signal is monitored using amperometry, allowing a significant improvement of the limit of detection compared to colorimetric methods such as Enzyme Linked Immunosorbent Assay (ELISA). Development of diagnostic tools for specific detection of such pathogens will contribute to more effective management of water quality through better monitoring, rapid assessment as well as identification of sources contributing to the spread of disease.

Incidence of *Vibrio parahaemolyticus* in Oysters of Central Luzon, Philippines

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Codex guidance recommends all shellfish-producing countries asses total and pathogenic Vibrio levels in commercial shellfish production areas. As no data existed on local Vibrio populations in the Philippines, we conducted a study to assess the prevalence and densities of *V. parahaemolyticus* populations in oysters from the major producing areas of Central Luzon, Philippines. Eight sampling stations were established and oysters were collected from these stations during the rainy (November 2011) and dry (April 2012) seasons. A most-probable number (MPN) enrichment followed by colony isolation on TCBS agar was used during both collection seasons. Presumptive *V. parahaemolyticus* colonies were identified by the presence of typical (sucrose negative) colonies on TCBS. Additionally, for samples collected during the dry season, MPN-real-time PCR (RTi-PCR) was employed for identification of total and pathogenic *V. parahaemolyticus*: the BAX® Vibrio Assay was used for total *V. parahaemolyticus*, while pathogenic *V. parahaemolyticus* was determined using a RTi-PCR assay that targets the thermostable direct hemolysin (tdh) and the tdh-related hemolysin (trh) genes. Presumptive *V. parahaemolyticus* were isolated in samples from seven of eight sites during the rainy season and all eight sites during the dry season. Total and pathogenic *V. parahaemolyticus* was detected in all samples collected during the dry season by RTi-PCR. Total *V. parahaemolyticus* densities ranged from 290-15,000MPN/g. Densities of tdh-positive *V. parahaemolyticus* ranged from 0.23-93 MPN/g, while trh levels ranged from 0.93-240 MPN/g. These levels are consistent with levels found in shellfish from countries that routinely report *V. parahaemolyticus* illness. Seafood-borne *V. parahaemolyticus* illness has not been reported in the Philippines, but this may be attributed to the absence of an established reporting system. The data presented here indicates the potential for *V. parahaemolyticus* illness from consumption of oysters harvested from the Central Luzon area.

Marine Biotoxin Monitoring

Keynote

A New High Resolution LC-MS Method for Identification, Quantification and Monitoring of Palytoxin-Like Compounds in Molluscs

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In the last decade, blooms of *Ostreopsis* ovata have been repeatedly reported along the Mediterranean coasts of Europe and North Africa posing serious risks to human health. Liquid chromatography-mass spectrometry (LCMS) played a key role in the identification of palytoxin and its new analogues, ovatoxin-a to -f, in the algal extracts. Ovatoxin-a generally represents the major component of *O. ovata* toxin profile, accounting up to 55% of the total toxin content, with the remaining 46% being constituted by the whole of the other ovatoxins, and palytoxin being present in very minute amounts . Occurrence of *O. ovata* may result in palytoxin contamination of seafood and, in order to prevent sanitary risks, the need exists to develop efficient extraction procedures to be coupled to rapid and sensitive detection methods of palytoxin -like compounds in seafood. In the present study, the best conditions for extraction of palytoxin-like compounds from seafood and their quantification by using liquid HR LC-MS are presented. The method includes a simple extraction procedure, a SPE (solid phase extraction) clean-up step and HR LC-MS detection in full MS mode; it allows to detect palytoxin-like compounds in shellfish at the tolerance level of 30 ug/kg of shellfish proposed by the European Food Safety Authority (EFSA). Application of the method to the monitoring of ovatoxins in shellfish collected along Italian coasts is also presented.

The Assessment of Paralytic Shellfish Poisoning Occurrence and Toxin Profiles in Bivalve Molluscs from the United Kingdom During the First Five Years of Official Control Monitoring Using Liquid Chromatography

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Paralytic Shellfish Poisoning (PSP) toxins are a family of saxitoxin analogues known to be potent neurotoxins and which are present in some blooms of marine phytoplankton. Bivalve molluscs may feed on toxic strains of plankton and periodically accumulate harmful levels of these toxins, resulting in a serious risk to the shellfish consumer. Consequently, to ensure consumer protection, monitoring of PSP toxins from designated shellfish harvesting areas is a statutory requirement. The European Union reference method is the mouse bioassay (MBA), but in recent years there has been a slow but steady move away to the use of alternative methods, particularly those employing liquid chromatography with fluorescence detection (LC-FLD). Following a period of method refinement and validation at our laboratory, the AOAC 2005.06 LC-FLD method was implemented in Spring 2008 into the UK official control monitoring programme for the screening and quantitation of PSP toxins in mussels. Subsequent work resulted in the implementation of the method for all species of interest to the UK toxin monitoring programme. Providing greater sensitivity, and for some species improved accuracy than the MBA, the LC method is advantageous as it supplies additional information including early warning of toxic events and provides a full indication of toxin profiles present. With 5 years of monitoring by LC completed, this presentation assesses the implications of official control monitoring using LC methods and examines the data generated in the UK over this time period. Specific focus is given to the spatial and temporal distribution of toxic events, early warning statistics, and the distribution of different profile fingerprints around the coastline. The assessment will discuss the suitability of PSP testing methods (including commercial kits) available to the industry for end product testing and highlight the growing understanding of risk assessment in relation to PSP and shellfish harvesting within the UK.

Diagnostics for Saxitoxins Using Saxiphilins

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The saxitoxins are a globally distributed, naturally occurring contaminant which can cause fatal neurotoxicity if ingested by humans and other animals. They occur in freshwater cyanobacteria and marine microalgae, contaminating the water column and moving through the food chain as they are ingested and bioaccumulated. Saxiphilins are a protein found in the circulatory fluid of an increasing number of vertebrates and invertebrates. It is a transferrin, proteins known more for their iron binding and sequestration properties. Saxitoxins have only been found in the marine and freshwater environment, yet many terrestrial organisms possess this unusual saxitoxin binding protein in their blood, providing a mystery as to its biological role with a role in bioaccumulation or chemical defense being possible. Specifically, animals found to date to possess saxiphilin include lizards, amphibians, fish, spiders, scorpions, insects, crabs, centipedes, molluscs and onycophorans, most of which do not harbour or bioaccumulate saxitoxins. To satisfy the increased demand for rapid toxin tests, saxiphilin has been used to develop rapid, microtitre plate assays for the saxitoxins, and in a bench-top biosensor. This latter aspect was enabled by the biotinylation of saxitoxin creating a novel bifunctional analogue of these toxins. The original saxiphilin, that from the North American bullfrog, has also been expressed in a yeast vector with a His-tag and an expression signal enabling the large scale production, purification and coating of inert surfaces for biodetection. Many test samples contain a multitude of saxitoxin analogues with very different potencies and understanding how toxin mixtures behave in these diagnostic systems is critical to their wider deployment. A mathematical model that explains the behaviour of very complex saxitoxin mixtures has been developed and validated and may be extended to other bioactive chemicals. Each isoform of saxiphilin has a different pattern of sensitivity to the different saxitoxins and this is a potentially powerful property upon which to develop tests with a very broad coverage of all of the saxitoxins. By combining different isoforms with divergent sensitivities to the various classes of the saxitoxins, the spread of toxin binding strength against these isoforms may provide an indication of the classes of toxins present.

Persistence of YTXs in Shellfish During Two Successive Blooms of *Gonyaulax spinifera* and *Protoceratium reticulatum*. The Need of LC-MS/ MS in Monitoring Programmes

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Yessotoxins are regulated toxins in shellfish whose toxicity in humans is being reviewed. The present Maximum Permitted Level (MPL) in the European Union is 1000 ug.kg-1 of YTX equivalents. Presently, the official reference method is LC-MS/MS but YTXs can still be evaluated using the MBA by implementation of annex of the current Standard Operation Procedure (SOP) number
5. Currently the monitoring system for lipophilic toxins in Catalonia implements the MBA in routine but uses LC-MS/MS during specific situations at risk. During two successive blooms of the species *Gonyaulax spinifera* and *Protoceratium reticulatum* toxins of the family of YTXs were followed by MBA and LC-MS/MS. A positive result using the MBA for lipophilic toxins was obtained in july 2012 leading to closure of the harvesting area. LCMS/MS analysis confirmed presence of Yessotoxin below the MPL and traces of Okadaic acid demonstrating the previous MBA was a false positive result. Phytoplankton monitoring confirmed presence of *G. spinifera* at maximum densities of 1000 cells.L-1 and *Dinophysis saccculus* at densities of 60 cells.L-1. Implementation of annex of SOP 5 in order to manage the episode in the presence of YTXs gave negative results both for YTXs and for other lipophilic toxins. The shellfish area was consequently opened three days later. The bloom of *G. spinifera* was followed by a bloom of P. reticulatum at maximum densities of 1800 cells.L-1 leading to a change in toxin composition in mussels. Analysis performed by LC-MS/MS showed a decrease in YTX but an increase in Homo-YTX, 45-OH-YTX and 45-OH-HomoYTX. According to the current toxicity factors proposed by the European Food Safety Authority (EFSA) levels of total YTXs reached 985 ug.kg-1. During these two successive episodes, implementation of the LC-MS/MS method favoured comprehension of the toxins present in shellfish for the correct management of the shellfish production area.

Virus Uptake and Elimination Dynamics

Keynote

Shellfish and Norovirus: What Do We Know?

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Noroviruses (NoVs), being the most common viral agents of acute gastroenteritis in humans, are discharged at high concentrations into the environment. As they are very resistant to inactivation, the sanitary consequences are contamination of food including molluscan shellfish. Bivalve molluscan shellfish, such as oysters filtering large volumes of water as part of their feeding activities, are able to accumulate and concentrate different types of pathogens, including noroviruses, from fecal human pollution. Since noroviruses are known to attach to carbohydrates of the histo-blood group family, we conducted a panel of tests using immunohistochemical analysis and demonstrated a specific binding to digestive ducts (midgut, main and secondary ducts and tubules), involving carbohydrate structures with a terminal N-acetylgalactosamine residue in alpha linkage (A-like carbohydrate structure indistinguishable from human blood group A antigen) for NoV GI particles.Extending these tests to other NoV strains showed that the diversity in terms of carbohydrate-binding specificities,is reflected in oyster ligands. The GI.1 and GII.4 strains differed in that the latter recognized a sialic acid-containing ligand, present in all tissues, in addition to the A-like ligand of the digestive tract shared with the GI.1 strain. Furthermore, a seasonal effect on the expression of these ligands was detected, most visible for the GI.1 strain, with a clear impact on bioaccumulation efficiency and tissue distribution in oysters.

To complement this approach, analysis of shellfish related outbreak data worldwide show an unexpected high proportion of NoV GI strains. These observations contribute to explain the GI/GII bias observed in shellfish-related outbreaks compared to other outbreaks. We can conclude that oysters are not just passive filter, but can selectively accumulate norovirus strains based on virus carbohydrate ligands shared with humans.

Seasonal Behaviour of Sewage-Derived Norovirus upon Discharge of Sewage Effluent into Coastal Waters

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Norovirus is the leading cause of shellfish-associated gastroenteric illness worldwide. Bivalve shellfish can concentrate noroviruswhen growing waters are contaminated with human faecal matter. According to the European Food Safety Authority, production of shellfish in areas which are not faecally contaminated is the most effective control measure. However, norovirus has been shown to behave differently to typically adopted faecal indicators, which may not adequately contain the risk from human viruses. Identification of suitable waters requires an understanding of norovirus behaviour upon discharge of sewage into coastal waters and identification of areas which remain consistently uncontaminated. However, direct recovery and concentration of viral pathogens from coastal waters is problematic, often requiring large sample volumes and providing only a time-specific measure of contamination. Therefore, this study exploited the accumulation potential of *Mytilus edulis* and employed a molecular method derived from the proposed international standard for quantification of norovirus within digestive tissues.

In March 2012 an array of sentinel moorings was deployed, centred about the outfall of a wastewater treatment plant operating only secondary treatment without UV or other disinfection. Caged mussels were sampled monthly for information regarding exposure to norovirus and *E. coli* and accumulation/elimination kinetics. The spatial pattern of norovirus contamination observed in caged mussels was used to validate the existing hydrodynamic model of the effluent plume and to confirm pronounced seasonality. In April, high levels of norovirus were detected within the modelled plume, whilst norovirus impact upon nearshore bathing waters and offshore waters identified for potential bivalve production, was not observed during the study period. A different spatial pattern was observed for *E. coli*: Nearshore sentinels demonstrated considerable contamination, suggesting secondary or multiple, possibly non-point, sources of *E. coli* or differential behaviour post-discharge.

The results from this ongoing study may generate important recommendations for the local shellfish industry.

Human Enteric Viruses inAustralian Bivalve Molluscan Shellfish

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Outbreaks of gastroenteritis, caused by norovirus-contaminated shellfish, occur worldwide and have occasionally been linked to shellfish of Australian origin. There is, however, a paucity of data on norovirus occurrence in Australian shellfish. This, together with outbreaks and the impending introduction of international policies for norovirus in shellfish, provided impetus to investigate the prevalence of norovirus in Australian oysters.

Norovirus and *E. coli* occurrence in Australian oysters from six growing areas in the three main oyster producing states (two areas per state) was assessed. Oysters were sampled on four occasions, through four seasons, during 2010 and 2011 and norovirus GI, GII and *E. coli* prevalence determined. Norovirus GII was detected in two of 120 (1.7 %) samples. Norovirus GI was not detected. Five samples (4.2 %) exceeded the regulatory level of 230 *E. coli* per 100g. No human illnesses due to norovirus-contaminated oysters were reported during the survey period.

In a spatial and temporal study of norovirus and *E. coli* in oysters after a raw sewage overflow into a river estuary, no Hepatitis A virus was detected.

The results of sequence analysis of the virus strains detected in the norovirus-positive oyster samples will be presented and their significance discussed.

Bivalve Purification – Do Inspection and Approval Procedures Contribute to Reduction of Risk For Norovirus?

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Norovirus is the principal agent of bivalve shellfish– associated gastroenteric illness worldwide. One option for controlling the risks associated with shellfish originating from moderately polluted areas is for them to undergo purification in tanks of clean seawater to purge themselves of sewage contaminants. Whilst bacteria are rapidly and effectively removed during this process it is well documented that purified shellfish shown to be free of *E. coli* (the statutory indicator) have been associated with outbreaks of norovirus. Reliance on end product testing for *E. coli* following purification would therefore not appear to guarantee 'safe' shellfish. European Regulation EC 853/2004 is not overly prescriptive in terms of how bivalve purification should be carried out. This paper will describe the current approach adopted in England and Wales to the statutory approval of purification systems required under these Regulations. This approach is intended to optimise the removal of all pathogens (including norovirus) and not just *E. coli*, the statutory bacterial indicator. Examples of good, and poor, practice will be discussed. However, there is limited quantitative data on the removal of norovirus from shellfish under purification conditions to establish the relationship between operational practices and parameters and norovirus risk. Preliminary investigations are described applying the recently developed quantitative CEN standard method on norovirus behaviour in *Crassostrea gigas* purified in a standard small scale recirculating commercial system in comparison with FRNA bacteriophage and *E. coli*. Parameters evaluated include water disinfection system (ozone and UV), time and temperature. Results are discussed in relation to further evidence needs for policy development.

Biotoxin Uptake and Elimination Dynamics

Keynote

DissolvedAzaspiracids are Absorbed and Metabolized By Blue Mussels (*Mytilus edulis*)

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Azaspiracid (AZA) poisoning has been reported following consumption of contaminated shellfish. The direct relation between mussel contaminations and a small Dinophyceae, *Azadinium spinosum*, has been shown recently. The organism produces AZA1 and -2, while AZA3 and other analogues are metabolic products formed in shellfish. We evaluated whether mussels are capable of accumulating AZA1 and -2 from the dissolved phase, and compared the toxin profiles of these mussels at 24 h with profiles of those exposed to *A. spinosum*. We also assessed the possibility of preparative production of AZA metabolites by exposing mussels to semi-purified AZA1. Mussels were exposed to dissolved AZA1+2 (crude extract) at 7.5 and 0.75 µg L-1, dissolved AZA1+2 (7.5 µg L-1) in combination with Isochrysis affinis galbana, and with lysed and live *A. spinosum* cells at 1 × 105 and 1 × 104 cell mL-1. Digestive glands (DG), gills and remaining flesh (RF) were then dissected and analysed. Mussels accumulated AZAs in excess of the regulatory limit except at the lower levels of dissolved AZAs. The toxin profile of the mussels varied significantly with treatment. The gills contained 42–46% and the DG 23–24% of the total toxin load using dissolved AZAs, compared to 3–12% and 75–90%, respectively, in mussels exposed to live *A. spinosum*. Exposure of mussels to semi-purified AZA1 produced the metabolites AZA17 (16.5%) and AZA3 (1.7%) after 4 days of exposure, but the conversion efficiency was too low to justify using this procedure for preparative isolation. These observations prompted us to search in the literature for other examples of toxin uptake from the dissolved phase. Information on brevetoxins, saxitoxins and microcystins shows that other aquatic organisms also absorb toxins through this route. Recommendations for further studies are given to clarify the quantitative importance of the uptake from the dissolved phase.

Putative Vectoral Transfer Dynamics and Biotransformation of Azaspiracids in Components of Marine Planktonic Food Webs

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In recent years the cause of azaspiracid poisoning (AZP) in humans has been linked to the presence of the dinoflagellate *Azadinium* as the proximal source of azaspiracids (AZAs). In particular, the species *A. spinosum* is known to produce a few AZA analogues, which are then subject to biotransformation in bivalve molluscs. Such accumulation and subsequent biotransformation processes can yield >30 AZA analogues, many of unknown mammalian toxicity. Although association of AZAs with *Azadinium* species is now uncontested, bloom dynamics and transfer kinetics and pathways of AZAs into bivalve molluscs remain poorly described. Blooms of *Azadinium* species have not been reported to coincide closely in time and space with maxima and persistence of AZA toxins in bivalve shellfish. This may reflect observational deficiencies in toxic plankton and toxin monitoring programmes but also opens the possibility of alternative AZA sources (i.e., cryptic AZA-producing species) or toxin vectors, e.g. transfer via the pelagic food web. Thus in principle AZAs could accumulate in bivalve shellfish following feeding upon AZA bound to suspended particulates or via plankton vectors (e.g., copepods, tintinnids or other microplankton grazers) that have fed upon toxigenic *Azadinium* cells. Here we explored the possibility of such vectoral transfer in grazing experiments conducted with toxigenic *Azadinium* together with various grazers, including within natural phytoplankton assemblages. Preliminary experiments with copepods indicated a general negative grazing selection against *Azadinium* in comparison with other plankton within the same size range. Furthermore, according to LC-MS/MS analysis, there was little evidence of substantial AZA accumulation over extended time intervals (≥24 h) and few signs of significant biotransformation of AZAs as were observed from bivalve molluscs. Whereas these preliminary experiments do not contradict the possibility of vectoral transfer of AZAs to bivalves they tend not to provide strong support for this mechanism, at least for prey-predator combinations thus far selected.

The classification of shellfish aquaculture areas and investigation of Diarrheic Shellfish Toxins using SPATT in Jiaozhou bay Yellow Sea China.

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In recent years the output of shellfish in China has increased significantly. The government pays much attention to shellfish safety especially during the peak aquaculture periods. Thus sanitation rules for aquaculture areas have been developed for both domestic consumers and to meet export requirements. The classification rules of aquaculture areas will be discussed. Diarrheic Shellfish Toxins (DSTs) are an important factor when considering shellfish safety. An investigation was undertaken using SPATT in Jiaozhou Bay, Yellow Sea, China. The main DST toxins, OA, DTX-1, PTX were analyzed both in seawater and shellfish, and the variation in toxin content across different seasons and areas will be discussed.

Alexandrium inAustralian coastal waters: Temperature and Saxitoxin Accumulation in Commercial Shellfish

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Accumulation of paralytic shellfish toxins (PST), produced by marine planktonic dinoflagellates, can occur in all major commercial shellfish species. Aside from the potential risk to human health, aquaculture industries have reported severe economic losses. Members of the genus *Alexandrium* are known PST producers. Since 2005, there has been an apparent increase in reports of *Alexandrium* blooms in New South Wales (NSW), with species causing over 50% of algal related shellfish harvest zone closures. Our current knowledge of the distribution of the species in NSW is examined with an emphasis on high-risk zones and the potential for the development of predictive models.

Globally, the increase in frequency and intensity of harmful algal events has been linked to climate change. It is expected that increases in seawater temperature will influence both the distribution and abundance of *Alexandrium* species, and the dynamics of toxin uptake in shellfish. To date, it is unknown how increases in temperature will affect commercial bivalves in the presence of PST producing dinoflagellates. To investigate this, commercial species of oysters (Sydney rock and diploid and triploid Pacific oysters) were fed with cultures of *Alexandrium minutum* (up to 300 cells ml-1) over a period of 17 days at two constant temperatures (22°C and 27°C). The effect of ambient temperature on PST uptake and metabolism in the bivalves is presented and the role of polyploidy in influencing toxin metabolism is also discussed.

Risk Assessment

Keynote
Microbiological Risk Assessment (MRA) – Useful or Just Feel-Good?

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In the mid-1990s risk assessment became a discipline in its own right – as an extension of HACCP (severityof the hazard and likelihood that it would occur). In 1999, the FAO undertook the first risk assessment on seafood, assembling an expert consultation on the trade impact of Listeria in fish products. The FAO consultancy spawned a series of risk assessments commissioned by FAO and WHO, known as the Joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA), intended to provide advice to Codex. In the seafood arena, a JEMRA team assessed risks of vibrios in oysters, clams, prawns and finfish. Over the past decade, governments and organisations have undertaken numerous risk assessments on a number of hazard:product pairings – but to what effect? The present paper reviews seafood risk assessments and questions why risk managers seem unable to embrace risk estimates unless the likelihood of an adverse event is disappearingly low. It is noted that, as this abstract was being written, an Italian court has convicted six scientists of manslaughter for underestimating the likely effect of an earthquake which, when it came, killed 309 people in L'Aquila. The case prompts the question: will the six years in jail and the $10 million in costs and damages incurred by the six scientists prove a deterrent to the future of risk assessment in general, and of MRA in particular?

Risk Assessment to Paralytic Shellfish Poisoning (PSP) Due to the Bivalves Consumption of the Population in Nha Trang City, Vietnam

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The present study is the first in Vietnam to evaluate the risk of consumer to PSP (paralytic shellfish poisoning) due to the bivalves consumption. To achieve the shellfish contamination data, the PSP concentrations were determined by the High Performance Liquid Chromatography (HPLC). The results show that the PSP concentrations in bivalves are lower than the limits fixed by the Vietnamese and international regulations. In associating the consumption and contamination data by the probabilistic approach with the aid of @Risk® software, the exposures of studied population to PSP due to consumption of bivalves are low. The comparisons of these levels with the ARfD (Acute Reference Dose) of PSP established by EFSA (European Food Safety Authority) permit to conclude that there is no risk concerning the exposure to PSP due to the bivalves consumption of consumer in Nha trang city, Vietnam.

