Gilbert Sauvé *Editor*

Molluscan Shellfish Safety

Springer

Molluscan Shellfish Safety

Gilbert Sauvé
Editor

Molluscan Shellfish Safety

Proceedings of the 8th ICMSS,
Charlottetown, PEI, Canada,
June 12–17, 2011

Springer

Editor
Gilbert Sauvé
Canadian Food Inspection Agency
Ottawa, ON
Canada

ISBN 978-94-007-6587-0 ISBN 978-94-007-6588-7 (eBook)
DOI 10.1007/978-94-007-6588-7
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2013941043

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The organizers and myself were very pleased to welcome you to the 8th edition of the International Conference on Molluscan Shellfish Safety, held in Charlottetown, Prince Edward Island, from June 12 to 17, 2011.

Despite the tough economic times, there was a good turnout with representatives from various fields. This allowed us to make this 8th edition one of reflection on the scientific basis of requirements imposed to those who wish to export molluscs, often developing countries, as well as those economically stronger countries that would like to import more. The former can produce more with limited technological support while the latter develop and require the use of sophisticated and expensive technologies for their internal production. Managing this dichotomy is an ongoing challenge and our risk management measures need to find a balance in effectively minimising risk without unrealistic costs.

It is no coincidence that the conference logo features a long bridge – symbolic of the way people reach one another. Let's try to keep those bridges open without requiring excessive tolls.

We especially wish to thank the Food and Agriculture Organization (FAO) for their help with more than ten participants from developing countries who were given the opportunity to present the results of their research at the conference.

As in previous years, the 2011 ICMSS developed a program centred on the key themes and made it possible for everyone to attend more than 50 oral presentations and view 55 poster presentations. A special thank you to all these experts for enlightening us and enabling meaningful discussions. What other reasons would there be for us to meet every two years but to learn more, create new relationships and encourage new partnerships?

An event such as the ICMSS can only happen with the support of strong organizations. Members of various organizing committees were recruited from the ranks of the Canadian Food Inspection Agency and the Department of Fisheries, Aquaculture and Rural Development within the province of Prince Edward Island. Members of the ICMSS International Advisory Committee cannot be forgotten for their support from the beginning.

We appreciated the resources and funding received from several organizations, especially the Government of Prince Edward Island (the above mentioned Department and the Department of Environment, Energy and Forestry) and Fisheries and Oceans Canada. Several other valuable private and institutional sponsors are listed on the following pages. I sincerely thank each and every one of them.

I hope that all the peer-reviewed scientific articles, presented here, will inspire you and stimulate further research that will deepen our understanding of the production, processing and marketing of safe shellfish for the well-being of all populations.

Thank you to everyone and we will meet again in Australia in 2013.

Lead Organizer of the ICMSS 2011 Gilbert Sauvé

Préface

C'est avec grand plaisir que les organisateurs et moi-même vous avons accueillis pour cette huitième édition de la Conférence internationale sur la sécurité des coquillages qui s'est tenue à Charlottetown sur l'Île-du-Prince-Édouard du 12 au 17 juin 2011.

Malgré un contexte économique difficile, la participation a été bonne et nous avons pu rencontrer des représentants de tous les champs d'expertise. Cela nous a permis de faire de cette huitième édition celle d'une réflexion sur les fondements scientifiques des exigences imposées à ceux qui veulent exporter des mollusques, souvent les pays en voie de développement, et ceux qui voudraient en importer plus, c'est-à-dire les pays économiquement plus solides. Les uns peuvent produire plus avec le support de technologies simples alors que les autres développent et exigent le recours aux technologies sophistiquées et chères qu'ils utilisent pour leur propre production. Les défis résident dans la manière de gérer cette dichotomie et dans une recherche de l'équilibre dans la gestion du risque qui permette de minimiser le risque tout en évitant les coûts prohibitifs.

Ce n'est pas un hasard si le logo de la conférence intègre un long pont – symbole du moyen par lequel les peuples se rejoignent. Tentons de garder ces ponts ouverts sans exiger des droits de passage excessifs.

Je tiens à adresser un remerciement spécial à l'Organisation pour l'Agriculture et l'Alimentation (FAO) pour l'aide apportée à plus de dix participants de pays en voie de développement qui ont ainsi pu venir présenter les résultats de leur recherche à la conférence.

Comme lors des années précédentes, l'ICMSS 2011 a développé un programme centré sur les thèmes-clé, ce qui a permis à chacun d'assister à plus de 50 présentations orales et de lire environ 55 présentations sur affiches. Merci à tous ces spécialistes de nous avoir transmis leur connaissance et d'avoir susciter des échanges fructueux. Pour quelles autres raisons nous réunissons-nous aux deux ans sinon pour apprendre, créer de nouveaux liens et susciter de nouvelles coopérations?

Un événement comme l'ICMSS ne s'organise qu'avec l'aide d'une solide organisation : les membres des différents comités ont été recrutés dans les rangs de l'Agence Canadienne d'Inspection des Aliments et du Ministère des Pêches,

de l'Aquaculture et du Développement Rural de la province de l'Île-du-Prince-Édouard. Sans oublier, bien sûr, les membres du comité consultatif international qui nous ont épaulé depuis le début.

En plus des ressources humaines, nous avons reçu des fonds de plusieurs organisations dont les principales sont le gouvernement de l'Île-du-Prince-Édouard (le ministère nommé plus haut et le ministère de l'Environnement, de l'Énergie et des Forêts) et Pêches et Océans Canada. Les autres commanditaires institutionnels et privés sont énumérés aux pages suivantes. Je les en remercie tous.

Je souhaite que l'ensemble des articles scientifiques, évalués par les pairs, qui forme le corps des actes de la conférence saura vous inspirer et susciter de nouvelles recherches qui feront progresser nos connaissances sur la manière de produire, traiter et mettre en marché des coquillages sains pour le bien-être de toutes les populations.

Merci à tous et revoyons-nous en Australie en 2013.

Organisateur principal de l'ICMSS 2011 Gilbert Sauvé

Acknowledgements

We wish to express our deep appreciation to people who have directly or indirectly collaborated to make this conference a success and this book a reference for the specialist in this field of work.

Remerciements

Nous désirons exprimer nos sincères remerciements aux personnes qui directement et indirectement ont collaboré à faire de cette conférence un succès et de ce livre une référence pour les spécialistes du domaine.

Reviewers / Réviseurs

John A. AASEN BUNAES
Norwegian School of Veterinary Science
Norway

Solomon AKLILU
Canadian Food Inspection Agency
Canada

Zouher AMZIL
French Research Institute for Exploitation of the Sea (IFREMER)
France

Les E. BURRIDGE
Fisheries and Oceans Canada, Coastal Ecosystem Science Division
Canada

Hélène COUTURE
Health Canada, Evaluation Division, Bureau of Microbial Hazards
Canada

Pablo DE LA IGLESIA
Institute for Food Research and Technology (IRTA)
Spain

Jorge DIOGENE FADINI
Institute for Food Research and Technology (IRTA)
Spain

Arne DUINKER
The National Institute of Nutrition and Seafood Research (NIFES)
Norway

Robyn EDWARDS
Canadian Food Inspection Agency
Canada

J.M. FREMY
Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du travail (ANSES)
France

Ana GAGO-MART´iNEZ
EU Reference Laboratory for Marine Biotoxins, University of Vigo
Spain

Katsuji. HAYA
Fisheries and Oceans Canada
Canada

Philipp HESS
French Research Institute for Exploitation of the Sea (IFREMER)
France

Joanne HEWITT
Institute of Environmental Science and Research (ESR)
New Zealand

Wendy HIGMAN
Centre for Environment, Fisheries and Aquaculture Science (CEFAS)
United Kingdom

Hussein HUSSEIN
Health Canada, Evaluation Division, Bureau of Microbial Hazards
Canada

Iddya KARUNASAGAR
Products, Trade and Marketing Service, Food and Agriculture Organisation (FAO)
Italy

Valentin KOUAME,
Health Canada, Evaluation Division, Bureau of Microbial Hazards
Canada

Patrick LASSUS
French Research Institute for Exploitation of the Sea (IFREMER)
France

Dot LEONARD
CEO, Ocean Equities LLC
USA

Carlos LIMA DOS SANTOS
International Consultant, Rio de Janeiro-RJ
Brazil

Annie LOCAS
Canadian Food Inspection Agency, Office of Food Safety and Recall
Canada

John D. MARTELL
Fisheries and Oceans Canada, Aquaculture Science Branch
Canada

Pearse MCCARRON
National Research Council Canada
Canada

Cath MCLEOD
South Australian Research and Development Institute
Australia

Kazuaki MIYAGISHIMA
World Organization for Animal Health (OIE)

Cory MURPHY
Canadian Food Inspection Agency, Dartmouth Laboratory
Canada

Mitsuaki NISHIBUCHI
Kyoto University
Japan

Gary RICHARDS
Agricultural Research Service, United States Department of Agriculture
USA

Christopher ROBERTS
Environment Canada, Marine Water Quality Monitoring – Atlantic
Canada

Shawn M.C. ROBINSON
Fisheries and Oceans Canada
Canada

Wade ROURKE
Canadian Food Inspection Agency, Dartmouth Laboratory
Canada

Krista THOMAS
National Research Council Canada
Canada

Andy TURNER
Centre for Environment, Fisheries and Aquaculture Science (CEFAS)
United Kingdom

David J. WILDISH
Fisheries and Oceans Canada
Canada

Jacquelina WOODS
Gulf Coast Seafood Laboratory, U.S. Food and Drug Administration
USA

ICMSS 2011 COMMITTEES / COMITÉS DE L'ICMSS 2011

Steering Committee / Comité directeur

Neil MACNAIR[1]
Terence MCRAE[2]
Kelly CANTELO[1]
Gilbert SAUVÉ[2]
Rod PENNEY[2]
Kim GILL[1]

Organizing Committee / Comité organisateur

Gilbert SAUVÉ[2]
Kim GILL[1]

---

[1]Prince Edward Island, Department of Fisheries, Aquaculture and Rural Development / Ministère des Pêches, de l'Aquaculture et du Développement rural de l'Île-du- Prince-Édouard.

[2]Canadian Food Inspection Agency / Agence canadienne d'inspection des aliments.

John WHITE[2]
Robyn EDWARDS[2]
Maria IANNI[2]
Scott BRADLEY[1]
Sarah LALIBERTÉ[2]
Muchigi GITHII[2]
Tracey SINGLETON[3]
Tracy STRETCH[3]
Shannon MURNAGHAN[3]
Angie GALINDO[2]

Scientific and Program Committee / Comité scientifique et du programme

Robyn EDWARDS[2]
Enrico BUENAVENTURA[4]
Jeff VAN DE RIET[2]
Jeff FARBER/Hélène COUTURE[4]
Kim GILL[1]
Aaron RAMSAY[1]
Jennifer MARTIN[5]
Maurice LEVASSEUR[6]
Sarah LALIBERTÉ[2]

International advisory committee / Comité consultatif international

Lahsen ABABOUCH
Food and Agriculture Organization (FAO)
Italy / Italie

Phil BUSBY
New Zealand Food Safety Authority
New Zealand / Nouvelle-Zélande

---

[3]Versatile Management Group.
[4]Health Canada / Santé Canada.
[5]Fisheries and Oceans Canada / Pêches et Océans Canada.
[6]Laval University / Université Laval.

Cyr COUTURIER
The Fisheries and Marine Institute of Memorial University of Newfoundland
Canada

Patrick LASSUS
French Research Institute for Exploitation of the Sea (IFREMER)
France

David LEES
Centre for Environment, Fisheries and Aquaculture Science (CEFAS)
United Kingdom / Royaume-Uni

Dorothy L. LEONARD
CEO, Ocean Equities LLC
United States / États-Unis

Rowena LINEHAN
Canadian Food Inspection Agency
Canada

Catherine MCLEOD
South Australian Research and Development Institute
Australia / Australie

Dinorah MEDINA
National Direction of Aquatic Resources (DINARA)
Uruguay

Mitsuaki NISHIBUCHI
Kyoto University
Japan / Japon

Gary RICHARDS
Agricultural Research Service, United States Department of Agriculture
United States / États-Unis

Joe SILKE
Marine Institute
Ireland / Irelande

Helen SMALE
Marlborough Shellfish Quality Programme Inc.
New Zealand / Nouvelle-Zélande

WANG Wei
General Administration of Quality Supervision, Inspection and Quarantine of China (AQSIQ)
China / Chine

ICMSS 2011 Conference Sponsors/Commanditaires de la conférence

Diamond/Diamant----------

Prince Edward Island CANADA — Fisheries, Aquaculture and Rural Development

Île-du-Prince-Édouard CANADA — Pêches, Aquaculture et Développement rural

Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

Prince Edward Island CANADA — Environment, Energy and Forestry

Île-du-Prince-Édouard CANADA — Environnement, Énergie et Forêts

Fisheries and Oceans Canada | Pêches et Océans Canada

Silver/Argent----------

Atlantic Canada Opportunities Agency | Agence de promotion économique du Canada atlantique

Canada

United States Food and Drug Administration

FDA

National Research Council – Conseil national de recherches Canada

NRC-CNRC Canada

Bronze ----------

PRINCE EDWARD AQUA FARMS INC.

Seafood Producers Association of Nova Scotia

CANADIAN AQUACULTURE INDUSTRY ALLIANCE | ALLIANCE DE L'INDUSTRIE CANADIENNE DE L'AQUACULTURE

Fisheries Council of Canada
Conseil Canadien des Pêches
www.fisheriescouncil.ca

Prince Edward Island AQUACULTURE ALLIANCE
SUPPORT. PROMOTE. GROW.

Centre for Environment, Fisheries and Aquaculture Science/Centre de science de l'environnement, de pêches et d'aquaculture. Weymouth, UK

Cefas

Special Partner/Partenaire spéciale----------

Food and Agriculture Organization of the United Nations (FAO)

FAO FIAT PANIS

Table of Contents/Table des matières

Associate Editors

Enrico Buenaventura
Heath Canada, Frederick G Banting Building – Floor: 4 – Room: E430, 251 Sir Frederick Banting Driveway, Tunney's Pasture Mail Stop: 2204E Ottawa, Ontario, Canada K1A 0K9
enrico.buenaventura@hc-sc.gc.ca

Hélène Couture
Heath Canada, Frederick G Banting Building – Floor: 4 – Room: E430, 251 Sir Frederick Banting Driveway, Tunney's Pasture Mail Stop: 2204E Ottawa, Ontario, Canada K1A 0K9
helene.couture@hc-sc.gc.ca

Robyn Edwards
Canadian Food Inspection Agency, 5th Floor, 1400 Merivale Road, Tower 2, Ottawa, Ontario, Canada K1A 0Y9
Robyn.Edwards@inspection.gc.ca

Muchigi Githii
Canadian Food Inspection Agency, 5th Floor, 1400 Merivale Road, Tower 2, Ottawa, Ontario, Canada K1A 0Y9.
Muchigi.Githii@inspection.gc.ca

Cory Murphy
Canadian Food Inspection Agency, 1992 Agency Drive, Dartmouth, Nova Scotia, Canada B3B 1Y9
Cory.Murphy@inspection.gc.ca

Dave Wildish
Fisheries & Oceans Canada, 531 Brandy Cove Road, St. Andrews, New Brunswick, Canada E5B 2L9
Dave.Wildish@dfo-mpo.gc.ca

John White
Canadian Food Inspection Agency, 690 University Avenue, Charlottetown, Prince Edward Island, Canada C1E 1E3
John.White@inspection.gc.ca

Contributors

K. Aarstad Department of Clinical Pharmacology, St. Olav's University Hospital, Trondheim, Norway

Ana Alonso Martinez Department of Biochemistry, Genetics and Immunology, Faculty of Sciences, University of Vigo, Vigo, Spain

Department of Biochemistry, Genetics and Immunology, Faculty of Sciences, University of Vigo, Vigo, Spain

C. Álvarez INTECMAR (Instituto Tecnolóxico para o Control do Medio Mariño de Galicia), Vilagarcía de Arousa, Spain

Zouher Amzil IFREMER, Nantes, France

Laboratoire Phycotoxines, IFREMER, Nantes, France

M. Angulo TRAGSATEC, Pontevedra, Spain

Nathalie Arnich Risk Assessment Department, ANSES – French Agency for Food, Environmental and Occupational Health & Safety, Maisons-Alfort, France

J. Blanco Centro de Investigacións Mariñas (Xunta de Galicia), Pontevedra, Spain

Ingeborg L.A. Boxman Food and Consumer Product Safety Authority (NVWA), Wageningen, The Netherlands

F. Buzin Station expérimentale, IFREMER, Bouin, France

M. Cardinal IFREMER, Nantes, France

François-Gilles Carpentier Food and Cellular Toxicology Laboratory, Université de Bretagne Occidentale (UBO), Brest, France

J.B. Castaing GEPEA, Saint Nazaire, France

Dean O. Cliver[†] School of Veterinary Medicine, Department of Population Health and Reproduction, University of California at Davis, Davis, CA, USA

S. Darriba INTECMAR (Instituto Tecnolóxico para o Control do Medio Mariño de Galicia), Vilagarcía de Arousa, Spain

J. Díez Consello Regulador Denominación de Orixe Mexillón de Galicia, Vilagarcía de Arousa, Spain

G. Durand IDHESA Bretagne Océane, Technopôle de Brest-Iroise, Plouzané, France

O. Garcia Department of Biochemistry and Molecular Biology, University of Santiago de Compostela, Santiago de Compostela, Spain

Gail E. Greening Food Group, Kenepuru Science Centre, Institute of Environmental Science and Research Ltd. (ESR), Porirua, New Zealand

Alejandra Beatriz Goya Marine Biotoxin Department, Mar del Plata Regional Laboratory, Agri-food Health and Quality National Service (SENASA), Mar del Plata, Argentina

Trude S. Guldberg Department of Clinical Pharmacology, St. Olav's University Hospital, Trondheim, Norway

T. Hatlen Department of Clinical Pharmacology, St. Olav's University Hospital, Trondheim, Norway

J. Haure Station expérimentale, IFREMER, Bouin, France

G. Hermann Marine Scotland Science, Aberdeen, Scotland, UK

C. Herrenknecht Nantes Atlantique Université, Nantes, France

P. Hess Laboratoire Phycotoxines, IFREMER, Nantes, France

Nantes Atlantique Université, Nantes, France

J. Hussenot Station expérimentale, IFREMER, Bouin, France

P. Jaouen GEPEA, Saint Nazaire, France

T. Jauffrais Laboratoire Phycotoxines, IFREMER, Nantes, France

Iddya Karunasagar Products, Trade and Marketing Service, Food and Agriculture Organization, Rome, Italy

J.P. Lacaze Marine Scotland Science, Aberdeen, Scotland, UK

Patrick Lassus IFREMER, Nantes, France

Jean-Charles Leblanc French Agency for Food, Environmental and Occupational Health & Safety, Risk Assessment Department, Maisons-Alfort, France

L. Le Grel LEMNA, Université de Nantes, Nantes Cedex 3, France

G. Limon IDHESA Bretagne Océane, Technopôle de Brest-Iroise, Plouzané, France

A. Longa Consello Regulador Denominación de Orixe Mexillón de Galicia, Vilagarcía de Arousa, Spain

Santiago Maldonado Environmental Laboratory, Office of the Secretary of Sustainable Development and Environment, Ushuaia, Argentina

C.F. Manso Departamento de Microbiología y Parasitología, CIBUS-Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

C. Marcaillou IFREMER, Nantes, France

A. Massé GEPEA, Saint Nazaire, France

Dinorah Medina Unidad de Certificación, National Direction of Aquatic Resources (DINARA), Montevideo, Uruguay

F. Mondeguer IFREMER, Nantes, France

Ulysses M. Montojo National Fisheries Research and Development Institute, Quezon City, Philippines

Ronald Jefferson A. Narceda National Fisheries Research and Development Institute, Quezon City, Philippines

Dominique Parent-Massin Laboratoire de Toxicologie Alimentaire et Cellulaire (EA 3880), Université Européenne de Bretagne - Université de Bretagne Occidentale (UEB-UBO), Brest Cedex 3, France

Cyndie Picot Laboratoire de Toxicologie Alimentaire et Cellulaire (EA 3880), Université Européenne de Bretagne - Université de Bretagne Occidentale (UEB-UBO), Brest Cedex 3, France

D. Polo Departamento de Microbiología y Parasitología, CIBUS-Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

Rosario J. Ragaza Post Harvest Research and Development Division, National Fisheries Research and Development Institute, Quezon City, Philippines

R. Raine The Ryan Institute, National University of Ireland, Galway, Ireland

Gary P. Richards United States Department of Agriculture, Agricultural Research Service, James W.W. Baker Center, Delaware State University, Dover, DE, USA

J.L. Romalde Departamento de Microbiología y Parasitología, CIBUS-Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

Marc Lawrence J. Romero Bureau of Fisheries and Aquatic Resources, Quezon City, Philippines

A.E. Rossignoli Centro de Investigacións Mariñas (Xunta de Galicia), Pontevedra, Spain

Alain-Claude Roudot Laboratoire de Toxicologie Alimentaire et Cellulaire (EA 3880), UFR Sciences et Techniques, Université de Bretagne Occidentale, Brest Cedex 3, France

F. Royer IFREMER, Nantes, France

Claudia Rozas Departamento de Sanidad Pesquera, SERNAPESCA, Valparaíso, Chile

Y. Ruiz Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain

N.E. Sabiri GEPEA, Saint Nazaire, France

Gielenny M. Salem Post Harvest Research and Development Division, National Fisheries Research and Development Institute, Quezon City, Philippines

F. San Juan Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain

V. Séchet IFREMER, Nantes, France

Laboratoire Phycotoxines, IFREMER, Nantes, France

Helen Smale Marlborough Shellfish Quality Programme Inc. (MSQP), Blenheim, New Zealand

Véronique Sirot Risk Assessment Department, ANSES – French Agency for Food, Environmental and Occupational Health & Safety, Maisons-Alfort, France

P. Suárez Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain

Hajime Toyofuku Department of International Health and Collaboration, National Institute of Public Health, Saitama, Japan

P. Truquet Laboratoire Phycotoxines, IFREMER, Nantes, France

A. Vidal Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain

Reivin T. Vinarao Post Harvest Research and Development Division, National Fisheries Research and Development Institute, Quezon City, Philippines

M.L. Vilariño Departamento de Microbiología y Parasitología, CIBUS-Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

Noime S. Walican National Fisheries Research and Development Institute, Quezon City, Philippines

Nathalie Wesolek Food and Cellular Toxicology Laboratory, Université de Bretagne Occidentale (UBO), Brest, France

A.M. Wilson The Ryan Institute, National University of Ireland, Galway, Ireland

Chapter 1
Recent International Efforts to Improve Bivalve Molluscan Shellfish Safety

Iddya Karunasagar

Global Production and International Trade in Bivalve Molluscs

Fish and fishery products continue to be one of the most important food commodities contributing to food security, livelihoods and to the economies of many countries in the world. According to FAO statistics, in 2008, nearly 81 % (115 million tonnes) of world fish production was destined for human consumption and the rest (27 million tonnes) were used for non-food purposes that included ornamental purpose (6.4 million tonnes), reduction to fish meal and oil (20.8 million tonnes), direct feeding for aquaculture, bait or pharmaceutical uses (FAO 2010). During 1976–2008, fishery trade grew at an average annual rate of increase of 8.3 % in value terms. In 2008, exports of fish and fishery products reached a record of US$102.0 billion, 9 % higher than 2007 (FAO 2010). Bivalve molluscs represented almost 10 % of the total world fishery production, but 26 % in volume and 14 % in value of the total world aquaculture production.

World bivalve mollusc production (capture + aquaculture) has increased substantially in the last 50 years, going from nearly 1 million tonnes in 1950 to about 14.6 million tonnes in 2010. Global production in tonnes during the last decade (2000–2010) is illustrated in Fig. 1.1. 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.

China is by far the leading producer of bivalve molluscs, with 10.35 million tonnes in 2010, representing 70.8 % of the global molluscan shellfish production

I. Karunasagar (✉)
Products, Trade and Marketing Service, Food and Agriculture Organization, Room F521, Viale delle Terme Di Caracalla, Rome, Italy
e-mail: Iddya.Karunasagar@fao.org

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_1,

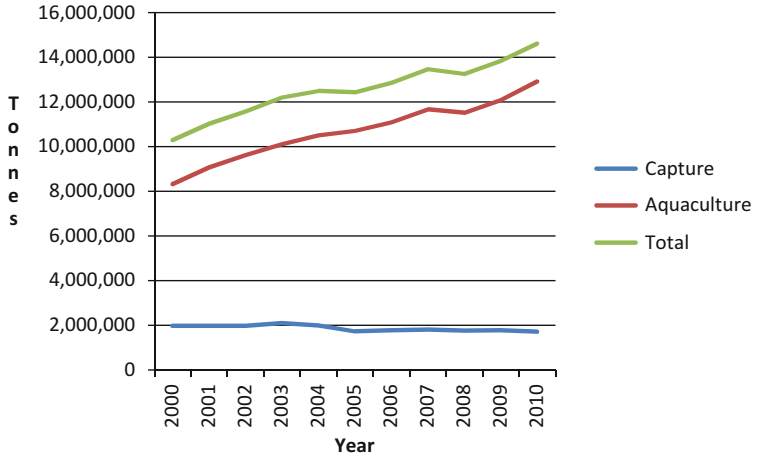


Fig. 1.1 Global production of bivalve molluscs during the decade 2000–2010 (FAO 2012a)

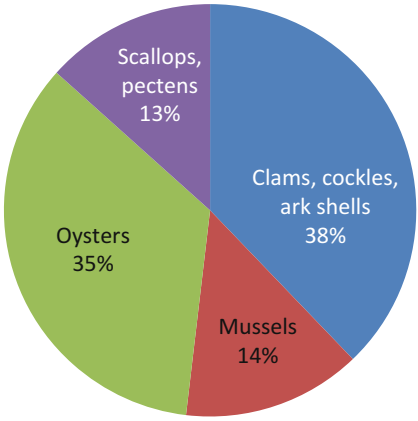


Fig. 1.2 Contribution of various species for bivalve aquaculture production (FAO 2012a)

and 80 % of the global bivalve mollusc aquaculture production. All of the Chinese bivalve production is cultured. Chinese bivalve mollusc production has steeply increased during the last 30 years, from a mere 178,000 tonnes in 1970. The increase was particularly strong in the 1990s, with an average growth rate of 17.6 % per year. Other major bivalve producers in 2010 were Japan (819,131 tonnes), the USA (676,755 tonnes), the Republic of Korea (418,608 tonnes), Thailand (285,625 tonnes), France (216,811 tonnes) and Spain (206,003 tonnes).

By species, the bivalve mollusc production from aquaculture in 2010 consisted of 38.0 % clams, cockles and ark shells, 35.0 % oysters, 14.0 % mussels and 13.0 % scallops and pectens (Fig. 1.2), with an impressive growth in the production of oysters, clams, cockles and ark shells since the early 1990s.

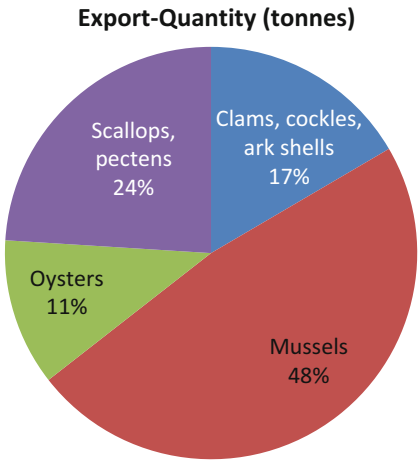


Fig. 1.3 Contribution of various species for export in terms of quantity (FAO 2012a)

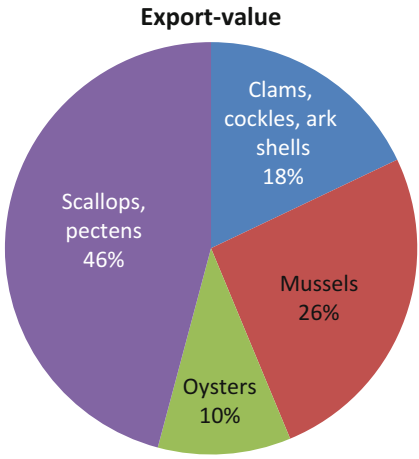


Fig. 1.4 Contribution of various species of bivalves for export in terms of value (FAO 2012a)

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 % (Fig. 1.3). Scallops are the most important species with 46 % of the value (Fig. 1.4), followed closely by mussels (26 %).

The causes of non-compliance at border inspection posts could be used as good indicators of problems in international markets. There have been 52 alerts for bivalve molluscs in the EU Rapid Alert System for Foods and Feeds (RASFF) during 2009, 78 in 2010 and 68 in 2011. The major causes (Fig. 1.5) were related to hygiene (*Escherichia coli* exceeding limits), biotoxins, viruses (norovirus), pathogenic bacteria (*Salmonella*) and other causes (labeling, organoleptic etc.).

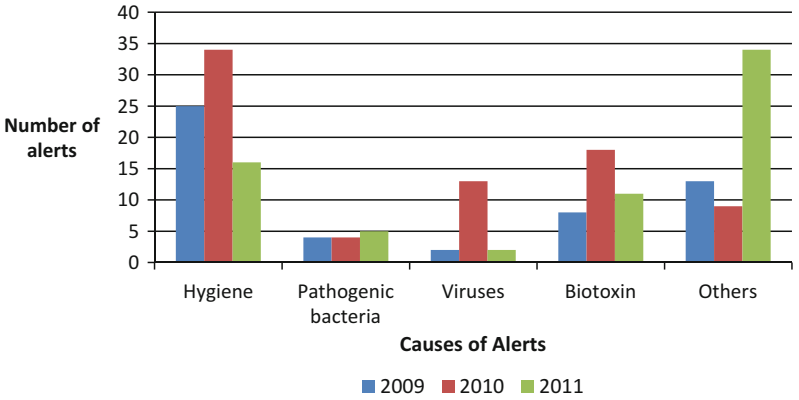


Fig. 1.5 Number of rapid alerts with respect of bivalve molluscs due to various causes (Data from RASFF database)

Safety Record of Bivalve Molluscs

The safety record of bivalve molluscs that are managed by sanitary surveys has been fairly good. According to database of the US Center for Science in Public Interest (CSPI), during the decade of 1999–2008, there were 792 seafood associated outbreaks involving 6,337 cases (CSPI 2012). Of these, 118 outbreaks involving 1,444 cases were due to bivalve molluscs. The causes of illnesses range from bacteria (*V. parahaemolyticus*) to viruses (norovirus), to biotoxins. In the EU, during 2009, out of 977 foodborne outbreaks for which a cause could be verified, 35 (3.6 %) were linked to shellfish including crustaceans and molluscs (DeWaal et al. 2012).

Scientific Advice Related to Codex Work on Bivalve Molluscs

Pathogenic Vibrio *spp. in Bivalve Molluscs*

While reviewing the hygiene provisions in the Draft Standard for Live and Raw Bivalve Molluscs developed by the Codex Committee on Fish and Fishery Products (CCFFP), the 38th Session of Codex Committee on Food Hygiene (CCFH) noted that the standard included various microbiological limits. The basis for the proposed limits was not established and there was no agreement on what the limits should be. CCFH requested FAO/WHO to use risk assessment on *V. parahaemolyticus* to provide scientific guidance to CCFFP and raised the following risk management question: "Estimate the risk reduction from *V. parahaemolyticus* when the total

Table 1.1 Predicted and reported illness due to *V. parahaemolyticus* following consumption of oysters in selected countries (FAO/WHO 2011a)

Production region for which data was available	Annual cases predicted by the model for the country	Epidemiological records of *V. parahaemolyticus* illness following oyster consumption
Wallis Lake, Australia	91	Two cases in 18 years, two large outbreaks from other seafood sources
Orongo Bay, New Zealand	0	None during 1997–2002 from oysters; several outbreaks from other seafood sources
Hiroshima Bay, Japan	66	13 during 1998–2004
British Columbia, Canada	186	212 in decade 1997–2006

number of *V. parahaemolyticus* or the number of pathogenic *V. parahaemolyticus* ranges from absence in 25 g to 1,000 CFU or MPN per gram".

The FAO/WHO risk assessment for *V. parahaemolyticus* in seafood (FAO/WHO 2011a, b) estimated the risk in four countries for which some data was available: Japan, New Zealand, Canada and Australia. With the exception of New Zealand, the model predicted higher levels of illness in other regions than are recorded in the countries notifications (Table 1.1).

In the FAO/WHO risk assessment, four factors were used to model exposure (a) level of pathogenic *V. parahaemolyticus* in oysters at harvest (b) effect of postharvest handling and processing (c) ability of the organism to multiply to an infective dose (d) number of pathogenic *V. parahaemolyticus* consumed. However, local data for the regions mentioned in Table 1.1 were available only with respect to amount of oysters harvested, time oysters out of water, air and water temperature. The relation between temperature and *V. parahaemolyticus* multiplication in oysters was based on US data, so also was the proportion of *V. parahaemolyticus* that are pathogenic, oyster consumption pattern, and illness under-reporting factor. Some of the differences observed between the predicted illness and epidemiological data could be due to variations in the above mentioned factors in the model in different geographical regions. For example, Eyles et al. (1985) reported that *V. parahaemolyticus* does not multiply in Sydney rock oysters stored at 30 °C for 7 days.

Further, in trying to address the CCFH question on risk reduction achieved when different criteria were applied, the FAO/WHO risk assessment also considered the impact of applying criteria on product rejection. This indicated that application of 100 CFU/g of *V. parahaemolytucs* criterion would reduce predicted illness by 96–99 % in Australia, New Zealand and Japan, but this would lead to rejection of 67, 53 and 16 % of products in these three countries respectively. This highlights the relation between application of specified target and baseline levels of *V. parahaemolyticus* in oysters in different geographical regions and that establishment of international limits for *V. parahaemolyticus* in oysters may have greater impact on product rejection in some countries.

Considering these findings of the FAO/WHO risk assessment for *V. parahaemolyticus*, CCFH decided to develop the Code of Hygienic Practice for control of pathogenic *Vibrio* spp. in seafoods, which has now been adopted (CAC/GL-73, 2010). During the process of drafting this Code, it was brought to the attention of the Codex member countries that United States has developed a risk reduction tool that can be used to estimate risk when environmental conditions like environmental temperature varies. While finalizing the Code, the 41st session of the CCFH recognized the need to provide countries with tools to assist them in the implementation of the guidelines under the various conditions that exist in different regions and countries. Such tools are envisioned to support countries in their efforts to use risk-based approaches in the selection of control measures appropriate for their seafood species, primary production and post-harvest practices. As the tool developed by the United States of America is based on the conditions and data of the USA, its broader application could not be recommended without a review of its validity when applied to non-USA scenarios. In light of this, the CCFH requested FAO/WHO to convene an Expert Meeting with the following terms of reference:

- Conduct validation of the predictive risk models developed by the United States of America based on FAO/WHO risk assessments, with a view to constructing more applicable models for wide use among member countries, including adjustments for strain virulence variations and ecological factors;
- Review the available information on testing methodology and recommend microbiological methods for *Vibrio* spp. in order to monitor the levels of pathogenic *Vibrio* spp. in seafood and/or water; and
- Conduct validation of growth rates and doubling times for *V. parahaemolyticus* and *V. vulnificus* in *Crassostrea virginica* (eastern or American oyster) using strains isolated from different parts of the world and different bivalve molluscan species.

FAO/WHO conducted this Expert meeting during September 13–17, 2011 at Rome. The Expert meeting evaluated the US risk calculator with a view of determining the context to which it is applicable and the potential modifications that may be needed for application, in other geographical regions. The Meeting concluded that *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. It was noted that besides temperature, there may be other ecological factors affecting the levels of *V. parahaemolyticus* in oysters. For example, the study done in India (Deepanjali et al. 2005) shows that in a tropical environment, where temperatures are favourable for *V. parahaemolyticus*, variations in levels occur and that this may be influenced by other ecological factors. The report of Eyles et al. (1985) that *V. parahaemolyticus* does not multiply in Sydney rock oysters even at 30 °C suggests that the tool based on multiplication of *V. parahaemolyticus* in American oysters (*Crassostrea virginica*) cannot be directly applied to other bivalve

species. Therefore, to develop a tool that is applicable to other regions or other bivalve species, or to answer risk management questions other than postharvest refrigeration, it would be necessary to first modify the existing FAO/WHO risk assessment model or develop a new model that considers and evaluates other factors such as salinity, strain differences and bivalve species related factors. This would require data from different regions and involving different bivalve species. It was also noted that the *Vibrio vulnificus* calculator tool is less likely than is 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 (FAO/WHO 2012b).

Regarding monitoring the level of pathogenic *V. parahaemolyticus* in seafood or water, the Expert meeting noted that monitoring on an ongoing basis could be expensive but consideration could be given to undertaking a study over the course of a year and using this as a means to establish a relationship between total and pathogenic *V. parahaemolyticus* and *V. vulnificus* in the seafood and abiotic factors such as water temperature and salinity. Once such a relationship is established for the harvest area of interest and measuring abiotic factors may be a more cost effective way to monitor. Further, the meeting noted that monitoring seawater may be of limited value, because there is no linear relation between the levels of *V. parahaemolyticus* and *V. vulnificus* in seawater and bivalves and the relation may vary depending on region and species of bivalves. Since a range of methods from conventional culture based to molecular methods are available, the meeting found the identification of a single method for monitoring really challenging and of limited value as molecular methods are evolving rapidly. The method selection would depend on the specific purpose of the monitoring activity, the cost, the speed with which results are required and the technical capacity of the laboratory. The meeting recommended evaluating the performance criteria of methods, so that a decision on a "fit for purpose" method could be facilitated.

The growth model for *V. parahaemolyticus* in the FAO/WHO risk assessment is based on data for the American oyster (*C. virginica*). The Expert meeting (FAO/WHO 2012b) noted that the model was also appropriate for estimating growth in at least one other oyster species (*Crassostrea gigas*) but is not appropriate for predicting growth in the Sydney rock oyster (*Saccostrea glomerata)*. It was also noted that the *V. parahaemolyticus* model currently used would over estimate growth at higher temperatures (e.g., >25 °C) in live oysters. The model was based on studies that were primarily undertaken using natural populations of *V. parahaemolyticus* as these are considered to be the most representative. While data are limited and inconsistent with respect to the impact of strain on growth rate, recent studies in live oysters are suggestive of differences between *tdh*/*trh* positive (pathogenic) populations versus total or non-pathogenic populations of *V. parahaemolyticus*. Since there is no data to evaluate the performance of the growth models in any other oyster species or other filter feeding shellfish or other seafoods, its use in other products cannot currently be supported, and if used should be done with clear understanding of the associated uncertainty (FAO/WHO 2012b).

The 42nd Session of the Codex Committee on Food Hygiene recommended FAO/WHO to continue work in a stepwise manner as follows:

- **Step 1**: 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;
- **Step 2**: 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;
- **Step 3**: 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; and
- **Step 4**: 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 become available.

In order to recommend test methods, a Joint FAO/WHO Expert Consultation held in Ottawa during October 2011 identified the following major end uses of *Vibrio* methodology: (a) harvest area monitoring (b) end product monitoring (c) postharvest process verification (d) outbreak investigation (e) performing growth studies in bivalve molluscs. The performance characteristics considered were: (a) whether the methods yielded qualitative or quantitative results (b) ability to detect pathogenic and stressed cells (c) possibility of strain characterization (d) volume of test sample required (e) availability of supplies their and cost (f) skill level required to perform the tests. The recommendations of this expert meeting are being used by FAO/WHO to develop a guidance document for use by *Vibrio* testing laboratories in member countries.

Salmonella *Criterion in Bivalve Mollusc Standard*

The 29th Session of CCFFP, while adopting the Standard for Live and Raw bivalve molluscs with a microbiological criterion for *Salmonella* (n = 5; c = 0; m = 0/25 g) asked FAO/WHO to address the following question: “in the context of harvesting area monitoring for faecal contamination and lot contamination, estimate the risk mitigation for *Salmonella* in bivalve molluscs when different sampling plans and microbiological criteria are applied”. Based on a review of the available scientific information, FAO prepared a discussion paper that was presented to the 30th Session of CCFFP highlighting the following points:

- Estimation of risk mitigation requires data from risk assessments. Currently, neither national nor international risk assessments for *Salmonella* in bivalve molluscs or other fish and fishery products are available.

Table 1.2 Data on the prevalence of *Salmonella* in bivalve molluscs at market level (FAO/WHO 2011b)

Region and year	No of single samples	% positive	No of batches	% positive	Reference
EU, 2007	1,009	0.6 %	3,214	0.9 %	EFSA Journal 3: 223, 2009
EU, 2008	330	0.9 %	5	0	EFSA Journal 8:1496, 2010
EU, 2009	499	3.4 %	294	1 %	EFSA Journal 9: 2090, 2011
US, 2007	395	1.5 % are positive by culture method and 8.6 % by more sensitive PCR method	–	–	DePaola et al., Appl. Environ Microbiol., 76: 2574, 2010

- The Appropriate Level of Protection and related Food Safety Objectives/ Performance Objectives should be defined before a relevant sampling plan is recommended.
- In the absence of these being defined, the assessment is based on the fact that there is no evidence of a particular public health problem with regard to non-typhoidal salmonellosis associated with the consumption of bivalve molluscs.
- Given the data on the predictive ability of faecal bacterial indicators for *Salmonella* spp., routine monitoring of harvesting areas for this pathogen would have very limited value.
- Proper application of current classification procedures and associated controls in control programmes should provide adequate protection.
- Current Codex Code of Practice for fish and fishery products does not recommend pathogen monitoring on a routine basis.

While considering these points, the 30th Session of CCFFP asked FAO/ WHO to:

- Undertake a risk assessment to determine whether there is a significant public health risk from *Salmonella* associated with the consumption of bivalve molluscs and
- Evaluate whether a criterion for *Salmonella* is meaningful to ensure adequate consumer health protection.

FAO/WHO started working on these two points with an Electronic Expert Working Group that considered these questions and noted that in areas that are managed by sanitary surveys, the prevalence of *Salmonella* in bivalve molluscs is generally low. Table 1.2 indicates the prevalence reported in bivalve molluscs at market level in the EU and US. Prevalence ranges from 0 to 3.4 % in EU data was available for either single samples or for batches from which five samples were tested as per EU regulation. The data of DePaola et al. (2010) indicate that the prevalence detected depends on methodology used for detection. While culture based method yielded a prevalence of 1.5 %, molecular testing raised it to 8.6 % (Table 1.2).

Table 1.3 The number of samples required, for a given level of confidence, at various frequencies of contamination (FAO/WHO 2011b)

		Confidence required that the test result is correct				
	Prevalence	50 %	90 %	95 %	99 %	99.9 %
		Number of samples that must be tested:				
Acceptable proportion of contaminated samples	≤1 in 10	7	22	29	44	66
	≤1 in 100	69	229	299	459	688
	≤1 in 1,000	693	2,301	2,995	4,603	6,906
	≤1 in 10,000	6,932	23,025	29,957	46,050	69,080
	≤1 in one million	693,148	2,302,594	2,995,750	4,605,202	6,908,723

Thus, the sampling plan for any microbiological criterion should be able to provide the desired level of confidence at these low levels of prevalence. Table 1.3 shows the relation between prevalence levels and the number of samples that need to be tested to give various levels of confidence. To be able to detect a prevalence level of 1 % at 95 % confidence, 299 samples need to be tested.

Salmonellosis associated with bivalve molluscan shellfish is rarely reported, as indicated by epidemiological data. In US, since 1990, there have been three reported outbreaks: one outbreak in 2003 involving six cases of typhoid fever associated with a single oyster lease which was determined to be caused by overboard discharge by immigrant workers on harvesting vessel; an outbreak caused by *S. typhimurium* involving steamed clams affected five people in a private home in 1991 and another outbreak caused by *S. typhimurium* involving molluscan lapas affected four people in a retirement home in 1990. In UK, there were three bivalve molluscan shellfish associated outbreaks between 1992 and 2007. An outbreak caused by *S. enteritidis* involved cockles in 1997; another outbreak caused by *S. enteritidis* involved scallops and king prawns in 2001; and the third outbreak caused by *S. typhimurium* involved mussels in 2005. In France, there were 31 *Salmonella* outbreaks associated with molluscan shellfish between 1996 and 2005, of which 19 were due to two common serovars (6 due to *S. typhimurium* and 13 due to *S. enteritidis*). A study in 1997 showed that France accounts for 28 % of EU bivalve mollusc consumption (Girard and Mariojouls 2000).

While the available epidemiological information suggests very low incidence of salmonellosis, it must be noted that the extent to which this reflects the actual situation is difficult to determine. Among other factors, the available data is limited to certain geographical areas, bivalve molluscs may not be adequately considered in investigating outbreaks of salmonellosis and information on the extent to which bivalves are consumed raw is lacking. For those areas where information is available, estimates of underreporting of salmonellosis have been made, and the multipliers range from 6.9 for Australia (Hall et al. 2006) to 3.2 in England (Wheeler et al. 1999) and 29 in the US (Scallan et al. 2011). While the limited epidemiological record makes it difficult to determine the extent of the problem, given that we are essentially dealing with a ready-to-eat food, the issue of under-reporting cannot be dismissed without further investigation.

FAO/WHO convened an Expert Consultation in Ottawa in October 2011, the results of which were presented to the 43rd Session of CCFH. The Expert Meeting noted that in parts of the world where harvesting of bivalve molluscs for direct human consumption is controlled through sanitary surveys using fecal coliforms or *Escherichia coli*, 0.5–2 % samples could be positive for *Salmonella*, but epidemiological evidence from these regions indicate that there have been very few outbreaks (in the order of one every few years) and usually involving relatively small number (<10) of consumers. Thus the Expert Meeting concluded that bivalve molluscs harvested from areas controlled through sanitary surveys do not cause frequent outbreaks of salmonellosis. The Expert Meeting used two approaches to address the question on the usefulness of the criterion. The first, based on available data, looked at the incremental value of having a *Salmonella* test over using the fecal indicator (fecal coliform/*E. coli*) test. This indicates that performing a *Salmonella* test in addition to the *E. coli* test would increase the number of unacceptable lots detected from 9.0 to 9.5 %. The second theoretical approach was based on the performance of the following sampling plan n = 5, c = 0, m = 0/25 g. This sampling plan cannot reliably detect contamination level of less than 2–5 cells of *Salmonella*/200 g serving (which translates to an estimated risk of 1 in 200 servings). Thus the assurance provided is that the risk is less than 1 in 200 and epidemiological data indicates that it is much lower than that. Therefore, the conclusion of the Expert Meeting was that the *Salmonella* criterion provides little or no additional protection than that achieved by current risk management strategy using fecal indicators.

Taking into consideration this information, the 43rd Session of CCFH agreed to recommend to the Committee on Fish and Fishery Products (CCFFP) to remove the criterion for *Salmonella* (Section I-6.5) from the *Standard for Live and Raw Bivalve Molluscs* (CODEX STAN 292–2008) and to include in the *Code of Practice for Fish and Fishery Products*, Section 7.2.2.2, the following: "When appropriate, taking into account the epidemiological situation as indicated by the results of environmental monitoring and/or other surveillance, the competent authority may decide to implement a criterion for *Salmonella*."

Conclusions

Bivalve molluscs constitute an important segment of the seafood industry and production by aquaculture has been growing at a significant pace. Live and raw bivalve molluscs are a high value commodity and while developing standards and codes of practices for this commodity, there were a number of scientific issues such as biotoxins, pathogenic *Vibrio* spp. and *Salmonella* that came up for advice by FAO/WHO. The risk assessment performed by FAO/WHO has led to the development of the Codex Guidelines on the application of general principles of food hygiene to the control of pathogenic *V*ibrio species in seafood. The development of risk management tool for *V. parahaemolyticus*, that has wide geographical application, requires more data from different regions.

Acknowledgements I am grateful to Stefania Vannuccini, Zhou Xiaowai and Luca Garibaldi from Fisheries Statistics and Information Service for providing the data on bivalve production and trade.