Keywords: risk assessment, bivalves, PSP, paralytic shellfish poisoning

Risk Assessments to Underpin the South Australian Shellfish Program

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As it is well documented, shellfish are a high risk food as they bio accumulate a variety of contaminates from the aquatic environment. In Australia the majority are eaten raw or only slighty cooked. Many risks are associated with the consumption of shellfish from harvesting areas; entric viruses, microbiology – indicator species, *E. coli*, biotoxins and chemicals – heavy metals. As the State shellfish control authority it is essential that risk assessments are performed to ensure risk management strategies are implemented so contaminated shellfish do not reach the end consumer.

There are also other risk assessments associated with international markets that may not pose a risk within the domestic markets. The following presentation will focus on the risk assessments performed by the South Australian Shellfish Quality Assurance Program to provide consumer protection and allowing the development of a sustainable shellfish industry domestically and internationally.

Discovery of *Cryptosporidium* and *Giardia* in ContaminatedAmerican Oysters from Prince Edward Island, Canada:Are Current Canadian Shellfish Sanitation Program Guidelines Enough?

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Cryptosporidium parvum and *Giardia duodenalis* are zoonotic parasites commonly found in livestock that are capable of causing gastroenteritis in humans. Both parasites bio-accumulate in filter feeding bivalves and are retained for longer periods than bacteria. The Canadian Shellfish Sanitation Program (CSSP) guidelines report shellfish harvesting sites as prohibited, approved, or conditionally approved/conditionally restricted for fishing based on a multitude of factors, but areas that are temporarily closed due to sewage contamination may reopen after bacteriological quality of the water and shellfish has again met the approved area standards. Opportunistic samples of American oysters (*Crassostrea virginica)* collected from various locations within the prohibited sector of the Hillsborough River system in Prince Edward Island, Canada, were collected in 2011 for initial parasitological analysis. Results reveal that between 12-77% and 0-46% of oysters were positive for Cryptosporidium and Giardia, respectively, with the highest parasite burden present in sites that were within close proximity to the sewage outflows. Due to the high parasite prevalence and recurring sewage treatment facility overflows into this system, it is prudent to investigate whether current growing site classifications should incorporate parasitological analyses in areas subject to frequent human or environmental fecal contamination. A more detailed sampling of sites was conducted in 2012, including locations within the prohibited, conditionally restricted, and approved regions, at different time periods. The prevalence of *Cryptosporidium* and *Giardia* in these samples will be reported at this meeting and will provide the first evidence regarding the effectiveness of current CSSP site classification guidelines for the prediction of protozoan parasites in high risk areas.

HAB Monitoring, Management and Mitigation (I)

Keynote

Bivalve Biotoxins Monitoring, an Integrated Approach

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Since measures were initially conceived to avoid placing toxic shellfish on the consumer marketplace, there has been a continuous development of several advanced monitoring and management methodologies. At the same time impacts on public health from toxic shellfish have been widely reported, leading to increased scientific and regulatory attention. In each toxic outbreak we learn lessons, so by now existing technologies and methodologies should guarantee consumer safety while also protecting industry from these events with appropriate management programmes.

Today the diversity of data available to regulatory authorities include very precise measurements of toxin concentration in shellfish and chemical analogues of these toxins, quantification of toxin containing phytoplankton by cell counts, and molecular technology. Additional information include in situ estimation of blooms by fluorescence biomass, models of physical oceanographic parameters combined with biological and biogeochemical processes, and the availability of satellite products giving synoptic snapshots of chlorophyll, temperature, currents and sea state.

The global population consuming cultured shellfish is increasing, and the role of cultured shellfish as a source of nutrition for human consumers is increasing in importance each year. The protection of consumers from the risk of known biotoxins in shellfish is essential. However, management programmes can only be successful when they go beyond the legislation and take all known risks into account and then apply rigid regulatory programmes in order to assure human protection. Utilising information from research programmes and ongoing monitoring informs the regulators of existing risk and any potential novel or emerging risks which require evaluation. Combining statutory requirements with additional information from diverse and in some cases novel sources is necessary to protect both consumer and industry from outbreaks, and to promote the development of the shellfish industry in supplying the global markets.

What is in the Bucket? Molecular Diversity Assessment of Diversity

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Assessments of biodiversity and the detection of potential harmful species are crucial for monitoring programs. Within the last years harmful algae blooms (HABs) are increasing in terms of frequency and intensity. Beside the impact of HABs on ecosystem functioning, also services such as in the dynamic field of aquaculture are particularly affected. More and more molecular markers and tools have been developed and applied to detect and quantify HAB species. However, in most cases the approaches are species targeted and can only detect previously defined species. For aquaculture industry it is important to have a frequent inventory of the potential hazard biodiversity even for rare species which might become only abundant under certain climatic and hydrographic. Here we present an amplicon sequencing based approach, where diversity can be assessed on species/taxa level even for rare species which were not known to be present before. A data set from the North Sea is used to show the work flow and demonstrate the outcome of a detailed study on dinoflagellate diversity with emphasis on the Amphidomataceae including the toxigenic Azadinium species. A diverse set of dinoflagellate species were detected in all samples and we identified 3 potentially new species (OTUs) within this family pointing out a higher risk of more unknown azaspiracid-producing species in the North Sea. This information in turn allows for a specific search for known and new related toxins in these new taxa by chemical methods such as LC-MS/MS.

Morphological, Molecular and Toxicological Characterization of the Diatom Genus *Pseudo-nitzschia* In SoutheasternAustralian Coastal Waters

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Pseudo-nitzschia H Peragallo is a marine diatom genus found worldwide in polar, temperate, subtropical and tropical waters. It includes toxigenic representatives that produce domoic acid (DA), a neurotoxin responsible for Amnesic Shellfish Poisoning (ASP) and the causative organism for at least three human deaths in eastern Canada in 1987 (from the consumption of cultured mussels). Whilst unequivocal identification of this genus is difficult using routine light microscopy, more detailed studies have revealed 37 species worldwide with 14 of these confirmed as potential DA producers. In this study we characterized the species of *Pseudo-nitzschia* found in south-eastern coastal waters of Australia for unambiguous and future identification. Clonal isolates were sub-sampled for i) light and transmission electron microscopy; ii) DNA sequencing, based on the nuclear-encoded partial LSU and ITS- ITS1, 5.8S and ITS2 rDNA regions and, iii) DA production as measured by liquid chromatography–mass spectrometry (LC-MS/MS). More than 10 Australian species of *Pseudo-nitzschia* were confirmed morphologically and molecularly, with *P. micropora* reported for the first time in the southern hemisphere. Toxigenic species included *P. multistriata* (11 pg DA cell-1) and *P. cuspidata* (25.4 pg DA cell-1), the latter species being responsible for a 16 week closure of Sydney rock oyster *(Saccostrea glomerata)* harvest areas in Wagonga Inlet, New South Wales, in 2010 (max. concentration of 34 mg DA kg-1 oyster tissue). Cultures of *P. micropora*, tested for DA production for the first time, proved non-toxic. Species resolution and knowledge on toxicity of local *Pseudo-nitzschia* spp. has important implications for monitoring and management of shellfish harvest areas.

Aquatic Microbial Early Warning System

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Aquaculture production is compromised by a range of factors including biosafety, water quality and other environmental conditions. The ability to manage these adverse situations depends on the time available to implement mitigation options like controlling feed, harvesting or even medicating the cultured shellfish. A key to improve management is therefore to improve forecasts of environmental conditions. In the following we present a project that delivered an improved forecast of organism blooms (pathogenic and HABs) that threaten aquaculture production, thereby improving the management options.

Over the past two years a project in Singapore made use of advanced monitoring and modelling tools to improve predictions of microbial (bacterial, phyto- and zooplankton) community behaviour in fresh and marine water. We adopted a genomic-based analysis approach of microbial aquatic communities in combination with a detailed (weekly and biweekly sampling) monitoring of physical, chemical and biological parameters. The results were used to develop molecular probes of keystone organisms indicative of community composition changes. The molecular probes were implemented on an automatic environmental sample processor (ESP) from Spyglass that allows a semi-quantitative assessment of several organisms using sandwich hybridization. Next detailed physically based hydrographical and ecological modelling using the MIKE 3 and ECOLAB platform was used to establish a numerical description of the environment. The models were calibrated to the monitoring data and validated against a detailed monitoring campaign conducted towards the end of the project. The established near-real time monitoring system together with the advanced modelling tools significantly improved ecological forecasts compared to conventional technology. The system components are now available to develop detailed forecasting systems for the aquaculture industry.

Risk Management Approaches

Keynote

Virtual Shellfish Quality Assurance Managers – A Future Possibility?

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Tasmania has long been using remotely sensed salinity, rainfall and river-flow data to aid in shellfish growing area management. Advances in ICT have provided a platform to amalgamate the sensor data with the shellfish QA management system to provide a cost effective method of determining the safety of harvesting from each growing area in real time. Furthermore, a predictive capability for the near future will be developed, based on weather and flood predictions.

This decision support tool is being developed as part of SenseT: an ambitious project to achieve substantial social, economic and environmental benefits by harvesting information from a wide range of sensor networks, in a single, large scale system. The open access to information in a single system creates opportunities for innovation and economies of scale.

An increasing array of in situ monitoring tools are becoming available, opening up the possibilities of real time management of many different issues. Combining ecosystem models with live sensor data will not only aid in the management of a current issue in a growing area (such as a toxic microalga), but also indicate when and where this problem might spread to an adjacent growing area, and help determine the appropriate scaling up of monitoring regimes analysis regimes to cover this risk. The concept of SenseT could be applied equally to biotoxin or microbial management: the only limit is the type and number of sensors available, and sensor technology is continuously advancing.

Projects like SenseT will benefit both recreational and commercial shellfish harvesters through a web-based display the current situation. Public health warnings will be readily accessible, closure notices and predictive warnings will become automated. Both public health safety and industry profitably benefit from these new advances.

How far can SenseT and projects like it go towards replacing shellfish QA managers? There are always limits to virtual decision tools when applied to real life situations: data interpretation is more complex than it appears on the surface. However, SenseT has capability for machine learning, providing continuous improvement in management guidance based on information fed in by the "live" shellfish quality assurance manager. Perhaps in the future the role of the shellfish QA manager could change significantly, alongside their virtual partner?

Evaluation of the Sanitary Status of the Dutch Shellfish Production Waters Over a 6 Year Period

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The EU has prescribed specific rules for the control of shellfish production area's (EC No 854/2004) in order to ensure that harvesting area's and their shellfish product do not contain micro organisms and toxic substances in quantities considered dangerous to human health. In the Netherlands a monitoring program is set up according to these regulations, in which the production areas are monitored year round, for *E. coli*, toxic phytoplankton and marine biotoxins.

The sanitary status of the Dutch production waters is evaluated using results achieved over a 6-year monitoring period (2006-2012). In addition to the monitoring plan, presence of viruses in two production areas is discussed

E. coli: According to the EU classification criteria, most Dutch production waters are classified as A. Results over de past 6 years justifies the A-classification. Occasional violation of 230 CFU/100g was always followed by a compliant result of a second sample a few days later.
Phytoplankton and biotoxins: Presence of potential toxic algae is used as an early warning system for presence of biotoxines. ASP en DSP algae are the most predominantly phytoplankton in the Dutch production waters. DSP blooms occur yearly. Occasional ASP bloom with violation of criteria in 2006, 2008 and 2010 was observed. These algae blooms did not lead to the presence of toxins in the shellfish harvested from those areas.

Viruses: Two areas were investigated for the presence of viruses, one in 2007-2008 and one in 2012. Norovirus results showed that no positive batches were found if analysed in pools of 10 animals. When analysed individually, 5% of mussels and oysters were contaminated with norovirus GGII. Hepatitis E virus was found in 4% of the samples, whereas human adenoviruses and human parechoviruses were found in 1% of the samples. Norovirus GGI and enterovirus were not detected.

Molluscan Shellfish Safety Issues in the Pacific Islands: Case Studies from Fiji and New Caledonia

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Bi-valve mollusc aquaculture constitutes the most valuable aquaculture industry among Pacific island countries, however it is for round pearl production from blacklip oyster *Pinctada margeritifera*. Large-scale commercialised fisheries or aquaculture production and export of molluscs for food is conspicuous more by its absence. A major constraint is lack of capacity to assure food safety of edible bivalve molluscs. Major rivers in Fiji support robust fisheries for freshwater mussel *Battissa violacea*, which is a reliable livelihood opportunity for village women with few alternative sources of regular income. Periodically there are cases of shellfish poisoning which leads to this fishery being closed for a time. This shellfish has export potential to Pacific islanders living in Australia and New Zealand, however export licence applications are routinely declined because of the uncertain phyto-sanitary status. Lack of capacity in shellfish food safety thereby results in economic losses for Fiji. Meanwhile in New Caledonia a successful trial of scallop spat collection has led to aquaculture of two species of scallops, *Bractehlamys vexillum* and *Mimachlamys gloriosa.* For two years now, local communities have been farming these scallops commercially in submerged cages. A simple and user friendly depuration system, combined with periodical water quality control protocols, has been implemented in order to avoid food safety concerns. There is also a Pacific oyster *Crassostrea gigas* farm operating in New Caledonia. The approaches being taken toward shellfish safety, to enable these new seafood industries to become established in New Caledonia, are here described.

HAB Monitoring, Management and Mitigation (II)

Keynote

HarmfulAlgal Blooms in theAustralian Region

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While microalgal blooms in a strict sense are completely natural phenomena [*Trichodesmium* cyanobacterial blooms reported by Captain Cook in 1770], since the 1980s their impacts on Australian public health, tourism, and fisheries have increased in frequency, intensity and geographic distribution. To a major extent this reflects increased scientific awareness [e.g. *Anabaena, Cylindrospermopsis* and *Microcystis* cyanobacterial toxins in drinking water reservoirs; pinnatoxins in SA oysters]. In other cases, algal bloom problems reflect increased utilisation of coastal waters for aquaculture and fisheries [fish-killing *Chattonella marina* raphidophytes in Port Lincoln, dinoflagellate *Dinophysis* Diarrhetic Shellfish Toxins in NSW pipis, diatom *Rhizosolenia amaralis* causing bitter mussels in Port Phillip Bay]. Eutrophication has only rarely been rarely invoked as a causative factor [haptophyte *Prymnesium parvum* in NT barramundi ponds, Lyngbya majuscula cyanobacterial "swimmers itch" in Moreton Bay]. Other harmful species have been newly introduced via ship ballast water discharge [dinoflagellate *Gymnodinium catenatum* causing Paralytic Shellfish Poisoning in Tasmania] or exhibit significant range expansions in relationship to climate change [dinoflagellates *Gambierdiscus cf.toxicus* into NSW, *Noctiluca scintillans* red tides into the Southern Ocean]. Algal blooms may also pose unexpected problems for desalination plants. Heightened scientific and regulatory attention has triggered the development of many new technologies (molecular probes, remote sensing) and approaches for monitoring (continuous plankton recorder) and management (clay flocculation) of algal bloom phenomena.

A Short Term HAB Forecasting System for the Irish Mariculture Industry.

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Recent advancements in molluscan shellfish biotoxin monitoring methods and technologies have resulted in improved regulation to ensure produce is safe for human consumption. In Ireland, results from the INAB accredited national biotoxin monitoring programme are distributed to the aquaculture industry, regulatory agencies and public via the internet, SMS and fax within 24-48 hours of laboratory sample receipt. In order for fish farmers to manage the extraction and sale of their product in a sustainable manner, managers want to be able to anticipate the onset of HAB events. A HAB alert system would enable farms to improve harvesting practices (i.e. get bivalve products onto market prior to intoxication event) and ensure that protective measures (e.g. installation of aeration systems) are implemented on time when a HAB is imminent. Such improved decision on risk and crisis management will encourage industry growth.
The EC FP7 funded project "ASIMUTH" seeks to develop a short term HAB forecasting (2-3 day) system for countries (Scotland, Ireland, France, Spain and Portugal) situated in NE Atlantic. In this paper we discuss a web portal "HAB Decision Support System" designed to distribute information (satellite images, model forecasts, ground data) to end users via expert interpretation of results. The success of the 2012 Irish field campaign will be examined. This includes a collaboration with the Irish coast guard to detect high biomass blooms in Irish shelf waters and model their movements. A survey dedicated to assist the validation of the Irish bio-physical models (Karenia mikimotoi and Dinophysis spp.) using satellite imagery, a small autonomous underwater vehicle glider (CTD, oxygen probe and fluorometer on board), ADCP, drogued drifter and traditional CTD casts will be presented.

The First Report on the Detection of Yessotoxin From *Gonyaulax spinifera* in the Benguela Current Upwelling System.

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The Benguela current is one of the four major coastal upwelling currents on the eastern boundary of the ocean basins. This upwelling system provides nutrient-rich deep water to the euphotic zone. This enhances the growth of phytoplankton and can thus facilitate the proliferation of harmful algal blooms towards the end of the upwelling season. One such bloom consisting predominantly of dinoflagellates was detected during late summer of 2011 in the coastal embayment of Walvis Bay, Namibia (23˚S, 14˚30"E). The phytoplankton species composition during this bloom included potentially toxic *Gonyaulax spinifera*. Environmental parameters as well as biological samples (oysters, mussels and phytoplankton) were taken during this event, and the toxin content was assessed using an LC/MS-MS method and DSP MBA for the oysters. Yessotoxin (YTX) and the analogues 45-OHYTX and homo-YTX were the dominant toxins present in the mussel samples at concentrations of up to 5.4 mg YTX equivalents/kg shellfish tissue. The YTX profile of the phytoplankton samples was dominated by Homo-YTX and 45-OH YTX, with YTX accounting for less than 10% of the total YTX content. The highest levels produced were 156.0pg cell-1 with homoYTX being produced at 96.0pg cell-1 . The homoYTX content is three times that of the other homoYTX producing *G spinifera* from the Adriatic sea. This report details the first detection of YTX and its analogues in shellfish samples as well as *G. spinifera* in the Benguela current upwelling system. It also details the first report of *G spinifera* producing 45-OH YTX as one of the dominant YTX analogues.

First Reported Closure of a Classified Mussel ProductionArea in Northern Ireland Due to DomoicAcid

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Testing for Domoic Acid has been part of the Northern Ireland Monitoring programme since 1997 when it was introduced for farmed scallops. It was extended to wild scallops, mussels and oysters in 1999 and whilst levels above the regulatory limit have frequently been detected in whole scallops, the closure in July 2012 is the first to affect mussel production. Northern Ireland has eight production areas centred on the main sea inlets of the coast of Northern Ireland. Approximately 40 beds are classified of which 27 are mussel production areas and the remainder comprise oyster, clam and scallop beds. In accordance with EC 854/2004, water samples are taken fortnightly throughout the year from beds which are in production. Tissue samples for biotoxin analysis are taken from each bed monthly, with sampling rotated to ensure the entire water body is sampled twice per month. Northern Ireland production areas have experienced closures due to biotoxin levels above the regulatory limit in most years however the frequency is low, ranging from 2% of samples in 2008 to 4% of samples tested in 2011. These closures were due to the detection of lipophilic toxins in mussels and oysters and the detection of Domoic Acid in scallops. From mid-June 2012 pseudo-nitzschia counts began to rise at a number of sampling points in Belfast Lough. By mid-July 2012 counts were above 150,000 cells per litre initiating additional water and tissue sampling. Domoic Acid levels peaked at 28ug/g, breaching the regulatory limit and resulting in the closure of the harvesting area. The report illustrates the key role that phytoplankton and tissue monitoring plays in protecting public health and supporting the aquaculture industry, even when the assessed risk of a toxic event is low.

The Sanitary Survey Approach

Moving Toward Scientific Consensus on the Microbiological and Management Aspects of International Shellfish Safety Systems

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Two of the principal shellfish safety systems in the world are those of the United States (US) National Shellfish Sanitation Programme (FDA Guide for the Control of Molluscan Shellfish) and the European Union (EU) Food Hygiene Regulations (principally Regulations (EC) 853/2004 and 854/2004). These systems are not only applied in their own jurisdictions but also in other countries that need to comply with their export requirements. While the programmes are in accordance with the Live and Raw Bivalve Molluscs Section of the Codex Code of Practice for Fish and Fishery Products, there are a number of differences in the scientific and technical approaches and practical implementation between the two systems. This has implications not only for trade between the US and the EU but also for countries that wish to export to both. Co-operative initiatives have taken place to establish clear understanding of the basis for the differences in technical approaches, to advance scientific consensus, and to facilitate moves toward comparability and equivalency. Efforts to date have focused on the approaches employed for shellfish area classification and management. Findings from these endeavours will be reviewed, primarily with regard to sanitary surveys and microbiological monitoring used for establishing shellfish area classifications and management practices that ensure consumer safety.

Sanitary Wastewater SystemAssessments in Canadian Bivalve Molluscan Shellfish Harvest Areas

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Sanitary wastewater systems discharging to bivalve molluscan shellfish harvesting areas were assessed to determine appropriate shellfish area classification under the requirements of the Canadian Shellfish Sanitation Program. The objective of the technical assessments included predicting the extent of impacts following failures under. Assessments included: reviewing the operating history and status of the collection and treatment systems; the collection of samples of influent, post-treatment/pre-disinfection effluent and final disinfected effluent to determine representative fecal coliform concentrations; hydrometric drogue studies of receiving waters; and detailed hydrometric modelling to predict system failure impacts under varying operating and environmental conditions. The assessment results provided a greater range of shellfishery management options in adjacent harvesting areas, with increased confidence in wastewater impact prediction.

A Preliminary Analysis of Risk Factors Involved in the Faecal Pollution of Shellfish Harvesting Areas of the Marche Region By Using Geographical Information System.

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Introduction:

The sanitary survey in shellfish production areas is a compulsory request in every regulation of developed countries. The relevant authorities can use different tools to clarify how and how much the different sources of pollution affect the shellfish harvesting areas. The EU Regulation doesn't give details on how the competent authority must consider all the information found during the sanitary survey. The Istituto Zooprofilattico Umbria and Marche through its Regional Epidemiological Observatory in collaboration with the Italian National Reference Centre for Microbiological and Chemical Control of live bivalve molluscs, worked on an evaluations of individual data of each area for various microbiological parameters. Aim of this work is to show the efficacy of Geographical Information System to identify main human and animal factors affecting faecal contamination of mollusc production areas.

Methods:

The study area are molluscs natural beds and breeds located along the Marche region coast (Italy), for a total of 180 km in length and that extends over a surface of 835 km2. Harvesting areas has been identified, on the basis of coordinates points assigned to natural beds and reported in the regional legislation. Descriptive statistics of Escherichia coli level and the percentage of presence of Salmonella spp. in clams *(Chamelea gallina)* and mussels *(Mytilus galloprovincialis*) collected between Jan 2012 and Dec 2012 have been used to identify most contaminated areas. A Geographical Information System (GIS) has been used to identify risk factors spatially located in correspondence of the main contaminated areas and involved in faecal contamination. On the basis of bibliography we investigated main risk factors for faecal contamination: the number of inhabitants, livestock of rural poultry and trophic load of each hydrographic area. Trophic load provides an estimate of just nitrogen and phosphorus, elements derived from activities in civil, agricultural (livestock included) and industrial and potentially released to the environment.
A GIS of the Marche region, central Italy, was constructed utilizing vector data layers of the hydrographic sectors boundaries as base map (source: Cartographic Office of the Marche region), data about organic pollution related to the catchment areas of the Marche region, the human census geographic units source: (Cartographic Office of the Marche region), and the georeferenced rural poultry farms with their productive data obtained by the Regional Veterinary data bank (SIVA web-based Veterinary Information System ver. 1.6.4 of the Marche region). Data about risk factors evaluated have been categorized on 4 groups on the basis of their 25th 50th and 75th centiles of distribution. Samples were analyzed by the MPN method - ISO 16649-3.
For descriptive statistics of *E. coli* levels, geometric mean has been used. For descriptive statistics of *Salmonella* spp., positive percentage has been evaluated All analyses were conducted using the Stata ® 11.1 statistical software package. Maps were done by using Map Info Professional Version 7.5® G.I.S. statistical software package

Results:

A total of 927 samples has been analyzed for search of *Salmonella* spp. and *E. coli* in clams and mussels (Table. 1). In clams (*Chamelea gallina*) geometric mean of *E. coli* is
91.58 (CI 95% 74.76 - 112.17); in molluscs geometric mean of *E. coli* is 13.70 (CI 95% 11.86 - 15.83). Percentage of positive samples for *Salmonella* spp. of each species detected is reported in table 2.

Discussion and conclusions:

Thematic maps are a valid tool to identify harvesting areas with higher level of faecal contamination and to evaluate their spatial correlation with animal and human waste from corresponding catchment areas. The use

of GIS is useful to investigate the presence of point sources of pollution, to support classification and monitoring activities of the production areas of bivalve molluscs and to select factors to be analyzed by statistical models.

Conducting Hydrographic Dye Studies toAssess Pollution Impacts on Shellfish Growing Areas

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Since its inception in 1925, the National Shellfish Sanitation Program (NSSP) has relied upon dilution as a principal means to minimize the presence of enteric
pathogenic microorganisms in shellfish growing areas. The U.S. Food and Drug Administration (FDA) has served as the nation's competent authority since 1970, and during this period FDA has continued to assist States and international MOU countries with technical support and training for shellfish safety, as specifically sanctioned under the U.S. Public Health Act. Part of the assistance and training offered by FDA involves designing and conducting hydrographic dye studies used to reliably determine the impact of pollution sources on shellfish growing areas. Some of the most important examples of such studies are assessments of wastewater treatment plant (WWTP) discharges in waters proximal to shellfish growing waters. Determining the impacts from these under normal operating as well as failure conditions is vital for assuring shellfish safety. Another important example are assessments of tributary influences to growing areas to determine rainfall and river stage levels that will result in adverse impacts to downstream shellfish growing areas. FDA also conducts assessments of the size and location of marina buffer zones and evaluations of other water based activities to determine the potential of discharges from these to impact shellfish growing areas. Additionally, hydrographic dye studies have been used in conjunction with pollution source tracking surveys to determine possible sources that may have been associated with an illness outbreak associated with shellfish identified from a shellfish growing area. Recent advances in technologies as well as decreases in equipment costs have allowed hydrographic dye studies to become much more facile and cost effective to perform. Dye studies in conjunction with computer modeling now allow for the most comprehensive assessment of nearly every pollution event situation.

Remediation of Impacted Production Areas

Keynote

New Zealand Little-Neck Clams – Health Risks Following Earthquake Disturbances

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Little-neck clams *(Austrovenus stutchburyi,* the common NZ cockle) are abundant in many estuaries in the South Island of New Zealand where they are collected for food and bait. In many areas shellfish are unsuitable for human consumption because of contaminant levels in the tissues. In February 2011, Christchurch City was rocked by several large earthquakes and sand volcanoes, formed from liquified sediment, appeared on the surface of the Avon-Heathcote Estuary/Ihutai. The estuary also received increased nutrient inputs when the city infrastructure failed and large quantities of raw sewage was discharged into the estuary. Although the sediments became anoxic, the clams in the shellfish beds survived the disturbance and there has been a gradual recovery over time. We know that cockles accumulate trace metals and microbes from the environment and we predicted that this would increase as a result of earthquake disturbance. Water quality data and shellfish samples have been collected regularly by local authority monitoring programmes. This presentation will evaluate the effects of both earthquake disturbance and the removal of treated waste water from the Estuary on cockle health and safety. Finally we will attempt to answer the question - when will the cockles in this Estuary be safe to eat?

"Application of Risk Analysis for Ensuring Food Safety After Fukushima Nuclear Accident - Focused on Molluscan Shellfish Safety"

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The Great East Japan Earthquake, the magnitude-9.0, occurred at 14;46 (Japan standard time) on 11 March 2011, with 15,870 confirmed deaths and 2,846 still mission people. The same day, an accident occurred at the Fuskuhima Dai-Ichi Nuclear Power Plant as a result of the large earthquake and tsunami which swept toward the plant about 1 hour after the Earthquake, with over 14 m in height. All the operating boiling water reactors were automatically shutdown upon the quake, but overheated due to inoperable nuclear fuel cooling system. Partial core melt and hydrogen explosions took place, followed by radionuclide release outside reactors. The Ministry of Health, Labour and Welfare (MHLW) set the provisional regulatory values on 17 March 2011 in line with the "index relating to the restriction of food intake" derived by the Nuclear Safety Commission under the assumption of nuclear power plant accidents. Since the Food Safety Basic Law requires risk assessments before establishing any regulatory limits, MHHW requested the Food Safety Commission (FSC, Governmental risk assessment body under the Cabinet Office) to assess the validity of the provisional regulatory values on 20th March 2011. On the 29th March, the FSC concluded that the ongoing measures based on the provisional regulatory values are effective enough to ensure food safety for human consumption. Further the provisional regulatory value for radio-iodine in seafood was additionally set on the 5th April, because fish contaminated at levels of concern began to emerge. Based on current scientific knowledge, commodities that met the provisional regulation values are considered to be safe, and in fact food safety is basically secured. However, to achieve further food safety and consumer confidence and based on the results of the risk assessment performed by the FSC, MHLW decided to reduce the maximum permissible dose from 5mSv/year to 1mSv/year, and revised and enforced the new limits for radionuclides in food on 1st of April 2012. The government of Japan has taken various risk management options concerning radionuclides in foods, such as monitoring, restricting the distribution of foods exceeding regulatory limits, and disclosing monitoring results. As for fishery products, monitoring has been conducted in East Japan on a weekly basis since March, 2011. Currently, radionuclide levels in many species are below detectable limits or below the regulatory limit. Therefore, species which have exceeded the limit have been selected as priority monitoring targets. As of September 25 2013, 87.8% of samples (15,958 out of 18,183) are below 100Bq/kg in total. 73.3 % (4,996 out of 6,816) and 96.4% (10,962 out of 11,367) of samples are below the regulatory limit in Fukushima and other prefectures, respectively. Most of the fish that exceeded the limit are bottom fish and fresh water fish caught in limited area (ex. Miyagi, Fukushima, Ibaraki). As for monitoring results for molluscan shellfish, radionuclide levels in the Hen-clam *(Peudocardium sachalinense)* were relatively high among molluscan shellfish species, with a highest values of 950 Ba/Kg from Hen-clam harvested in Iwaki City in Fukushima in June 2011. Monitoring results indicated continual decrease in radionuclide levels in all the molluscan species. The level in all molluscan shellfish samples including Peudocardium sachalinense harvested in 2013 decreased to less than 32 Ba/Kg (below the regulatory limit), while most of the samples were less than 15 Ba/kg. As for risk communication, the Government of Japan has been organizing a series of risk communication events with all the stakeholders including farmers and consumers to promote better understanding of the risk associated with radionuclides, risk management activities including the scientific background of the regulatory limits, monitoring results and restricting the distribution of foods exceeding regulatory limits. By applying the risk analysis framework, it is thought that risks associated with radionuclides in food have been managed.

On-Site Sewerage Management Adjacent to Shellfish Harvest Areas in NSW

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On-site sewerage management systems (OSMS) have been implicated in several shellfish harvest area closures in NSW over the last 10 years and remain one of the most significant risks to the NSW oyster industry. Since 2006 NSW DPI has played an important role in assessing applications for new OSMS adjacent to shellfish harvest areas through the referral provisions contained in NSW State Environmental Planning Policy 62. Highlights and lowlights of this experience will be presented and conclusions on best management practice for OSMS will be drawn from this practical experience and from the outcomes of the NSW Parliamentary Committee on Environment and Regulation inquiry into the management of domestic wastewater.

Improving the Management of the Risk of Human Enteric Viruses in Shellfish at Harvest

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A series of eight retrospective case studies from a total of five commercial oyster growing areas in New Zealand and New South Wales, Australia, was undertaken to identify the fundamental reasons why the current bivalve shellfish classification and management systems can fail to protect consumers from human enteric viral contamination in shellfish at harvest. The case studies were based on norovirus illness events since 1990 in growing areas with Shellfish Quality Assurance Programmes. A framework to assist in structured data collection and analysis was developed by identifying control points. Case study analysis included analysis within cases, cross-case analysis, and in instances in which there were several cases in one growing area, analysis within growing areas. The study showed that as a result of the disparity between science and the regulatory framework, the manner in which faecal coliform indicators are used in the current shellfish quality assurance programme fails to consistently predict the risk of enteric virus contamination in shellfish, placing a high level of reliance on the sanitary survey component of the programme. Across the cases, several common factors were found to have contributed to the failure of the sanitary survey component to protect consumers against viral illness. This paper will highlight new potential frameworks identified during the project for improved management strategies for shellfish growing areas. There will also be recommendations on priorities for future work to develop and implement improved management strategies to protect shellfish from viral contamination.

Poster Presentation Abstracts

(In alphabetical order of presenter's surname)

Optimum Use of Selective Agars to Quantify *Vibrio vulnificus* Levels in Oysters

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Vibrio vulnificus (Vv) is responsible for 95% seafood-borne related deaths in the U.S. with an infective septicemia dose for susceptible populations of <100 organisms. It is therefore important to be able to isolate and identity it even at low numbers. The Food and Drug Administration (FDA) recommends the use of the most probable number (MPN) method to detect and enumerate Vv in shellfish. This is a two-step protocol: enrichment, followed by culture on a choice of selective agars: Thiosulfate Citrate Bile Salts Sucrose, modified Cellobiose Polymyxin Collistin or Cellobiose Collistin (CC). Selective chromogenic media such as CHROMagar™ (CA) have demonstrated superior sensitivity and specificity compared with conventional selective agars. We therefore compared the performance of CA with CC on naturally contaminated Pacific oysters (*Crassostrea gigas*). We also evaluated using a crosschecking step to minimize the number of false positives. Samples (171) were harvested from five North Island NZ farms between December 2009 and February 2012 and analysed according to the FDA method. Four presumptive colonies from CA were streaked onto CC and vice-versa for cross-checking. Presumptive colonies from both agars were then confirmed by PCR. Vv was present in 28 samples: 16 detected on agars plus 7 and 5 exclusively detected on CA or CC, respectively. Thus using CA in parallel with CC increased detection by 25%. Positive samples ranged from 0.36 to 2.1x103 MPN/g but most samples (17) had <10 MPN/g. Exclusive detections of Vv from either CA or CC usually occurred when <10 MPN/g were present (4/7 in CA and 4/5 in CC).The cross-checking step resulted in a 49% decrease in false positives for CA and 78% for CC, reducing the need for further confirmation by PCR or biochemical tests.

Collaboration Between NRCC and IRTA to Produce a New Certified Reference Material for Homoyessotoxin

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Yessotoxins (YTXs) are lipophilic marine toxins characterized by sulphate moieties and a ladder-shape polyether backbone. YTXs are regulated by the European Union with a maximum permitted level of 1 mg YTX equivalents/kg meat in shellfish products. For routine laboratories working under quality standards such as that of ISO 17025, certificate reference materials (CRMs) are needed in order to provide accurate results as well as to estimate the uncertainty of measurements. The Measurement Science and Standard division (formerly CRMP Program) of the National Research Council of Canada (NRCC) is a well-established international supplier of CRMs for marine toxins. Because of the wide range of marine toxin CRMs needed by laboratories internationally NRCC frequently collaborates with other research groups around the world in order to meet requirements. Previous studies performed at IRTA identified a strain of the marine dinoflagellate

P. reticulatum, isolated from the Ebro Delta (South Catalonia, Mediterranean Sea), that produced large amounts of homoYTX. Collaboration between NRCC and IRTA was commenced aimed at producing a new CRM for hYTX. First, large-scale cultures of the *P. reticulatum* were grown and the particulate fraction (69% of total toxin) was harvested by filtration. The dissolved fraction remaining in the culture media was recovered by dispersion of the sorbent resin DIAON HP20 for 24h (31% of total toxin). Subsequently, consecutive orthogonal chromatographic fractionation protocols based on size-exclusion chromatography and reversed-phase chromatography were applied on preparative and semi-preparative scales, using LC-MS/MS for monitoring the target analyte as well as interferences. Approximately 5 mg of hYTX was eventually isolated, and purity was established through LC-MS and qNMR methods. From this material a CRM was produced with an assigned certified value and uncertainty. CRM-hYTX has been released in 2012 and is now available for all routine and research laboratories around the world.

Persistence of Diarretic Shellfish Poisoning (DSP) Toxins in Shellfish During Two Successive Blooms of *Dinophysis* Spp. The Need of Lc-Ms/ Ms In Monitoring Programmes.

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We studied in depth two consecutive blooms of *Dinophysis* spp. in the Alfacs Bay of the Ebro Delta (Catalonia, Spain, NW Mediterranean Sea) from January 9th to April 4th, 2012. During the blooms, the Maximum Permitted Level (MPL) of okadaic acid (OA) in mussels (160 µg/kg) was exceeded during more than 70 days, involving serious economic losses for the closed harvesting area. The current reference method for DSP toxin control is LC-MS/MS, but the mouse bioassay (MBA) can still be performed. More than one hundred samples of shellfish were collected from several locations of the bay and analyzed using both control methods, comprising three species (mussels, oysters and clams). The correlation between MBA and LC-MS/MS results was consistent. Moreover, the presence of dissolved lipophilic toxins in the water column was determined by LC-MS/MS using solid-phase adsorption toxin tracking devices (SPATTs) and water filters. LC-MS/MS analysis revealed the maximum OA concentration surpassed 400 µg/kg in mussels. Phytoplankton monitoring confirmed the presence of *Dinophysis* spp., reaching a maximum density of 2000 cells.L-1 and a maximum production of 85 pgOA/cell and 58 pgPTX2/cell. The LCMS also revealed the presence of other lipophilic toxins such as cyclic imines and yessotoxins. The implementation of the LC-MS/MS provided invaluable information of the qualitative and quantitative toxin production by *Dinophysis* spp., and its accumulation in shellfish during a natural bloom. The data from phytoplankton analysis, toxin concentration analysis and environmental monitoring provided a better understanding of the blooms of *Dinophysis* spp. and were integrated into a predictive model that will improve the management of the shellfish production areas in the Ebro Delta.

Protein Phosphatase Inhibition Assays for Diarrheic Lipophilic Toxins: Determination of Inhibition Equivalency Factors, Analysis of Shellfish Samples and Comparison With Lc-Ms/ Ms

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Okadaic acid (OA) and dinophysistoxins (DTX-1 and DTX-2) are lipophilic toxins produced by microalgae of the *Dinophysis* and Prorocentrum genera. These phycotoxins are accumulated mainly in the digestive glands of shellfish and are responsible for the diarrheic shellfish poisoning (DSP) syndrome in humans. In order to guarantee shellfish safety and protect human health, the development and application of rapid, fast and sensitive methods for their determination is necessary. The protein phosphatase inhibition assay (PPIA) is a promising screening tool to be run in parallel to the official methods, but requires in-depth characterisation and performance evaluation before its approval as an official control method to be used in monitoring programs. This work presents the application of two PPIAs, with a recombinant and a wild-type PP2A, to the determination of DSP toxins in shellfish. DTX-1 and DTX-2 certified standards (NRC) were used for the study of their respective inhibitory potencies on both enzymes. The inhibition equivalency factors (IEFs) were established (1.1 and 0.9 for DTX-1, and 0.4 and 0.6 for DTX-2, for recombinant and wild-type PP2A, respectively). The PPIAs were applied to the determination of OA equivalent contents in spiked and naturally-contaminated shellfish samples. Results were compared to those provided by LC-MS/MS analysis (method recently approved for the official control), after application of the IEFs, showing good agreement. Results derived from the analysis of shellfish samples demonstrate the applicability of the developed PPIAs not only as screening tools but also for the quantitative determination of the toxin equivalent contents.

Analytical Evaluation of Emerging Toxins Present in Shellfish and Phytoplankton From Certain Areas of the Spanish and Portuguese Coasts

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It is well known that marine biotoxins are worldwide distributed having an increased socioeconomic impact. These toxins have been extensively evaluated over the last few years and big progress has been made not only in the development of analytical methodologies for their control, but also in their toxicological evaluation. Nevertheless there is still a lack of knowledge about new and emerging toxins that might occur in different areas, probably due to the increase of the maritime traffic, climatic changes and several other factors, described in the literature. These emerging toxins include Ciguatoxins, Palytoxins, Spirolides and Cycles Imines, most of them are responsible for human intoxications with neurological, gastrointestinal and cardiovascular symptoms , some of which result in elevated mortality and long-term morbidity, but the lack of standards and reference materials made difficult to progress in their toxicological evaluation as well as in the method development for their control. These toxins are recently emerging in Europe and although there are EFSA recommendations for them, the European legislation is still limited, mainly because of the limited epidemiological data. The appearance of these toxins in certain areas of Spain and Portugal and their analytical evaluation constitutes the aim of this work. . The lack of standards and reference materials, is the main limitation for the development of efficient analytical methods, nevertheless efforts are being made to find these materials to be able to develop good analytical methods for the control of these toxins. This work shows the application of LC coupled to tandem Mass Spectrometry (UPLC- MS/MS) for the analysis of these toxins in phytoplankton, fish and bivalve samples from Portugal and Spain. The method used had been previously developed and optimized by this research group. Some of the samples were also analyzed by MBA, but the symptoms observed in the mice were not conclusive, particularly for the fish samples contaminated with ciguatoxins. The results obtained in this work confirm the presence of these toxins in different areas of Spain and Portugal, nevertheless further studies are still required to improve the knowledge about the identity of some of the toxins, particularly in the case of Ciguatoxins, on the other hand, more toxicological studies would be also required to justify the interest of developing and improving analytical methods for these toxins, to be able to recommend the EU authorities to establish regulations for them.

Norovirus Detection and Quantitation in Bivalve Shellfish from New Zealand and Overseas

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Norovirus contamination of shellfish can cause outbreaks of gastroenteritis which may lead to international trade restrictions for shellfish. In 2007, an ISO 17025 accredited method for norovirus detection in bivalve shellfish was established in New Zealand. Over 700 shellfish samples have since been analysed for norovirus presence. Analysis of Pacific oysters, mussels, cockles and clams from New Zealand, Australia, Korea and China was carried out for water and shellfish quality monitoring programmes, outbreak investigations, product clearances, export screening and following sewage discharge events. Following proteinase K digestion to recover norovirus from shellfish digestive tissue (DT), samples were analysed using separate RT-quantitative PCR (RT-qPCR) for norovirus GI and GII. Norovirus concentrations (genome copies/g DT) were calculated using plasmid standards.

Norovirus was detected in 368/709 (52%) of shellfish analysed, including 330/639 (52%) New Zealand shellfish samples. Both norovirus GI and GII were detected in 162/709 (23%) of samples. Most positive samples contained low levels of norovirus GI or GII (<80 genome copies/g DT). Very high levels (> 1000 genome copies/ g DT) of norovirus GI were detected in 41/341 (12%) samples and in 107/341 (31%) norovirus GII positive samples.

Norovirus was detected in 48/74 (65%) shellfish samples associated with outbreak investigations. Of these, both norovirus GI and GII were detected in 22 samples, GI only in 4 samples and GII only in 22 samples implicated in outbreaks. Norovirus GI and GII in shellfish implicated in outbreaks ranged from >10,000 genome copies/g DT in 8 samples to <80 genome copies/g DT in 29 samples. No samples with very high norovirus (>10,000 genome copies/g DT) originated in New Zealand, but given the frequent presence of low norovirus levels in outbreak related samples and the low infectious dose of norovirus, all norovirus-positive shellfish must be regarded as a public health risk.

Collective Ciguatera Fish Poisoning in Paris, France, Due to Tropical Fish From Guadeloupe, French West Indies

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We describe two clusters of ciguatera fish poisoning (CFP) due to fish caught in Guadeloupe and consumed in Paris after being transported by plane in a cool-box. The first cluster involved 5 individuals from 12 to 43 years old who were admitted for gastro-intestinal symptoms after consuming a barracuda *(Sphyraena barracuda)* 24 hours before. Three patients exhibited neurologic symptoms (paresthesias, headache, dizziness). Two children did not present symptoms after consuming one bite. CFP was suspected. Intravenous mannitol was administered in two patients. All recovered in several days. The second cluster involved a 56 years old couple. The man exhibited paresthesia and muscle weakness after consuming a grey sapper *(Lutjanus griseus)* at 7 occasions whereas his wife exhibited milder symptoms after one meal. She recovered quickly but neurological symptoms persisted for more than 2 months in the man. In both cases, fish leftovers were sent to the National Reference laboratory for ciguatoxins analysis by mouse bioassay (Vernoux, 1994). The results were positive; for the barracuda, 2/2 mice died within 24h suggesting a high content of ciguatoxins whereas for the grey sapper, the 2 mice lose 20% of their weight in 24h, suggesting that it was less contaminated than the barracuda. In our study, the diagnosis of CFP was supported by detection of ciguatoxins in the fish consumed which is rarely reported in France metropolitan. CFP is a major public health problem in Pacific and Caribbean tropical waters. Some imported species may contain ciguatoxins. Moreover, since 2008, fish caught in Canaries and Madeira Islands were also reported to be responsible of CFP. However, ciguatera may be undiagnosed in most cases as physicians working in temperate countries are not aware of the symptoms and leftovers of contaminated fish are rarely analysed. Physicians and public health authorities should be more informed about this issue.

Keywords: fish poisoning, ciguatera, ciguatoxins.