References

CSPI (2012) www.Cspinet.org/new/201303251.html

Deepanjali A, Sanath Kumar H, Karunasagar I, Karunasagar I (2005) Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the Southwest coast of India. Appl Environ Microbiol 71:3575–3580

DePaola A, Jones JL, Woods J, Burkhardt W, Calci KR, Krantz JA, Bowers JC, Kasturi K, Byars RH, Jacobs E, Williams-Hill D, Nabe K (2010) Bacterial and viral pathogens in live oysters: 2007 United States market survey. Appl Environ Microbiol 76:2754–2768

DeWaal CS, Roberts C, Catella C (2012) Outbreak Alert, 1999–2008. Center for Science in the Public Interest. http://www.cspinet.org/foodsafety/PDFs/Outbreak_Alert_1999-2008.pdf

EFSA (2009) The Community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. *The EFSA Journal* (2009), 223

EFSA (2010) The Community summary report on trends and sources of zoonoses and zoonotic agents and foodborne outbreaks in the European Union in 2008. EFSA J 8(1):2496

EFSA (2011) The European Union summary report on trends and sources of zoonoses and zoonotic agents and foodborne outbreaks in 2009. EFSA J 9(3):2090

Eyles M, Davey G, Arnold G (1985) Behaviour and incidence of *Vibrio parahaemolyticus* in Sydney rock oysters (*Crassostrea commercialis*). Int J Food Microbiol 1:327–334

FAO (2010) The state of the world fisheries and aquaculture. FAO, Rome, 197 pp

FAO/WHO (2011a) Risk assessment of *Vibrio parahaemolyticus* in seafood, Microbiological risk assessment series 16. FAO/WHO, Rome, 183 pp

FAO/WHO (2011b) Interim report of the electronic expert group on *Salmonella* in bivalve molluscs. ftp://ftp.fao.org/ag/agn/jmra/CRD_12_Interim_report.pdf

FAO (2012a) FAO fisheries and aquaculture information and statistics service. FAO, Rome

FAO/WHO (2012b) Risk assessment tools for *Vibrio parahaemolyticus* and *Vibrio vulnificus* associated with seafood. Microbiological risk assessment series 20 (in press)

Girard S, Mariojouls C (2000) Analysis of the French demand for oysters and mussels, within the European market. http://oregonstate.edu/dept/iifet/2000/papers/girard.pdf

Hall G, Raupach J, Yohannes K (2006) An estimate of under-reporting in foodborne notifiable diseases: *Salmonella*, *Campylobacter*, Shiga Toxin producing *Escherichia coli* (STEC). NCEPH working paper 52. http://nceph.anu.edu.au/Publications/Working_Papers/WP52.pdf

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M, Roy SL, Jones JJ, Griffin PM (2011) Foodborne illness acquired in the United States – major pathogens. Emerg Infect Dis 17:16–22

Wheeler JG, Sethi D, Cowden JM, Wall PJ, Rodrgues LC, Tompkins DS, Hudson MJ, Roderick PJ (1999) Study of intestinal infectious disease in England: rates in the community, presenting to general practice and reported to national surveillance. BMJ 318:1046–1050

Part I
Management and Regulation/Gestion et la réglementation

Chapter 2
Evolution of PSP Toxicity in Shellfish from the Beagle Channel (Tierra del Fuego, Argentina): An Overview

Alejandra Beatriz Goya and Santiago Maldonado

Introduction

The Beagle Channel is an interoceanic passage located in the southernmost region of South America, spanning over 280 km with a general east–west direction, connecting the waters of the Pacific and Atlantic oceans (Fig. 2.1). The international boundary between Chile and Argentina goes almost through the centre of the channel. The biggest settlement on the channel is Ushuaia in Argentina followed by Puerto Williams in Chile, two of the southernmost settlements of the world.

Shellfish harvesting activities for human consumption have been carried out for many years in this area. The existence of accessible natural beds (from intertidal and subtidal zones) and the quality of the molluscs harvested have been the main factors for the exploitation of this resource by fishermen. The first experience with culture of mussels (*Mytilus edulis chilensis*) in Argentinean waters of the Channel began in the early 1990s with excellent results. In recent years the number of production units has been increasing, using raft culture or long-line culture systems. Mussel production in the Beagle Channel can be considered as the southernmost production sites in the world (Ministerio de Agricultura).

Monitoring of marine biotoxins in molluscs is an essential Federal requirement before marketing (Reglamento de Inspección de Productos). PSP monitoring was implemented in Argentina in 1980, with the first recorded PSP outbreak occurring off the Atlantic coast of Peninsula Valdes (Elbusto et al. 1981) due to an

A.B. Goya (✉)
Marine Biotoxin Department, Mar del Plata Regional Laboratory, Agri-food Health and Quality National Service (SENASA), Aviso Dorrego y Víctimas del '46. Banquina Puerto, Mar del Plata CP 7600, Argentina
e-mail: agoya@senasa.gov.ar; abgoya@gmail.com

S. Maldonado
Environmental Laboratory, Office of the Secretary of Sustainable Development and Environment, Ushuaia, Argentina

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_2,

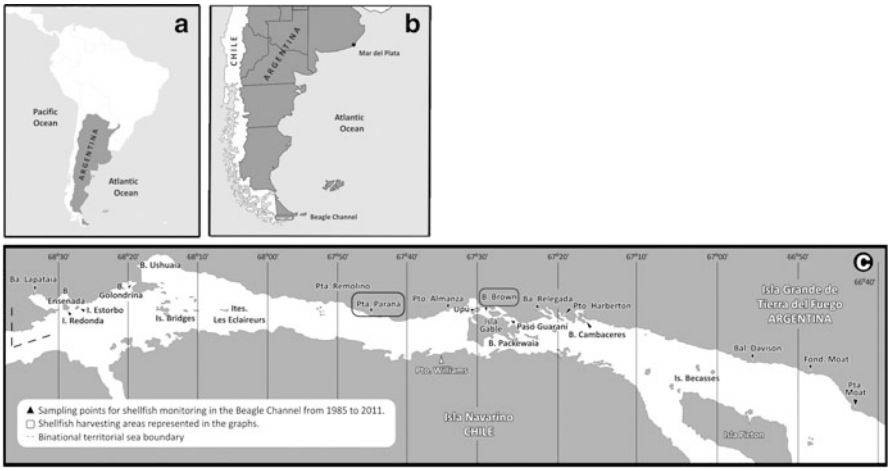


Fig. 2.1 Map of Beagle Channel, Tierra del Fuego, Argentina. (**a**) Continental view of Argentina; (**b**) Close up view of Argentina; (**c**) Magnification of the *encircled* area in (**b**)

Alexandrium tamarense bloom (Carreto et al. 1981). In early years, all mollusc samples collected along the Argentine Sea, both from coastal waters and offshore, were sent to SENASA Laboratory in Mar del Plata for the analysis of PSP by the mouse bioassay method (AOAC International 2000). As this procedure started to be implemented by laboratories of the Patagonian provinces, each of them began to perform analyses of their own samples. The province of Tierra del Fuego started to send samples to the Mar del Plata laboratory since 1985. From 1995 to 1998 analyses were performed in parallel by the official laboratories of Ushuaia and Mar del Plata. Since then, all samples for PSP monitoring from the Channel have been analyzed in the Ushuaia laboratory.

From 1985 to early 1992, there were no outbreaks of PSP toxicity in shellfish, and no detectable PSP levels were recorded in most of the samples from Beagle Channel. But in mid-January, 1992 an increase in toxicity was detected in the mussel *Aulacomya ater* sampled at Upú Island (~54°53′S–67°30′W) which reached levels of 365 μg STX eq.100 g^{-1} and a few days later molluscs reached high PSP levels in other points of the channel. In February, PSP levels of 120,440 μg STX eq.100 g^{-1} were detected by us in *Mytilus edulis chilensis* mussels from Bahía Golondrina (54°49′S–68°19′W), which to date represents the highest PSP concentration recorded worldwide (Carreto and Benavides 1993). An exceptional bloom of *Alexandrium catenella* was recorded in the channel waters with high toxic cell concentrations, which produced discoloration and bioluminescence phenomena (Benavides et al. 1995). Coinciding with this bloom, unusual die-offs of marine fauna were observed. The occurrence of a large number of dead seabirds floating adrift (penguins, cormorants, ducks) was reported, with mass mortalities of several species of clams which were deposited on the coastal beaches, and even mortalities

of sessile organisms like large sponges (Vinuesa 1993). The data showed that an *A. catenella* bloom began during October/November 1991 in southern Chilean waters and it was detected for the first time in the Strait of Magellan. Then, it spread south and eastwards affecting Beagle Channel waters (Lembeye 1992).

Since this exceptional outbreak, detectable PSP toxin levels have been recorded every year in molluscs from Beagle Channel, with levels exceeding the safety limit being generally recorded in late spring and summer. Some poisoning in humans has occurred due to consumption of shellfish collected by residents sampling near the affected areas. In October, 2009, PSP levels increased in some harvesting areas, and in December shellfish harvesting was banned along the entire Beagle Channel. Unlike previous years, many areas had to remain closed until winter due to an unusual persistence of toxicity in shellfish. A similar pattern was recorded during the following year.

Purpose

The aim of this article is to document the multi-year (1985–2011) trend of PSP levels in bivalve mollusc samples obtained from the Argentinean coasts at Beagle Channel, in shellfish harvesting areas where monitoring activities are conducted. By showing how episodes of PSP toxicity have evolved through time, the reported data are expected to be a suitable aid to develop predictive models and adjust monitoring strategies for dealing with PSP toxicity episodes.

Materials and Methods

Samples of *Mytilus edulis chilensis* and *Aulacomya ater* were obtained at different points of the Beagle Channel (Fig. 2.1) between August, 1985, and May, 2011. They were shipped for analysis to the Mar del Plata Regional Laboratory of SENASA and/or to the Environmental Laboratory of Ushuaia. The technique used for PSP detection was the mouse bioassay (AOAC International 2000) using the albino mouse strain CF1. These animals are regularly supplied to the two laboratories from the central mouse breeding facilities of SENASA in Buenos Aires. Sample toxicity was expressed in μg of saxitoxin-equivalent per 100 g of meat (μg STX eq.100 g^{-1}). Conversion factor (CF) value applied in both laboratories was 0.19. The limit of detection (LOD) of the bioassay was within the 30–35 μg STX eq.100 g^{-1} range.

The established Federal guideline of 80 μg STX eq.100 g^{-1} was applied to determine whether harvesting areas were to be open or closed (Reglamento de Inspección de Productos).

Results

High levels of toxicity that occurred during the summer of 1992 throughout the Channel, declined gradually in subsequent months, although they rarely fell below 80 μg STX eq.100 g^{-1}. The toxicity levels reported in molluscs from several areas of the channel during February 1992 reached particularly high values in Bahía Golondrina and Bahía Ensenada (Table 2.1).

From that exceptional year onwards there was an upward trend in PSP toxicity in shellfish sampled from October to November, with peaks in December, January or February (Table 2.2), with a subsequent decrease during the autumn. An exception was the peak of toxicity recorded in March 1996 in Isla Redonda, although in mid-April it had fallen to 59 μg STX eq.100 g^{-1}.

In 2009, toxin levels in shellfish began to rise slightly in October, with increases in November and December, when shellfish harvesting was banned along the entire Beagle Channel. During January and February, 2010, high PSP levels were recorded, reaching a toxicity peak in late February (5,600 μg STX eq.100 g^{-1}). The toxicity decreased slowly during the autumn and early winter. At several points the values remained above 80 μg STX eq.100 g^{-1} up to the end of July and several collection areas had to remain closed for longer than in previous years. In the period spanning from spring 2010 to summer 2011 a similar pattern was observed, with high concentrations of PSP between January and February, and retention levels in molluscs above the safety limit being recorded until May.

Figures 2.2 and 2.3 show the results in samples from two areas of the Channel, Bahía Brown and Punta Paraná, where this pattern is readily observable.

In Table 2.2, the two lines for years 2009 and 2010 represent a two-step rise in PSP levels with a first peak detected in Dec 14, 2009 which was followed by a plateau ending in a second peak in Feb 22, 2010. PSP levels fell back to safe levels in July, 2010.

Table 2.1 Levels of PSP recorded from several zones along the Beagle Channel during February 1992

Date	Molluscs	Zone	PSP level
02/02/92	*Aulacomya ater*	I. Upú	4,342
02/02/92	*M. edulis chilensis*	Pta. Paraná	5,524
02/04/92	*M. edulis chilensis*	Is. Bridges	44,119
02/05/92	*M. edulis chilensis*	B. Golondrina	120,846
02/07/92	*M. edulis chilensis*	B. Ensenada	86,201
02/18/92	*M. edulis chilensis*	Almanza	22,120
02/18/92	*M. edulis chilensis*	Ea. Harberton	22,647
02/20/92	*M. edulis chilensis*	Moat	51,984
02/20/92	*Aulacomya ater*	P. Guaraní	12,632
02/24/92	*M. edulis chilensis*	B. Lapataia	48,790

PSP levels are expressed in μg STX eq.100 g^{-1}

Table 2.2 Zones from the Beagle Channel where maximum levels of PSP were recorded each year and dates when lower PSP rises were recorded after the peak

Date	Molluscs	Zone	Maximum PSP Level	Decreasing PSP post peak	
				Date	Level
Nov, 1985	*M. edulis chilensis*	Almanza	37	Nov, 1985	ND
March, 1991	*Aulacomya ater*	Almanza	36	April, 1991	ND
Feb 5, 1992	*M. edulis chilensis*	B. Golondrina	120,846	Aug, 1992	493
Dec 21, 1993	*M. edulis chilensis*	I. Redonda	7,263	July, 1994	103
Dec 17, 1994	*M. edulis chilensis*	Is. Bridges	9,675	No data	No data
Jan 18, 1995	*M. edulis chilensis*	B. Lapataia	4,412	Oct, 1995	ND
March 4, 1996	*M. edulis chilensis*	I. Redonda	1,234	April, 1996	59
Jan 6, 1997	*M. edulis chilensis*	Ea. Harberton	278	April, 1997	ND
Jan 5, 1998	*M. edulis chilensis*	P. Guaraní	402	April, 1998	ND
Jan 7, 1999	*M. edulis chilensis*	B. Cambaceres	391	March, 1999	ND
Dec 4, 2000	*Aulacomya ater*	I. Upú	976	April, 2001	61
Jan 4, 2001	*M. edulis chilensis*	Ea. Harberton	875	March, 2001	39
Dec 29, 2002	*M. edulis chilensis*	Bal. Davison	1,629	Jan, 2003	119
Nov 11, 2003	*M. edulis chilensis*	B. Lapataia	1,505	Feb, 2004	48
Jan 7, 2004	*M. edulis chilensis*	B. Brown	2,870	March, 2004	ND
Jan 25, 2005	*M. edulis chilensis*	Punta Paraná	192	Feb, 2005	57
Dec 4, 2006	*M. edulis chilensis*	B. Brown	281	Dec, 2006	42
Dec 18, 2007	*M. edulis chilensis*	B. Packewaia	962	Feb, 2008	41
Jan 8, 2008	*M. edulis chilensis*	B. Brown	5,441	March, 2008	ND
Dec 14, 2009	*M. edulis chilensis*	B. Brown	2,196	Jan, 2010	1,425
Feb 22, 2010	*M. edulis chilensis*	B. Brown	5,600	July, 2010	62
Feb 22, 2011	*M. edulis chilensis*	Punta Paraná	3,571	May, 2011	46

PSP levels are expressed in μg STX eq.100 g^{-1}
ND non detectable level

Discussion

For several years there were no outbreaks of toxicity in molluscs from the Beagle Channel. It is probable that several factors converged at the end of 1991 which favored the onset of the toxic bloom that would occur the following year. Cyst production facilitates species dispersal as well; blooms carried into new waters by currents depositing "seed" populations to colonise previously unaffected areas (Mons et al. 1998). Cyst of *A. catenella* from Southern Pacific waters could have been conveyed by currents flowing into waters of the channel. Decysting has to take place, requiring a temperature of 5 °C or higher (Mons et al. 1998). Temperatures in the Channel waters are above 5 °C starting in October, reaching an 8 °C average in December and January (Luchini and Wicki 2002), helping the germination of the cysts. Nutrient concentrations do not seem to affect the success or rate of germination, but light, salinity and oxygen are important to varying degrees (Anderson 1998). Oxygen can have a dramatic effect on cyst germination, and most dinoflagellate species have an absolute requirement for oxygen during germination

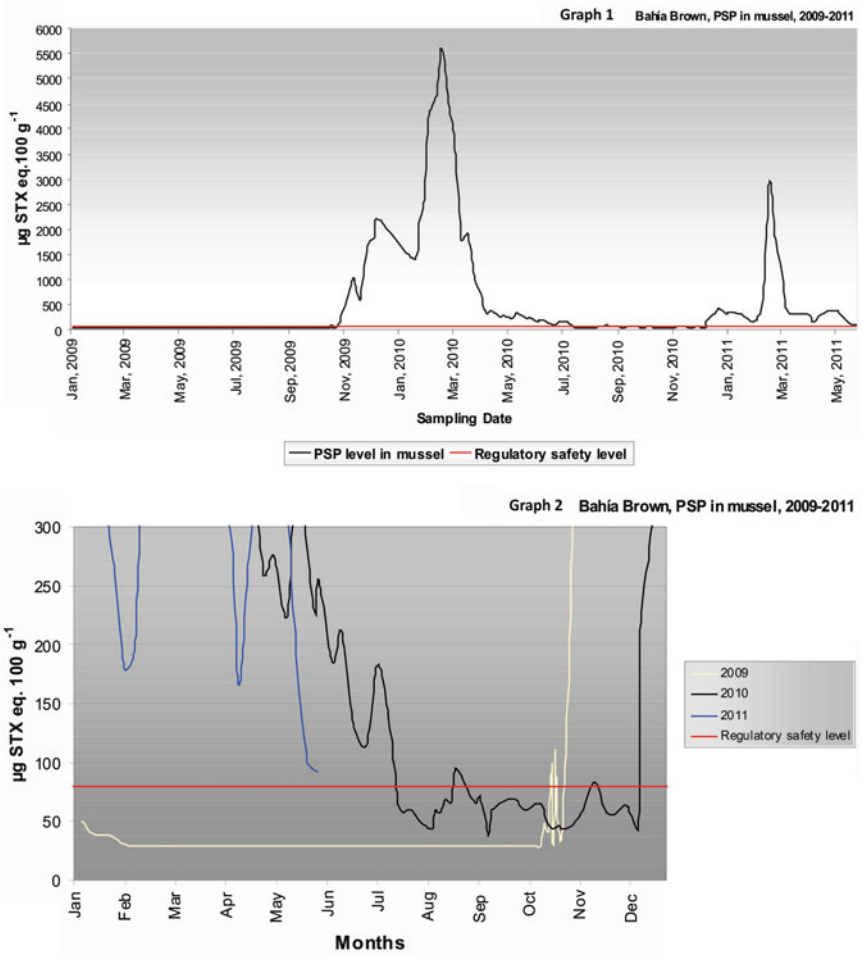


Fig. 2.2 PSP toxicity levels recorded in Bahía Brown's mussels. *Graph 1*: PSP levels from January, 2009, to May, 2011. *Graph 2*: Yearly toxicity levels shown as overlapping plots

(Anderson 1998). Studies conducted in the channel show a high level of oxygenation of its coastal waters (Luchini and Wicki 2002), and the sunlight period is longer during summer.

The toxic bloom of *A. catenella* which occurred in 1992 had exceptional features, namely, high cell density (821 cell.mL^{-1} in Lapataia Bay) and very high toxin content per cell (325 pg STX eq.cell^{-1}). The extreme mussel toxicity levels seem to be a consequence of those factors (Benavides et al. 1995). Even though the peaks of toxicity recorded in February, 1992 progressively declined in the following months, they remained above the safety limit during the autumn and winter. These residual levels of toxicity were still high at the beginning of spring and began to

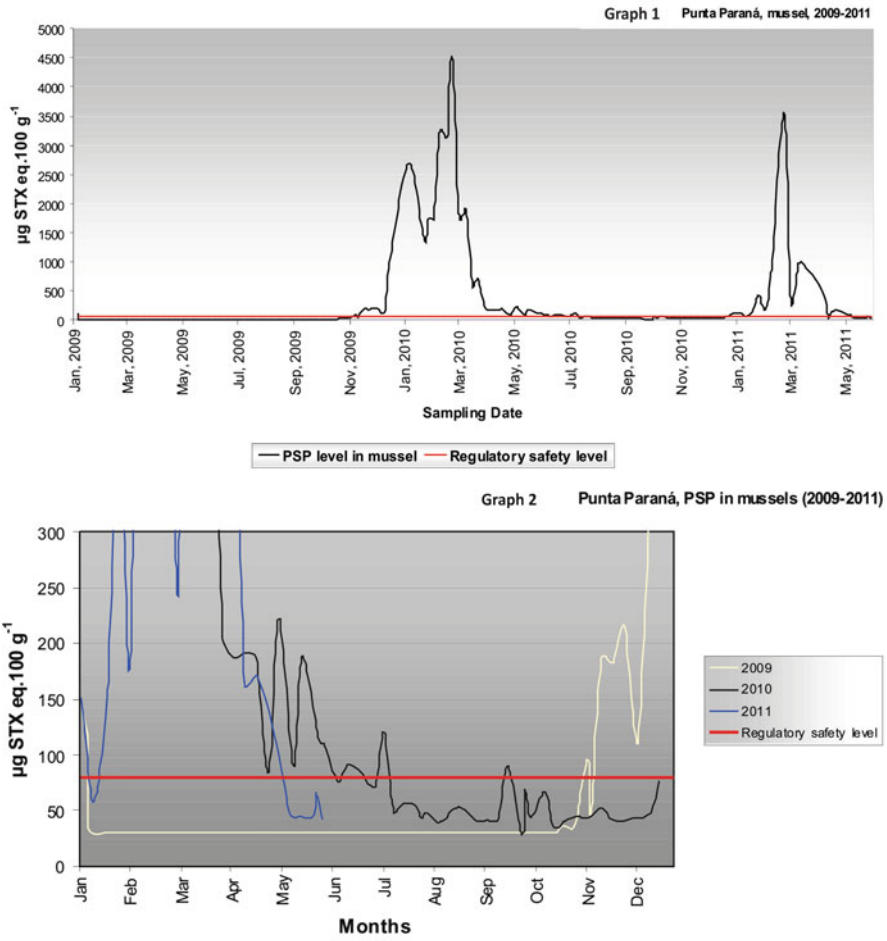


Fig. 2.3 PSP toxicity levels recorded in Punta Paraná's mussels. *Graph 1*: PSP levels from January, 2009, to May, 2011. *Graph 2*: Yearly toxicity levels shown as overlapping plots

increase again during that season, probably because of a new bloom of *A. catenella*. However, the levels reached in the summer of 1993 were of lower magnitude than in the previous summer.

The rate of loss varies with season and low water temperatures apparently retards toxin loss. Furthermore, the detoxification rate is highly dependent on initial or peak levels of toxicity (Mons et al. 1998). As it has been described for other Mytilid species, natural detoxification processes in *M. chilensis* and *A. ater* appeared to be mainly related to elapsed time (after the bloom) and maximum PSP concentration acquired by the shellfish (Molinet et al. 2010).

According to a study conducted during 2006 and 2007 (Almandoz et al. 2011), low phytoplankton densities were present in the channel during fall and winter. The phytoplankton was mainly composed of unidentified tiny phytoflagellates. By late spring, large species of dinoflagellates increased in biomass, representing about 50 % of the total phytoplankton biomass in December. One of the species identified was *A. catenella*.

PSP outbreaks in molluscs which began in the spring of 2009 and 2010 reached toxicity peaks just in late February 2010 and 2011. These findings may indicate that episodes of toxic blooms could have extended in time. On the other hand, the persistence of unusually high toxicity through the autumn and early winter could be due to low water temperatures which, by lowering shellfish metabolic activity, decrease the rate of shellfish detoxification.

Conclusions

Until 1992, the Beagle Channel was considered to be free of toxicity episodes caused by biotoxins, which impacted positively on the commercial harvesting activities of molluscs. Harvest volumes were significant and in the years 1989, 1990 and 1991, it exceeded coastal fish catches (Subsecretaría de Recursos Naturales y Ambiente Humano, Tierra del Fuego, Argentina n.d.). The toxic bloom which occurred in January 1992 constituted a watershed in the sanitary management of shellfish harvesting areas. Since then, monitoring activities have intensified, and a large number of samples need to be analyzed periodically in order to safeguard public health. Additionally, due to an unusual persistence of toxicity in molluscs during 2010 and 2011, many areas had to remain closed until winter. A consequence of this situation is that shellfish harvesting periods have been shortened, with consequential economic losses for producers. The potential sanitary consequences call for a closer monitoring of this phenomenon. Effective monitoring plans for molluscs have been carried out by sanitary authorities, who have implemented all necessary safety measures to protect public health. However, there is little information on the phytoplankton species involved in toxic outbreaks, and there is no current data on toxin profiles in contaminated molluscs. Future toxin monitoring efforts should include regular analysis of phytoplankton samples along with the monitoring of toxins in shellfish as well as retrieval of data that provide further insight into the nature of toxic events.

Acknowledgments The authors thank Andrea Rossini, Débora Bellonio, Ana Luz Candelo and Sergio Ledo for their help with data processing. The authors are also grateful to Ms. Silvina Parma for valuable assistance with maps design as well as the valuable contribution provided by the staff of the Centre for Documentation and Information (CdeI) of SENASA.

References

Almandoz GO, Hernando M, Ferreyra G, Schloss I, Ferrario M (2011) Seasonal phytoplankton dynamics in extreme southern South America (Beagle Channel, Argentina). J Sea Res 66:47–57

Anderson DM (1998) Physiology and bloom dynamics of toxic Alexandrium species, with emphasis on life cycle transitions. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms, vol G 41, NATO ASI series. Springer, Berlin/Heidelberg, pp 29–48

AOAC International (2000) Official method 959.08 paralytic shellfish poison: biological method. In: Horwitz W (ed) Official methods of analysis of AOAC International, 17th edn. AOAC International, Gaithersburg, pp 59–61

Benavides H, Prado L, Díaz S, Carreto JI (1995) An exceptional bloom of *Alexandrium catenella* in the Beagle Channel, Argentina. In: Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C (eds) Harmful algal blooms. Lavoisier, Paris, pp 113–119

Carreto JI, Benavides HR (1993) World record of PSP in Southern Argentina. Harmful Algal News, IOC/UNESCO 5(2). From: http://www.ioc-unesco.org/hab

Carreto JI, Lasta ML, Negri RM, y Benavides HR (1981) Los fenómenos de marea roja y toxicidad de moluscos bivalvos en el Mar Argentino. Contrib Inidep N°399, 55 pp. Mar del Plata, Argentina

Elbusto CA, Ballabene AC, Campero CM, Ramírez EE, Villanueva CR (1981) Toxina Paralizante de los Moluscos del Mar Argentino. Acta Bioquim Clin Latinoam XV(3):447–456

Lembeye G (1992). Major PSP outbreak in Chile, 1991–1992. Harmful Algal News, IOC/UNESCO 5(1–2). From: http://www.ioc-unesco.org/hab

Luchini L, Wicki GA (2002) Evaluación del potencial para acuicultura en la Provincia de Tierra del Fuego (revisión). Información básica. Secretaría de Agricultura, Ganadería, Pesca y Alimentos (SAGPyA), 29 pp

Ministerio de Agricultura, Ganadería y Pesca de la Nación, Dirección de Acuicultura. El Cultivo de lo smoluscos bivalvos marinos en Argentina. From: http://www.minagri.gob.ar/SAGPyA/pesca/acuicultura/01=Cultivos/04-acuicultura_marina/_archivos/000001

Molinet C, Niklitschek E, Seguel M, Díaz P (2010) Trends of natural accumulation and detoxification of paralytic shellfish poison in two bivalves from the Northwest Patagonian inland sea. Rev Biol Mar Oceanogr 45(2):195–204. doi:10.4067/S0718-19572010000200001

Mons MN, Van Egmond HP, Speijers GJA (1998) Paralytic shellfish poisoning: a review. RIVM Report 388802–005, June 1998. RIVM, Bilthoven, 47 pp

Reglamento de Inspección de Productos, Subproductos y Derivados de Origen Animal (Decreto 4238/68) Cap. XXIII Productos de la Pesca, Apartado 23.24: Moluscos bivalvos destinados a consumo humano. http://www.senasa.gov.ar/Archivos/File/File753¬Capitulos.pdf

Subsecretaría de Recursos Naturales y Ambiente Humano, Tierra del Fuego, Argentina (n.d.) Recursos Naturales: La Pesca Marítima en Tierra del Fuego. From: http://www2.medioambiente.gov.ar/sian/tfuego/book.htm

Vinuesa JH (1993) Marea Roja en el Canal Beagle. Contrib Cient CADIC N°16, 1–34. Ushuaia, Tierra del Fuego, Argentina

Chapter 3
Storage and Detoxification of Bivalve Molluscs as a Tool in a Marketing Strategy

J. Haure, J. Hussenot, F. Buzin, Patrick Lassus, C. Marcaillou, F. Mondeguer, V. Séchet, F. Royer, Zouher Amzil, M. Cardinal, L. Le Grel, A. Massé, N.E. Sabiri, J.B. Castaing, and P. Jaouen

Introduction

In France, as in some other European countries, there is strong evidence that toxic algal blooms are increasing in frequency (Cordier et al. 2000; Lassus et al. 2009), and are economically detrimental to the shellfish industry (Belin 2003). As a result, there has been increasing demand from shellfish farmers in recent years to develop industrial scale processes that would allow for continued sale of toxin-free shellfish during periods when harvesting is forbidden due to toxic events.

The 3-year project 'Comsaumol' was the result of an agreement between the French administration, shellfish industry representatives and research institutes. As early as 2007, a partnership charter was signed between the Agriculture and Fisheries Ministry, National Shellfish Farming Committee and IFREMER

J. Haure • J. Hussenot • F. Buzin
Station expérimentale, IFREMER, Polder des Champs, 85230 Bouin, France
e-mail: Joel.Haure@ifremer.fr; jerome.hussenot@ifremer.fr; florence.buzin@ifremer.fr

P. Lassus (✉) • C. Marcaillou • F. Mondeguer • V. Séchet • F. Royer • Z. Amzil • M. Cardinal
IFREMER, Rue de l'Ile d'Yeu, BP 21105 44311 Nantes, France
e-mail: patrick.lassus@ifremer.fr; claire.marcaillou@ifremer.fr; Florence.Mondeguer@ifremer.fr; veronique.sechet@ifremer.fr; florence.royer@ifremer.fr; Zouher.Amzil@ifremer.fr; mireille.cardinal@ifremer.fr

L. Le Grel
LEMNA, Université de Nantes, BP 52231 44322 Nantes, Cedex 3, France
e-mail: laurent.legrel@univ-nantes.fr

A. Massé • N.E. Sabiri • J.B. Castaing • P. Jaouen
GEPEA, 37 Bvd de l'Université, 44602 Saint Nazaire, France
e-mail: anthony.masse@univ-nantes.fr; Nour-Eddine.Sabiri@univ-nantes.fr; Jean-Baptiste.Castaing@univ-nantes.fr; Pascal.Jaouen@univ-nantes.fr.

V. Séchet • Z. Amzil
Laboratoire Phycotoxines, IFREMER, Rue de l'Ile d'Yeu, 44311 Nantes, France
e-mail: veronique.sechet@ifremer.fr; Zouher.Amzil@ifremer.fr

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_3,

(Institut Français de Recherche et d'Exploitation de la Mer) in order to support research work on toxic algae and their impact on aquaculture. The objective was to limit economic losses caused by toxic algae in the French shellfish industry. Several publications resulting from this project, offer more detailed methodological descriptions (Castaing et al. 2010, 2011; Gueguen et al. 2008, 2011; Le Grel and Le Bihan 2009; Marcaillou et al. 2010).

Two general scenarios have been observed: (i) sporadic and seasonal toxic events and (ii) long-term exposures to toxic algae. Implementation of generic land-based culture was thought to be the most promising solution to maintain shellfish trade during toxic events. Accordingly, two types of land-based culture were considered:

– If sporadic toxic blooms occurred, an early preventive storage of uncontaminated shellfish (either mussels or oysters) was considered as a means of avoiding contamination. Land-based procedures of either wet or dry storage were considered to be appropriate.
– In cases of long-term exposure of shellfish to toxic algae, industrial-scale detoxification systems were thought to be the best solution for producers, provided that detoxification was achieved, and that investment costs were exceeded by income generated from the sale of detoxified shellfish.

Tanks supplied with re-circulated seawater were used, as these are the most convenient systems for wet storage.

The Research and Development project was based on four workshops: (i) Water quality (seawater treatment, phycotoxin stability and bioavailability as dissolved compounds), (ii) Safe storage of non-toxic shellfish (in recirculating seawater), (iii) Detoxification (improving PSP and DSP detoxification yields using algal feed or physico-chemical treatments), (iv) economic analysis (regional oyster industry characterization, market bans, commercial losses and possible counter-measures). According to the different phycotoxin bioaccumulation patterns described in the literature (Bricelj and Shumway 1998) some experiments were focused on blue mussel, Pacific oyster, or the use of both species.

Material and Methods

Biological Materials

Toxic and Non-toxic Bivalves

Non-toxic or toxic market-sized mussels (*Mytilus edulis*) were collected from shellfish growers of Vilaine Bay. Market-sized Pacific oysters (*Crassostrea gigas*) were obtained from a producer in the Bay of Bourgneuf (French Atlantic coast), where they had been grown in safe conditions, in an area with no history of paralytic shellfish poisoning.

Toxic and Non-toxic Microalgae

Alexandrium minutum (AM89BM strain isolated from Morlaix Bay, France) was batch-cultivated (10 L-vessels) in temperature-controlled rooms (16 ± 1 °C), with a diurnal photon density flux of 50 ± 4 μE and a 12/12 h photoperiod. This toxic strain was also grown in continuous culture systems in two 100-L photobioreactors at 17 ° C, with an 8 L day^{-1} renewal of filtered natural seawater enriched with L1 medium (Guillard and Hargraves 1993). In both types of culture vessel used the *A. minutum* strain was producing GTX2, GTX3, dcGTX2 and dc GTX3, and reached a mean toxicity of 1 pg eq.STX $cell^{-1}$. *Prorocentrum lima* (strain PL4V from Vigo, Spain) was also batch-cultivated according to the same culture conditions as for *A.minutum*. Both strains were grown with F/2 medium (Guillard 1975). *P. lima* was producing AO and DTX1 and reached a mean toxicity of 10 pg eq.AO $cell^{-1}$. A non-toxic strain of *Skeletonema costatum* was cultured in Provasoli ES medium under the same conditions as the batch cultures.

Seawater Treatments

Seawater was filtered through either a sand filter or membranes. The sand filter was tested using three classes of sand with different mean grain sizes: 256 (size ranging from 26 to 496 μm), 389 (size ranging from 21 to 1,018 μm) and 392 μm (size ranging from 38 to 599 μm). *H. triquetra* suspensions containing 30,000 cells.mL^{-1} were fed into the sand filter at a flow rate equal to 3.5 $m^3/m^2/h$.

For the membrane test, an immersed membrane was used in dead-end filtration mode in seawater containing different concentrations (1,000 or 30,000 cells.mL^{-1}) of three micro-algae (*H. triquetra*, *A. minutum*, and *P. lima*). The hollow fibre surface area was equal to 0.37 m^2 and the average pore size equal to 0.2 μm. Air was injected at 676 $L \cdot h^{-1} \cdot m^{-2}$ below the fibre bundle.

During microfiltration or sand filtration of micro-algal suspensions, the mean filtrate was sampled in order to evaluate the retention rate RR expressed as:

$$RR = 1 - Cp/Cf, \tag{3.1}$$

where Cp is the concentration in the filtrate at the end of filtration and Cf is the concentration in the feed water.

Toxin Stability and Bioavailability Experiments

For experiments on DSP or PSP toxin bioavailability to shellfish, mussels were placed in 160-L capacity flume tank equipped with an air-lift system. Seawater was re-circulated continuously in the flume. Every flume tank contained four baskets

filled with 1 kg mussels each and water temperature was regulated at 16 °C. Several treatments were set up at the start of the experiment, in which the mussels were exposed to: (i) intact *A. minutum* or *P. lima* cells (toxic control); (ii) cell fragments (lysate) recovered by centrifugation, after cell lysis by sonication in a culture volume equivalent to that used in the toxic control; and (iii) dissolved toxins recovered in the supernatant obtained after centrifugation made under the same conditions as in (ii). Additionally, control mussels were exposed to *S. costatum* (a non-toxic algal species).

Detoxification Trials

For trials on PSP-contaminated shellfish using non-toxic feed algae, either oysters or mussels were placed in seawater tanks for a 4 day- acclimation period (16 ± 0.5 °C) and then put in experimental 150-L raceways or in individual 800-mL boxes, depending on the different detoxification conditions being tested.

When using H_2O_2 as a chemical treatment to improve phycotoxin breakdown, 60 kg of DSP-contaminated mussels were distributed between six baskets immersed in 700-L tanks. Seawater used in the re-circulated flume was filtered and kept at a constant temperature (16 °C). Toxic mussels were either exposed to seawater supplemented with a daily dose of hydrogen peroxide (trials) or kept in pure filtered seawater (controls). Oxidant addition (to an accuracy of 1 ppm) was regulated by a data-logger and computer system which provided a continuous recording of H_2O_2 concentrations, with a platinum redox probe.

Safe Storage of Uncontaminated Shellfish

Oysters or mussels (180-kg) were stored in re-circulated systems at 16 °C and fed *Skeletonema costatum* for 35 days. The excretion of three dissolved nitrogen compounds was monitored for each of the re-circulation systems, the first equipped with a Pozzolana biofilter and the second without. The total ammonia level was measured by the indo-phenol blue method (Solorzano 1969), nitrite was quantified by the Greiss reaction (Bendschneider and Robinson 1952) and nitrate measured using a Merkoquant Kit®. Mortality rate, total body weight and soft tissues dry weight were measured for both systems.

Chemical Analysis

PSP-toxin stability was assessed in natural or artificial seawater over 10–15 days by liquid chromatography coupled with a fluorometer (LC-FLD). Toxic substances were added to sea-water either as certified standards or as *Alexandrium minutum* cell

lysates. Each shellfish sample was acidified at pH <3.5 with 5 N HCl solution to stabilize the toxins. The analysis was then performed by LC/FLD, according to the method developed by Diener et al. (2006). *A. minutum* toxin content was estimated by the Oshima HPLC method (1995). Contrary to the method commonly used to quantify PSPs, the Diener method measures GTX and STX in a single step. Toxins were separated on a Gemini reversed phase C18 chromatographic column 5 μm, (250 × 4.6 mm) at 20 °C. For each test solution, three samples were injected three times to determine each toxin concentration and the associated variation coefficients during each experiment.

For DSP toxins, homogenized mussel digestive glands (2 g aliquots) were extracted with 8 ml of a 95/5 methanol/water solution. Then, the extract (1 ml aliquot) was tested by alkaline hydrolysis for the detection of okadaic acid (OA) derivatives (diol- or fatty acid-esters). The hydrolysed extract was ultra-filtered on an 0.2 μm membrane and 5 μl of the filtrate was injected on a Hypersil MOS C8 column (50 × 2 mm × 3 μm) and analysed by a triple quadripole mass spectrometer (API 2000, MRM positive ion mode). OA concentrations were quantified by comparing the response obtained with a calibration curve based on OA reference solution (IBM/NRC, Halifax, Canada).Toxin quantification in mussels exposed to H_2O_2 detoxification was applied daily in triplicate: digestive gland samples were stored in liquid nitrogen until the end of the experiment. Later, they were thawed, ground, extracted with acetone and then analyzed by LC-ESI-MS/MS (Ion Trap, Finnigan LCQ). DTXs were quantified according to a procedure described by Mondeguer et al. (2010).

Economic Analysis

The first objective was to develop a methodology capable of assessing the financial losses that toxic algae cause to the shellfish industry. Since the aim of the newly devised methods was to minimize these losses, the benefit they offer needs to be compared with the investment and running costs of them in a cost-benefit analysis. It was considered that in the case of a harvesting closure in a bay, sales would be delayed, thus causing the usual markets to be lost. This scenario means that shellfish would have to be sold on the wholesale market instead. Thus, the financial loss per kg is the gap between the expected retail price and the actual wholesale price.

As a consequence the total financial loss L is determined as follows:

$$L = (Pr - Pw)Q^{*}d \tag{3.2}$$

where Pr is the expected price (usually the retail price), Pw the wholesale price, Q the daily sales in kg and d the number of days of closure.

Our case study was the oyster farming industry in Bourgneuf Bay (French Atlantic coast). On the basis of a field survey, three groups of farmers were defined

(Le Grel and Le Bihan 2009) according to a number of criteria, among which the most relevant in terms of phycotoxin contamination were: (i) the size of the oyster farm and (ii) the producer's perception of risk:

- Group 1: farms located in several areas, with high production levels to meet demands from different distribution networks;
- Group 2: family farms with local outlets and no investment dynamics, run by farmers who are nearly retired;
- Group 3: young farmers running family concerns in a growth phase.

Results

Water Treatment

The first objective was to remove any toxin producing cells from the re-circulating seawater system. Two possible filtration processes were tested:

- Sand filters, which are already on the market, cheap and generally favoured by the producers
- Membrane filters, which are more expensive but more reliable.

Practically all micro-algae were trapped during the first hour of sand filtration, but after 7 h running this was reduced to 35 % (Fig. 3.1), when using sand of the largest size (from 21 to 1,018 μm mean particle diameter) in a column of 1 m high. The results indicated that a back-wash was needed to ensure a high and steady

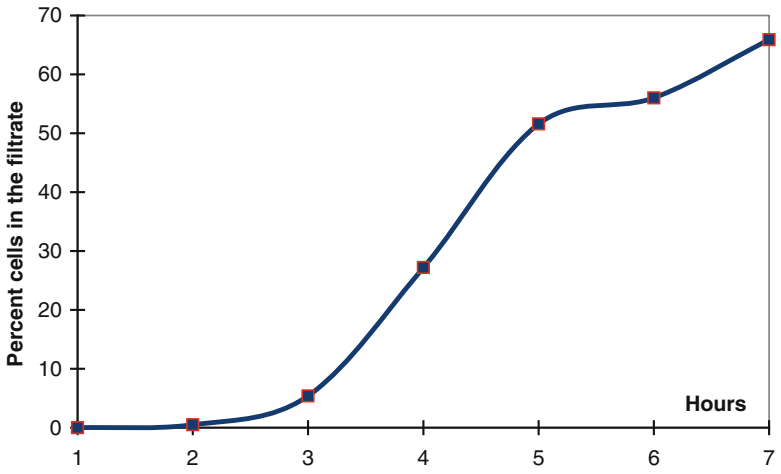


Fig. 3.1 Percentage of toxic algae found in the filtrate after 8 h filtration on a commercial sand filter (grain size: 21–1,018 μm)

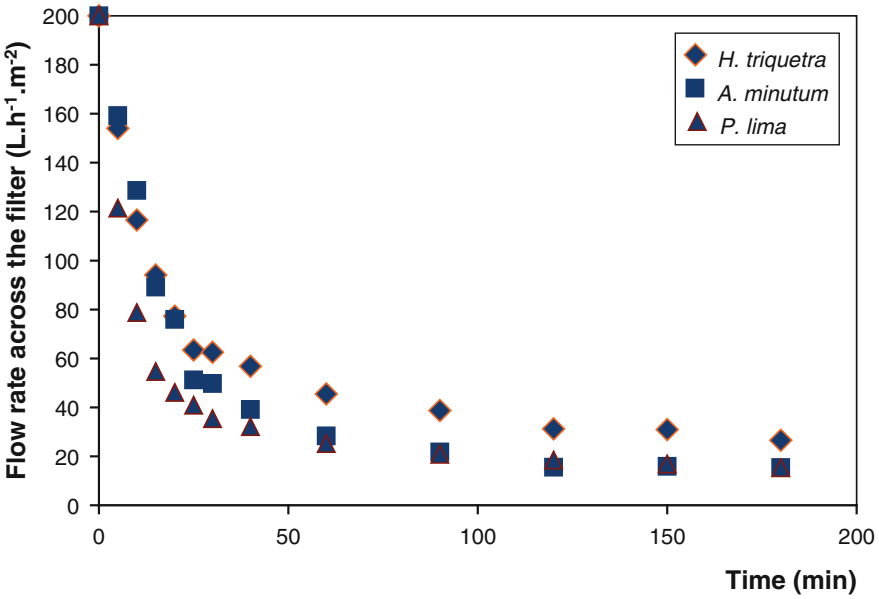


Fig. 3.2 Flow rates during membrane micro-filtration (0.2 μm) of three micro-algal suspensions at 30,000 cells.mL^{-1} *H. triquetra, A. minutum, P. Lima* (control) and *Skeletonema costatum* continuous supply (trial)

particle rejection rate over time. Filtration rates obtained with this grain size could be increased by the use of filters placed in series. The best retention, 99 % after 4.5 h of filtration, was obtained with the finest sand, which had a grain size ranging from 26 to 496 μm. It was also noted that the appearance of micro-algae in the treated water was delayed when bed thickness was increased (Sabiri et al. 2011).

For membrane filtration, 99 % of the micro-algae were retained regardless of whether the suspension consisted of *A. minutum*, *H. triquetra* or *P. lima* cells, and independent of cell concentration (1,000 or 30,000 cells.mL^{-1}) (Castaing et al. 2010). In the case of sand filtration of *A. minutum*, *H. triquetra* and *P. lima* suspensions at 30,000 cells.mL^{-1}, the flow rate decreased during the first hour of filtration, then stabilized (Fig. 3.2). Membrane fouling depended on the micro-algal species and compounds present in the supernatant, and was mainly due to organic compounds (Castaing et al. 2011). In addition, mixing diatoms (1,000 cells.mL^{-1}) with toxic algae to mimic natural populations did not significantly modify the observed drop in flow rates.

Phycotoxin Stability and Bioavailability

SEM observations of cells caught on either sand or membrane filters unambiguously demonstrated that cell walls were broken and that, as a consequence, intracellular

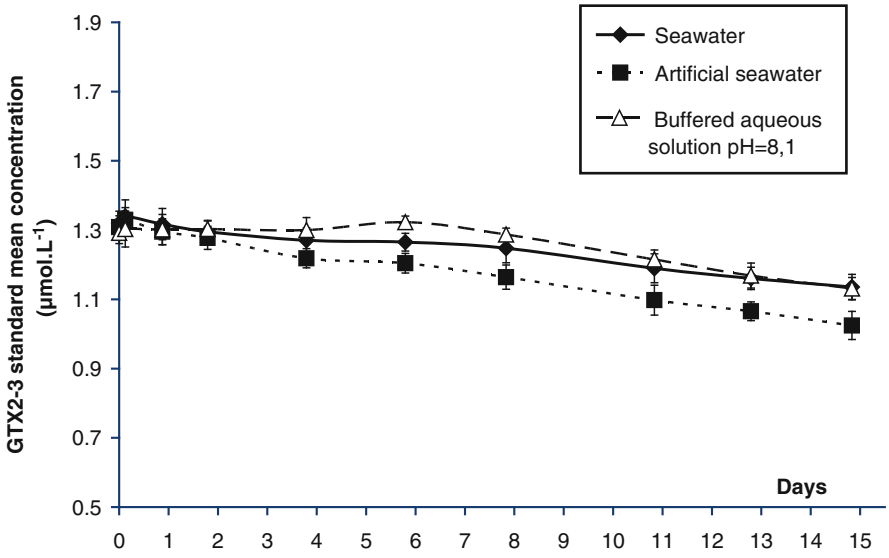


Fig. 3.3 Comparative stability of gonyautoxins from lysed toxic cells in different seawater media

phycotoxins could be released into the seawater once the cells lysed, especially as dissolved toxic compounds. As a first step in analysing this problem, PSP toxins were checked for their stability after being added as pure compounds (standards) to either natural or artificial seawater. No significant decrease was observed in STX or analogues (GTX2-GTX3) over 11 days. As a second step, phycotoxins obtained from induced cell lysis were added to different seawater-based media. Dissolved DSP toxins released into natural seawater from contaminated mussels stored in detoxification tanks were detected using SPATT (Solid Phase Adsorption Toxin Tracking) bags (Fux et al. 2008). The time-course analysis revealed no increase in the release of toxins from mussels after 1 week (first sample taken after 1 week). Similarly, PSP toxins extracted from lysed *A. minutum* cells were introduced as dissolved compounds into either untreated seawater or filtered, L1-enriched, seawater. No significant decrease was observed in either STX (data not shown here) or GTX2-GTX3 epimers (Fig. 3.3) over 14 days.