Enumeration and Presence of *Salmonella enterica* Serotypes from Shellfish in Open Market Study in Chile 2012

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The south of Chile harbours around 1000 shellfish farm coexisting with an important Salmon industry. According to the WHO FAO recommendations concerning the risk of non-typhoidal salmonellosis associated with molluscan bivalve consumption, we evaluate the presence and abundance of Salmonella spp. in shellfish produced in Chile. A total of 49 samples were collected by duplicated over the summer in 2012 and from November 2011 to March 2012. Four different shellfish were analyzed: *Protothaca taca, Mytilus chilensis*, *Choromytilus chorus* and *Aulacomya ater.* Samples were obtained from Open Market in the city close to the shellfish farm in Region de los Lagos. The methodology used was inmunoconcentrations screening using Vidas (BioMerieux) and Most Probable Number for the enumeration of positive samples. Only the two duplicates of one sample of *Protothaca taca* were positive for salmonella by VIDAS. *Salmonella enterica serovar Typhimurium* was isolated from one of two samples. The enumeration was positive in the format of 1 to 0,001 g with 0.036 MPN/g. These results showed a low incidence of *Salmonella* in shellfish harvested in these areas with low level of the pathogen in the positive samples. A preliminary screening methodology before the enumeration may be recommended. Acknowledgement: Students from DUOC and Universidad Tecnológica Metropolitana and CDC Europa and Universidad de Santiago de Compostela. Project CORFO 09CN14-5951

Isolation of *Vibrio parahaemolyticus* of *Patella (Patella) caerulea fragilis* in The Tidalbusheher (Persian Gulf, Iran)

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Introduction and Objectives

Patellidae is a taxonomic family of sea snails or true limpets, marine gastropod molluscs in the clade Patellogastropoda. Hard substrate species graze on algae, algal spores, detritus or bacteria; some species live on and consume macroscopic algae. The purpose of this study of in *Vibrio parahaemolyticus* isolated from species *Patella (Patella) caerulea fragilis (Patellidae)* of Busheher Port in the South Coast Persian Gulf, Iran.

Methods

One hundred *Patella (Patella) caerulea fragilis* were collected from Busheher in south coast of Persian Gulf (Iran) during October to December 2011. The samples were transferred to the laboratory in appropriate conditions. In the laboratory as a first step, 300 ml of alkaline peptone water (APW) was added to 25 g of homogenized intracellular tissues *Patella (Patella) caerulea fragilis* and incubated at 37°C. On the basis of colony shape on TCBS agar, catalase activities, motility and sensitivity and other biochemical tests described. A more complete genus identification was obtained using the API 20E test.

Results:

Some Vibrio species such *Vibrio parahaemolyticus* (Blue to green centered colonies) were present as well as in intracellular tissues *Patella caerulea fragilis.*

Conclusion:

The isolation of some potential pathogenic vibrio species shows the importance of Vibrio research to estimate water quality and to avoid transmission of infection to man and to other marine organism. Also, The circulation of *Vibrio parahaemolyticus* was medium in the tidal Busheher.

Keywords: *Vibrio parahaemolyticus, Patella (Patella) caerulea fragilis*, Persian Gulf.

Study of red tide of Harmful Dinoflagellate *Cochlodinium polykrikoides* on the Gastropoda and Fishes in Coastal Water Northern Persian Gulf (Bushehr and Qeshm Island)

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Introduction and Objectives

Red tide is a phenomenon caused by algal *Cochlodinium polykrikoides* (planktonic dinoflagellate species) blooms during which algae become so numerous that they discolor coastal waters (hence the name “red tide”). Before the rains, four to five million units of algae could be found in a liter of Persian Gulf water but after the spring rains, the number decreased to 10,000 units per liter. *C. polykrikoides* is a planktonic species. The algal bloom may also deplete oxygen in the waters and/or release toxins that may cause illness in humans and other animals aquatic. The target of study the in the effect of red tide by harmful dinoflagellate *C.polykrikoides* on the gastropoda and fishes in the Coastal Bushehr Port (Persian Gulf,Iran).

Materials and Methods

The strains of *C.polykrikoides* were isolated in June /July 2009 in the Coastal Water Northern Persian Gulf (Bushehr and Qeshm Island), during a bloom of this species.

Results

The results showed that *Cochlodinium polykrikoides* as one of 337 species of phytoplankton living in the Gulf’s waters, when bloom patches form sporadically around Bushehr until Qeshm Island. Also, these phytoplankton are always present in seawater, but only when their population density reaches a certain critical mass (about 1,000,000 cells/l) the HAB is said to occur. In some areas in Iran (Bosheher and Qeshm Island), concentrations of nine million to 27 million individual microorganisms per liter was reported. It is non-toxic to fish and mollusca, nevertheless, fishes and mollusca may be killed as a result of oxygen depletion which is known to occur simultaneously with HAB. Also, the invertebrates that died during the Northern Persian Gulf bloom included herbivores and carnivores from multiple phyla including mollusks, echinoderms and crustaceans. Finally, this phenomenon consequently may seriously threaten water resource and aquatic life in the region. These results suggest that the inactivation of gill transport-related enzymes activities, the fall in blood pO and abnormal 2 secretion of gill mucus by the *C. polykrikoides* may be one of the principal causes of fish kill. In winter this species to remain dormant in the waters.

Conclusion

The intensive anthropogenic activities, under the shadow of climate change can lead to many negative environmental consequences, among them is the alteration of phytoplankton populations which in turn can lead to the phenomenon of red tide or the Harmful Algal Bloom (HAB). The long term persistence of *C. olykrikoides* blooms may be caused by a succession of different ribotypes in the same area. However, further studies should be done about this planktonic dinoflagellate species area.

Keywords: Red tide, Harmful Dinoflagellate, *Cochlodinium polykrikoides*, Gastropoda, Persian Gulf.

Reproduction, Growth and Production of *Amiantis umbonella* (Lamarck, 1818) (Bivalvia: Veneridae) on Northern Coast of The Persian Gulf, BandarAbbas, Iran

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Amiantis umbonella is distributed throughout the coast of the northern Persian Gulf. This study of 893 clams provides data on reproduction, growth and production for the period April 2007 to March 2008 from two transects along the Bandar Abbas coast. Histological preparations showed six stages of gametogenic development: resting stage (Stage 0), early active (Stage I), late active (Stage II), ripe (Stage III), partially spawned (Stage IV) and spent (Stage V). The specimens were gonochoric and showed synchronized spawning. The reproductive cycle commenced in September and finished in March with one major spawning event in January which correlated with lower sea temperature. Von Bertalanffy growth parameters for the sample were asymptotic length ($L\infty$) =58-62 mm, growth constant (k) =0.28-0.29 yr-1 and length zero (t0) =-0.48--0.47. The mean annual clam abundance, mean biomass and production were 10 individuals. m-2, 5.7 g Shell Free Dry Weight (SFDW) m-2 and 0.495 g Shell Free Dry Weight (SFDW) m-2 yr-1, respectively.

Keywords: reproduction, growth, production, *Amiantis umbonella*, Persian Gulf.

Risk Governance in the Baltic Sea Region – Alien Species Case Study

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Alien species (AS) pose a global threat to marine ecosystems, thus they are addressed in international regulatory frameworks and require coordinated and consistent course of action from all the stakeholders involved. In the Baltic Sea region the legislation and policy is still being developed. Hence, the role of science in the context of IAS risk governance is often underlined. The results of the study based on an extensive desk research, consultations and interviews with stakeholders from different Baltic countries, showed that the science-policy interaction in this field is explicitly related to the risk assessment area, monitoring activities and providing tools for planning further actions. Among the most important findings was the need for the rapid and transparent information flow from science to the management level, supported by the development of conceptual models for communication, clear allocation of roles and responsibilities at each stage of response and the design of long-term programmes involving all stakeholders. Since our knowledge on the consequences of introductions is often limited and so far there is no legally binding instrument available, science should provide a comprehensive base for supporting Ecosystem Approach to Management of AS by integrating ecological principles into the risk governance measures.

The Effect of Environmental Toxicity and Neoplasia on the Ecophysiological Condition of Mussels *(M. trossulus)* FromAquaculture (S. Baltic)

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The aim of this work is to present the effect of environment quality on physiology and condition of *M. trossulus* from aquaculture. The coastal waters are often places of strongly opposite type of human activities such as shipyard industry, mining, agriculture and urbanization on one side, and tourism, fishery including intensive aquaculture and nature conservation on the other. The increasing human economic activity is polluting marine ecosystems with different type of contaminants (metals, PCBs, WWA), including EDs and pharmaceuticals. Moreover, more than 100000 chemical products are transported from the catchment area directly to the coastal zones of the seas and are deposited in sediments. Their synergic effect remains unknown.

The ecophysiological performance of the cultured mussels living on the ropes on different depths (from 1 to 10m below the water surface) and exposed on harsh environmental conditions was estimated on the basis of growth rate, scope for growth, respiration rate, condition index and mortality rate. No direct relationships between specific contaminant (Pb, Hg, Cu, Cd, PCB, WWA) and ecophysiological performance of the mussels was observed. Only cumulative toxicity of all toxicants present in the sediments, performed with a ToxAlert 100 kit (Merck®), showed the relationship with scope for growth and condition index. The growth rate, scope for growth and condition index was dependent on the depth from where the mussels were analysed. The general condition of mussels on the surface was better than those from the bottom.

The histological analyses of mussel soft tissue showed the presence of gills degeneration and necrosis of filaments. The origin of this cancerous changes (neoplasia) remains unknown, however many authors suggest a cause-and-effect relationship with environmental pollution. The average prevalence of gill erosion was higher in mussels living on the deeper part of rope, what can suggest the effect of sediment toxicity/pollution. Such disease may indicate a decrease in the immunological resistance of organisms to infections caused by harmful factors in the ambient environment. The effect of disseminated neoplasia on the ecophysiological performance of mussels, show a lower condition index and limited physiological performance. It could also increase the mortality rate, especially after the reproduction of females.

Appendix A
ICMSS 2013 Industry Session: Learning Lessons; PST Event in Tasmania

ICMSS 2013 Industry Session: Learning Lessons; PST Event in Tasmania

Chair: Prof. Gustaaf Hallegraeff, UTAS: Institute for Marine and Antarctic Studies.
Rapporteur: Dr. Hazel Farrell, SIMS, Sydney

Invited speakers:

- Alison Turnbull, Manager, Tasmanian Shellfish Quality Assurance Program at Department of Health and Human Services, Tasmania.
- Phil Lamb, Managing Director at Spring Bay Seafoods, Tasmania.
- Dr. Chris Bolch, UTAS: Australian Maritime College.

International Observers: Dr Philipp Hess, IFREMER, France; Prof Ana Gago-Martinez, University of Vigo, Spain.

Background

During October 2012, an unprecedented *Alexandrium* toxic dinoflagellate bloom affected more than 200km of coastline on the eastern seaboard of Tasmania, Australia. The event resulted in widespread closures of both commercial and recreational bivalve growing areas, rock lobster, scallop and crab fisheries and sparked a national and international recall of mussels, due to their contamination by paralytic shellfish toxins (PST). No human illnesses related to the event were confirmed. The total economic loss to the affected fisheries has been estimated at $12 million. As part of the ICMSS conference, industry members, government representatives and research scientists were invited to an open discussion, sponsored by the Australian Seafood Cooperative Research Centre (CRC), in order to discuss the development and impact of the bloom, the genetics and toxicology of the species, management of the event and the economic impacts.

The Chair (GH) noted that the discussion would provide a valuable opportunity to assess how well the incident was managed and what could have been done differently. An Incident Review of the event is being undertaken by the relevant stakeholders and is due for completion by 1 July 2013. This ICMSS meeting would allow stakeholders to identify priorities for management and research. Parallels were drawn to the improvements and advances made by the NSW shellfish industry drawn from a Hepatitis A outbreak in Wallis Lake during 1997.

Management of the *Alexandrium* event

Alison Turnbull set the scene for the monitoring procedures in the lead up to the event and also provided a brief summary of previous HAB events in the region. Aquaculture areas along the eastern coast of Tasmania had been assigned a risk classification for biotoxins. Areas that had never been affected by algal blooms were considered to be low risk. High-risk areas were concentrated in the southeast of the state due to the seasonal occurrences of *Gymnodinium catenatum* blooms during the austral autumn (March-May) and occasionally during spring (September - November). Aquaculture regions were typically classified as medium risk zones if they ever had a harvest closure due to a HAB event. Traditionally, the east coast of Tasmania has had a very low number of harvest closures. As host to one of the largest marine farms affected by the 2012 bloom event, Spring Bay (medium risk area) was classified according to a historical summary of HAB events. Since regular algal monitoring began in 2001, there were no closures due to diarrhetic shellfish toxins (DST) or amnesic shellfish toxins (AST) at Spring Bay. Occurrences of *G. catenatum* were also rare for the region with small events in both 2004 and in 2005 that caused two-week closures. One other high sample was observed in 2008 but flesh tests were non-toxic. *Gymnodinium catenatum* usually occurred in March and the over-winter/early spring period (June-October) was considered to be low-risk. During this low period, water samples were collected on a monthly basis and sampling was increased to fortnightly from October. Prior to 2012, there had been one event of *Alexandrium*

catenella, which did not cause any PST in Spring Bay. While *Alexandrium tamarense* was listed on the action level table within the Biotoxin Management Plan for the Tasmanian Shellfish Quality Assurance Program (TSQAP), its presence had not been confirmed in samples prior to 2012.

Phytoplankton samples were collected on 14/10/2012 and 21/10/2012 by Spring Bay Seafoods, as part of their prescribed monitoring program. Due to a delay at the analytical lab and an initial species misidentification, the samples were not confirmed to contain *Alexandrium* by the lab until 1/11/2012. Paralytic shellfish toxins were first detected by the Japanese import-testing program from mussels that were harvested on 21/10/2012. On 29/10/2012, (late afternoon) TSQAP were notified by Phil Lamb (Spring Bay Seafoods) that the mussels had tested positive in Japan and by 30/10/2012 it was confirmed that the toxin levels detected exceeded the Japanese health limits. The Spring Bay growing area was closed. Early on 31/10/2012 it was established that this limit was equivalent to the toxin levels deemed unfit for human consumption in Australia (0.8 mg/kg shellfish flesh) and that the exported mussel samples exceeded this value. At the time of the notification from Japan, Phil Lamb was in Sydney coincidentally with retained mussel samples from the same harvest date and was able to transfer them to Advanced Analytical Laboratories in Sydney for toxin analysis. On 1/11/2012 these mussels were confirmed to contain PST levels above the regulatory limit. A withdrawal of shellfish from the market had begun on the 31/10/2012, however following the confirmation, Spring Bay Seafoods commenced the formal recall of the contaminated product in domestic and international markets.

The time line of available toxicity and cell concentration results indicated that the bloom peak had been missed during sampling, and had likely occurred in the weeks prior to the 21/10/2012. The toxin results reached 10mg/kg shellfish flesh and matched those reported by the Japanese toxin analysis. From the monitoring data it was apparent that the bloom hit the whole of the east coast simultaneously, rather than seeding from adjacent areas. The results showed toxin and cell numbers decreasing rapidly following the high toxicity peak. As a precautionary measure within the biotoxin management plan, the harvest closure action levels for *Alexandrium* had been set at 500 cells l^{-1}, to allow the regulators to assess any potential impacts of any such event. During the collapse of the bloom, available data indicated that this level could be altered to 1,000 cells l^{-1}. This data and a subsequent event of non-toxic algae (putative *Alexandrium* spp.) during February 2013 resulted in an amendment to the biotoxin management plan.

It was confirmed that the toxin profile from the mussels did not match the known toxin profile of *Gymnodinium catenatum* and an initial taxonomic assessment of the cells described the species as being *A. tamarense*. However, genetic analysis indicated that the species was related to *A. catenella*. Until further analysis the species has been classed as *A. tamarense* Group IV.

TSQAP staff and Spring Bay Seafoods liaised with the public health officers and representatives of the communicable diseases section of the Tasmanian Department of Health and Human Services (DHHS). A public hotline was set up to monitor case definitions of the event. There were 15 reported illnesses. Most of these cases were assessed as not consistent with PSP. There were no clinically referred cases. Two potential cases were identified, however they were unconfirmed as both individuals had preexisting conditions with similar symptoms.

The bloom affected all of the shellfish growing areas on the east coast. *Alexandrium tamarense* was also detected on the west coast although the regional circulation patterns do not account for the movement of this organism from the east coast. During the *Alexandrium* event, a *G. catenatum* bloom occurred on the south coast resulting in mixed blooms and further harvest closures. Several public health warnings, including those banning recreational fishing, were issued and are still current at the time of the meeting.

Other recreational and commercial fisheries were affected by the *Alexandrium* event. As these fisheries had not been severely impacted by biotoxins in the past, there were no management

entities or schemes in place. Abalone and scallop closures were enforced from 2/11/2012. The Tasmanian abalone industry had experienced PST issues during prior *G. catenatum* events and this was beneficial in communicating the 2012 event. The recreational rock lobster fishery had been open prior to the bloom occurring. The commercial season was postponed until the PST content of rock lobsters was assessed. Following testing and the subsequent confirmation of PST content in the viscera it was agreed that the fishery would remain closed. Other species were tested to alleviate public concern and PSTs were not detected in abalone, periwinkle, flat head, sea urchins, squid and banded morwong. Scallops rock lobster and giant crab (the latter from 300m depth off St Helens) were found to contain toxins. This presents serious logistical issues for future biotoxin events. Recreational and commercial fishing from the continental shelf were reopened on 9/2/2013 for rock lobsters and crab with only 3 ½ weeks remaining for the season. The scallop fishery never reopened within the seasonal window. Toward the end of the bloom there was some opportunity for harvesting of scallops. However due to logistical issues, with sampling and testing within a limited time frame, it was decided by those involved to redirect their efforts to other fisheries.

The bloom event created a paradigm shift in biotoxin management in Tasmania. Due to the toxic nature of the species, the widespread distribution of the bloom from an offshore source and the likelihood of the formation of cyst beds, the east coast is now considered to be high risk for future HAB events.

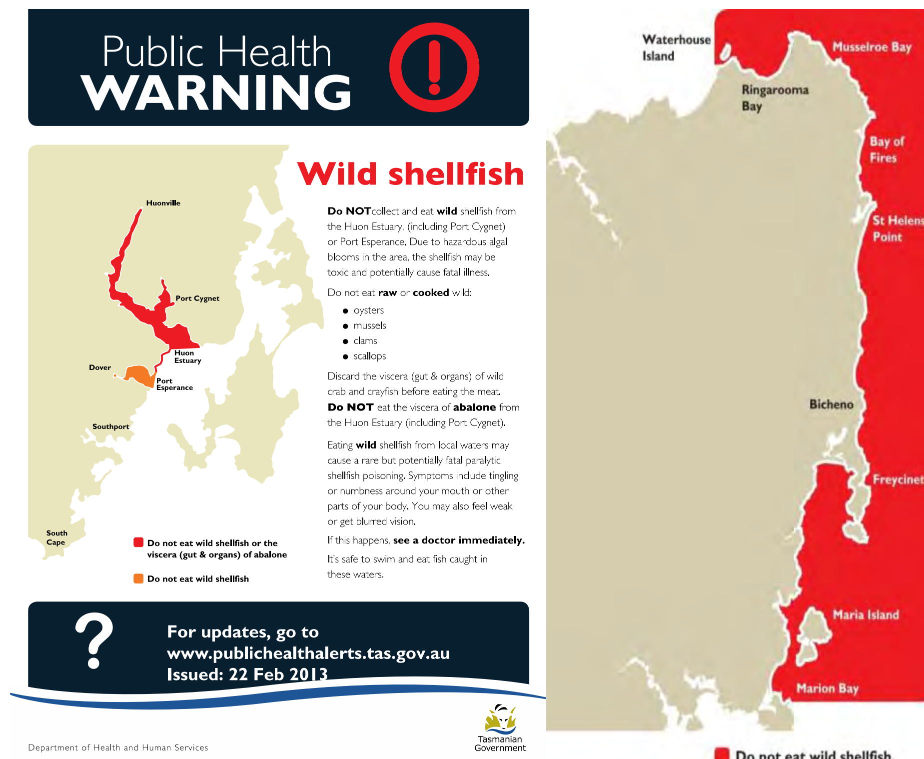

Figure. 1: Traditionally biotoxin problems in Tasmania were exclusively caused by *Gymnodinium catenatum* and mostly confined to the Derwent and Huon estuaries (left); In Oct-Nov 2012 an unprecedented novel *Alexandrium tamarense* bloom event caused seafood closures along the entire East Coast of Tasmania (right).

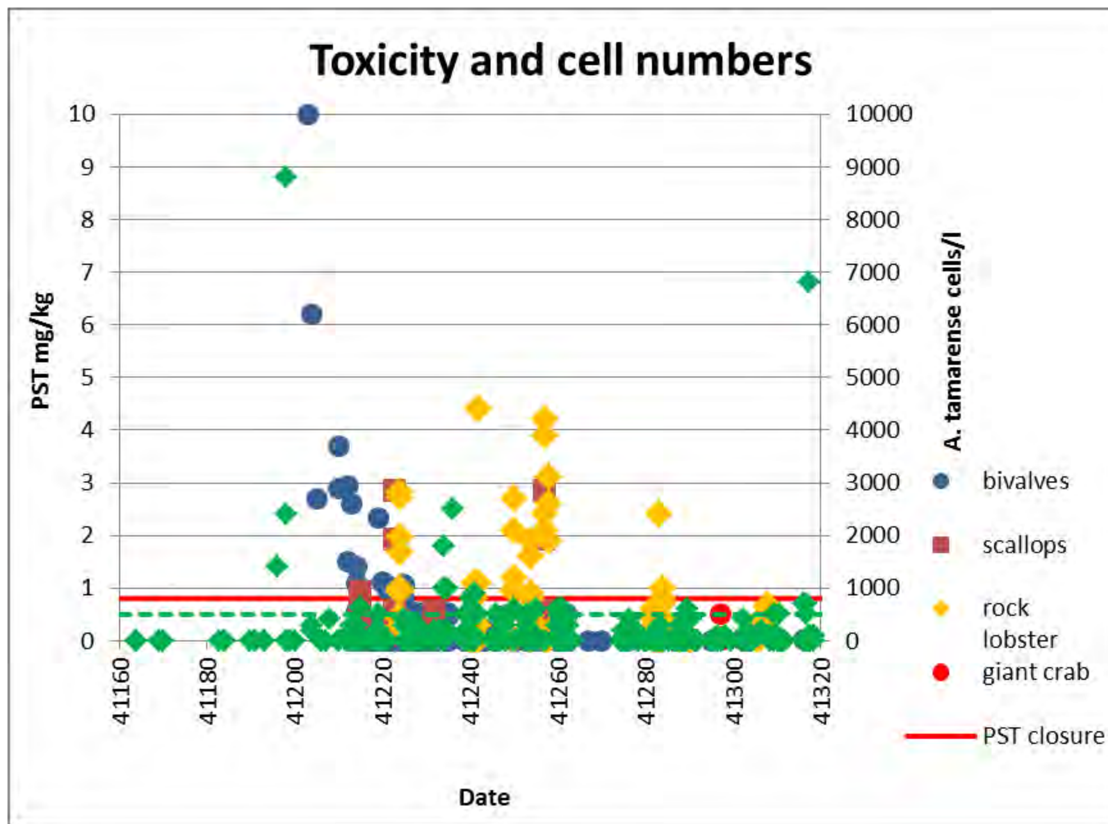

Figure 2: Time series of *Alexandrium tamarense* cell counts (green diamonds) along the Tasmanian East Coast , and PST toxins in bivalves (blue dots), rock lobster digestive tracts (yellow diamonds), including a single analysis on giant crab (red dot). Data by A. Turnbull, Tas. Dept of Health and Human Services

Significant changes are being implemented to the biotoxin management plan with baseline monitoring taking place including weekly algal monitoring and monthly toxin uptake data. The trigger levels for flesh testing are being assessed with samples for toxin analysis currently being sampled on a fortnightly basis. It is also recognized that tighter controls are needed for laboratory turn around times. A biotoxin management program for multiple fisheries species is needed.

More recent events saw a bloom (ca. 9,000 cells l^{-1}) of a putative *Alexandrium* occurring in Feb 2013. However, there was no toxicity observed in shellfish flesh samples. The biotoxin management plan did not have a contingency for this and a high cell concentration of a potentially harmful species resulted in a harvest area closures.

During a three-week closure period a large amount of information was collected. This, along with data collected during the 2012 event allowed the biotoxin management plan to be modified.

The efforts of the DHHS Environmental Health and Communicable diseases and Protection unit were acknowledged. Also credited were members of ASQAAC (including representatives from New Zealand) Cath McLeod at SafeFish, along with Australian research scientists (Shauna Murray at UTS/SIMS, Chris Bolch and Gustaaf Hallegraeff at UTAS).

The TSQAP program manager identified the following future needs:

- Research on short and long term time scales
- Management support – lack of staff and skills to deal with a large program. Suggestion for a management team for big events on a national level. Need to upskill the lab on species identification and confirm the species type.
- Communications- were happy with communications during such a large event but could do better for future events

The first step forward is a review of the event, which has been cofunded by stakeholders, including all fishery and aquaculture sectors.

HAB incident review (due 1 July 2013)

- Enhance risk management by industry and controlling authorities to underpin the public health and market access issues
- To mitigate the business risk
- Assess the current risk management system and its significance for other sectors
- Identify reasons for non compliance
- Reform official response to trading partners
- Identify opportunities for improvement to the national QA manual
- Review and revise cross sector response strategies
- Risk based framework for prioritizing Research & Development – tactical, targeted and inform risk management decisions.

For the review the combined steering committee is represented by industry and control authorities including:

Cath Mcleod (Project Manager)
Al Campbell (NZ, marine biotoxin management)
Andrew Pointon (Food safety and market access specialist)
Cath Nicholls (Communication aspect, including emergency response)
David Hudson (economist, financial implications and cost to industry)

Questions:
Clarification was requested on the timing of delay in the notification of the toxin. The Japanese toxin results were based on the harvest on 21/10/2012. This was not the date of first PST detection.

Lyndon Llewellyn commented that toxicity in the crabs was not surprising, as on other occasions crabs have been found to contain toxin of unknown origin.

How far away are the plankton analytical lab from PCR machines?
Discussions are underway about getting equipment and quality assurance, in talks with Chris Bolch (UTAS) and Shauna Murray (SIMS/UTS).

How do other countries manage abalone and rock lobster during PST events?
Canada, South Africa have issues but no representatives were present at this meeting – Hillary Reville (DPIPWE Wild Fisheries Management) to follow up.

Delay in sample analysis was due to a fish kill priority; the initial result was delayed 14 days. This is a government funded lab that is under-resourced due to government cutbacks.

Industry perspective
Phil Lamb provided background information on Spring Bay Seafoods and their operations. The farm is the largest in the area with three marine farm leases between Triabunna and Maria Island comprising a 1700 hectare lease area for mussel long lines. The farm has a number of sites for phytoplankton sample collection with their location depending on which sections of the farm are undergoing harvest. The leases are exposed to oceanic water conditions due to the wide nature of the passage (approx. 10km). Water flow is 1-3 knots and bottom depth is ca. 25m. The company's hatchery produces mussel spat and oyster spat and harvesting and processing are carried out on the adjacent site. Live musssel products are packed in vacuum bags, bulk packs, and net bags for shipping. Newer lines of product have been developed (e.g. marinated and pickled mussels) and during October 2012 the company launched two new cooked products under the Coles brand. The company is also a supplier for Costco Australia and Japan. At the time of the bloom, the mussels were being exported to six international markets as well as domestically (all states except WA).

The harvesting of the mussels, which were unknown to be toxic, took place on 21/10/2012. The product arrived in Japan on 24/10/2012 and was sold throughout the week. Late on the 29/10/2012 notification from Costco Japan was received that the mussels had tested positive for high levels of PST. At this time Phil was attending a promotional event at the Sydney Costco.

This was the first recall for the company. By the time the results were confirmed in Australia a formal recall was put in place. Domestic and

international recalls were coordinated by the company and Food Standards Australia and New Zealand (FSANZ). The recall was complex as there was a range of affected products with a widespread market (Prior to the event over 30 tonne of mussel product had been sold . During the recall approx. 10 tonnes were recovered; the balance was consumed. Due to the perceived risk to human health, while the recall was being organized (i.e. Arranging lists of product, use by/harvest dates, customers affected, markets that received the product) the DHHS issued a public announcement at noon on 2/11/2012.

The recall procedure involved a large amount of interaction with both Coles (in Australia) and Costco (globally) as they were large stakeholders and some of recalled product had been branded as Coles merchandise. Stakeholder engagement also involved liaising with other aquaculture producers in the local area (and throughout Australia) and informing the public to address concerns about the recall. Initially it was thought that the contamination just involved mussels.

Recall costs have been estimated to be in excess of $110,000 and incudes the cost of the initial product, recall costs, communications, public relations.

The recall involved a large amount of reporting to FSANZ and included an interim and final report eventually finalized in Dec 2012. A series of reports were required and finalized in January. Since the event, there has been investigations and consultation with the Australian Dept. of Agriculture, Fisheries and Forestry (DAFF), DHHS, Tasmanian Dept. of Primary Industries (DPI), Australian Mussel Industry Association (AMIA) and TSQAP.

Lost sales during the closure were estimated at $750,000 by Spring Bay Seafoods. Coles were understanding and supportive towards the event and their branded product was relaunched in stages commencing on December 2012; Approx. 25 out of 42 permanent staff members were put on forced annual leave and 8 casuals were laid off. The 125 mt mussels that were scheduled for harvest in November created significant management issues for the company to address and to avoid further losses. The company lost market access to Japan (whose import authority imposed 100% test and hold requirement, which can only be lifted after >300 "clear" shipments or 2 years).

Phil Lamb provided the following summary of issues for consideration and review :

- Lab delays and initial analysis of samples was incorrect
- New problem species, and limited experience with *Alexandrium* events in the area
- Frequency of testing was based on the area being a medium risk class
- Limited understanding of the species and its behaviour for this area
- Limited resources of TSQAP due to the widespread nature of the event.
- Gaining understanding of the relationship between the cell counts and toxicity levels delayed re-opening of farms even though the mussels depurated quickly (management plan was subsequently changed).

Lessons learned/issues to be addressed

- Cell counts and lab accountability, review capabilities and resources – TSQAP program review.
- More frequent water samples tests and meat tests for customer confidence (cost vs. benefit).
- Quick responses required along with an adaptive biotoxin management plan.
- Further abilities for testing (future developments for genetic based testing being considered)
- Desire for a quality assurance program to meet and exceed the standards.
- It is a positive step that the testing and harvest criteria has now been changed based on meat testing results being "clear" during high cell counts of apparently toxic species.
- It is important to try and clarify the uncertainty around the species identification and once it is confirmed to prepare for future events.

Questions:

During the questions that followed his talk it was noted that the media and publicity following the event was very intense but that Spring Bay Seafoods had dealt with the media in a very positive manner. Phil responded that he had had taken an open and honest approach and he had benefited from the advice from media consultants who had advised neighboring businesses and also from staff at TASSAL . The Spring Bay Seafood website, social media websites (Facebook and Twitter) along with emails and press releases were used to keep the public informed about what was happening at the farm and with the product. Alison Turnbull commented that this really assisted in alleviating public concerns. Phil responded by noting that the company regained 95% of sales in the month following the event. It was also noted during Phil's presentation that in the weeks after the PST event the company regained market share quickly and attracted positive promotion from renowned restaurateur Tetsuya Wakuda.

The impact on other fisheries was queried and it was explained that the initial concerns of other farmers and markets were rapidly dissipated. Other industries/markets within Australia reopened quickly after the initial "knee jerk" reaction. All of the company's previous export markets (except Japan) were reopened following effective representation from DAFF. No illnesses were reported in Japan or elsewhere. Costco (Japan) contacted 600 customers in a number of days during the recall.

Now algae and toxin monitoring takes place on a weekly basis at Spring Bay and of the company's volition.

Identity and distribution of the causative species

Chris Bolch acknowledged a group of researchers (Shauna Murray, Gustaaf Hallegraeff, Miguel de Salas) involved in efforts to identify and characterise the causative species and genotype. To begin, a brief background on identifying *Alexandrium* was provided. *Alexandrium tamarense* is difficult to distinguish from other similar species such as *A. catenella* and *A. fundyense*, and relies on examination of the shape and arrangement of the thecal plates that cover the surface of the cells It takes a lot of experience and practice to visualize these cell features. The presence of a ventral pore on the first apical plate is a key distinguishing characteristic.

Globally, strains of these three species are divided into 5 genetic groups of varying toxicity referred to as Group I to Group V. Group V(low toxicity) and Group IV (toxic) are both previously known from southern Australian waters. Toxic Group I, which contains*Alexandrium catenella /fundense/tamarense* morphotypes are limited to higher latitudes of the northern and southern hemispheres (Lilly et al., 2007). Chris showed a global mean sea-surface temperature map indicating that Group I genotypes are associated with coastal shelf and shelf edge environments primarily below the 15°C isotherm. In the Southern Hemisphere, suitable environments exist at the tip of South Africa, southern South America, Eastern Tasmania, and the southern half of the south Island of New Zealand.

Chris outlined known problematic species of the *Alexandrium tamarense* complex in SE Australia

- *A. catenella* has been known from NSW since as early as the 1930s and has been reported more widely and frequently over time across southeastern Australia. It is not known whether this is as a result of natural spreading or if the species was already widely distributed but cryptic (low cell numbers). . A sediment sample collected from Spring Bay in 1997 contained cysts of *A. catenella*, and cultures established were typed as Group IV (temperate Asian clade) and toxic. A low-level bloom of this species occurred in 2004 in Spring Bay.

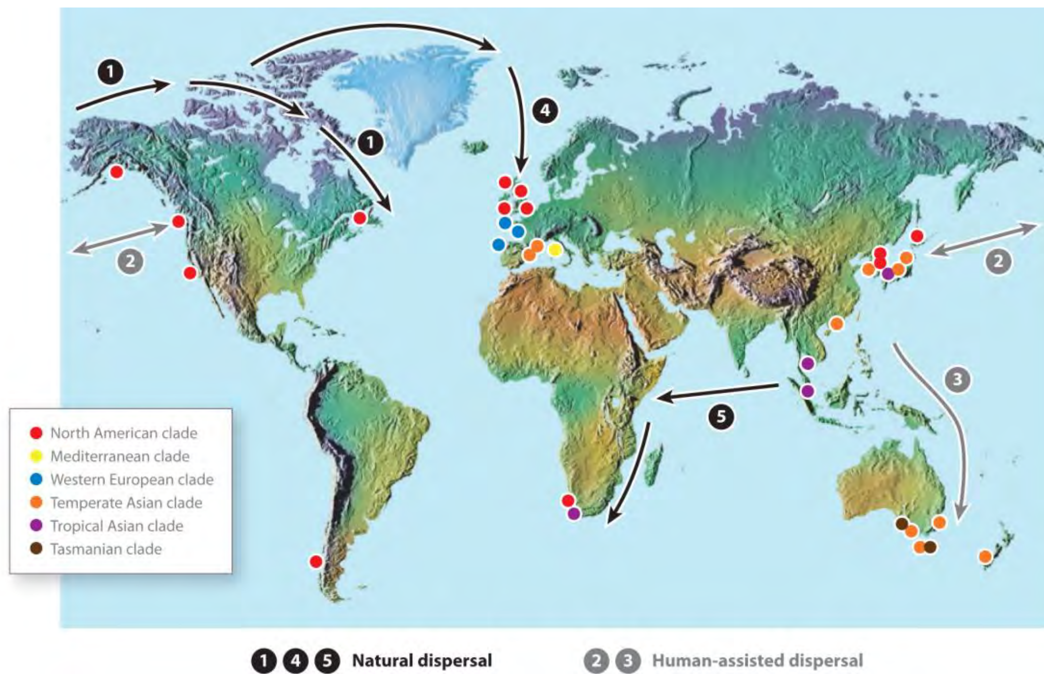

Fig. 3. Globally *Alexandrium tamarense/catenella* occurs as 5-6 genotypes with different toxic potencies and toxin profiles. East Coast Tasmanian populations may represent 2-3 different genotypes, one of which is mostly nontoxic. This severely limits the value of plankton monitoring and calls for routine application of genetic probes (Illustration from Anderson, Cembella, Hallegraeff 2012. *Ann Rev. Mar. Sc.)*

- The history of *Alexandrium tamarense* in Australia is more complex. It was first detected in fixed plankton samples from St Helens in 1987. Cultures were established in 1988 from cysts collected on the Tasmanian north coast which proved to be non-toxic, but have since been shown to produce very low levels on some saxitoxins. The genotype of other *A. tamarense* blooms across a range of other mainland locations have rarely been genotyped or cultured therefore the status of most mainland populations is unknown, but have not been linked to shellfish toxicity and were presumed to low toxicity Group V genotypes. Up to 2012 all Australian *A. tamarense* genotypes tested have been determined to be low toxicity Group V low-toxicity (Tasmanian clade). Past cyst studies of cultures established from cyst collected in 1995 from Spring Bay revealed a toxin profile similar to that known for Group I toxic (North American clade), however the culture was lost before it could be genotyped. When considered in total, the historical data indicate that toxic genotypes of *A. tamarense* may have been present along the eastern Tasmanina coast from as early as 1987, however its association with shellfish toxicity is unprecedented.

In other regions of the globe, *A. tamarense* tends to be offshore phenomena, typically associated with upwelling regions near the shelf-edge and slope. In early November 2012 during the tail of 2012 PST toxicity event in Tasmania, Chris Bolch and staff from Spring Bay Seafoods carried out a single cross-shelf transect of 5 sampling stations from Spring Bay. Samples were taken from the surface (integrated 0-7 m) and in the 20-40m range, and a net tow also collected from 0-15m for culturing. Cells were detected only at two inshore stations (Spring Bay, and Oakhampton Bay at the north end of Mercury Passage), and the outermost station beyond the shelf-edge. No cells were detected at three stations on the shelf. The low cell concentrations at the surface and deep sampling, and the presence of a significant amount of cells in the 15m net haul, suggests that the population maximum was located somewhere between 5m and 40m at the off-shelf station.

Initial on board examination detected cells resembling *A. tamarense*, however these were difficult to observe clearly on board, and on return

to shore the samples were in poor condition (cells shed their theca under stress) and identification could not be confirmed on-site. On return to AMC laboratories in Launceston, the net samples were diluted and "revived" by addition of algal culture medium and overnight incubation. The next morning, samples contained a mixed community of diatoms (dominated by diatoms including *Pseudo-nitzschia* species) and mobile dinoflagellates dominated by a *Scrippsiella* species, but with a considerable population of *Alexandrium* cells as a sub-dominant dinoflagellate.

Thirty five single cell culture isolates were established. Twenty one isolates incubated at 22-24 °C. died within 48h. All 10 isolates incubated at 16-18 °C. survived and resulted in subsequent establishment of 8 on-going *Alexandrium* cf. *tamarense* cultures.
The surviving cultures from offshore samples prefer high salinity (35ppt) and temperatures less than 20°C. Surface water temperature at the offshore station at the time of sampling was 15°C.

All other live inshore samples collected by Chris during the bloom period contained few or no *A. tamarense* cells. When they were observed in samples, they generally contained contracted and degenerate cell contents suggesting that they were not experiencing an optimal environment for their growth and survival. None of the attempted inshore cultures survived and were likely dead at the time of isolation. In contrast, observation of the suspected *A. tamarense* cells collected beyond the shelf-edge while on-station appeared to be in a much healthier condition and culturing success was high when incubated at appropriate temperatures (18 °C).

Preliminary results of the toxin analysis indicated a per cell toxicity of 16 fmol STX per cell, within the middle of the range published for toxic *A. tamarense*. The PST congeners from dinoflagellates identified were GTX1,4 (90%), C1,2 (7.3%) and NEO (2.7%). Oyster and and mussel PST toxin profiles varied somewhat between but were consistent within shellfish species. Results from the mussel flesh analysis varied considerably between oysters and mussels but on average were dominated by GTX2,3 (40-60%), C1,2 (17-43%) and GTX1,4 (2.3-12%), with minor amounts of dcGTX2,3 (5-7%) NEO (1.5-2.6%) and STX (1.2-4.5%) and dcSTX (0-3.3%). Shifts in toxin profile were evident in cooked product and appeared related to the type of preparation/seasoning – indicating that cooked product presents different risk for consumers. The differences between the cells and mussel/oyster flesh toxin profiles may be the result of biotransformation of toxins by the mussels, however, as there were delays during transit to Advanced Analytical Australia labs in Sydney, it seems more likely that acid or heat conversion in transit may be responsible for the differences.

The toxin profile in shellfish from the 2012 event did not match the toxin profile of *A. catenella* Group IV type strains previously known from Australia and Tasmania , but it is admitted that PST profiles are known to vary considerably .The toxin profile was instead similar to that of the *A. tamarense* culture isolated and tested for toxicity in 1997, and similar to that known for Group I Northern Asian genotypes. Chris indicated that toxin analyses of additional cultures by both Advanced Analytical Australia labs in Sydney, and Cawthron Institute (New Zealand, Tim Harwood) is planned to confirm the preliminary findings.

Data from LSU-rDNA sequencing indicated that that 2 of the 8 cultures were almost identical to Group I toxic strains known for northern high latitudes of the northern atlantic and North Pacific. Shauna Murray at UTS carried out PCR analysis on DNA from mussels from Spring Bay samples which had been confirmed as toxic, and found results that were most similar to an *Alexandrium catenella/tamarense* Group IV. This raises the question as to whether 2012 east coast bloom populations consisted of both Group I and IV genotypes both with proportions and distribution varying in space and time. Comparison of toxin data from cultures and shellfish also support this hypothesis. Further work is underway to confirm these findings.

Chris also mentioned the complexities we now face in identifying *Alexandrium* species. It is impossible to visually distinguish Group I toxic and Group V low toxicity genotypes, and difficult

during routine monitoring to distinguish single cells of Group IV. *A. catenella* from either *A. tamarense* type. There are also similarities in fixed samples between other co-ocurring non-toxic species such as, *A. affine*, *A. ostenfeldii* and *A. margalefii* and *Gonyaulax hyalina.* Some of the discrepancies in cell counts and shellfish toxicity noted during early 2013 may be accounted for either by the presence of low toxicity Group V *A. tamarense* or mis-identification of other related non-toxic species.

Questions:
The question was raised about the temperature that Chris' samples were found at and if temperature could indicate potential toxicity. The temperature was 14.9°C. Temperature boundaries are a broad indication of suitable habitat at larger spatial scales but seasonal extension of cool coastal currents beyond the 15 °C isotherm may provide suitable habitat along lower latitude coastlines .

The point was raised that species can have both toxic and non-toxic strains and the presence/misidentification of other similar species can account for toxin profiles not matching. It is important to know what species are present. Australia is now a high-risk area and molecular detection needs to be part of future risk management. Some of the required work has been funded/supported over the last few years by the NSW Industry and other sources so developments are underway. Further testing and validation is required but we are at least not starting from scratch.

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Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M. (2012). Annual Reviews in Marine Science 4: 143–76.

Lilly, E.L., Halanych, K.M. & Anderson, D.M. (2007). Journal of Phycology. 43: 1329–1338

Appendix A
ICMSS 2013 Industry Session: Learning Lessons; PST Event in Tasmania

ICMSS 2013 Industry Session: Learning Lessons; PST Event in Tasmania

Chair: Prof. Gustaaf Hallegraeff, UTAS: Institute for Marine and Antarctic Studies.
Rapporteur: Dr. Hazel Farrell, SIMS, Sydney

Invited speakers:
- Alison Turnbull, Manager, Tasmanian Shellfish Quality Assurance Program at Department of Health and Human Services, Tasmania.
- Phil Lamb, Managing Director at Spring Bay Seafoods, Tasmania.
- Dr. Chris Bolch, UTAS: Australian Maritime College.

International Observers: Dr Philipp Hess, IFREMER, France; Prof Ana Gago-Martinez, University of Vigo, Spain.

Background
During October 2012, an unprecedented *Alexandrium* toxic dinoflagellate bloom affected more than 200km of coastline on the eastern seaboard of Tasmania, Australia. The event resulted in widespread closures of both commercial and recreational bivalve growing areas, rock lobster, scallop and crab fisheries and sparked a national and international recall of mussels, due to their contamination by paralytic shellfish toxins (PST). No human illnesses related to the event were confirmed. The total economic loss to the affected fisheries has been estimated at $12 million. As part of the ICMSS conference, industry members, government representatives and research scientists were invited to an open discussion, sponsored by the Australian Seafood Cooperative Research Centre (CRC), in order to discuss the development and impact of the bloom, the genetics and toxicology of the species, management of the event and the economic impacts.

The Chair (GH) noted that the discussion would provide a valuable opportunity to assess how well the incident was managed and what could have been done differently. An Incident Review of the event is being undertaken by the relevant stakeholders and is due for completion by 1 July 2013. This ICMSS meeting would allow stakeholders to identify priorities for management and research. Parallels were drawn to the improvements and advances made by the NSW shellfish industry drawn from a Hepatitis A outbreak in Wallis Lake during 1997.

Management of the *Alexandrium* event
Alison Turnbull set the scene for the monitoring procedures in the lead up to the event and also provided a brief summary of previous HAB events in the region. Aquaculture areas along the eastern coast of Tasmania had been assigned a risk classification for biotoxins. Areas that had never been affected by algal blooms were considered to be low risk. High-risk areas were concentrated in the southeast of the state due to the seasonal occurrences of *Gymnodinium catenatum* blooms during the austral autumn (March-May) and occasionally during spring (September - November). Aquaculture regions were typically classified as medium risk zones if they ever had a harvest closure due to a HAB event. Traditionally, the east coast of Tasmania has had a very low number of harvest closures. As host to one of the largest marine farms affected by the 2012 bloom event, Spring Bay (medium risk area) was classified according to a historical summary of HAB events. Since regular algal monitoring began in 2001, there were no closures due to diarrhetic shellfish toxins (DST) or amnesic shellfish toxins (AST) at Spring Bay. Occurrences of *G. catenatum* were also rare for the region with small events in both 2004 and in 2005 that caused two-week closures. One other high sample was observed in 2008 but flesh tests were non-toxic. *Gymnodinium catenatum* usually occurred in March and the over-winter/early spring period (June-October) was considered to be low-risk. During this low period, water samples were collected on a monthly basis and sampling was increased to fortnightly from October. Prior to 2012, there had been one event of *Alexandrium*

catenella, which did not cause any PST in Spring Bay. While *Alexandrium tamarense* was listed on the action level table within the Biotoxin Management Plan for the Tasmanian Shellfish Quality Assurance Program (TSQAP), its presence had not been confirmed in samples prior to 2012.