Dissolved PSP or DSP toxin bioavailability to mussels was tested in re-circulated tanks. When intact (toxic control) *A. minutum* or *P. lima* cells were supplied to mussels, toxin bioaccumulation in tissues was very rapid. Contrarily, dissolved PSP or DSP toxins obtained from sonicated cells did not induce significant bioaccumulation in mussel digestive gland (Fig. 3.4), independent of which fraction was considered (filtrates, lysed cells or filtrate + non-toxic cells).

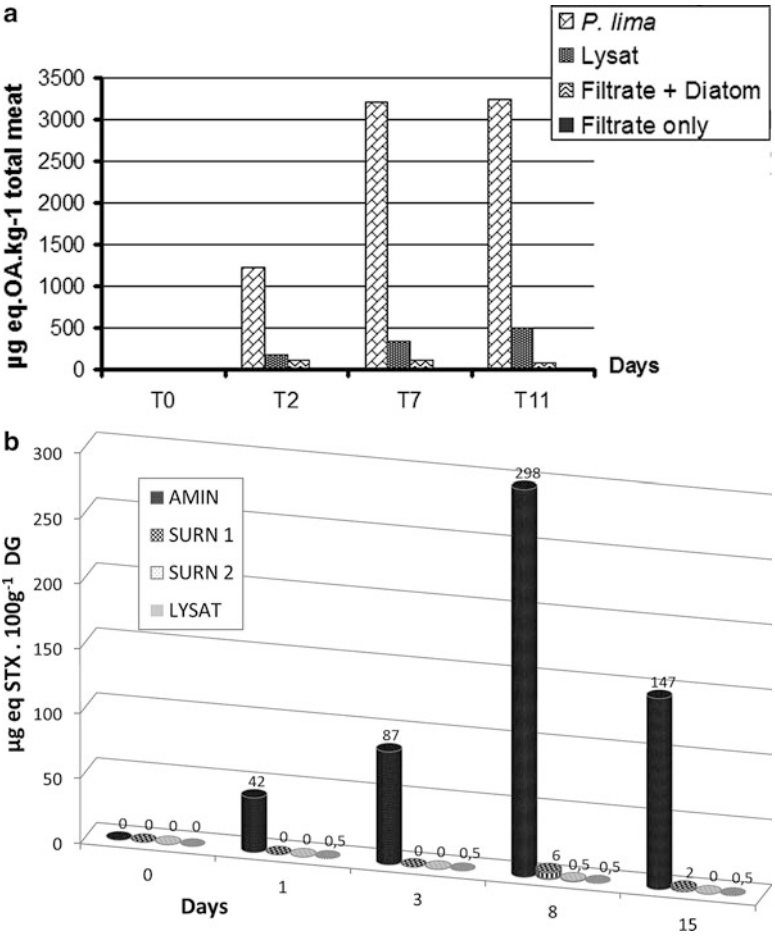


Fig. 3.4 (**a**) Mussels exposed to *P. lima* toxins through different pathways: *P. lima* culture, lysate, filtrate alone or with diatom culture added. There was little or no retention of dissolved DSP toxins in the shellfish meat. (**b**) PSP toxin concentration in mussel digestive glands after feeding toxic control treatment with *A. minutum* cultures (AMIN*):* Surn 1: supernatant resulting from culture filtration, together with supernatant of centrifuged pellets, ultrasound treated: Surn 2: supernatant resulting from culture filtration with *Skeletonema costatum* cells added: Lysat: *A.minutum* cellular debris following ultrasound treatment and supernatant recovery by centrifugation

Safe Storage of Uncontaminated Shellfish

Regarding oysters no significant differences were observed between the 'Pozzolana' (biological filters) and control tanks in terms of mortality (less than 5 % over 24 days), total body weight or soft tissue dry weight. Moreover, continuous monitoring of nitrogen excretion in the re-circulated water system revealed a natural

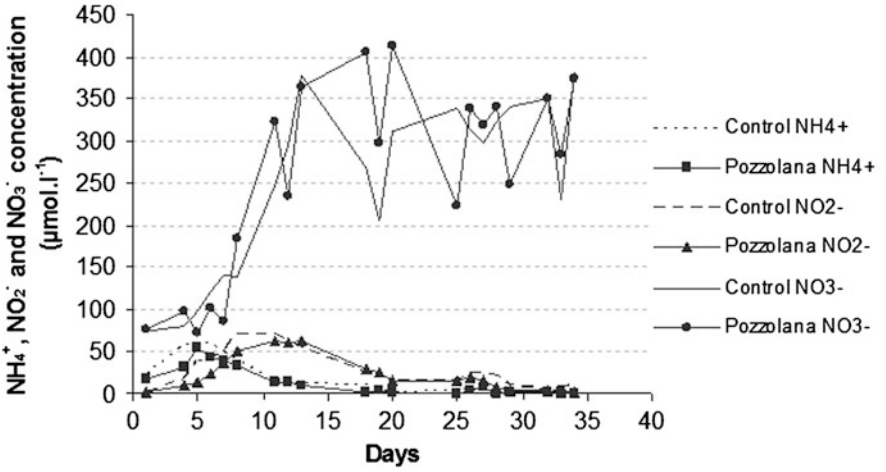


Fig. 3.5 Storage of the oyster *Crassostrea gigas* in re-circulated system for 5 weeks: evolution of the total ammoniacal nitrogen, nitrite and nitrate concentration measured in a re-circulated system equipped with a Pozzolana biofilter compared with a control (without biofilter)

nitrification (Fig. 3.5) throughout the experiment, regardless of treatment, with maximum N-NH_4^+/NH_3 values during the first 4 days of the trial, followed by a rapid conversion toward N-NO_2^-, and then N-NO_3. Results for mussels are still being processed, but N-NH4+ values are far higher than for oyster.

Detoxification

By experimentally supplying shellfish with organic, toxin-free food particles (mass-cultured *S. costatum*), detoxification time was significantly reduced for PSP-contaminated oysters or DSP-contaminated mussels (Fig. 3.6). However, although the detoxification time for oysters does meet industry requirements (less than 1 week), it appears that active feeding of toxin-free micro-algae did not succeed in reducing DSP toxin content of mussels below the safety threshold for >20 days (Marcaillou et al. 2010).

In an attempt to optimise DSP detoxification in mussels hydrogen peroxide (H_2O_2) was tested for its potential enhancement of toxin degradation, and its known short lifetime in seawater. For DSP-contaminated mussels that received a 2 ppm H_2O_2 treatment by sequential addition (2×2 h) vs. continuous addition, encouraging results were observed: a 95.7 % drop in toxin content occurred within 10 days for an initial DTX toxin level of 11.6 µg OA g^{-1} digestive gland. Detoxification time still remained too long (15 days), however, for the higher initial DTX concentration (28 µg OA g^{-1} DG).

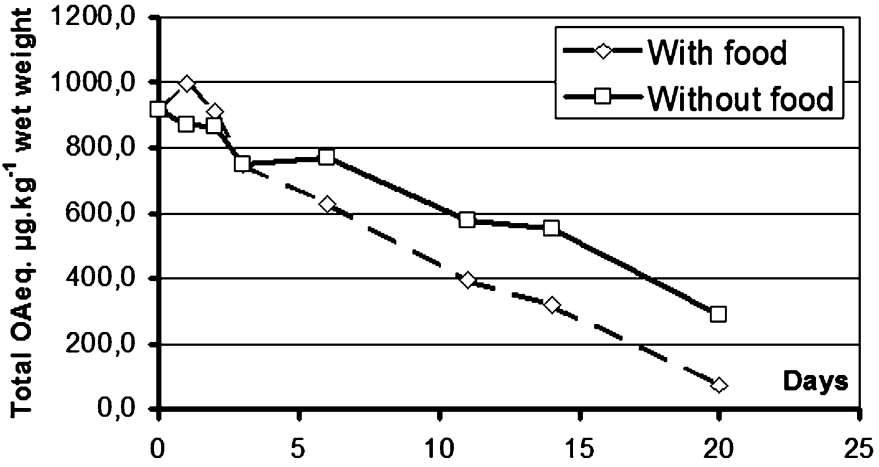


Fig. 3.6 DSP toxin detoxification patterns as a function of feeding conditions of contaminated-mussels: *without food* (control) or *with food* (continuously supplied *Skeletonema costatum*)

Economic Analysis

An economic analysis using a very simple model was performed on Microsoft Excel software, which compared the investment and running costs of the various methods with the short-term commercial losses they prevented. For each method and each type of farmer, one can determine the number of closure days for which the losses equal the costs. This type of analysis can be considered as a public and private decision-making tool since it provides information for private investors and for public policy measures. For instance, a shellfish grower from Bourgneuf Bay subjected to a 4-month harvesting closure during summertime would suffer a total cost of 5,887 euros because of costs associated with safe storage or detoxification. In this case, it appeared that no farm in any of the groups we examined had recorded a loss of this magnitude, sufficient to recover the investment and running costs of a filtration system. However, if two farmers of 'group 2' pooled their equipment, they would benefit from such a filtration system. It is worth remembering that in our example the total annual losses in the Bourgneuf oyster industry exceeded 1 million euros.

Many methods of safe storage or detoxification were assessed. The results can be broadly summarised as follows: investment costs were a key factor, so safe storage systems must use existing equipment, in order to reduce the financial costs, especially for membrane filters. Although sand filters are much cheaper and thus attractive, their efficiency remains to be validated.

Discussion

To ensure a high quality seawater supply for land-based systems designed for shellfish safe storage or detoxification, a primary goal was to remove any toxic cells from the seawater supply. It was shown that micro-filtration membranes were more efficient than sand filters for removing micro-algae. Both membrane and sand filter units were supplied with seawater containing toxic micro-algae at a flow rate of 2 m^3/h. An economic evaluation showed that for a ban on shellfish harvesting lasting 21 days, the annual running costs would be approximately equal to 1,470 or 6,100 euros $year^{-1}$ for the sand filter and the membrane process, respectively.

Another objective of this work was to define industrial processes that would enable the storage of market-sized shellfish in good condition, to ensure high survival rates and maintain the initial flesh quality. It appeared that oysters could be stored dry (Buzin et al. 2011) or wet for 3 weeks, before being sold, regardless of the process used.

As observed in other studies (Chen and Chou 2001; Lassus et al. 1999, 2005), the detoxification trials performed here showed that, with active feeding of contaminated shellfish with non-toxic micro-algae, a reduction in the time required to complete detoxification to a level below the safety threshold can be achieved. With this simple process, a 60 % drop in PSP toxin content can easily be obtained within 5 days in oysters. However, as PSP toxic events have become rare in France in the last 10 years, there is less need to research this type of toxin today. Likewise, a 90 % drop in DSP toxin content can be obtained by feeding non-toxic micro-algae to mussels within 3 weeks, but this result is hardly better than that observed under natural conditions. Thus in this case, the detoxification treatment is not cost effective for the producer. Finally, when using oxidizing agents, such as H_2O_2, for phycotoxin removal, it gave encouraging results for DSP toxins, provided that initial toxin concentrations were not too high. A future challenge will be to understand how lipophilic toxins are metabolised in mussels.

Preventative shellfish safe storage must include adaptation of existing equipment to reduce investment costs and future solutions must overcome the spatial constraints often faced by producers. The main limiting factor is the low capital investment in the industry, while energy costs represent much of the operating costs. A simple model working in Excel software was developed and successfully used to simulate the economic impact of 113 days of harvesting closure on cash flows for three different groups of producers (which were defined in a survey of Bourgneuf Bay farmers). For some oyster farmers, pooling filtration equipment would offer a real advantage by sharing some of the financial losses caused by market closures. The loss assessment is a short term and simplified one: it does not take into account various factors such as shellfish mortality, price variations or wage savings due to changes in work organisation during a market closure. In order to produce a more generalised economic model it could be improved by integrating these additional factors, as well as estimates of the closure frequency. This study provided the opportunity to test various mitigating measures, and apply cost benefit analyses in a case-study of the French shellfish industry.

Acknowledgments The authors are grateful to COSTE, an Industry-supervised programme that fostered this study, and to Basse-Normandie, Bretagne, Pays-de-la-Loire, Poitou-Charentes and Languedoc-Roussillon regional bodies for their financial support.

References

Belin C (2003) Bilan sur 20 ans des interdictions administratives de vente et de ramassage de coquillages, pour la présence de phycotoxines, sur le littoral français, 1984–2003. Ifremer Publish, Brest, 79 pp

Bendschneider K, Robinson RJ (1952) A new spectrophotometric method for the determination of nitrite in sea water. J Mar Res 11:87–96

Bricelj MV, Shumway SE (1998) Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics and biotransformation. Rev Fish Sci 6:315–382

Buzin F, Baudon V, Cardinal M, Barillé L, Haure J (2011) Cold storage of Pacific oysters out of water: biometry, intervalval water and sensory assessment. Int J Food Sci Technol 46: 1775–1782

Castaing JB, Massé A, Pontié M, Séchet V, Haure J, Jaouen P (2010) Investigating submerged ultrafiltration (UF) and microfiltration (MF) membranes for seawater pre-treatment dedicated to total removal of undesirable micro-algae. Desalination 253:71–77

Castaing JB, Massé A, Séchet V, Sabiri NE, Pontié M, Haure J, Jaouen P (2011) Immersed hollow fibers microfiltration (MF) for removing undesirable micro-algae and protecting semi-closed aquaculture basins. Desalination 276:386–396

Chen CY, Chou HN (2001) Accumulation and depuration of paralytic shellfish poisoning toxins by purple clam *Hiatula rostrata* Lighttoot. Toxicon 39:1029–1034

Cordier S, Montfort C, Miossec L, Richardson S, Belin C (2000) Ecological analysis of digestive cancer mortality related to contamination by diarrhetic shellfish poisoning toxins along the coasts of France. Environ Res 84:145–150

Diener M, Erler K, Hiller S, Bernd C, Luckas B (2006) Determination of Paralytic Shellfish Poisoning (PSP) toxins in dietary supplements by application of a new HPLC/FD method. Eur Food Res Technol 224:147–151

Fux E, Marcaillou C, Mondeguer F, Bire R, Hess P (2008) Field and mesocosm trials on passive sampling for the study of adsorption and desorption behaviour of lipophilic toxins with a focus on OA and DTX1. Harmful Algae 7(5):574–583

Gueguen M, Bardouil M, Baron R, Lassus P, Truquet P, Massardier J, Amzil Z (2008) Detoxification of pacific oyster *Crassostrea gigas* fed on diets of *Skeletonema costatum* with and without silt, following PSP contamination by *Alexandrium minutum*. Aquat Living Resour 21:13–20

Gueguen M, Baron R, Bardouil M, Truquet P, Haberkorn H, Lassus P, Barille L, Amzil Z (2011) Modelling of paralytic shellfish toxin biotransformations in the course of *Crassostrea gigas* detoxification kinetics. Ecol Model 222:3394–3402

Guillard R (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, pp 22–60

Guillard RRL, Hargraves PE (1993) *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia 32:234–236

Lassus P, Bardouil M, Masselin P, Naviner M, Truquet P (1999) Comparative efficiencies of different non-toxic diets in detoxification of PSP-contaminated oysters (*Crassostrea gigas* Thunberg). J Nat Toxins 9:1–2

Lassus P, Bardouil M, Baron R, Bérard JB, Masselin P, Truquet P, Pitrat JP (2005) Improving detoxification efficiency of PSP-contaminated oysters (*Crassostrea gigas* Thunberg). Aquac Eur 3–6

Lassus P, Gowland D, Mckenzie D, Kelly M, Braaten B, Marcaillou-Martin C, Blanco J (2009) Industrial scale detoxification of phycotoxin-contaminated shellfish: myth or reality? In: Busby P (ed) Proceedings of the 6th international conference on molluscan shellfish safety, Blenheim NZ, March 2007, Miscellaneous series71. The Royal Society of New Zealand, Wellington, pp 289–297

Le Grel L, Le Bihan V (2009) Oyster farming and externalities: the experience of the Bay of Bourgneuf. Aquac Econ Manag 13:112–123

Marcaillou C, Haure J, Mondeguer F, Courcoux A, Dupuy B, Pénisson C (2010) Effect of food supply on the detoxification in the blue mussel, *Mytilus edulis*, contaminated by diarrhetic shellfish toxins. Aquat Living Resour 23:255–266

Mondeguer F, Elie N, Truquet P, Savar V, Dupuy B, Lassus P, Penisson C, Haure J, Amzil Z, Hess P (2010) Oxidant treatment (H_2O_2) effect on detoxification process in DSP-contaminated mussels (Etude de l'effet d'un traitement oxydant (H_2O_2) sur la décontamination de moules contaminées DSP). In: Contractual report IFREMER Nantes, 09/5 210 251/YF. IFREMER, Nantes

Oshima Y (1995) Post-column derivatization liquid chromatographic method for paralytic shellfish poisoning in Tohoku district. J AOAC Int 78:528–532

Sabiri NE, Castaing JB, Massé A, Jaouen P (2011) Performance of a sand filter in removal of micro-algae from seawater in aquaculture production systems. Environ Technol. doi:10.1080/09593330.2011.587027

Solorzano L (1969) Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol Oceanogr 14:799–801

Chapter 4
Molluscan Shellfish Safety in South America

Dinorah Medina, Alejandra Beatriz Goya, and Claudia Rozas

Introduction

Molluscan shellfish, particularly bivalve molluscs, are an important resource for South American countries that intend to export or are exporting at present to important markets such as the European Union or the USA. Most of these bivalve molluscs are wild, although in the last years the growth of the aquaculture industry has been seen in countries such as Chile, Peru and Brazil. Nevertheless, the requirements of the destination markets sometimes limit the opportunities for export, due to the stringent sanitary requirements. A label with country of origin could be an identity factor that brings the opportunity to be recognized at a worldwide level. In some South American countries, there still is no specific regulation for molluscan shellfish safety and its sanitary control.

That is why the sanitary authorities of exporter's countries have to invest in human resources and in laboratory equipment to implement the necessary safety controls required for molluscan safety. Staffs require training on the dynamic regulations of the importing market, and on equipment and methods which also need to meet current international regulations. All these items need important economic

D. Medina (✉)
Unidad de Certificación, National Direction of Aquatic Resources (DINARA), Constituyente 1497, Montevideo, Uruguay
e-mail: dmedina@dinara.gub.uy

A.B. Goya
Marine Biotoxin Department, Mar del Plata Regional Laboratory, Agri-food Health and Quality National Service (SENASA), Aviso Dorrego y Víctimas del '46. Banquina Puerto, Mar del Plata CP 7600, Argentina
e-mail: agoya@senasa.gov.ar; abgoya@gmail.com

C. Rozas
Departamento de Sanidad Pesquera, SERNAPESCA, Victoria, 2532 Valparaíso, Chile
e-mail: crozas@sernapesca.cl

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_4,

investments, which are very difficult to afford in developing countries. In spite of these difficulties, some South American countries have successfully implemented the requirements and are either authorized or should soon be authorized to export to developed countries. The EU is the main destination market for live or frozen molluscs. In order to verify the compliance with the European Union regulation in force, missions of the FVO (Food and Veterinary Office) evaluate the performance of the Competent Authorities of the exporter countries "in situ".

Based on EU regulation, current requirements are those stated in the Hygienic package that comprises Regulations (CE) 852/2004 (10), 853/2004 (11) and 854/2004 (12), referred to general, specific and official controls on foods. In addition, the Directive 923/79/CEE relates to the quality of molluscan growing waters. Besides these regulations, there is the legal framework: (CE) 1881/2006 (7), (CE) 2073/2005 (5), (CE) 2074/2005 (6), and (CE) 333/2007 (8) which refer to maximum limits of contamination, microbiological criteria and analysis methods for foodstuff. For biotoxins Decision 2002/225/EC (4) is applied; Directive 2001/22/EC (3) for heavy metals; Regulation (CE) 854/2004, Annex II which refers to production zones (delimitation and classification of growing zones), and Regulation (CE) 853/2004 Annex III, refers to the harvest from authorized zones, identification documents and reception controls at depuration and expedition centres (Fig. 4.1).

Argentina

In Argentina the molluscs of commercial interest are mainly from wild beds, which are spread along the continental shelf. The shellfish aquaculture industry is not yet developed on a large scale, although in recent years some companies have begun to develop the culture of oysters and mussels. At present Argentina does not export live or frozen bivalve molluscs to the European Union. Only the scallop adductor muscle, free of viscera and gonads processed and frozen on board of factory vessels, can be exported to the EU, USA and Canada. The "Fishery Federal Law" of Argentina states that Provinces are sovereign for the resources that inhabit the coastal waters up to 12 nautical marine miles. For this reason each Province is responsible for sanitary shellfish monitoring within its jurisdiction. Since the 1980s, the Provinces have carried out plans for monitoring of Paralytic Shellfish Poison (PSP) either in commercial harvested shellfish or for molluscs in coastal zones accessible to residents and tourists. In 2000 the SENASA (National Animal Health and Agrifood Quality Service) updated its legislation and included the operating rules for the harvest of bivalve molluscs for human consumption in the chapter of fishery products (1). This was based on the Directive 91/492/CEE of 15 July 1991 (laying down the health conditions for the production and placing on the market of live bivalve molluscs) in force at the time. Due to some difficulties in its application, the Regulation was modified in 2006 and aligned with the Regulations 853/2004 (11) and 854/2004 (12) of the European Union.

Fig. 4.1 Implementation of sanitary controls in South America

Through the Provincial Competent Authorities, each Province defines the maritime shellfish areas that will be assessed for classification. The final classification of A, B or C is given by the National Authority (SENASA), according to sanitary quality based on chemical and bacteriological analysis. Currently, four provinces – Buenos Aires, Río Negro, Chubut and Tierra del Fuego – have classified areas for shellfish harvesting which correspond to "A" class. In other areas with potential commercially important volumes of molluscs, classification assessments have begun. In order to maintain their classification level, the provinces monitor molluscs for the presence of Paralytic Shellfish Poison (PSP) toxins, Amnesic Shellfish Poison (ASP) toxins and the Lipophilic Toxins complex (okadaic acid and dinophysistoxins, yessotoxins, pectenotoxins and azaspiracids) in authorized

laboratories. Production areas are closed and bans for harvesting are established when biotoxin levels in molluscs are unsafe for human consumption. One of the major problems related to mollusc harvesting in Argentina is the extent of its coastline (over 5,000 km); distances to the control centers and means of transportation are important restrictions for the prompt shipment of samples for analysis. Moreover there are few authorized laboratories in relation to the number of samples to be analyzed. These two issues have become greater difficulties considering the rapid increase in the number of classified zones for shellfish harvesting.

At present, the laboratories in Argentina are working intensively to become accredited for different analytical techniques for monitoring marine toxins. The central laboratory of SENASA has been accredited for the analysis of ASP toxin by HPLC method. Official bioassay methods are used for the detection of PSP and the Lipophylic toxin group. With respect to toxin bioassays, it is important to mention that there are not enough intercalibration exercises offered by international laboratories. The importation of saxitoxin standard (for use in laboratory calibration) was also a problem in Argentina for several years because it is considered a chemical hazard. No less important is animal welfare in the laboratory: in Argentina there are specific regulations and training courses offered by EU experts on this issue.