Phytoplankton samples were collected on 14/10/2012 and 21/10/2012 by Spring Bay Seafoods, as part of their prescribed monitoring program. Due to a delay at the analytical lab and an initial species misidentification, the samples were not confirmed to contain *Alexandrium* by the lab until 1/11/2012. Paralytic shellfish toxins were first detected by the Japanese import-testing program from mussels that were harvested on 21/10/2012. On 29/10/2012, (late afternoon) TSQAP were notified by Phil Lamb (Spring Bay Seafoods) that the mussels had tested positive in Japan and by 30/10/2012 it was confirmed that the toxin levels detected exceeded the Japanese health limits. The Spring Bay growing area was closed. Early on 31/10/2012 it was established that this limit was equivalent to the toxin levels deemed unfit for human consumption in Australia (0.8 mg/kg shellfish flesh) and that the exported mussel samples exceeded this value. At the time of the notification from Japan, Phil Lamb was in Sydney coincidentally with retained mussel samples from the same harvest date and was able to transfer them to Advanced Analytical Laboratories in Sydney for toxin analysis. On 1/11/2012 these mussels were confirmed to contain PST levels above the regulatory limit. A withdrawal of shellfish from the market had begun on the 31/10/2012, however following the confirmation, Spring Bay Seafoods commenced the formal recall of the contaminated product in domestic and international markets.

The time line of available toxicity and cell concentration results indicated that the bloom peak had been missed during sampling, and had likely occurred in the weeks prior to the 21/10/2012. The toxin results reached 10mg/kg shellfish flesh and matched those reported by the Japanese toxin analysis. From the monitoring data it was apparent that the bloom hit the whole of the east coast simultaneously, rather than seeding from adjacent areas. The results showed toxin and cell numbers decreasing rapidly following the high toxicity peak. As a precautionary measure within the biotoxin management plan, the harvest closure action levels for *Alexandrium* had been set at 500 cells l^{-1}, to allow the regulators to assess any potential impacts of any such event. During the collapse of the bloom, available data indicated that this level could be altered to 1,000 cells l^{-1}. This data and a subsequent event of non-toxic algae (putative *Alexandrium* spp.) during February 2013 resulted in an amendment to the biotoxin management plan.

It was confirmed that the toxin profile from the mussels did not match the known toxin profile of *Gymnodinium catenatum* and an initial taxonomic assessment of the cells described the species as being *A. tamarense*. However, genetic analysis indicated that the species was related to *A. catenella*. Until further analysis the species has been classed as *A. tamarense* Group IV.

TSQAP staff and Spring Bay Seafoods liaised with the public health officers and representatives of the communicable diseases section of the Tasmanian Department of Health and Human Services (DHHS). A public hotline was set up to monitor case definitions of the event. There were 15 reported illnesses. Most of these cases were assessed as not consistent with PSP. There were no clinically referred cases. Two potential cases were identified, however they were unconfirmed as both individuals had preexisting conditions with similar symptoms.

The bloom affected all of the shellfish growing areas on the east coast. *Alexandrium tamarense* was also detected on the west coast although the regional circulation patterns do not account for the movement of this organism from the east coast. During the *Alexandrium* event, a *G. catenatum* bloom occurred on the south coast resulting in mixed blooms and further harvest closures. Several public health warnings, including those banning recreational fishing, were issued and are still current at the time of the meeting.

Other recreational and commercial fisheries were affected by the *Alexandrium* event. As these fisheries had not been severely impacted by biotoxins in the past, there were no management

entities or schemes in place. Abalone and scallop closures were enforced from 2/11/2012. The Tasmanian abalone industry had experienced PST issues during prior *G. catenatum* events and this was beneficial in communicating the 2012 event. The recreational rock lobster fishery had been open prior to the bloom occurring. The commercial season was postponed until the PST content of rock lobsters was assessed. Following testing and the subsequent confirmation of PST content in the viscera it was agreed that the fishery would remain closed. Other species were tested to alleviate public concern and PSTs were not detected in abalone, periwinkle, flat head, sea urchins, squid and banded morwong. Scallops rock lobster and giant crab (the latter from 300m depth off St Helens) were found to contain toxins. This presents serious logistical issues for future biotoxin events. Recreational and commercial fishing from the continental shelf were reopened on 9/2/2013 for rock lobsters and crab with only 3 ½ weeks remaining for the season. The scallop fishery never reopened within the seasonal window. Toward the end of the bloom there was some opportunity for harvesting of scallops. However due to logistical issues, with sampling and testing within a limited time frame, it was decided by those involved to redirect their efforts to other fisheries.

The bloom event created a paradigm shift in biotoxin management in Tasmania. Due to the toxic nature of the species, the widespread distribution of the bloom from an offshore source and the likelihood of the formation of cyst beds, the east coast is now considered to be high risk for future HAB events.

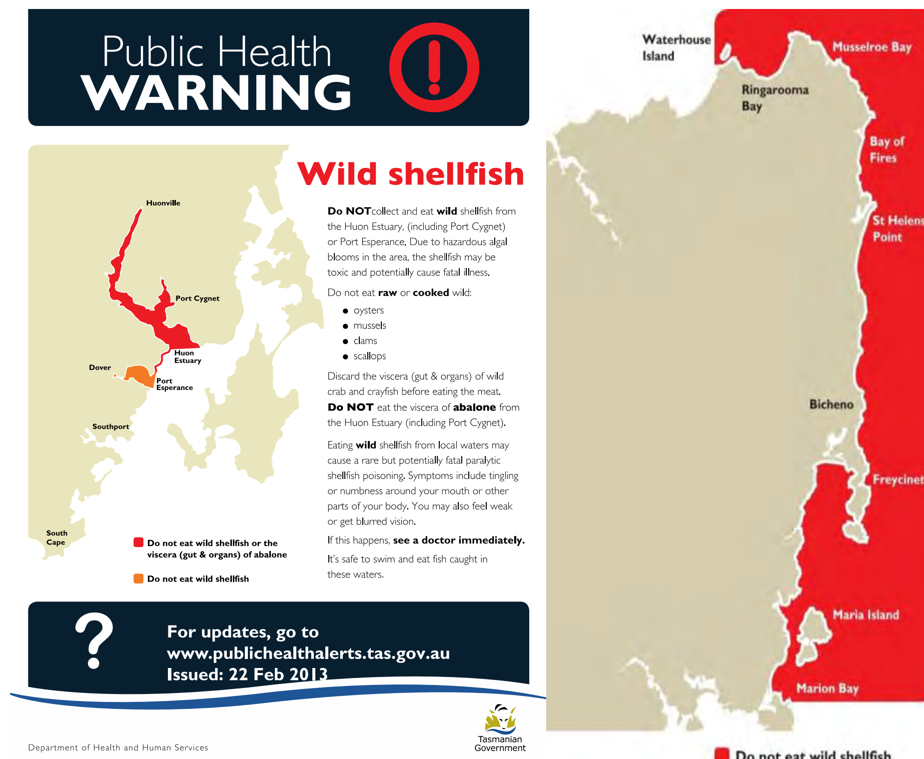

Figure. 1: Traditionally biotoxin problems in Tasmania were exclusively caused by *Gymnodinium catenatum* and mostly confined to the Derwent and Huon estuaries (left); In Oct-Nov 2012 an unprecedented novel *Alexandrium tamarense* bloom event caused seafood closures along the entire East Coast of Tasmania (right).

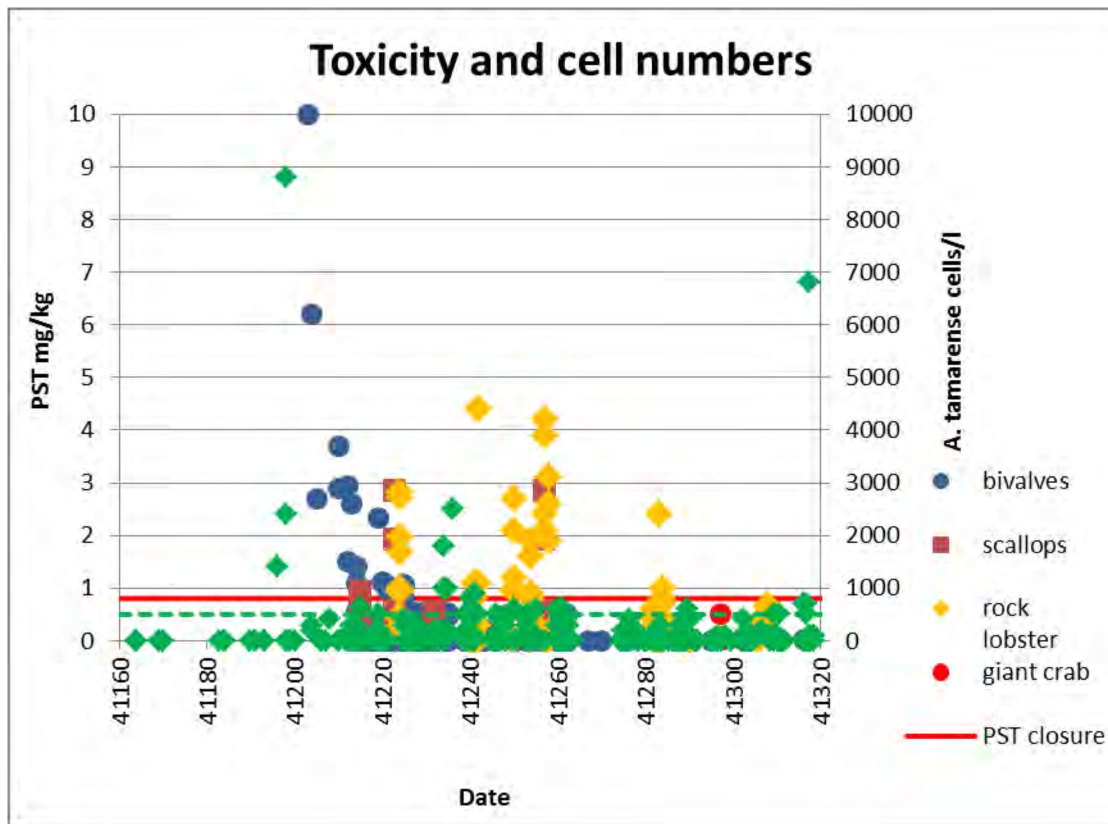

Figure 2: Time series of *Alexandrium tamarense* cell counts (green diamonds) along the Tasmanian East Coast , and PST toxins in bivalves (blue dots), rock lobster digestive tracts (yellow diamonds), including a single analysis on giant crab (red dot). Data by A. Turnbull, Tas. Dept of Health and Human Services

Significant changes are being implemented to the biotoxin management plan with baseline monitoring taking place including weekly algal monitoring and monthly toxin uptake data. The trigger levels for flesh testing are being assessed with samples for toxin analysis currently being sampled on a fortnightly basis. It is also recognized that tighter controls are needed for laboratory turn around times. A biotoxin management program for multiple fisheries species is needed.

More recent events saw a bloom (ca. 9,000 cells l^{-1}) of a putative *Alexandrium* occurring in Feb 2013. However, there was no toxicity observed in shellfish flesh samples. The biotoxin management plan did not have a contingency for this and a high cell concentration of a potentially harmful species resulted in a harvest area closures.
During a three-week closure period a large amount of information was collected. This, along with data collected during the 2012 event allowed the biotoxin management plan to be modified.

The efforts of the DHHS Environmental Health and Communicable diseases and Protection unit were acknowledged. Also credited were members of ASQAAC (including representatives from New Zealand) Cath McLeod at SafeFish, along with Australian research scientists (Shauna Murray at UTS/SIMS, Chris Bolch and Gustaaf Hallegraeff at UTAS).

The TSQAP program manager identified the following future needs:

- Research on short and long term time scales
- Management support – lack of staff and skills to deal with a large program. Suggestion for a management team for big events on a national level. Need to upskill the lab on species identification and confirm the species type.
- Communications- were happy with communications during such a large event but could do better for future events

The first step forward is a review of the event, which has been cofunded by stakeholders, including all fishery and aquaculture sectors.

HAB incident review (due 1 July 2013)

- Enhance risk management by industry and controlling authorities to underpin the public health and market access issues
- To mitigate the business risk
- Assess the current risk management system and its significance for other sectors
- Identify reasons for non compliance
- Reform official response to trading partners
- Identify opportunities for improvement to the national QA manual
- Review and revise cross sector response strategies
- Risk based framework for prioritizing Research & Development – tactical, targeted and inform risk management decisions.

For the review the combined steering committee is represented by industry and control authorities including:

Cath Mcleod (Project Manager)
Al Campbell (NZ, marine biotoxin management)
Andrew Pointon (Food safety and market access specialist)
Cath Nicholls (Communication aspect, including emergency response)
David Hudson (economist, financial implications and cost to industry)

Questions:
Clarification was requested on the timing of delay in the notification of the toxin. The Japanese toxin results were based on the harvest on 21/10/2012. This was not the date of first PST detection.

Lyndon Llewellyn commented that toxicity in the crabs was not surprising, as on other occasions crabs have been found to contain toxin of unknown origin.

How far away are the plankton analytical lab from PCR machines?
Discussions are underway about getting equipment and quality assurance, in talks with Chris Bolch (UTAS) and Shauna Murray (SIMS/UTS).

How do other countries manage abalone and rock lobster during PST events?
Canada, South Africa have issues but no representatives were present at this meeting – Hillary Reville (DPIPWE Wild Fisheries Management) to follow up.

Delay in sample analysis was due to a fish kill priority; the initial result was delayed 14 days. This is a government funded lab that is under-resourced due to government cutbacks.

Industry perspective
Phil Lamb provided background information on Spring Bay Seafoods and their operations. The farm is the largest in the area with three marine farm leases between Triabunna and Maria Island comprising a 1700 hectare lease area for mussel long lines. The farm has a number of sites for phytoplankton sample collection with their location depending on which sections of the farm are undergoing harvest. The leases are exposed to oceanic water conditions due to the wide nature of the passage (approx. 10km). Water flow is 1-3 knots and bottom depth is ca. 25m. The company's hatchery produces mussel spat and oyster spat and harvesting and processing are carried out on the adjacent site. Live musssel products are packed in vacuum bags, bulk packs, and net bags for shipping. Newer lines of product have been developed (e.g. marinated and pickled mussels) and during October 2012 the company launched two new cooked products under the Coles brand. The company is also a supplier for Costco Australia and Japan. At the time of the bloom, the mussels were being exported to six international markets as well as domestically (all states except WA).

The harvesting of the mussels, which were unknown to be toxic, took place on 21/10/2012. The product arrived in Japan on 24/10/2012 and was sold throughout the week. Late on the 29/10/2012 notification from Costco Japan was received that the mussels had tested positive for high levels of PST. At this time Phil was attending a promotional event at the Sydney Costco.

This was the first recall for the company. By the time the results were confirmed in Australia a formal recall was put in place. Domestic and

international recalls were coordinated by the company and Food Standards Australia and New Zealand (FSANZ). The recall was complex as there was a range of affected products with a widespread market (Prior to the event over 30 tonne of mussel product had been sold . During the recall approx. 10 tonnes were recovered; the balance was consumed. Due to the perceived risk to human health, while the recall was being organized (i.e. Arranging lists of product, use by/harvest dates, customers affected, markets that received the product) the DHHS issued a public announcement at noon on 2/11/2012.

The recall procedure involved a large amount of interaction with both Coles (in Australia) and Costco (globally) as they were large stakeholders and some of recalled product had been branded as Coles merchandise. Stakeholder engagement also involved liaising with other aquaculture producers in the local area (and throughout Australia) and informing the public to address concerns about the recall. Initially it was thought that the contamination just involved mussels.

Recall costs have been estimated to be in excess of $110,000 and incudes the cost of the initial product, recall costs, communications, public relations.

The recall involved a large amount of reporting to FSANZ and included an interim and final report eventually finalized in Dec 2012. A series of reports were required and finalized in January. Since the event, there has been investigations and consultation with the Australian Dept. of Agriculture, Fisheries and Forestry (DAFF), DHHS, Tasmanian Dept. of Primary Industries (DPI), Australian Mussel Industry Association (AMIA) and TSQAP.

Lost sales during the closure were estimated at $750,000 by Spring Bay Seafoods. Coles were understanding and supportive towards the event and their branded product was relaunched in stages commencing on December 2012; Approx. 25 out of 42 permanent staff members were put on forced annual leave and 8 casuals were laid off. The 125 mt mussels that were scheduled for harvest in November created significant management issues for the company to address and to avoid further losses. The company lost market access to Japan (whose import authority imposed 100% test and hold requirement, which can only be lifted after >300 "clear" shipments or 2 years).

Phil Lamb provided the following summary of issues for consideration and review :

- Lab delays and initial analysis of samples was incorrect
- New problem species, and limited experience with *Alexandrium* events in the area
- Frequency of testing was based on the area being a medium risk class
- Limited understanding of the species and its behaviour for this area
- Limited resources of TSQAP due to the widespread nature of the event.
- Gaining understanding of the relationship between the cell counts and toxicity levels delayed re-opening of farms even though the mussels depurated quickly (management plan was subsequently changed).

Lessons learned/issues to be addressed

- Cell counts and lab accountability, review capabilities and resources – TSQAP program review.
- More frequent water samples tests and meat tests for customer confidence (cost vs. benefit).
- Quick responses required along with an adaptive biotoxin management plan.
- Further abilities for testing (future developments for genetic based testing being considered)
- Desire for a quality assurance program to meet and exceed the standards.
- It is a positive step that the testing and harvest criteria has now been changed based on meat testing results being "clear" during high cell counts of apparently toxic species.
- It is important to try and clarify the uncertainty around the species identification and once it is confirmed to prepare for future events.

Questions:

During the questions that followed his talk it was noted that the media and publicity following the event was very intense but that Spring Bay Seafoods had dealt with the media in a very positive manner. Phil responded that he had had taken an open and honest approach and he had benefited from the advice from media consultants who had advised neighboring businesses and also from staff at TASSAL . The Spring Bay Seafood website, social media websites (Facebook and Twitter) along with emails and press releases were used to keep the public informed about what was happening at the farm and with the product. Alison Turnbull commented that this really assisted in alleviating public concerns. Phil responded by noting that the company regained 95% of sales in the month following the event. It was also noted during Phil's presentation that in the weeks after the PST event the company regained market share quickly and attracted positive promotion from renowned restaurateur Tetsuya Wakuda.

The impact on other fisheries was queried and it was explained that the initial concerns of other farmers and markets were rapidly dissipated. Other industries/markets within Australia reopened quickly after the initial "knee jerk" reaction. All of the company's previous export markets (except Japan) were reopened following effective representation from DAFF. No illnesses were reported in Japan or elsewhere. Costco (Japan) contacted 600 customers in a number of days during the recall.

Now algae and toxin monitoring takes place on a weekly basis at Spring Bay and of the company's volition.

Identity and distribution of the causative species
Chris Bolch acknowledged a group of researchers (Shauna Murray, Gustaaf Hallegraeff, Miguel de Salas) involved in efforts to identify and characterise the causative species and genotype. To begin, a brief background on identifying *Alexandrium* was provided. *Alexandrium tamarense* is difficult to distinguish from other similar species such as *A. catenella* and *A. fundyense*, and relies on examination of the shape and arrangement of the thecal plates that cover the surface of the cells It takes a lot of experience and practice to visualize these cell features. The presence of a ventral pore on the first apical plate is a key distinguishing characteristic.

Globally, strains of these three species are divided into 5 genetic groups of varying toxicity referred to as Group I to Group V. Group V(low toxicity) and Group IV (toxic) are both previously known from southern Australian waters. Toxic Group I, which contains*Alexandrium catenella /fundense/tamarense* morphotypes are limited to higher latitudes of the northern and southern hemispheres (Lilly et al., 2007). Chris showed a global mean sea-surface temperature map indicating that Group I genotypes are associated with coastal shelf and shelf edge environments primarily below the 15°C isotherm. In the Southern Hemisphere, suitable environments exist at the tip of South Africa, southern South America, Eastern Tasmania, and the southern half of the south Island of New Zealand.

Chris outlined known problematic species of the *Alexandrium tamarense* complex in SE Australia

- *A. catenella* has been known from NSW since as early as the 1930s and has been reported more widely and frequently over time across southeastern Australia. It is not known whether this is as a result of natural spreading or if the species was already widely distributed but cryptic (low cell numbers). . A sediment sample collected from Spring Bay in 1997 contained cysts of *A. catenella*, and cultures established were typed as Group IV (temperate Asian clade) and toxic. A low-level bloom of this species occurred in 2004 in Spring Bay.

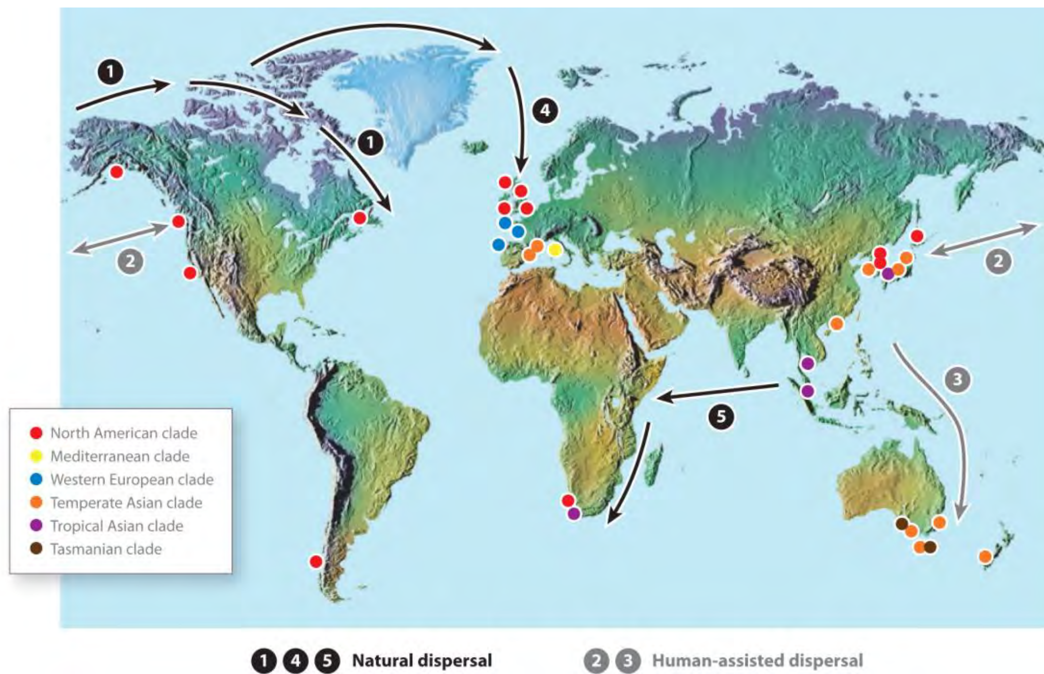

Fig. 3. Globally *Alexandrium tamarense/catenella* occurs as 5-6 genotypes with different toxic potencies and toxin profiles. East Coast Tasmanian populations may represent 2-3 different genotypes, one of which is mostly nontoxic. This severely limits the value of plankton monitoring and calls for routine application of genetic probes (Illustration from Anderson, Cembella, Hallegraeff 2012. *Ann Rev. Mar. Sc.)*

- The history of *Alexandrium tamarense* in Australia is more complex. It was first detected in fixed plankton samples from St Helens in 1987. Cultures were established in 1988 from cysts collected on the Tasmanian north coast which proved to be non-toxic, but have since been shown to produce very low levels on some saxitoxins. The genotype of other *A. tamarense* blooms across a range of other mainland locations have rarely been genotyped or cultured therefore the status of most mainland populations is unknown, but have not been linked to shellfish toxicity and were presumed to low toxicity Group V genotypes. Up to 2012 all Australian *A. tamarense* genotypes tested have been determined to be low toxicity Group V low-toxicity (Tasmanian clade). Past cyst studies of cultures established from cyst collected in 1995 from Spring Bay revealed a toxin profile similar to that known for Group I toxic (North American clade), however the culture was lost before it could be genotyped. When considered in total, the historical data indicate that toxic genotypes of *A. tamarense* may have been present along the eastern Tasmanina coast from as early as 1987, however its association with shellfish toxicity is unprecedented.

In other regions of the globe, *A. tamarense* tends to be offshore phenomena, typically associated with upwelling regions near the shelf-edge and slope. In early November 2012 during the tail of 2012 PST toxicity event in Tasmania, Chris Bolch and staff from Spring Bay Seafoods carried out a single cross-shelf transect of 5 sampling stations from Spring Bay. Samples were taken from the surface (integrated 0-7 m) and in the 20-40m range, and a net tow also collected from 0-15m for culturing. Cells were detected only at two inshore stations (Spring Bay, and Oakhampton Bay at the north end of Mercury Passage), and the outermost station beyond the shelf-edge. No cells were detected at three stations on the shelf. The low cell concentrations at the surface and deep sampling, and the presence of a significant amount of cells in the 15m net haul, suggests that the population maximum was located somewhere between 5m and 40m at the off-shelf station.

Initial on board examination detected cells resembling *A. tamarense*, however these were difficult to observe clearly on board, and on return

to shore the samples were in poor condition (cells shed their theca under stress) and identification could not be confirmed on-site. On return to AMC laboratories in Launceston, the net samples were diluted and "revived" by addition of algal culture medium and overnight incubation. The next morning, samples contained a mixed community of diatoms (dominated by diatoms including *Pseudo-nitzschia* species) and mobile dinoflagellates dominated by a *Scrippsiella* species, but with a considerable population of *Alexandrium* cells as a sub-dominant dinoflagellate.

Thirty five single cell culture isolates were established. Twenty one isolates incubated at 22-24 °C. died within 48h. All 10 isolates incubated at 16-18 °C. survived and resulted in subsequent establishment of 8 on-going *Alexandrium* cf. *tamarense* cultures.
The surviving cultures from offshore samples prefer high salinity (35ppt) and temperatures less than 20°C. Surface water temperature at the offshore station at the time of sampling was 15°C.

All other live inshore samples collected by Chris during the bloom period contained few or no *A. tamarense* cells. When they were observed in samples, they generally contained contracted and degenerate cell contents suggesting that they were not experiencing an optimal environment for their growth and survival. None of the attempted inshore cultures survived and were likely dead at the time of isolation. In contrast, observation of the suspected *A. tamarense* cells collected beyond the shelf-edge while on-station appeared to be in a much healthier condition and culturing success was high when incubated at appropriate temperatures (18 °C).

Preliminary results of the toxin analysis indicated a per cell toxicity of 16 fmol STX per cell, within the middle of the range published for toxic *A. tamarense*. The PST congeners from dinoflagellates identified were GTX1,4 (90%), C1,2 (7.3%) and NEO (2.7%). Oyster and and mussel PST toxin profiles varied somewhat between but were consistent within shellfish species. Results from the mussel flesh analysis varied considerably between oysters and mussels but on average were dominated by GTX2,3 (40-60%), C1,2 (17-43%) and GTX1,4 (2.3-12%), with minor amounts of dcGTX2,3 (5-7%) NEO (1.5-2.6%) and STX (1.2-4.5%) and dcSTX (0-3.3%). Shifts in toxin profile were evident in cooked product and appeared related to the type of preparation/seasoning – indicating that cooked product presents different risk for consumers. The differences between the cells and mussel/oyster flesh toxin profiles may be the result of biotransformation of toxins by the mussels, however, as there were delays during transit to Advanced Analytical Australia labs in Sydney, it seems more likely that acid or heat conversion in transit may be responsible for the differences.

The toxin profile in shellfish from the 2012 event did not match the toxin profile of *A. catenella* Group IV type strains previously known from Australia and Tasmania , but it is admitted that PST profiles are known to vary considerably .The toxin profile was instead similar to that of the *A. tamarense* culture isolated and tested for toxicity in 1997, and similar to that known for Group I Northern Asian genotypes. Chris indicated that toxin analyses of additional cultures by both Advanced Analytical Australia labs in Sydney, and Cawthron Institute (New Zealand, Tim Harwood) is planned to confirm the preliminary findings.

Data from LSU-rDNA sequencing indicated that that 2 of the 8 cultures were almost identical to Group I toxic strains known for northern high latitudes of the northern atlantic and North Pacific. Shauna Murray at UTS carried out PCR analysis on DNA from mussels from Spring Bay samples which had been confirmed as toxic, and found results that were most similar to an *Alexandrium catenella/tamarense* Group IV. This raises the question as to whether 2012 east coast bloom populations consisted of both Group I and IV genotypes both with proportions and distribution varying in space and time. Comparison of toxin data from cultures and shellfish also support this hypothesis. Further work is underway to confirm these findings.

Chris also mentioned the complexities we now face in identifying *Alexandrium* species. It is impossible to visually distinguish Group I toxic and Group V low toxicity genotypes, and difficult

during routine monitoring to distinguish single cells of Group IV. *A. catenella* from either *A. tamarense* type. There are also similarities in fixed samples between other co-ocurring non-toxic species such as, *A. affine*, *A. ostenfeldii* and *A. margalefii* and *Gonyaulax hyalina.* Some of the discrepancies in cell counts and shellfish toxicity noted during early 2013 may be accounted for either by the presence of low toxicity Group V *A. tamarense* or mis-identification of other related non-toxic species.

Questions:

The question was raised about the temperature that Chris' samples were found at and if temperature could indicate potential toxicity. The temperature was 14.9°C. Temperature boundaries are a broad indication of suitable habitat at larger spatial scales but seasonal extension of cool coastal currents beyond the 15 °C isotherm may provide suitable habitat along lower latitude coastlines .

The point was raised that species can have both toxic and non-toxic strains and the presence/misidentification of other similar species can account for toxin profiles not matching. It is important to know what species are present. Australia is now a high-risk area and molecular detection needs to be part of future risk management. Some of the required work has been funded/supported over the last few years by the NSW Industry and other sources so developments are underway. Further testing and validation is required but we are at least not starting from scratch.

References

Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M. (2012). Annual Reviews in Marine Science 4: 143–76.

Lilly, E.L., Halanych, K.M. & Anderson, D.M. (2007). Journal of Phycology. 43: 1329–1338

Appendix B
FDA Dye Study Report

As part of the 9th ICMSS, a dye tracing workshop was held on Friday 22nd of March, facilitated by FDA engineers with expertise in water modelling. The workshop involved:
- Adding Rhodamine WT dye into Brisbane Water, Gosford (4 hour injection),
- Taking readings in order to map out dispersion patterns and dye flow,
- Analysis of results and debrief at the end of the field component.

This study simulated a sewage discharge and provided valuable information on the impact on the oyster leases within the area. With this information closures can be limited to the affected area, ensuring that public health is protected and industry impact minimized.

U.S. Food and Drug Administration
CENTER FOR FOOD SAFETY AND APPLIED NUTRITION
U.S. Department of Health and Human Services

Hydrographic Study of Brisbane Water Area in Australia – Demonstration Project for the International Conference on Molluscan Shellfish Sanitation

Report of Findings from the March 22, 2013 Study

FDA Technical Assistance and Training Project

Reported by:
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
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Shellfish and Aquaculture Policy Branch
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TABLE OF CONTENTS

1.0 INTRODUCTION ... 3
1.1 Executive Summary ... 3
1.2 Study Objectives ... 3
1.3 General Description of Study Design ... 4

2.0 METHODS ... 4
2.1 Dye Standard Preparation and Fluorometer Calibration ... 4
2.2 Dye Injection ... 5
2.3 Dye Tracking ... 5
2.4 Dilution Analysis – Dye Readings from Submersible Fluorometer ... 6

3.0 RESULTS ... 6
3.1 Background Readings ... 6
3.2 Dye Injection ... 6
3.3 Travel Time ... 7
3.4 Dye Readings from Tracking Fluorometers ... 7
3.5 Projections of Dilution vs. Distance ... 8
3.6 Dye Readings from Submersible Fluorometer – Dilution Estimates and Anticipated Fecal Coliform Concentrations During a Raw Sewage Failure ... 8

4.0 CONCLUSIONS AND RECOMMENDATIONS ... 9

LIST OF FIGURES (APPENDIX)

Figure 1: Travel Times of Dye Excursion Determined Using Boat Tracking Data from RAFT-MAP
Figure 2: Dye Concentrations Recorded by RAFT-MAP on March 22, 2013
Figure 3: Dye Concentration Points Used in Regression Analysis
Figure 4: Regression Analysis of Dilution vs. Distance from Injection Site
Figure 5: Dilution Estimates with Increasing Distance form Dye Injection Site
Figure 6: Dye Concentration Data Collected by Submersible WET Labs 2416 Fluorometer

1.0 INTRODUCTION

1.1 Executive Summary

The U.S. Food and Drug Administration (FDA) and the New South Wales (NSW) Food Authority performed a hydrographic dye study demonstration in the Brisbane Water system near Sydney, Australia as part of the 9th International Conference on Molluscan Shellfish Sanitation. The goals of the study were to train conference participants on how to conduct a dye study and assess the results and to determine the time of travel and dispersion and dilution of the dye from a potential sewage failure site to an oyster growing area in Brisbane Water.

The results of the study indicate that it would take at least 14 hours and 45 minutes for dye to travel from the injection site in the northeast sector of Brisbane Water to the oyster growing area, even if dye were to travel the shortest distance from the site to the growing area. The dye took over a full tidal cycle to reach the growing area and was not detected in the growing area or anywhere near the growing area during the dye injection or subsequent boat tracking on March 22, 2013.

FDA deployed a submersible fluorometer within the oyster growing area to determine how long it took dye to reach the growing area and what the dilution levels in the growing area were. The first apparent peak in the dye readings recorded by the submersible fluorometer took place approximately 12 hours after the dye injection, but the readings took place near the limit of detection of the fluorometer, and thus it was difficult to ascertain if the readings were measurements of actual dye or just fluctuations in background levels. However, as indicated by the data, FDA was able to determine that a dilution of at least 1,000,000:1 was achieved in the growing area for a discharge of 71,760 liters (18,957 gallons) – the volume of simulated dye-tagged effluent discharged over a 5.2 hour period during the study. Therefore, FDA concludes that for raw sewage discharges of 71,760 liters or less from sources at or near the dye injection site, a sufficient level of dilution is achieved to mitigate the impact of pathogens and the shellfish growing area would not need to be closed to harvesting.

1.2 Study Objectives

A hydrographic dye study of a stormwater drain in the northeast sector of Brisbane Water (Murphy's Bay) was conducted on March 22-23, 2013 to assess the time of travel, dilution, and dispersion of a potential sewage failure in relation to an oyster growing area. This study was also conducted as part of the 9th International Conference on Molluscan Shellfish Sanitation (ICMSS), which was held from March 17 – 22, 2013 in Sydney, Australia, to train conference participants on how to perform hydrographic dye studies.

The study objectives were to:

(1) determine the notification time needed to respond to a raw sewage failure by closing the growing area
(2) determine the dilution level of sewage by the time it reaches the growing area
(3) determine under what conditions growing area closures may or may not be necessary

(4) teach study participants how to perform a dye injection, use fluorometers and mobile GIS to track dye, calculate dye concentrations and dilutions, and interpret results

Brisbane Water had previously experienced sewage failures that resulted in closures of the oyster growing area for long periods of time.

1.3 General Description of Study Design

The dye for the study was injected over 5.2 hours and remained in the Brisbane Water system for at least one day. Boat tracking with towed fluorometers on two boats was conducted to find the dispersion and dilution of the dye plume during daylight hours. Data was recorded with FDA's mobile geographic information system (GIS) software. Additionally, continuous dye readings were recorded by a single submersible fluorometer attached to a post within the oyster growing area. This fluorometer was collected three days after the dye injection. Data from the towed fluorometers was mapped and analyzed using the mobile GIS system and ArcGIS Desktop. Data from the submersible fluorometer was assessed to determine the time at which dye reached the growing area and the dilution level of the dye.

2.0 METHODS

2.1 Dye Standard Preparation and Fluorometer Calibration

The dye tracer used in this study was Rhodamine WT provided by the NSW Food Authority (Lot # A210H519). The Rhodamine WT dye was purchased from the Keystone Aniline Corporation and had a specific gravity of approximately 1.12 (20% as dry dye). Ten (10) standards were prepared from a sample collected from the 1:2 mix of Rhodamine WT dye and distilled water that was used in the dye injection. The standards were prepared by serial dilution, ranging from 100,000 parts per million (ppm) to 0.1 parts per billion (ppb).

The Rhodamine WT dye was detected and its concentrations in Brisbane Water were obtained using a combined total of three fluorometers. Two of these were WET Labs FLRHRT fluorometers (WET Labs, Inc., Philomath, OR) that were pulled behind a boat and used for tracking the dye during and shortly after the dye injection on March 22, 2013. The third unit was a WET Labs FLRHB fluorometer with internal batteries and memory that was attached to a post in the growing area after the boat tracking was concluded. This fluorometer was left in the water for three days to determine if any dye reached the growing area in subsequent tidal cycles.

The dye standards were used to develop calibration curves for FDA's WET Labs FLRHRT-586 and FLRHRT-2040 tracking fluorometers and FDA's WET Labs FLRHB-2416 submersible fluorometer. With the subtraction of background fluorescence levels in Brisbane Water, these curves were used to calculate part per billion (ppb) levels of dye based on the WET Labs' measured fluorescence units (FUs). Background readings were captured six days prior to the study, on March 16, 2013.

The y-intercept of the calibration curve was adjusted so that a "0.1 ppb" result read as a perfect "0.1" on the curve. The slope and x-axis values for the curve remained the same, but this

adjustment caused a slight addition of error (5-10% error) to the higher concentrations on the curve, such as 10 ppb and 100 ppb. However, higher accuracy at the lower end of the curve, 0.1 ppb, is more vital in order to optimize sensitivity in detecting the dye at low concentrations, as important data tends to fall within the 0.1-1 ppb range during FDA dye studies. Using a calibration curve adjusted in this manner is necessary when converting raw FU readings to ppb values if sensitivity in the 0.1-1 ppb range is critical for the study.

2.2 Dye Injection

To facilitate the pumping of dye, deionized water was added to the dye to create a 1:2 dye dilution mixture. For the dye injection, dye was injected at a constant rate into the stormwater drain over a 5.2 hour period from 7:20 AM – 12:30 PM on March 22, 2013. The dye was first observed on the water at 7:27 AM. A total of 15 Liters of the dye/water mixture was injected during the study. A Masterflex model 7553-20 variable speed peristaltic pump (Cole-Palmer Instrument Co.) was used to withdraw the tracer dye solution from a large plastic holding bin, using Masterflex Tygon L/S-14 tubing. A pump head size 7014 was used with a constant pumping rate of 50 ml/min which was maintained at about 225 revolutions/minute (rpm) head speed. A fire truck simulated a large rainfall event by adding water from a nearby hydrant into the stormwater drain at the same time the dye was being injected. The average pump rate of the fire truck was approximately 230 L/min. The initial concentration of the dye in the pumped water from the fire hose was determined using this flow average and the amount of dye injected over the course of the study.

2.3 Dye Tracking

The dye plume was followed on March 22, 2013 from 8:00 AM to 3:00 PM as it moved through Brisbane Water on an ebb tide using FDA's boat-towed WET Labs FLRHRT-586 and FLRHRT-2040 fluorometers. The fluorometers were linked to Panasonic Toughbook C-19 computers running FDA's mobile GIS system, the Real-Time Application for Tracking and Mapping (or RAFT-MAP). Two boats were used, with each instrument and computer on a different boat. Traverses of the dye plume were done from north to south and east to west and vice versa.

The calibration curve data for the each fluorometer was programmed into RAFT-MAP. This information was used by RAFT-MAP to convert raw dye readings from the fluorometer (in fluorescent units) into ppb concentration units. A five-point moving average was applied to the dye concentration data in RAFT-MAP to smooth out any false high or low readings. Dilution was calculated by dividing the initial concentration of dye injected at the stormwater drain by the final (five-point moving average) concentrations detected in the estuary. The associated GPS coordinates for each dye concentration data point were also recorded in RAFT-MAP. All of the data was displayed on a GIS map in real-time as it was being collected. Dye concentration data points were color-coded, with red, orange, and yellow points representing higher levels of dye, green points representing lower levels of dye, and white points representing the non-detection of dye. The data could also be viewed in a tabular format in the "Data View" feature of RAFT-MAP. Participants from the ICMSS conference were trained on how to use the RAFT-MAP program during the study.

All of the data and GIS layers stored in RAFT-MAP were downloaded as a geodatabase into ArcGIS Desktop v. 10.1 after the study. Using ArcGIS Desktop v. 10.1, FDA created refined GIS maps of the dye study data, including the 5-point moving average concentration points as well as other data of interest, such as travel times. A regression analysis of dilution vs. distance from the injection site was then conducted in SigmaPlot 10.0 using ten data points collected at different times in RAFT-MAP. This analysis was used to estimate the distance at which 100,000:1 and 1,000,000:1 dilution levels were achieved and to try and estimate the dilution level within the oyster growing area.

2.4 Dilution Analysis - Dye Readings from Submersible Fluorometer

The FLRHB-2416 submersible fluorometer was attached to a post within the growing area and programmed to record 30 seconds of data every 10 minutes. After the study, the fluorescence readings recorded by the FLRHB-2416 fluorometer were downloaded, converted to ppb using the fluorometer's calibration curve chart, and plotted in SigmaPlot.

A 60-point (one minute) moving average was applied to the dye concentration data to smooth out any false high or low readings. Dilution was calculated by dividing the initial concentration of dye injected by the final (60-point moving average) concentrations detected in Brisbane Water. Dilution level markers of 100,000:1 and 1,000,000:1 were added to the figure for comparison with the dye concentration readings. The submersible fluorometer had a pressure/depth sensor and the readings from this sensor were included in the figure to indicate changes in tidal cycles.

3.0 RESULTS

3.1 Background Readings

Background levels of fluorescence units (FUs) for the WET Labs FLRHRT-586 tracking fluorometer were measured as 77 - 89 FUs on average. These are normal background levels for an estuary system evaluated with the FLRHRT-586 fluorometer and are not considered indicative of excessive background levels, as are often seen in areas with high industrial activity. A background level of 87 was subtracted from the fluorescence readings during the dye studies, because this was the highest value that repeated often during background tracking.

Background levels of fluorescence units (FUs) for the WET Labs FLRHRT-2040 tracking fluorometer were measured as 49 - 52 FUs on average. The raw fluorescence readings recorded by the FLRHRT-2040 are normal background levels for an estuary system evaluated with this fluorometer. A background level of 52 was subtracted from the fluorescence readings during the dye studies, because this was the highest value that repeated often during background tracking. However, it was noted during the study that background levels were slightly lower within the oyster growing area (49 – 50 FUs) than outside the growing area (up to 52 FUs).

3.2 Dye Injection

A 15 liter mixture of Rhodamine WT dye and distilled water (1:1 ratio of dye to water) was prepared for the dye injection. Several fire trucks delivered water at a flow rate of ~230 L/min to

simulate a sewage effluent release. The dye injection began around 7:20 AM on March 22, 2013 and continued for 5.2 hours. Based on a flow of ~230 L/min from the fire hose simulating a rain event and using the concentration of dye in the jug (approximately 100,000,000 ppb) and the flow out of the jug (50 ml/min or 72 L/day), it was determined using a mass balance approach that the concentration of dye flowing out of the storm drain was 21,739 ppb. The total volume of dye-tagged water released in simulation of a sewage discharge was 71,760 liters (230 L/min x 60 min/hour x 5.2 hours), which equates to 18,957 gallons.

3.3 Travel Time

Figure 1 shows the extent of dye travel on an ebb tide on March 22, 2013. Dye was tracked from the injection site to the southern tip of the peninsula marked "End of Detectable Dye". The dye injection started at 7:20 AM and dye was first seen on the water at 7:27 AM. The dye excursion was then tracked until 3:04 PM, which is when the end of the detectable dye was recorded. As shown in the figure, higher concentrations of dye (>10 ppb, shown in red) traveled south at a rate of 0.117 km/h. Lower concentrations of dye (< 10 ppb) traveled south at rates of 0.213, 0.214, and 0.239 km/h or an average of 0.220 km/h. Dye movement in the western direction from the dye injection site consisted of dye concentrations in the range of 0.10 – 0.50 ppb and took place at a rate of 0.229 km/h.

Winds during the study were pushing the dye primarily in a southeast direction towards the shoreline. It's possible that under a different set of wind or tidal conditions, dye could be pushed in a more direct manner towards the growing area. A direct line measurement of distance from the injection site to the growing area is 3.525 km. The slowest speed of dye movement measured during the study was 0.117 km/h, which could result in dye reaching the growing area in 30 hours and 8 minutes. The fastest speed of dye movement was 0.239 km/h, which could result in dye reaching the growing area in 14 hours and 45 minutes in a worst-case scenario. In the event of a large-scale sewage failure in the vicinity of the dye injection site, there should be at least 14 hours and 45 minutes for authorities to respond and close the growing area.

3.4 Dye Readings from Tracking Fluorometers

Figures 2 and 3 represent the 5-point moving average concentration values for the dye study on March 22, 2013 as determined by the WET Labs 586 and the WET Labs 2040 FLRHRT boat tracking fluorometers. These figures were created using the geodatabase imported from FDA's mobile GIS program, RAFT-MAP, but the raw data collected by RAFT-MAP (in Excel sheets) can be provided upon request.

Only background levels were detected in and around the growing area. False positive readings caused by the boat moving too fast or other factors were removed from the data set. No dye was detected within the growing area during the period of boat tracking. Dye was detected up to the tip of the peninsula at the end of the day of tracking, around 3:04 PM, but was not detected beyond this point with the boat-towed fluorometers. Near-shore, the dye moved slowly to the south and remained concentrated at levels of 0.50 ppb or higher until the end of the day of tracking when it became more dilute as it approached the tip of the peninsula. Dye close to the

injection site also spread out in a westerly direction, but was diluted to levels of 0.10 – 0.50 ppb as it moved off-shore.

Figure 3 shows the dye concentration levels and locations of ten data points used in a regression analysis of dilution vs. distance from the injection site. Nine of the ten data points were collected between 12:05 PM and 12:42 PM on March 22, 2013 and are shown on the map with pink lines. The tenth data point is the farthest from the dye injection site and was collected about two hours later at 2:34 PM. This point is marked with a blue line on the map and was used to create a separate regression from the other data points since it more accurately reflects the dilution of the dye at a later point in time on the day of dye tracking.