A National Monitoring Program on Mollusc diseases has not yet been implemented. There is little knowledge of these diseases that could affect aquaculture production and development. In the 1990s there was an initiative in Rio Negro Province for aquaculture and export of native oysters (*Ostrea puelchana*) to the EU. In 1996 the Laboratory of Mollusc Parasitology and Histopathology (LABPATIBMP, SENASA LA 0116) was established. The laboratory is part of the Marine and Fishery Biological Institute “Alte. Storni”, is located at San Antonio Oeste, in the Rio Negro province, is a member of the SENASA network and is authorized to issue sanitary certifications for molluscs. On the other hand, since the end of the 1990s, the Parasitological Laboratory of the Patagonian National Centre of Chubut has been working on a significant research program on bivalve mollusc pathogens in the Argentinean Sea. This comprises (a) the massive mortality of the clam *Mesodesma mactroides* (distributed along the south occidental Atlantic coast from the south of Brazil to the south of Buenos Aires), (b) presence of *Perkinsus olseni* (Protozoa: Apicomplexa) and other parasites of the Uruguayan clam *Pitar rostrata*, (a pathogen which requires mandatory reporting to the OIE), and (c) histopathological study of commercially harvested Argentinean bivalve molluscs – scallop (*Aequipecten tehuelchus*), mussel (*Mytilus chilensis*), clam (*Protothaca antiqua*), clam panopea (*Panopea abbreviata*), clam (*Ensis macha*), Argentinean flat oyster (*Ostrea puelchana*) and false oyster (*Pododesmus rudis*). None of the parasites found are potentially hazardous human pathogens. There are not enough specialized professionals or a sufficient number of qualified diagnostic services on mollusc pathologies, to permit the establishment of relevant research lines on this topic.

Chile

Since 1989, the National Fisheries Service (SERNAPESCA) – the Competent Authority of Chile – has implemented the National Shellfish Sanitation Program (NSSP), which applies to the production areas situated along the Pacific coast where harvest is destined for export. One of the main targets of this program is to maintain permanent monitoring of the harvest areas, in order to ensure the safety of the resources, particularly with respect to marine biotoxins. The NSSP complies with the requirements of the sanitary authorities of the United States, Canada, European Union and New Zealand, which allows the country to export bivalve molluscs to these markets (2). Currently there are 162 production areas that participate in this Sanitation Program, 13 of which correspond to natural beds (wild) and 149 to aquaculture sites, which entails over 400 extraction sites. Ninety five percent of the total authorized areas are located in Los Lagos Region, an area that has been sporadically affected by episodes of Harmful Algal Blooms (HAB's) since 2002. The latest episode of HAB's, beginning in 2009, was due to the presence of PSP. The affected area comprises a large zone in the south of Chile (Aysen region) with significant toxin levels of PSP above the limit considered safe for human consumption. This episode affected the Quellón zone, at the south of Chiloé Island, where a large number of production areas authorized to export to the EU are located.

On February 27 2010, Chile was affected by an important earthquake, with severe damage to the local fishery industry in the regions of the Maule and Bio Bio; nevertheless, the major production areas located in the south of these regions were not affected. The major effects occurred in the region of Tubul (in the BioBio region) where the movement of submarine sediments did not permit harvesting of benthic species for some time. In March 2011, Chile was affected by a tsunami as a consequence of the Japan earthquake, resulting in economic detriment to the oyster and mussel culture. Fortunately, no chemical contamination was detected in the production areas. Presently, there is no evidence of significant contamination due to the presence of biotoxins in the production areas, only sporadic detection of diarrheic shellfish poison (DSP) during March and May 2011, in Seno del Roncaví and Butachauques islands, in the Lagos region. Historic surveys of phytoplankton in this area demonstrated the presence of *Protoceratium reticulatum*; the mouse bioassay symptoms seem to reveal the presence of Yessotoxins. If toxins are present in levels above standards stated by the CA, the harvest is banned in the affected areas.

Uruguay

In 1980, the Competent Authority of Uruguay, the National Direction of Aquatic Resources (DINARA) established a monitoring program for PSP biotoxins and phytoplankton in molluscs of the Atlantic coast of Uruguay. In the following

years, the program expanded to include the monitoring of the lipophilic group and ASP biotoxins (shellfish monitoring program). These molluscs are principally for domestic consumption, due to the small volumes harvested.

There is also a control program for molluscs for export, specifically for the clam *Pitar rostrata*, which comprises bacteriological, chemical and parasitological analysis, as well as the harvest area classification. Microbiological and phytoplankton analysis of the water were added to the control program in order to determine the presence of potential toxic algae, and the microbiological quality of the water; toxin analysis for PSP, ASP, and the DSP complex in the molluscs are also done. In 2000, three harvest zones located at three different depths (from 10 to 70 m) and more than 6 nautical miles offshore in the Atlantic Ocean were defined for the clam *Pitar rostrata*. It was the beginning of the microbiological studies of both water and clams for area classification, the determination of marine biotoxins and phytoplankton, and the control of chemical contaminants, as required by the EU regulation. To date, neither PSP, DSP complex, or ASP toxins, have been detected by the mouse bioassay (for the first 2 toxins) or HPLC method (for the latter) when monitoring clams from these areas.

The MPN standard ISO/TS 166493: 2005 is applied for enumeration of *E.coli* in the classification of the harvest areas, which are classified as "A" (<230 *E.coli*/100 g) zones (DINARA's microbiological analysis area). Heavy metal contaminants, such as cadmium (Cd), lead (Pb) and mercury (Hg), are determined by the Atomic Absorption Spectrophotometer (AAS) method (DINARA's chemical instrumental area) (14). Phytoplankton is monitored using qualitative and quantitative methods (DINARA's phytoplankton laboratory). With respect to the parasitological studies, the presence of the parasite *Perkinsus atlanticus* was determined in the clam *Pitar rostrata* (Fisheries Research Institute of the Faculty of Veterinary).

In January of 2002, an EC Decision authorized the export of frozen clams to the EU. Since the FVO mission of 2004, Uruguay has been permitted to export live bivalve molluscs to the EU (in this case the clam *Pitar rostrata*). Scallops without viscera, processed and frozen on board fishing vessels, were exported to the EU in the past. For the detection of norovirus and Hepatitis A virus, samples of molluscs were processed and kept frozen at the laboratory of DINARA in order to be analyzed based on the Polymerase Chain Reaction-Real Time (PCR-RT) protocols sent by the Santiago de Compostela University and with the collaboration of the University of Montevideo. The laboratory of DINARA is accredited (UNIT/ISO/IEC 17025:2005) (13) for *Salmonella*, *E.coli*, Cadmium (Cd), Lead (Pb), Mercury (Hg), and ASP toxin; due to its Management Quality System, and based on continuous system improvement, it is likely to be accredited for more techniques in the future.

Conclusions

According to Regulation (CE) 2076/2005, laboratories had to be accredited before December 31, 2009. As already stated, accreditation requires a significant investment for our countries. Besides this, other challenges lie ahead. They are stated below:

- In 2014 the EU reference mouse bioassay method used for the detection of the lipophilic toxins complex will be replaced by the LCMS/MS method, which means a large investment that our countries probably will not be able to afford (11).
- Regulations of the importing markets frequently change and methods and equipment have to adapt to these situations: this means additional investment.
- Human resources have to receive training on new regulations and methods, so enough qualified staff will be available.
- The high cost of intercalibration exercises, standards and reagents.
- Travel costs to participate in ICMSS and or other international congresses, and the language barrier, represent an important hurdle for many professionals from our countries to attend these events. In this regard, one of the proposals is to incorporate Spanish translation in these events, in order for the information to be accessible for scientists of Latin America countries.

These problems, common to Latin American countries, led to the creation of a network on shellfish safety in 2008. This network is called "Molluscs inoc", and has the support of the Infopesca organization (www.redmolluscus.infopesca.org). One of its goals is to reach agreements on safety standards on bivalve molluscs.

Acknowledgements We wish to thank the members of the "Molluscs inoc" network, and collaborators in general:
Argentina: Dr. Gabriel Meléndez, Lic. Liliana Figueroa and Dr. Gustavo Pérez Harguindeguy (SENASA); Lic. Marcela Álvarez; Dra. Marina Kroeck (LABPATIBMP); Dra. Florencia Cremonte and Nuria Vázquez (CENPAT); María Cielo Risoli (Professional illustrator)
Uruguay: Dirección Nacional de Recursos Acuáticos (DINARA): Fishery products analysis Laboratory (LAPP): Dra. Maria Salhi; I.Q. M.J. Pérez; MSc. Marta Odizzio; MSc. Silvia Méndez (phytoplankton laboratory); MSc. Alfredo Pereira (illustration) Faculty of Veterinary (Fisheries Research Institute): Dra. Estela Delgado

References

1. Argentina. Reglamento (Decreto 4238/68), Capítulo XXIII, Productos de la Pesca. 23.24.moluscos bivalvos destinados a consumo humano. http://www.senasa.gov.ar/Archivos/File/File753Capitulos.pdf
2. Chile. Procedure Manuals and Technical Norms (SMB/MP2, SMB/MP4, SMB/NT2, SMP/NT3) for export of bivalve molluscs for export. http://www.sernapesca.cl/index.php?option=com_remository&Itemid=246&func=select&id=63

3. Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods
4. Commission Directive 2001/22/EC of 8 March 2001, laying down the sampling methods and the methods of analysis for the official control of the levels of lead, cadmium, mercury and 3MCPD in foodstuffs
5. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs
6. Commission Regulation (EC) No 2074/2005 of 5 December 2005, laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004
7. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs
8. Commission Regulation (EC) No 333/2007 of 28 March 2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3MCPD and benzo(a)pyrene in foodstuffs
9. Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs
10. Regulation (EC) No 852/2004 of the European parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs
11. Regulation (EC) No 853/2004 of the European parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin
12. Regulation (EC) No 854/2004 of the European parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption
13. UNITISO/IEC 17025:2005: General requirements for the competence of testing and calibration laboratories
14. Uruguay. DECRETO 58/2011: Normas sanitarias para moluscos bivalvos vivos de exportación. Ministerio de Ganadería, Agricultura y Pesca

Chapter 5
Mitigating the Impacts of Paralytic Shellfish Poisoning During Harmful Algal Bloom Episodes in the Philippines

Ulysses M. Montojo, Marc Lawrence J. Romero, Ronald Jefferson A. Narceda, and Noime S. Walican

Introduction

Paralytic shellfish poisoning (PSP) due to harmful algal blooms (HABs; also called toxic red tides) continues to be a global food safety issue, particularly in Southeast Asia where the phenomenon is observed to be increasing in frequency and severity (Fukuyo et al. 2011). In the Philippines, PSP is among the greatest of marine poisoning hazards recorded in recent years (Table 5.1) and remains the major threat to the aquaculture industry and seafood consumers. Paralytic shellfish poisoning occurrences are mainly associated with blooms of toxic dinoflagellates, including *Pyrodinium bahamense* var. *compressum* (Hermes and Villoso 1983; Estudillo and Gonzales 1984), observed in a number of areas, and *Alexandrium minutum* (Bajarias et al. 2003), which was observed to be spreading in the Philippines (Fig. 5.1).

Filter feeders, such as molluscan bivalves, often become a vector of PSP toxins by ingesting toxic microalgae (Montojo et al. 2006). Human consumption of toxin-contaminated shellfish leads to acute poisoning and, in the worse cases, death (Furio and Gonzales 2002). The Philippine government, through the Bureau of Fisheries and Aquatic Resources (BFAR), recognized the importance of effective management of HABs by alleviating its related PSP impacts. The primary goal is to develop a protocol for timely and reliable public advisory in order to prevent its adverse effects on human health and the fishing industry.

At the same time, the National Fisheries Research and Development Institute (NFRDI), the research arm of BFAR, has implemented various PSP-related research

U.M. Montojo (✉) • R.J.A. Narceda • N.S. Walican
National Fisheries Research and Development Institute, 101 Mother Ignacia Avenue, South Triangle, Quezon City 1100, Philippines
e-mail: ulyssesmontojo@gmail.com

M.L.J. Romero
Bureau of Fisheries and Aquatic Resources, Elliptical Road, Diliman, Quezon City 1100, Philippines

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_5,

Table 5.1 Number of recorded marine poisoning incidences by type of poisoning or causative organisms, in the Philippines

Poisoning cases	Causative organisms	No. of victims	No. of deaths
Paralytic shellfish poisoning (1983–2010)	Shellfish exposed to toxic dinoflagellates	2,465	146
Ciguatera fish poisoning (1990–2010)	Coral reef fish	165	0
Tetrodotoxin poisoning (1990–2010)	Puffer and goby fish	150	14
Polycavernoside poisoning (2000–2010)	Brown seaweeds	10	5
Other marine poisonings (2000–2010)	Invertebrates	23	8

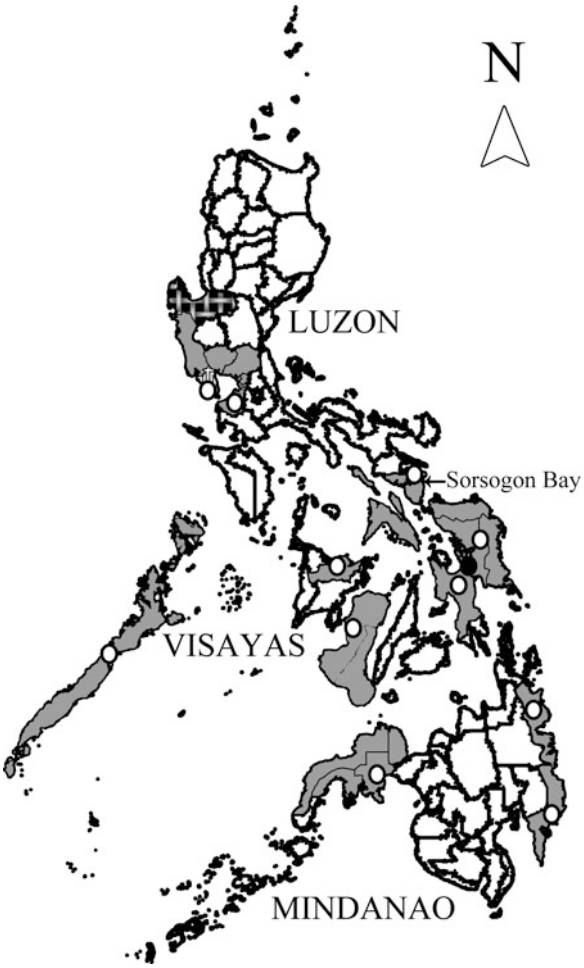


Fig. 5.1 Areas with recorded PSP outbreaks in the Philippines, from 1983 to 2010. ( ) *Pyrodinium bahamense* var. *compressum* (*Pbc*) blooms ( ) *Alexandrium minutum* blooms ( ) *Pbc* and *A. minutum* blooms

activities (Montojo et al. 2006, 2010, 2012; Narceda et al. 2011, unpublished) to help understand the PSP phenomenon and support the crafting of national programs and policies related to PSP management and monitoring.

This paper presents various management and research interventions aimed to mitigate impacts of PSP in the Philippines.

Centralized Versus Decentralized Monitoring Systems

After the first incidence of a toxic HAB in 1983, the BFAR established a centralized monitoring program for the early detection of toxic blooms, particularly those of *Pyrodinium* (Gonzales et al. 1989; Bajarias et al. 2006). The monitoring strategy is to identify the PSP causative algal species in the water samples and to analyze shellfish for PSP toxins. Toxic organisms are identified through morphological characteristics under the light microscope and shellfish toxicity is determined by mouse bioassay (MBA), using Method 959.08 of the Association of Official Analytical Chemists (1999). This method employs the injection of shellfish extract into the mouse and correlating the mouse death time with toxicity. Green mussels (*Perna viridis*) and, in some areas, thorny oysters (*Spondylus squamosus*), are used as indicator shellfish for PSP monitoring. The centralized monitoring system requires that the sample be brought to Manila for analysis and publication of results. However, the archipelagic nature of the country poses logistical problems, e.g. delays in the transport and analysis of samples, and consequently, information dissemination. In addition, some affected areas are not easily accessible. As a result, adverse effects, such as harvesting and selling of contaminated products, and sometimes death, have occurred before appropriate management interventions could be imposed.

In order to arrive at a more effective monitoring system, decentralization was implemented in 2002, in collaboration with the Japanese government. Twelve regional and local testing centers, strategically located in Luzon, Visayas and Mindanao, were established and have since made the fast tracking of results for public warnings possible (Bajarias et al. 2002). The decentralized monitoring system also uses the MBA for PSP toxin determination. As such, regional and local laboratories are required to maintain a mouse colony composed of a single strain, to ensure consistency.

Procedures to maintain test mice that are adapted to tropical climate conditions were provided to local laboratories (Montojo et al. 2002). However, it was sometimes difficult to maintain a mouse colony, especially for laboratories operated by Local Government Units (LGUs); as a result, only a few are operational. At present, alternative methods for screening shellfish samples for PSP toxins are being evaluated by BFAR, in collaboration with the North Pacific Marine Science Organization (PICES). The applicability of the ELISA PSP Kit (Abraxis) and PSP Rapid Test Kit (Jellett) are being tested (Relox et al. 2011, unpublished). ELISA is currently being evaluated at the BFAR Central Laboratory as a semi-quantitative

tool. This method was chosen for its much lower detection limit compared to the MBA, which allows for the early detection of PSP toxins. However, the presence of several PSP toxin analogues limits the quantitative accuracy of the method. The PSP Rapid Test Kit, on the other hand, is a qualitative method. Its application is intended for local laboratories because it eliminates the need for maintaining mice colonies. Both the aforementioned methods are intended for screening purposes; hence, positive samples must be confirmed using the MBA.

Should the screening procedures prove to be applicable, the LGUs will have the necessary tools to implement precautionary measures in the case of positive samples. Actions can be taken while samples are being confirmed for the presence of toxins.

Change in the National Regulation Limit for PSP Toxin

The Philippines previously implemented a very conservative regulatory toxicity limit of 40 μg STXeq/100 g of shellfish tissue for banning shellfish harvesting and selling the product for human consumption. This compares to the international limit of 80 μg STXeq/100 g (Philippine Guidebook 1999). With public safety as the primary concern, such policy is sound. However, this lower regulatory limit restricts the marketing of additional product, and thus has a negative impact on the livelihood of shellfish growers on small farms. The issue was addressed by raising the regulatory limit to 60 μg STXeq/100 g, through the FAO 235 Series of 2010 (BFAR 2010).

Information, Education and Communication (IEC)

Paralytic shellfish poisoning is primarily associated with consuming filter-feeding shellfish. However, during toxic red tide episodes, consumers worry not only about eating unsafe shellfish, but also about consuming other marine products as well. As a consequence, direct and indirect economic losses are incurred when PSP and HAB incidents are reported. One aspect of the management strategy is therefore to produce and disseminate outreach materials to educate the public about this natural phenomenon. Public meetings and information campaigns are conducted to allow a better understanding of the toxic red tide blooms. A number of handbooks, scientific posters and other outreach materials, also written in local dialects, have been published and circulated across the country (Figs. 5.2a–c and 5.3a–c).

A survey was also conducted in PSP-affected areas of Sorsogon Bay, using a prepared questionnaire, to determine the level of awareness among fishers and the community as a whole. The layman's term "toxic red tide", rather than "HAB", was used in the survey questions. Results indicated that the respondents have high level of awareness (92 %) of toxic red tides and its impacts. Among the sources

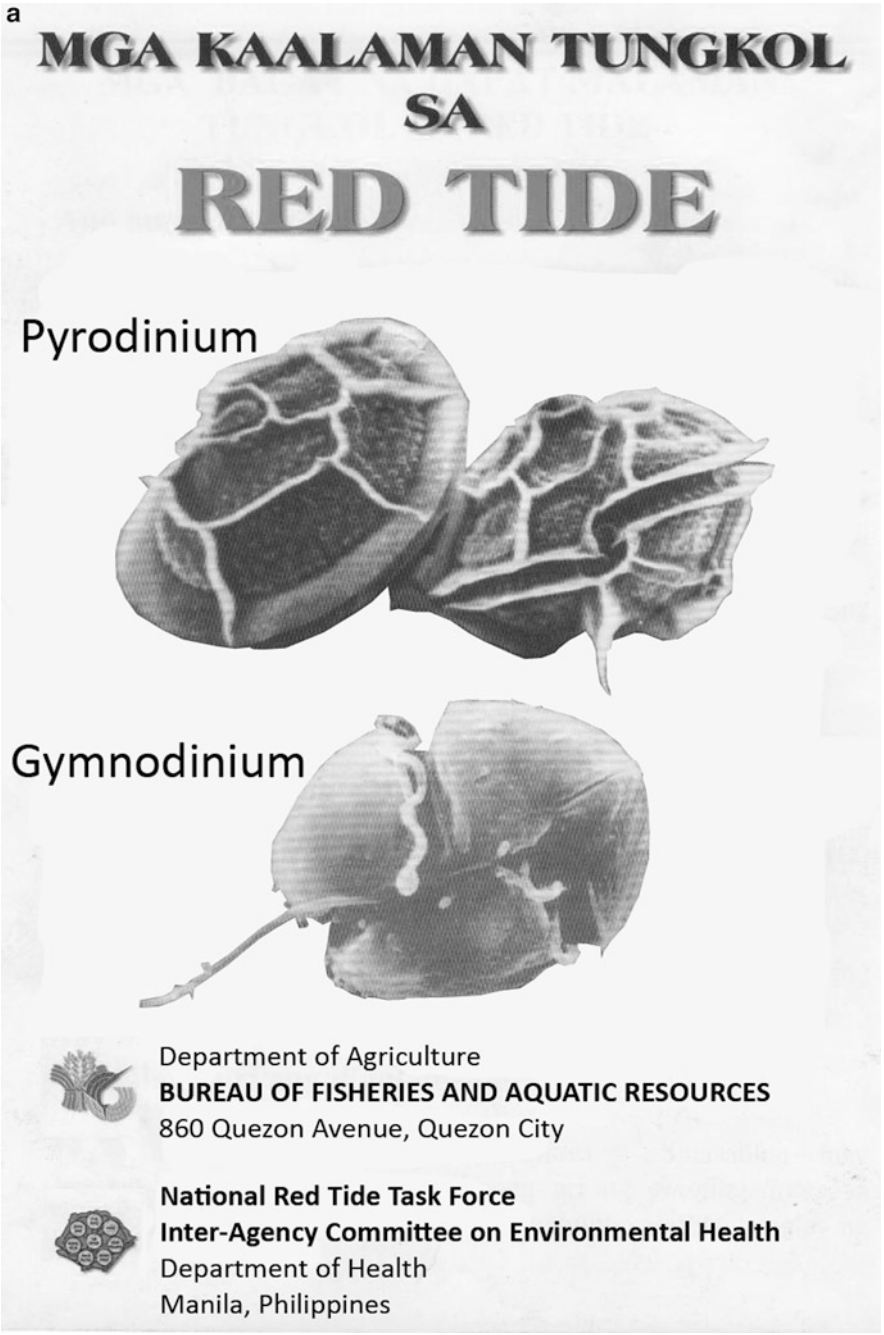
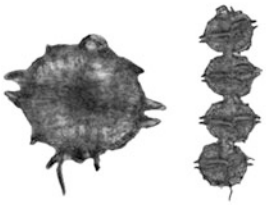


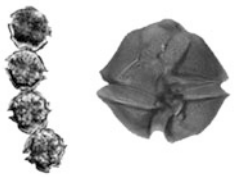
Fig. 5.2 Examples of PSP related outreach materials. (**a**) brochure on basic knowledge about red tide, produced in various dialects, which explains the occurrence, poisoning and possible health management, (**b**) information on causative organisms, (**c**) information on individual differences in PSP toxin accumulation and depuration among bivalve species, (**c**) information about safe consumption of other marine products

b

The causative organisms for paralytic shellfish poisoning in the Philippines



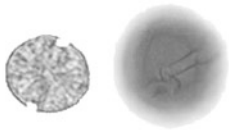
Pyrodinium bahamense var. compressum. Main causative organism of PSP in the country: toxin contents in culture cells: 165 to 402 fmol/cell: wild cells: 67 to 1624 fmol/cell: contains STX, neoSTX, dcSTX, B1 and B2 toxins.



Alexandrium tamiyavanichii. Toxin contents in culture cells:25 to 146 fmol/cell: contains GTX3, B1, C2 + 1 and B2 toxins: occurs in Manila Bay.

Gymnodinium catenatum.
Toxin contents in culture cells: 159 fmol/cell: contains C2 + 1, C4, B1 and B2 toxins: occurs in Manila Bay: sometimes co-occur with *P. bahamense* var. *compressum* and / or *A. tamiyavanichii*

Alexandrium minutum.
Caused lethal PSP in Bolinao Bay, Pangasinan: contains GTX1-4 toxins



Help ensure seafood safety, be vigilant...

Contact the Marine Fisheries Research Division
National Fisheries Research and Development Institute
940 Quezon Avenue, Quezon City 1103 Metro Manila
Tele Fax (02) 3725054 Email: um_montojo@yahoo.com

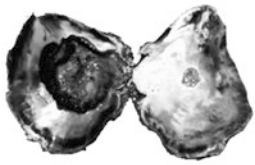
Fig. 5.2 (continued)

c

Alam nyo ba na sa tuwing panahon ng nakakalasong red tide may mga kabibe ang nagtataglay ng nakamamatay na lasong PSP subalit mabilis din itong nawawala kasabay ng pagdami at paglaho ng organismo sa dagat na sanhi nito...

Crassostrea sp.

Anadara antiquata



Placuna sella

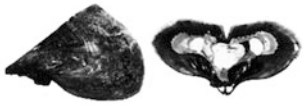
Isognomon ephippium

Perna viridis

Chama iostoma

...kaya masusing matiyagan ang antas ng lason bago ito kainin.
May mga kabibe namang nagtataglay ng mataas na antas ng lason sa mahabang panahon...

Spondylus squamosus



Atrina vexillum

...ang mga ito ay hindi maaring kainin kahit sa panahong wala ng red tide sa dagat...

NFRDI BFAR

Makipag-ugnayan sa Marine Fisheries Research Division
National Fisheries Research and Development Institute
940 Quezon Avenue, Quezon City 1103 Metro Manila
Tele Fax (02) 3725054 Email: um_montojo@yahoo.com

Fig. 5.2 (continued)

a

Republic of the Philippines
Department of Agriculture
Bureau of Fisheries and Aquatic Resources
PCA Compound, Elliptical Road, Diliman, Quezon City
Tel. Nos. 929-95-97 * 929-80-74

Shellfish Bulletin No. 19
Series of 2009
01 September 2009

Based on the latest laboratory results of the Bureau of Fisheries and Aquatic Resources (BFAR) and Local Government Units (LGUs), Shellfish collected at **Dumanquillas Bay in Zamboanga del Sur; Sorsogon Bay in Sorsogon and Bislig Bay in Bislig City, Surigao del Sur** are still **positive** for paralytic shellfish poison that is beyond the regulatory limit.

All types of shellfish and *Acetes sp.* or alamang gathered from the areas as shown above are NOT SAFE for human consumption. Fish, squids, shrimps, and crabs are safe for human consumption provided that they are fresh and washed thoroughly, and internal organs such as gills and intestines are removed before cooking.

The following areas continue to be **FREE from toxic red tides**: coastal waters of Cavite, Las Piñas, Parañaque, Navotas, Bulacan and Bataan in Manila Bay; coastal waters of Alaminos, Anda, Bolinao and Wawa, Bani in Pangasinan; Masinloc Bay in Zambales; coastal waters of Milagros and Mandaon in Masbate; Inner Malampaya Sound in Taytay and Honda Bay in Palawan; coastal waters of Pilar, President Roxas, Pontevedra, Panay, Roxas City, Ivisan and Sapian in Capiz; coastal waters of Pontevedra, in Negros Occidental; Irong-irong, Maqueda and Villareal Bays in Samar; Ormoc, San Pedro, Cancabato and Carigara Bays in Leyte; Biliran Waters in Biliran Province; Hinatuan and Lianga Bays in Surigao del Sur; Balite Bay in Mati, Davao Oriental; and coastal waters of Kabasalan in Sibuguey Bay, Zamboanga Sibugay; Juag Lagoon in Matnog, Sorsogon.

(signed) MALCOLM I. SARMIENTO, JR.
DIRECTOR

Fig. 5.3 Examples of public information during PSP outbreaks/HAB occurrence. (**a**) shellfish bulletin published in a national newspaper, (**b**) poster for a newly affected area, (**c**) information about safe consumption of other marine products

Fig. 5.3 (continued)

of information, television, radio and posters (78 %, 55 % and 28 %, respectively) were most frequently cited. In the survey, the respondents were asked to identify marine products that are safe for consumption. Fish were identified to be safe by most of the respondents (96 %) (Fig. 5.4). However, there seemed to be confusion with regards to the safety of other marine products. For example, only 30 %, 34 % and 30 % of the respondents considered squid, crabs and shrimp, respectively, to be safe. Ninety-six percent of the respondents considered shellfish to be the most toxic marine product during a toxic red tide bloom.

Their knowledge about the toxicity of various shellfish species was also investigated (Fig. 5.5). The majority of respondents (96 %) considered the carpet shell (*Paphia undulata*) to be safe for consumption during a red tide, an assumption that is correct.

In relation to PSP symptoms, severe headache (73 %), paralysis around the mouth (63 %) and vomiting (60 %) were the most-cited (Fig. 5.6).

Additional information was also gathered regarding myths, such as whether cooking or adding vinegar can remove or lessen the toxicity of contaminated shellfish. It is noteworthy that 95 % of the respondents were aware that such treatments are not effective. Lastly, the respondents' knowledge about the causes, and the possibility of predicting and preventing, toxic red tides was investigated (Fig. 5.7). Excessive nutrient loading and the deteriorating quality of the marine environment were cited as causes of toxic red tides. Seventy-five percent of the respondents believed that the occurrence of toxic blooms cannot be predicted, while 58 % believed that they can be suppressed. In reality, however, ways to suppress HABs are still being explored. It can be concluded that the information and education campaign has been mostly effective.

c

Republic of the Philippines
Department of Agriculture
BUREAU OF FISHERIES AND
AQUATIC RESOURCES

Fisheries Resources Management Division
Marine Biotoxin Section
860 ARCADIA BLDG QUEZON AVE., QUEZON CITY

Tel. No.: 332-0210
email add: bfar_nab@yahoo.com

Siguraduhin lamang na ang mga ito ay sariwa at:

* ang isda ay natanggalan ng hasang at lamang loob
* ang hipon ay natanggalan ng ulo
* ang pusit ay naalisan ng lamang loob
* ang alimasag ay natanggalan ng aligi sa tiyan

Fig. 5.3 (continued)

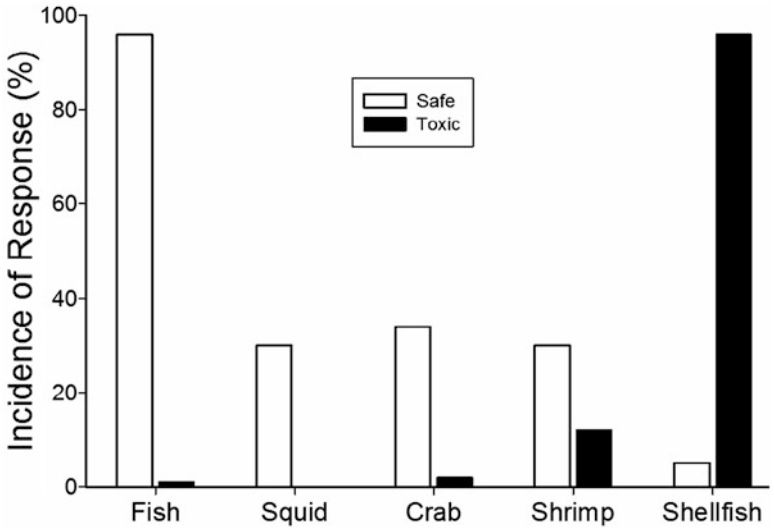


Fig. 5.4 Respondents' knowledge about safe (□) and toxic (■) marine products during toxic harmful algal blooms. Randomly selected individuals were surveyed using prepared questionnaires in three municipalities (Juban, Casiguran, Cambulaga) surrounding Sorsogon Bay, Sorsogon, Philippines. The question posed was "Which of these marine organisms are safe for consumption, or toxic, during a red tide?"

Research Interventions

Monitoring methods employ the use of indicator species to detect the presence of toxins in shellfish. Once the regulatory limit is reached or surpassed, a total ban of all shellfish harvesting is imposed. However, this very stringent policy results in closure of areas for harvest of certain shellfish species that may not, in fact, be above the regulatory limit for toxins. This leads to the collapse of the shellfish industry and great economic hardship to the growers. Scientific research was therefore required to help diminish further economic consequences.

Variation in Toxicity Among Shellfish Species

Research was carried out to address the concerns of the total ban, particularly in Sorsogon Bay. This bay supports a high diversity of bivalves, including epifaunal, partially-exposed benthic and infaunal species. The variation in toxin levels among shellfish species from different habitats and exposed to *P. bahamense* var. *compressum* during a natural bloom was investigated. Toxin accumulation for the epifaunal indicator species (*Perna viridis*) and the infaunal species (*Paphia undulata*) was also

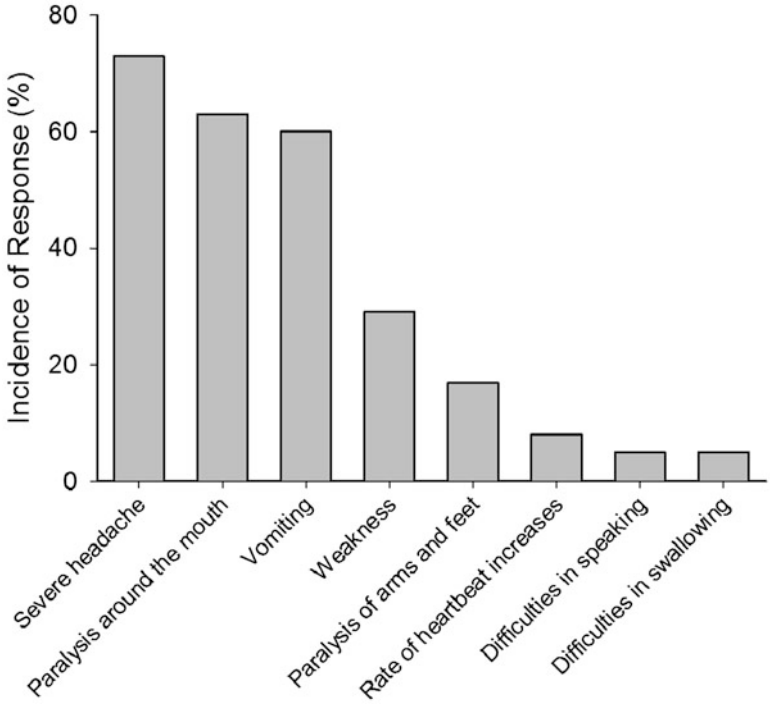


Fig. 5.5 Respondents' knowledge about the toxicity of various shellfish during toxic harmful algal blooms. The question posed was "Which of these shellfish are safe for consumption during a red tide?"

clarified under laboratory controlled conditions. Toxin profiles were identified and quantified by High Performance Liquid Chromatography (HPLC), according to the method of Oshima (1995).

Preliminary data revealed that there is a notable distinction in toxicities of shellfish from different habitats. Epifaunal species, including *Perna viridis*, exhibited high toxicity levels, while partially exposed benthic species (e.g. the pen shell, *Atrina pectinata*), although also showing high toxicity values, were less toxic. This indicates that shellfish from both of these environments are susceptible to PSP toxin contamination (Montojo et al. 2006, 2012). On the contrary, infaunal species, buried 20–200 mm in muddy substrates, showed no detectable PSP toxins. An exception was *Paphia undulata*, which showed traces of PSP toxins. Further study showed that PSP contamination of this species was only minimal, even at extremely high toxic algal cell concentrations, quite the opposite of the elevated toxicity values in *Perna viridis* (Montojo et al. 2010). It can also be inferred that the two shellfish species being of different habitat have different feeding habit as mentioned by Montojo et al. (2010). In a similar study involving domoic acid (Mafra et al. 2010), difference in domoic acid accumulation in *Mytilus edulis* and *Crassostrea virginica* was associated to the balance of uptake and elimination of toxins in shellfish tissue.

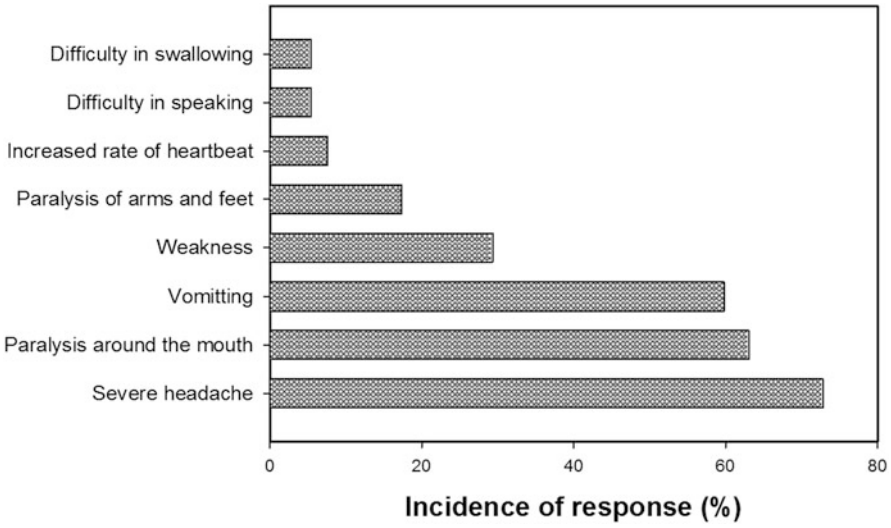


Fig. 5.6 Respondents' knowledge on PSP symptoms

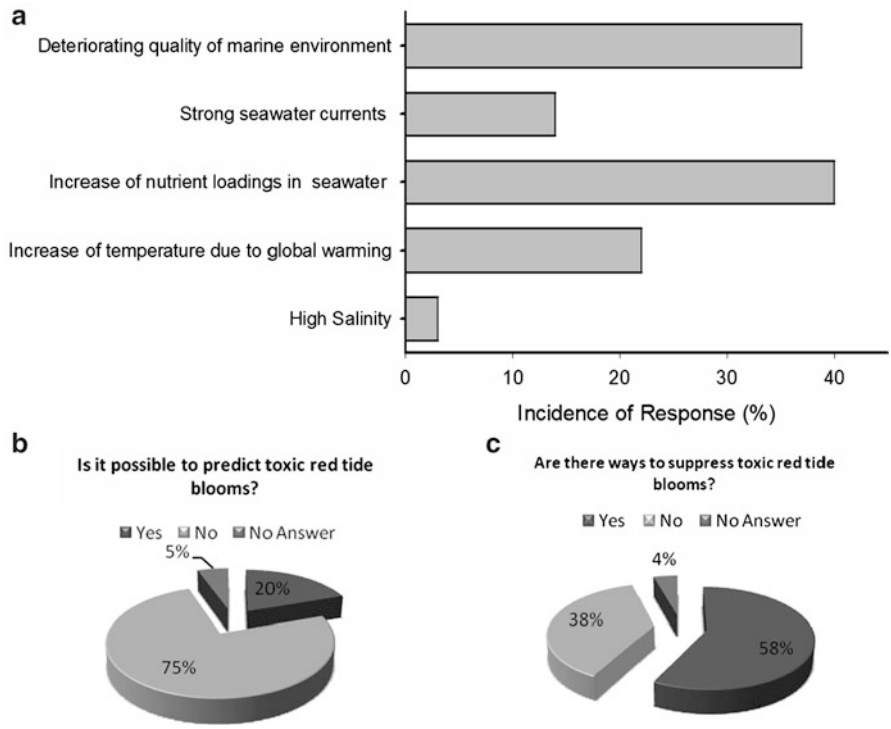


Fig. 5.7 Respondents' knowledge about the (**a**) possible causes, (**b**) the prediction and (**c**) suppression of harmful algal blooms

Investigations of other factors that can affect shellfish toxicity are being conducted. This includes: physiological transformation and seasonal variation of PSP toxins in different shellfish species, effect of substrate, distribution of PSP toxins in shellfish tissues and gut size.

Variation in Toxicity Among Shellfish Tissues

A study on the commercially important pen shell (*Atrina pectinata*) was carried out to determine the bioaccumulation and distribution of PSP toxins in different tissues. Remarkably, the bivalve's adductor muscle accumulated minimal levels of PSP toxins. This is in contrast to the high PSP levels observed in *Perna viridis* under the same conditions. The finding was validated by the standard MBA method regularly used for PSP monitoring, for adductor muscles collected during a toxic bloom (Narceda et al. 2011, unpublished).

Discussion

Declining Cases of PSP in the Philippines

The shifts in management strategies, combined with research interventions, have significantly contributed to reducing PSP impacts in the country. The above management and research interventions can therefore be considered as successful, despite the increasing number of areas contaminated by PSP toxins. Furthermore, a declining trend in poisoning cases was observed since the first incidence in 1983 (Fig. 5.8). Recent PSP cases that can be attributed to areas that have newly recorded blooms are an exception to this decreasing trend.

Decentralization of PSP monitoring, particularly in the conduct of laboratory sample analysis and results reporting, could provide autonomy to local officials when making management decisions. This could in turn lead into early issuance of shellfish bans and subsequent confiscations of contaminated shellfish products harvested from affected areas, before they could cause human harm. Consumers are now aware of the possible consequences of HABs and PSP in relation to food safety of marine products; prior to 1983 they were not.

Addressing Socio-economic Impacts of PSP: The Case of Sorsogon Bay

As a consequence of decentralization, the role of LGUs is recognized as being at the front line in response to PSP cases. It is also noteworthy that in recent years consumers have an increased awareness of the PSP phenomenon, as a result of information and education campaigns conducted at the local level. In 2009,

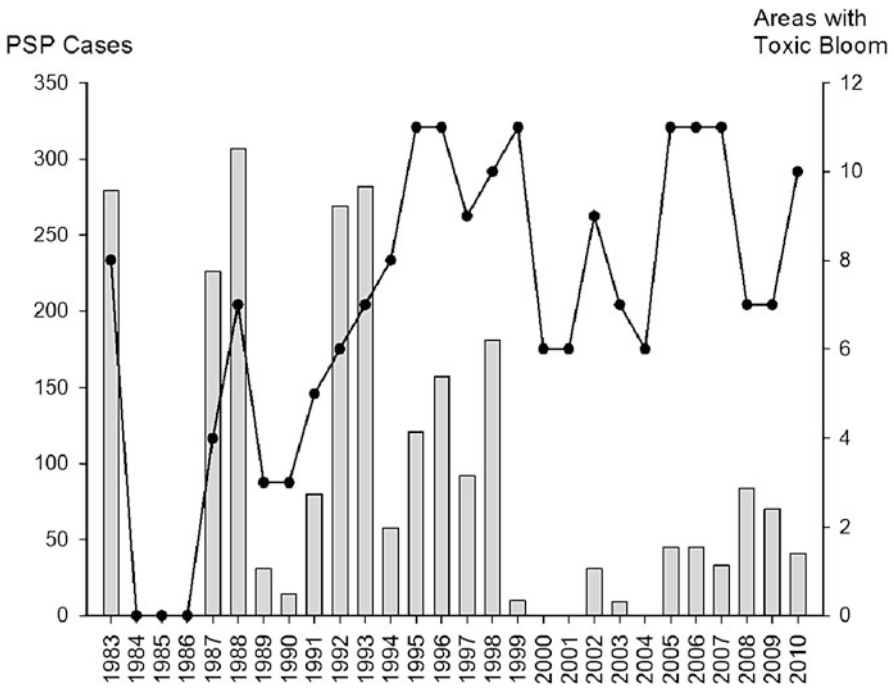


Fig. 5.8 Number of PSP cases (*bar graph*) and areas with toxic algal blooms (*line graph*) in the Philippines, from 1983 to 2010

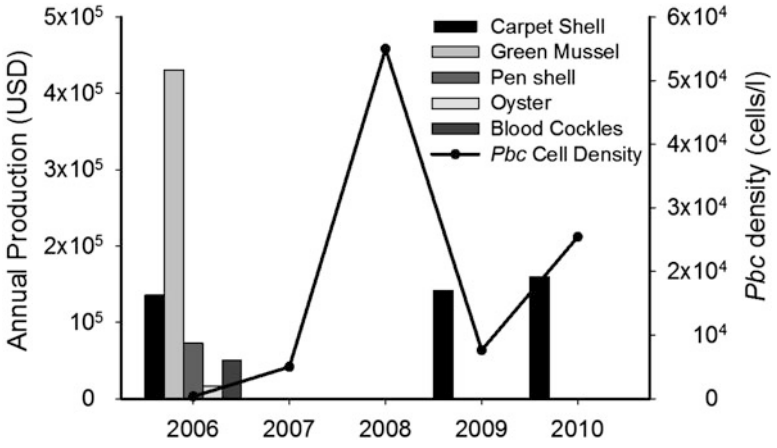


Fig. 5.9 Five-year production estimates in US dollar of five commercially important shellfish species (*bar graphs*) and the occurrence of *Pbc* cells (*line graph*) in Sorsogon Bay. Total ban on harvest of all kinds of shellfish resulted in almost zero production in 2007 and 2008. Production of carpet shell became possible after introduction of selective banning since 2009 (Source: Sorsogon Provincial Agriculture Office)

selective banning was introduced in Sorsogon Bay, where the carpet shell has been exempted from the ban on harvesting and transport. With this strategy, income generation became possible. Selective banning in Sorsogon Bay has been effective, while quality assurance procedures are still followed. Harvest of green mussels is prohibited but harvest and sale of carpet shells is allowed (Fig. 5.9), provided that carpet shell harvests are analyzed and certified to be free or within the allowable level of PSP toxins prior to shipment. Possible commercialization of the pen shell adductor muscle for import and export purposes can also help to alleviate income losses of local shellfish farmers, even during HAB episodes.

Commercialization of some species and parts of shellfish during HAB episodes is possible. However in the interest of food safety, proper risk assessment and regulation must still be done.

Acknowledgments We express our gratitude and appreciation to Ms. Mirriam Cayme, Mr. Valeriano M. Borja, Mr. Christoper Del Rosario, Ms. Mary Jeremie Faith F. De Jesus, BFAR-Region 5 and the Provincial Agricultural Office of Sorsogon for assisting us in the collection and analysis of the samples. We also thank Dr. Shigeru Sato of Kitasato University for providing PSP toxin standards, and the FAO for the partial travel grant to the first author to attend the 8th ICMSS, held at Prince Edward Island, Canada.