3.5 Projections of Dilution vs. Distance

Figure 4 shows a regression analysis of dilution vs. distance from the injection site (potential raw sewage discharge location) based on the ten high concentration points identified in Figure 3. The data points collected between 12:05 PM and 12:42 PM were plotted with a green dashed line representing the regression and the data point collected at 2:34 PM was plotted along with the first two data points using a blue dashed line to represent the regression. The equation for the latter regression is included in the figure, as FDA believes this regression to be a more accurate measure for predicting dilution levels beyond the locations where dye was tracked during the study. This regression represents the conditions later in the ebb tide when current velocities increased. Under these conditions, the dye moved more rapidly downstream from the injection site and in higher concentrations. Thus, the more conservative regression was used for dilution projections.

For the regression equation determined in Figure 4, 100,000:1 dilution is achieved around 2000 meters from the dye injection site and 100,000,000:1 dilution is achieved at less than 2800 meters from the dye injection site. The distance to the growing area from the injection site is about 3525 meters, so based on this analysis, FDA projects that greater than 1,000,000:1 dilution should be achieved within the growing area for sewage discharges of the same volume or less than the volume of dye injected. The map in Figure 5 shows how dilution is predicted to increase with increasing distance from the injection site, and it shows the location where 1,000,000:1 dilution is predicted to take place prior to a discharge reaching the growing area.

3.6 Dye Readings from the Submersible Fluorometer - Dilution Estimates and Anticipated Fecal Coliform Concentrations During a Raw Sewage Failure

Dye readings and pressure readings recorded by the submersible WET Labs FLRHB-2416 fluorometer are shown in Figure 6. Any readings at or below background levels were removed from the graphs after a 60-point moving average was applied.

As seen in the figure, no dye was detected at levels greater than 0.02 ppb, which is equivalent to approximately 1,000,000:1 dilution. The first apparent peak in the dye readings recorded by the submersible fluorometer took place approximately 12 hours after the dye injection, but these readings were less than 0.02 ppb and were close to the limit of detection of the fluorometer. The dye peaks did not correspond directly with the tidal cycles as indicated by the pressure data, and

FDA could not determine if the readings were measurements of actual dye or just fluctuations in background levels. The only result FDA can state with certainty in regards to the FLRHB-2416 data is that a greater than 1,000,000:1 dilution was achieved at the location of the fluorometer within the growing area. This finding supports the projection of greater than 1,000,000 dilution in the growing area from the regression analysis with the boat tracking data as described in Section 3.4.

Based on scientific literature and recent FDA studies, a fecal coliform value of 1.4×10^6 FC/100 ml is typically applied by FDA for a raw sewage discharge. For untreated sewage, FDA typically recommends a dilution level of 100,000:1 prior to the sewage reaching an approved growing area in order to achieve a fecal coliform level of 14 FC/100 ml or less in accordance with the U.S. National Shellfish Sanitation Program geomean standard for approved areas. If a raw sewage discharge occurred at or near the injection site in Brisbane Water, FDA estimates that the level of fecal coliforms in the oyster growing area from the discharge would be less than 1.4 FC/100 ml based on a greater than 1,000,000-fold dilution.

4.0 CONCLUSIONS AND RECOMMENDATIONS

FDA and the NSW Food Authority conducted a dye study on March 22, 2013 in Brisbane Water to train ICMSS conference participants on how to conduct dye studies in assessing pollution source impacts on shellfish growing areas and to determine the potential impact of raw sewage discharges on the oyster growing area for purposes of managing the area in the event of a failure. Both objectives were achieved during the study, with conference participants gaining a better understanding of the methods and tools used by FDA to conduct dye studies and the findings of the study being used to make recommendations for area management as described below.

Based on the results of the dye study, the dye was quickly diluted and dispersed in both southerly and westerly directions and did not reach the growing area during the 5.2 hour injection period or the 7 hour boat tracking period on March 22, 2013. Using the boat tracking data collected during the study, a regression analysis of dilution vs. distance from the dye injection site was performed to project the dilution of dye within the growing area in the case that the dye might reach the growing area at a later time. The projection indicated that dilution would be greater than 1,000,000:1 by the time dye reached the growing area. A submersible fluorometer placed within the growing area for approximately 3 days confirmed this projection in that dye readings indicated greater than 1,000,000:1 dilution at the site of the fluorometer.

For these reasons, FDA recommends that the oyster growing area could remain open when a failure occurs at or near the dye injection site and the volume of sewage discharged is 71,760 liters (18,957 gallons) or less. This volume is equivalent to the volume of dye-tagged simulated effluent released during the study. FDA does not have enough information to determine what would happen in the growing area if a higher volume of sewage were to be discharged. Based on information provided by the NSW Food Authority, it seems unlikely that a raw sewage failure of greater than 71,760 liters would occur in this area, but in such a scenario, FDA recommends closing the area as soon as possible and prior to 14 hours and 45 minutes, since this is the worst-case travel time estimated based on the fastest rate at which dye was moving during the study and the shortest distance from the injection site to the growing area.

Figure 1: Travel Times of Dye Excursion Determined Using Boat Tracking Data from RAFT-MAP

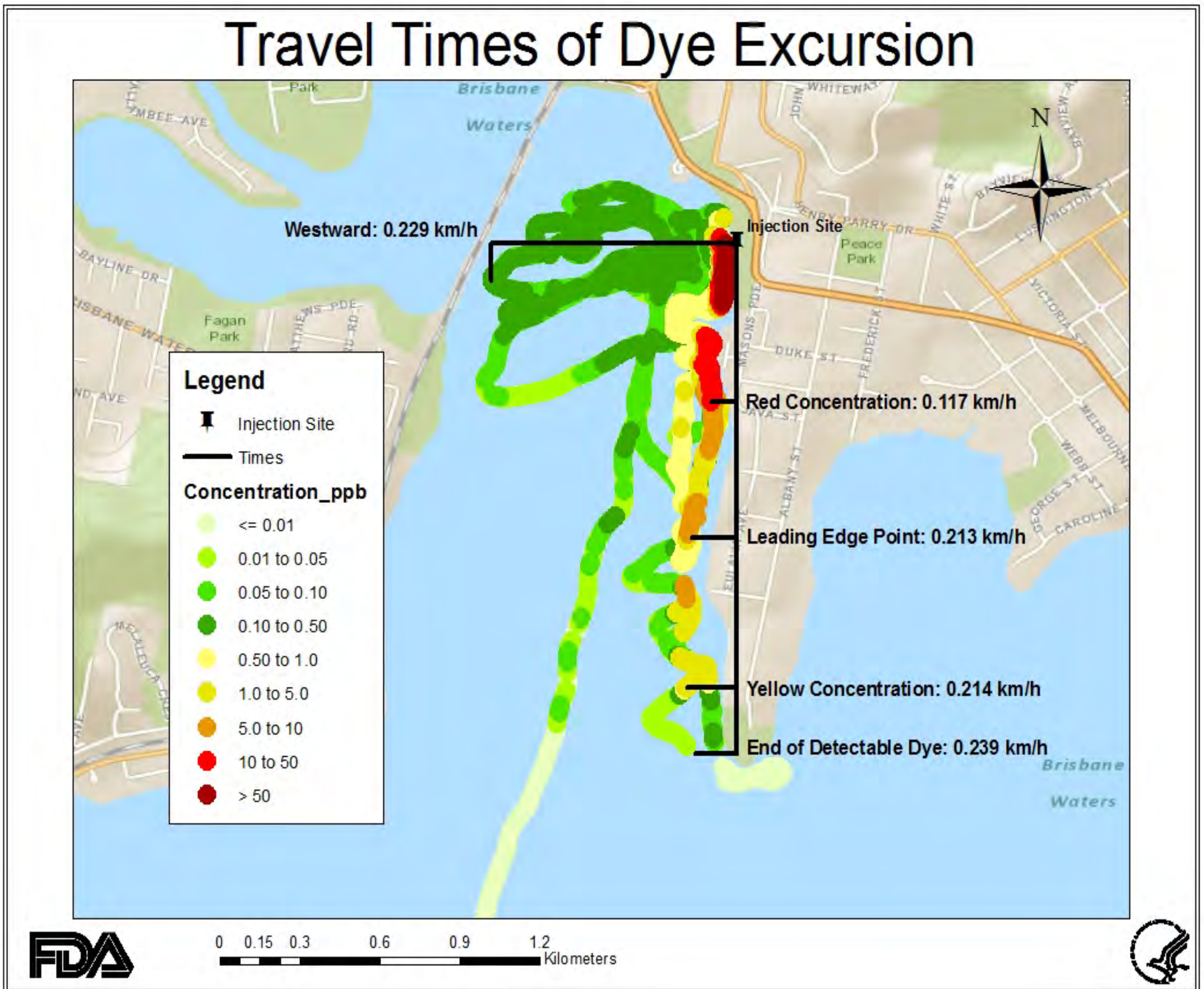


Figure 2: Dye Concentrations Recorded by RAFT-MAP on March 22, 2013

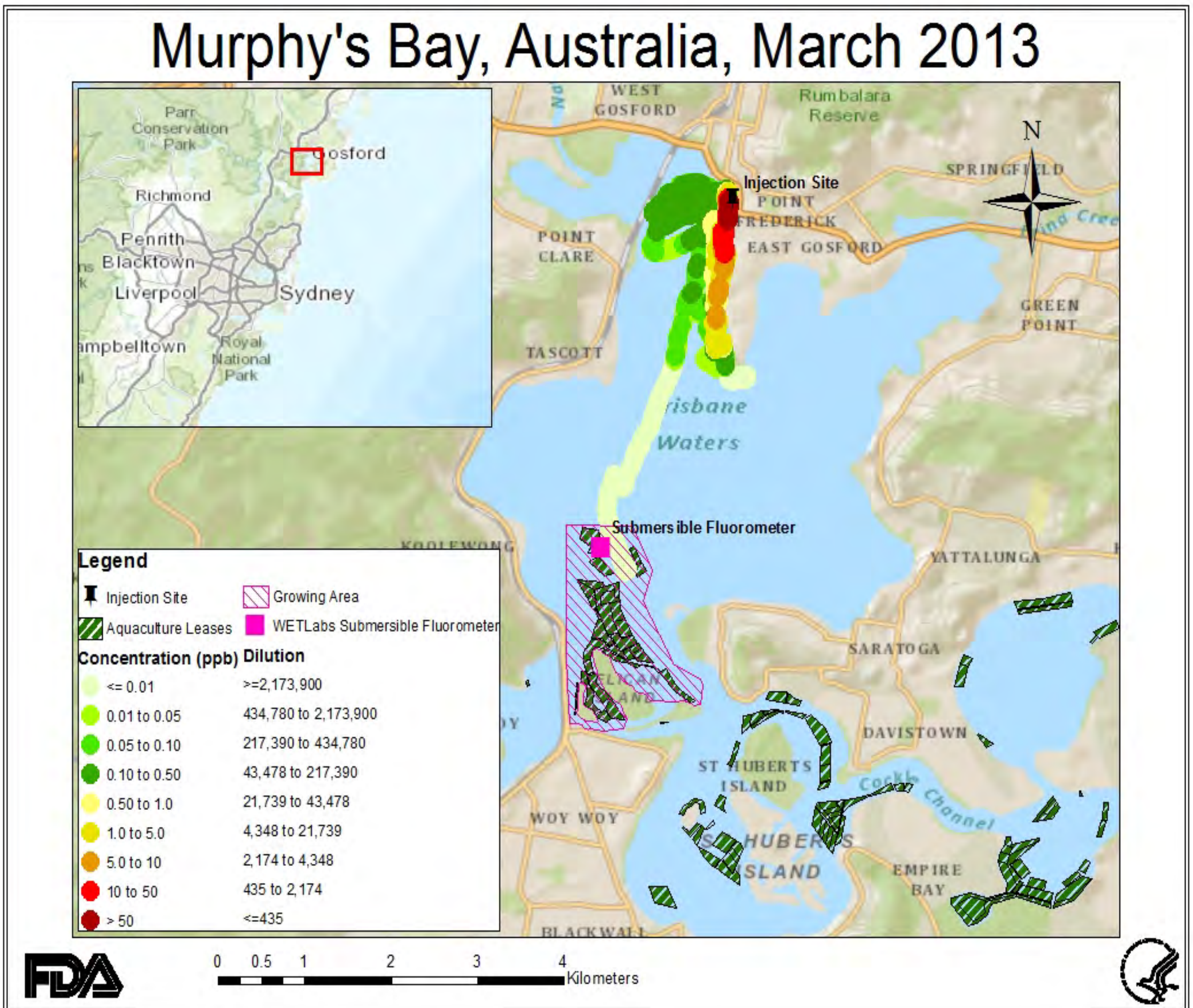


Figure 3: Dye Concentration Points Used in Regression Analysis

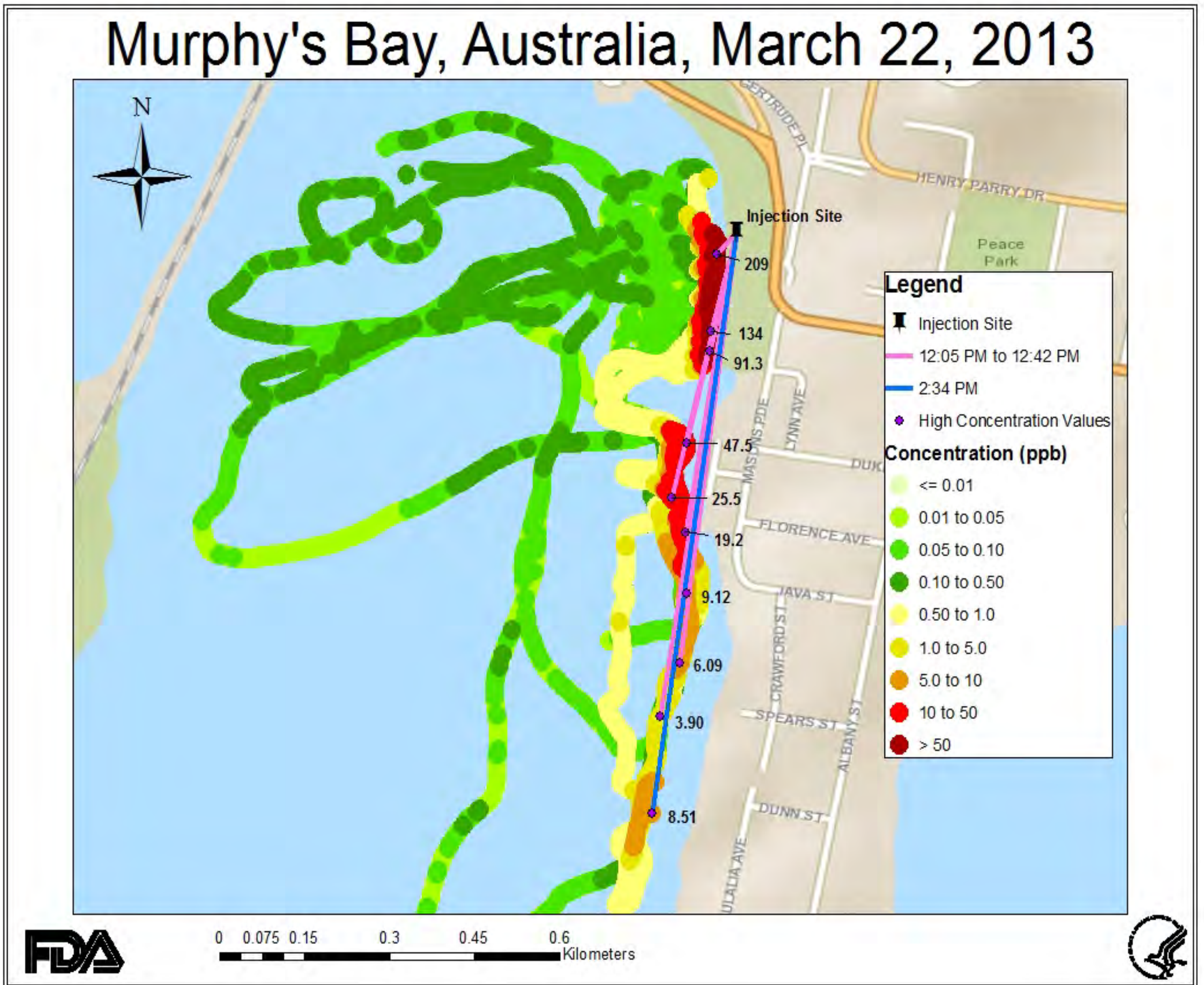
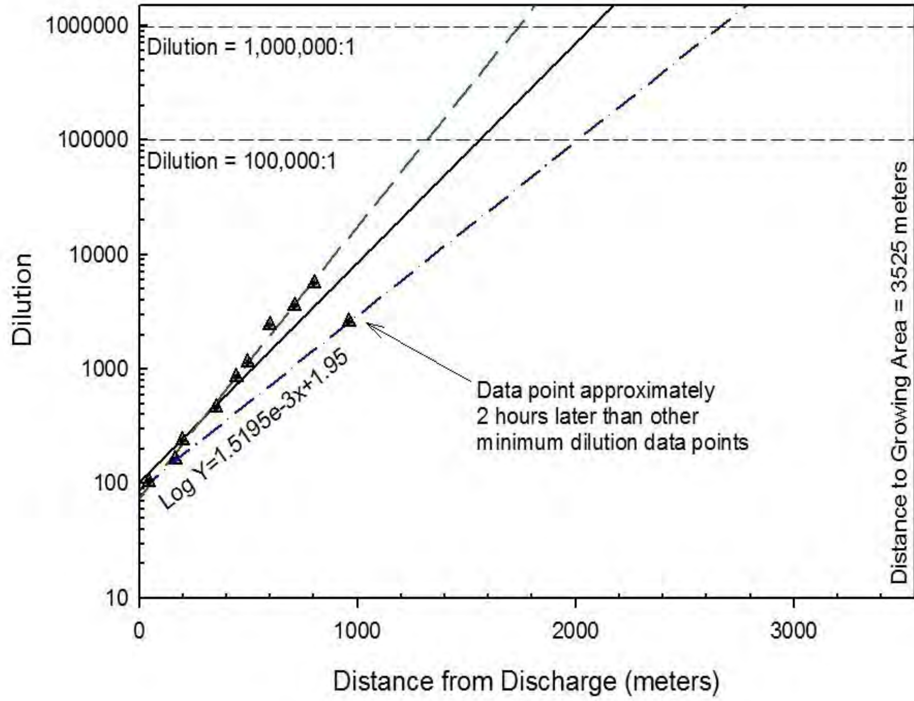


Figure 4: Regression Analysis of Dilution vs. Distance from Injection Site



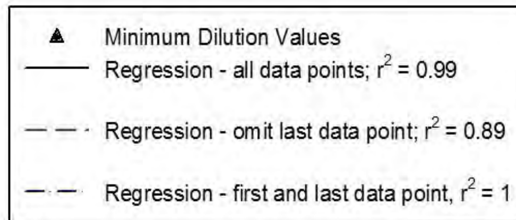


Figure 5: Dilution Estimates with Increasing Distance from Dye Injection Site

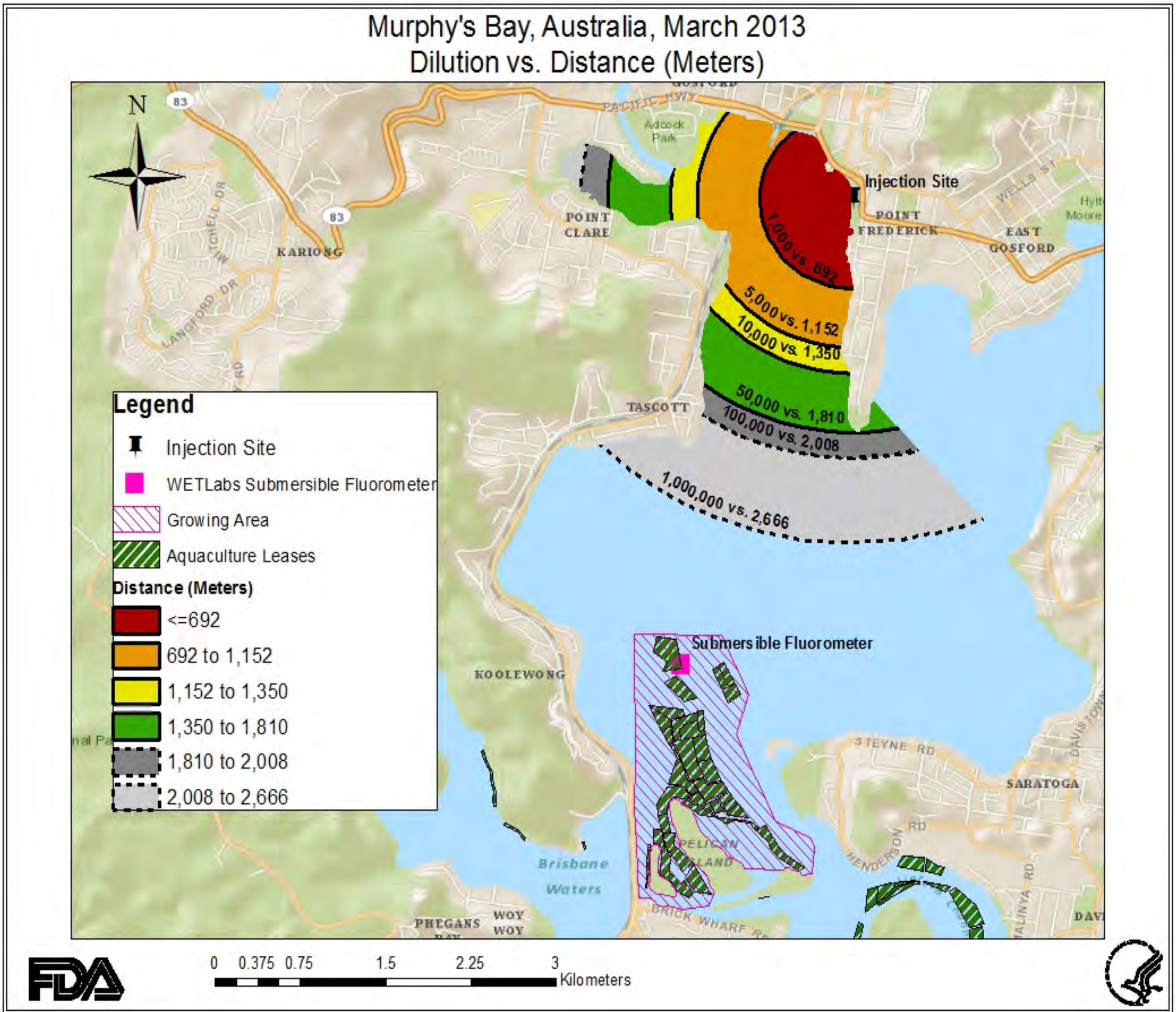


Figure 6: Dye Concentration Data Collected by Submersible WET Labs 2416 Fluorometer