References

Association of Official Analytical Chemists (AOAC) (1999) Official method 959.08 – Paralytic shellfish poison, biological method. In: Official methods of analysis, 1, 17th edn. AOAC International, Arlington

Bajarias FFA, Furio EF, Gonzales CL, Sakamoto S, Fukuyo Y, Kodama M (2002) Localizing PSP monitoring in the Philippines: a management option. In: Namba K (ed) Proceedings of the international commemorative symposium 70th anniversary of the Japanese Society of Fisheries Science, 68. The Japanese Society of Fisheries Science, Tokyo, pp 519–522

Bajarias FFA, Montojo UM, Relox J Jr, Sato S, Kodama M, Yoshida M, Fukuyo Y (2003) Paralytic shellfish poisoning due to *Alexandrium minutum*, Halim in Northwestern Philippines. In: Nitithamyong C (ed) Proceedings of the first joint seminar on coastal oceanography. Department of Marine Science, Faculty of Science, Chulalongkorn University, Chiang Mai, p 278

Bajarias FFA, Relox JR Jr, Fukuyo Y (2006) PSP in the Philippines: three decades of monitoring a disaster. Coast Mar Sci 30:104–106

Bureau of Fisheries and Aquatic Resources (BFAR) (2010) Fisheries Administrative Order No. 235. In: Safety and quality control standards for PSP. Retrieved 14 Dec 2011, from http://www.bfar.da.gov.ph/pages/Legislation/FAO/fao235.pdf

Estudillo R, Gonzales C (1984) Red tides and paralytic shellfish poisoning in the Philippines. In: White AW, Anraku M, Hooi KK (eds) Toxic red tides and shellfish toxicity in Southeast Asia. Southeast Asian Fisheries Development Center and International Development Research Center, Singapore, pp 52–79

Fukuyo Y, Kodama M, Omura T, Furuya K, Furio E, Cayme M, Lim PT, Dao VH, Kotaki Y, Matsuoka K, Iwataki M, Rujinard S, Thaithaworn L (2011) Ecology and oceanography of harmful marine microalgae. In: Nishida S, Fortes MD, Miyazaki N (eds) Coastal marine science in Southeast Asia. Terrapub, Tokyo, pp 23–48

Furio EF, Gonzales CL (2002) Toxic red tide and paralytic shellfish poisoning profiles in the Philippines. In: Gonzales CL, Sakamoto S, Furio EF, Ogata T, Kodama M, Fukuyo Y (eds) Practical guide in paralytic shellfish monitoring in the Philippines. GLYZ Printing House, Manila, pp 3–27

Gonzales CL, Ordonez JA, Maala AM (1989) Red tide: the Philippine experience. In: Okaichi T, Anderson DM, Nemoto T (eds) Red tides: biology, environmental science, and toxicology. Elsevier, New York, pp 45–48

Hermes R, Villoso EP (1983) A recent bloom of the toxic dinoflagellate *Pyrodinium bahamnese* var. *compressa* in the central Philippine waters. Fish Res J Philipp 8:1–8

Mafra LL Jr, Bricelj VM, Fennel K (2010) Domoic acid uptake and elimination kinetics in oysters and mussels in relation to body size and anatomical distribution of toxin. Aquat Toxicol 100:17–29

Montojo UM, Formeloza MA, Cruz NL, Candia DN (2002) Mouse bioassay methods for shellfish toxicity. In: Gonzales CL, Sakamoto S, Furio EF, Ogata T, Kodama M, Fukuyo Y (eds) Practical guide in paralytic shellfish monitoring in the Philippines. GLYZ Printing House, Manila, pp 77–99

Montojo UM, Sakamoto S, Cayme MF, Gatdula NC, Furio EF, Relox JR Jr, Sato S, Fukuyo Y, Kodama M (2006) Remarkable difference in accumulation of paralytic shellfish poisoning toxins among bivalve species exposed to *Pyrodinium bahamense* var. *compressum* bloom in Masinloc Bay, Philippines. Toxicon 48:85–92

Montojo UM, Romero MLJ, Borja VM, Sato S (2010) Comparative PSP toxin accumulation in bivalves, *Paphia undulata* and *Perna viridis* in Sorsogon Bay, Philippines. In: Lassus P (ed) Proceedings of the seventh international conference on molluscan shellfish safety. Ifremer, Nantes, pp 49–55

Montojo UM, Borja VM, Romero MLJ, Cayme MF, Sato S, Fukuyo Y, Kodama M (2012) Vulnerability of tropical molluscan shellfishes against PSP contamination during bloom of *Pyrodinium bahamense* var. *compressum*. Coast Mar Sci 35:64–66

Narceda RJA, Montojo UM, Cayme MF, Borja VM (2011) Remarkable difference in paralytic shellfish poisoning toxin distribution in tissues of pen shell *Atrina pectinata* exposed to toxic red tide bloom. Paper presented in the 26th Philippine Chemistry Congress, Cebu City

Oshima Y (1995) Post-column derivatization HPLC methods for paralytic shellfish poisons. In: Hallegraeff GM, Anderson DM, Cembella AD, Enevoldsen HO (eds) Manual on harmful marine microalgae. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp 81–94

Philippine Guidebook on Toxic Red Tide Management (1999) National Red Tide Task Force and Inter-agency Committee on Environmental Health, Manila

Relox J Jr, Arcamo S, Romero ML, Cabella L, Tuazon L, Carolino L (2011) Harmful algal blooms and marine biotoxin monitoring program in the Philippines. Paper presented at the 3rd National Harmful Algal Bloom (HAB) conference, Tagbilaran City, Philippines

Chapter 6
New Zealand Aquaculture Industry Initiative: Collingwood Case Study

Helen Smale

Introduction

This case study demonstrates the benefits of regulators, scientists, and industry managers collaborating, including where that collaboration goes beyond what we normally take to be the boundaries of growing water quality management. It explores an unexplained and previously undetected bacterial contamination of Collingwood growing areas that was detected by routine testing. To protect market access, a progressive tightening of the harvest criteria was imposed until the time open for harvest was reduced from 72 % to less than 50 %. This added significantly to test costs, impacted shellfish farming economics and compromised the operation of the processing factories because of interruptions to supply.

As managers of the water quality programme, we were confronted with two challenges:

1. Identify the source or sources of contamination; and
2. Design and implement a strategy to manage the contamination.

Each of us face different combinations of circumstances and the model adopted and specific conclusions may not have universal application but individual elements and subsequent developments will definitely prove of value to those having to manage similar situations. One thing that marine farming industries around the world share is that we all farm "the commons" – the public space. Ultimately we rely upon public good will and public cooperation to ensure access to our growing areas through appropriate resource management legislation and the maintenance of the quality of our growing waters. We also share that space with other users, including many who use it deliberately and legally to dispose of industrial, agricultural and

H. Smale (✉)
Marlborough Shellfish Quality Programme Inc (MSQP), PO Box 767,
Blenheim 7240, New Zealand
e-mail: msqp@xtra.co.nz

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_6,

municipal effluent. That effluent discharge can often arise tens if not hundreds of kilometres from the growing areas and the actual contamination of the water is often ephemeral but concentrated in the molluscs.

It is worth noting that, in contrast to some countries where once an area is classified for growing, certain legal protections of the water quality exist, no such protection currently exists in New Zealand.

Identifying the source of contamination was seriously constrained by the absence of any available methodology to link the bacteria found in the marine farm site samples with the potential sources of contamination. In retrospect that constraint took us down a valuable learning pathway and by applying the lessons learned alongside microbial source tracking that we now have available, we now have a powerful model for addressing the sorts of issues contained in this case study.

Explain the New Zealand User Pays System and Self Regulation

Two things distinguish New Zealand's approach to water quality management. First is that we operate under a user pays model whereby industry funds the services provided by the regulatory and science agencies. Second, industry, under oversight and audit by the regulators, self manages and directly funds large parts of the programme.

The net effect of these is a highly efficient and innovative system, an exceptionally high level of compliance and a high level of responsibility taken by industry for innovation related to maintaining water quality and addressing any issues that arise. It hinges around a collaborative approach where the constructive tensions between the scientists, regulators and industry drives continuous improvement – something that we are all very proud of.

Description of the Growing Area and Surrounding Influences

The growing area in question is located approximately 1.5 km offshore near Collingwood and to the North of the Aorere River Mouth/Ruataniwha Inlet in the north west of the South Island of New Zealand. This river, subject to substantial flow variations, discharges into the sea approximately 3 km south of this growing area. For over 90 % of the time, the Aorere's flow rate is less than 15 m^3/s. However floods are frequent and peak flow may reach 3,000 m^3/s then rapidly return to a low level, sometimes within a day. The river – due to its size, very large peak flows, and proximity to the marine farms – has a large influence on water quality at the Collingwood Farms.

The river catchment is 709 km^2 and the river winds its way to the sea through pristine native bush in Kahurangi National Park (including the popular Heaphy Track) and a rich alluvial valley used extensively for dairy, beef, sheep and deer farming.

The nearest settlement to the growing area is Collingwood, with a population of just 300 – but this may double in the peak summer holiday times. Collingwood has a sewage treatment facility that is controlled by the local Council. There are some local residential homes and a few holiday homes in the area, providing an additional population of approximately 30 people serviced by septic tanks. An adjacent black swan colony rounded out a wide range of potential of sources of contamination.

Collingwood marine farms occupy approximately 80 Ha, growing Greenshell mussels and producing around 4,000 tonnes pa. Significant expansion is planned in the area increasing the importance of solving this puzzle. The majority of production is exported and therefore subject to the regulatory requirements of domestic and export markets.

In 1994 this Growing Area was classified as conditionally approved (that is open subject to specified criteria) and has operated under differing harvest criteria since that time.

Shellfish harvesting is carried out year-round subject to sanitation and biotoxin closures. The initial harvest criteria was based on the level of the Aorere River. However, the currents and prevailing wind often took the river plume away from the farms. Frequently the shellfish farms would be closed under this criterion, but frustratingly farmers could clearly see the river plume well away from their farms and product. That led to investigation of the usefulness of salinity monitoring for the area based upon real time telemetered salinity data from buoys situated in the farms. Salinity monitoring based upon the rate of dilution of the sea water by the fresh water from the river was implemented in 1997, significantly increasing the number of days open for harvest.

Our quality programme requires ongoing monitoring to ensure that the harvest criteria continue to meet the regulatory standards. In 2002/2003 industry's routine monitoring began to detect with increasing frequency bacteria levels that exceeded the regulatory standards for human consumption.

To ensure ongoing compliance and market access, the harvest criteria was tightened several times between 2003 and 2004. This reduced the time open for harvesting from 72 % of the time to only 50 %. This threatened the viability of shellfish production in the area.

Warning Signs, Results and Options

The sanitary survey for the area had identified all potential and actual pollution sources. In the absence of any definitive evidence as to the pollution source or sources we had to consider:

Domestic and municipal discharges – this was an area of relatively low human population and human impact on the growing area was considered minimal, even though the treatment facility at Collingwood was not always operating correctly.
Wild life – feral animals in the National Park above the coastal verges were considered to be of low risk. A nearby black swan colony was also discounted.
Recreational users – Golden Bay is geographically remote and boating pressure is confined to short duration fishing excursions. There are no major marinas in the area.
Agricultural run off and stream contamination – Land use in the impacting catchment is primarily dairy farming. Previous research had identified that cows with access to creeks can cause substantial water pollution as they are inclined to defaecate when they enter the water.

The consensus between scientists and regulators was that the contamination was most likely attributable to farm stock crossings in creek beds and estuaries plus stand off areas adjacent to streams where the bacteria are subsequently washed into the waterways. However, due to the other possibilities, the source of contamination could not be determined with certainty and certainly not to the standard that any legal or even political approaches would require.

Resistance to Tackling the Problem

Dairy farming is New Zealand's largest industry by far and is the mainstay of the New Zealand economy. Fonterra is the principal dairy company in the country and is one of the two largest in the world. As a result, it wields considerable influence but at the same time – through what is known as the "Fonterra Accord" – is working towards more sustainable and environmentally responsible practices. It is particularly sensitive to allegations of "dirty dairying".

The first issue to confront was that permits to discharge dairy effluent had been issued by the local council, without bacterial standards. In the absence of conclusive evidence that the dairy farms were indeed the source of the bacteria being detected at the marine farm sites, the council was extremely reluctant to initiate action against the dairy farms, who of course were also tax payers and voters. Understandably, the dairy farmers themselves were also reluctant to initiate actions costing $50,000–$100,000 per farm, still in the absence of conclusive proof.

Two possible courses of action were available to us. The first was to pursue legal remedies to force the Council to in turn force the farmers to take action. New Zealanders tend to view litigation as action of last resort and the marine farmers were already bogged down in litigation costing the better part of $2 million over issuance of consents to farm shellfish in the general area. Further more, there was the matter of the legally issued consents to discharge the effluent.

There was also the concern that this was all taking place in a small, quite isolated rural community where neighbour would be pitted against neighbour, friend against friend.

As a result, we were tasked to pursue a collaborative approach involving the marine and terrestrial farmers, Fonterra, and the Council. There was no guarantee of success, but the upside was that, in the event of failure, the marine farming sector would not have unduly impacted its relationships with the community whose goodwill it relies upon and there would have been no expensive legal bills. Cost would be limited to staff time and the time commitment of the working group. In fact, as things turned out, the positive benefits to the industry's reputation and relationships with neighbours exceeded even our most optimistic expectations.

The first stage was to contact and meet with key players. The Council was supportive but restricted in both financial and personnel resources. We met with the country's principal environmental NGO, the Fish and Game Council, and they willingly joined the initiative. The importance of that participation should never be underestimated, as environmental groups are often opposed to marine farming on principle. We then approached Fonterra's Head Office and discussed the problem. They too were supportive in principle.

In February 2005, MSQP organised a helicopter flight over the area for Fonterra's environmental manager, the NGO and myself. Viewing the dairy and marine farms from the air provided a perspective that none of us could have gained from ground level and clearly opened the door to further action.

Council and Fonterra Research and Results

The next step was to hold a public meeting. We presented the issues and explained the impacts on the viability of the marine farms as well as recreational users of the water space. We explained that faecal coliforms came only from the gut of warm blooded animals and the reasons why the bacteria in the marine environment was such an issue for us and the community. Fonterra presented their process for implementing the Fonterra Dairy Accord. The Council presented data from their environmental monitoring. The meeting, after some considerable discussion and finger pointing as to who could be responsible, appointed a Working Group consisting of representatives from dairying, the Council and the marine farming sector, including myself.

Seven farms located along the coastal strip between the Aorere River mouth and Puponga (and most likely to be the cause of contamination) were selected for an initial study to be funded jointly by Fonterra and the council. This was intended to provide a broad overview of what was happening. A subsequent survey would address the remaining farms in the catchment. The dairy farmers were suspicious that the marine farming sector would use the information against them but, although still suspicious, they were persuaded to participate upon assurances that the results would remain anonymous. They were quite justifiably irritated that they were being asked to address these issues while complying with their discharge consents.

The study's aim was to determine the loading of faecal bacteria discharged in selected creeks and identify the specific activities on each of the seven farms in the study. At each farm the onsite activities were assessed including:

- All dairy farm effluent discharges to land and water
- regular stock crossings
- the extent of effluent build up on raceways and access the potential for discharge to waterways
- areas of stream bank crumbling/stock access to streams
- feed/stand off pads and stock management practices
- land drainage works, which may affect water quality

The farm specific results were provided to each of the farmers. A generic report was presented to the working group and at Dairy industry field days. The stage one results were so definitive that further study was abandoned and principles to manage and reduce discharge to the water ways developed and quickly rolled out to all the farms.

Fonterra developed a "Check List to minimise contamination of water by effluent" and were very proactive in visiting farms and persuading the farmers. Farmers were installing weeping walls, fencing off and planting waterways. The latter saw the marine farming industry contributing several thousands of dollars to the voluntary plant nursery that propagated and donated the trees planted in the waterway margins. Peer pressure soon became a powerful tool applied to the laggards by those farmers who had made the commitment and investment.

Towards the end of this process, the Cawthron Institute in NZ were researching the use of Microbial Source Tracking with their US partners Monterey Bay Aquarium Research Institute. Microbial source tracking as described in Cornelisen et al. (2011) was carried out to identify the source of the faecal contamination. Even though still in the research phase, the methodology was used to confirm our assumption that the bacterial source was bovine. This provided the dairy farmers with the confidence that changing their management practices would have the sought after benefits.

Outcomes

Beyond the farm discharge cleanup, a cascade of improvements were set in train. The local Council tightened up on non-compliances. They installed a UV treatment plant at the Collingwood sewerage plant, and a system of notification to MSQP in the event of any accidental discharges was developed, implemented and steadfastly adhered to.

During the process we forged strong relationships with various groups – some that had been anti marine farming in the past. They came to appreciate that we all share similar environmental goals. This factor is especially important as additional water space comes into production in the Golden and Tasman Bay areas.

An independent dairy farm environmental advisor is now working with the farmers in the Aorere and has drawn up environmental plans. In addition, a system was developed whereby a dairy farmer could self report accidental discharges to Fonterra who in turn notified MSQP. Fonterra then followed up with the farmer to mitigate future discharges. MSQP could then determine if the discharge would impact the shellfish sanitation and implement a voluntary closure ahead of the harvest of any affected product.

Conclusions

The net result of the project has been a strengthened community with committed self management and policing of an effluent problem, while harvest criteria has now been restored to the previous level. Pursuing a legal option in the first instance could have been expected to take many years and cost literally millions of dollars while pitting neighbour against neighbour, industry against industry. This case study provides an example of a sustainable development approach and offers further evidence of the value and efficacy of collaboration between scientists, regulators and industry.

The harvest criteria was able to be revised, such that the time open for harvesting improved from 50 % at the height of the problem to 79 %, better than the original 72 %. As an industry, marine farming is very unusual in that it operates in an environment subject to pollution by a wide variety of causes, some of which may originate hundreds of kilometres away, out of sight and too easily out of mind.

As regulators, scientists and industry it is our goal to optimise food safety of our products for our consumers. A basic premise of quality assurance is prevention of "defects" rather than their post occurrence detection. That implies mitigating risks as far back in the production chain as possible. In this instance, we were able to trace the risk factor to source and mitigate it there. While we had to rely largely upon a process of deduction and elimination, the availability now of microbial source tracking provides additional science to support efforts to identify and mitigate pollution sources.

Although the case study relates to a dairy farming environment in a rural location, the model and methodology, with adaptation could be applied in a variety of situations by industry and regulators when confronted with anthropogenic contamination of growing waters.

Reference

Cornelisen CD, Gillespie PA, Kirs M, Young RG, Forrest RW, Barter PJ, Knight BR, Harwood VJ (2011) Motueka River plume facilitates transport of ruminant faecal contaminants into shellfish growing waters, Tasman Bay, New Zealand. N Z J Mar Freshw Res 45:477–495

Chapter 7
Regulatory Perspective in Translating Science into Policy: Challenges in Utilizing Risk Assessment for the Elaboration of Codex Standards of Shellfish Safety

Hajime Toyofuku

Introduction

The Codex Alimentarius Commission or Codex is an intergovernmental food standard setting organization. Its importance increased after the formal recognition of the Codex as the reference international organization for food safety standards in the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS agreement). The SPS agreement includes provisions that require the use of science, including risk assessment, in standard setting. The specific provisions are as follow:

Article 2.2 Members shall ensure that any sanitary and phytosanitary measure is based on scientific principles.

Article 5 Members shall ensure that their sanitary or phytosanitary measures are based on an assessment, as appropriate to the circumstances, of the risks to human, animal or plant life or health, taking into account risk assessment techniques developed by the relevant international organizations.

In the assessment of risks, Members shall take into account available scientific evidence; relevant processes and production methods; relevant inspection, sampling and testing methods; prevalence of specific diseases or pests; existence of pest- or disease-free areas; relevant ecological and environmental conditions; and quarantine or other treatment (World Trade Organization 1995).

Codex strengthens its policies and operational practices with respect to the use of science in setting standards. In 1995, the Codex established a set of four

H. Toyofuku (✉)
Department of International Health and Collaboration, National Institute of Public Health, 2-3-6-Minami, Wako, Saitama 351-0197, Japan
e-mail: toyofuku@niph.go.jp

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_7,

statements of principles concerning the role of science in the Codex decision-making process and the extent to which other factors are taken into account (Codex 2011a). They are:

1. The food standards, guidelines and other recommendations of Codex Alimentarius shall be based on the principle of sound scientific analysis and evidence, involving a thorough review of all relevant information, in order that the standards assure the quality and safety of the food supply.
2. When elaborating and deciding upon food standards Codex Alimentarius will have regard, where appropriate, to other legitimate factors relevant for the health protection of consumers and for the promotion of fair practices in food trade.
3. In this regard it is noted that food labelling plays an important role in furthering both of these objectives.
4. When the situation arises that members of Codex agree on the necessary level of protection of public health but hold differing views about other considerations, members may abstain from acceptance of the relevant standard without necessarily preventing the decision by Codex.

Further the Codex (2011b) established the following "Statements of principle relating to the role of food safety risk assessment":

1. Health and safety aspects of Codex decisions and recommendations should be based on a risk assessment, as appropriate to the circumstances.
2. Food safety risk assessment should be soundly based on science, should incorporate the four steps of the risk assessment process, and should be documented in a transparent manner.
3. There should be a functional separation of risk assessment and risk management, while recognizing that some interactions are essential for a pragmatic approach.
4. Risk assessment should use available quantitative information to the greatest extent possible and risk characterizations should be presented in a readily understandable and useful form.

At the request of Codex, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) undertook a series of joint expert consultations to elucidate the basic principles of risk analysis between 1995 and 1998.

For over 50 years, FAO and WHO have been the international sources of scientific advice on matters related to chemical food safety. FAO/WHO scientific advice provides the basis for food standards, guidelines and codes of practice developed by the FAO/WHO Codex Alimentarius Commission (World Health Organization 2011).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is an international expert scientific committee that is administered jointly by FAO and WHO. It has been meeting since 1956, initially to evaluate the safety of food additives. Since 1999, and at the request of the CAC, FAO and WHO have initiated

a series of joint expert consultations to assess risk associated with microbiological contamination of foods (JEMRA). For hazards which are not covered by the standing risk assessment body, such as biotoxins, biotechnology, etc., ad hoc expert consultations were convened (FAO/WHO 2003).

In short, Codex is an international risk management body, while the FAO and WHO provide scientific advice and risk assessments to Codex and its member countries. Within the Codex system, committees prepare draft standards for submission to the Commission. There are two kinds of committees, (i) so called general subject committees whose work has relevance for all Commodity Committees and applies across the board to all commodity standards and (ii) so called Commodity Committees which develop standards for specific foods or classes of food. One of the examples of general subject committees is the Codex Committee on Food Hygiene (CCFH) which provides concepts and principles of food hygiene and microbiological food safety in general, or specific foods or groups of foods; and endorse or reviews relevant provisions in Codex commodity standards. One of the examples of Commodity Committees is the Codex Committee on Fish and Fishery Products (CCFFP). Provisions on food hygiene in fish related standards prepared by CCFFP are reviewed and endorsed by the CCFH.

Purpose

This paper reviews the process of risk analysis, mainly to see how Codex subsidiary bodies use outputs from risk assessment bodies to produce Codex documents, and identifies good practices, as well as areas needing improvement.

Method

To achieve the purpose mentioned above, three examples were selected: (1) marine biotoxins in a standard bivalve mollusc, (2) *Salmonella* in bivalve molluscs, and (3) *Vibrio parahaemolyticus* in seafood. Each case was reviewed in the following manner: (1) risk assessment questions posed, (2) outcomes from the risk assessment bodies analysed, and (3) how the Codex subsidiary bodies understood, interpreted and utilized the outcomes of risk assessments for elaborating the Codex documents, by reviewing reports of the sessions of Codex subsidiary bodies, particularly CCFH and CCFFP. These examples were selected because: (i) they are related to shellfish safety, (ii) CCFFP and CCFH requested scientific advice/risk assessments from the FAO/WHO, (iii) may indicate some problems/challenges and suggest further improvements.

Results

Marine Biotoxins in Bivalve Molluscs

Marine biotoxins includes poisonous substances naturally present in fish and fishery products or accumulated by the animals feeding on toxin-producing algae, or in water containing toxins produced by such organisms. At the 25th session of the CCFFP in 2002, the CCFFP asked FAO and WHO to provide scientific advice on marine biotoxins in conjunction with its work on the Proposed Draft Standard for Live and Processed Bivalve Molluscs (Codex 2002), and at its 26th session in 2003, CCFFP made the following more specific requests:

- Provide scientific advice to the CCFFP to enable the establishment of maximum levels in shellfish for toxins (paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), poisoning caused by azaspiracid (AZP), neurotoxic shellfish poisoning (NSP)-toxins, and yessotoxins and pectenotoxins);
- Provide guidance on methods of analysis for each toxin group;
- Provide guidance on monitoring of biotoxin-forming phytoplankton and bivalve molluscs (including sampling methodology);
- Provide information on geographical distribution of biotoxin-forming marine phytoplankton (Codex 2003).

The FAO, WHO and the Intergovernmental Oceanographic Commission of UNESCO (IOC) held a Joint *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs in Oslo, Norway in 2004, which generated a report that addressed the aforementioned requests. The report considers all available data, mainly derived from published and validated studies. Structured marine biotoxin risk assessments (based on prescribed methods) were conducted and were included in the report, along with guidance on methodology. The conclusions are summarized in Table 7.1 and should be reconsidered when further published findings become available (Toyofuku 2006).

At the 27th session of the CCFFP in 2005 Codex (2005), the CCFFP agreed to establish a physical Working Group (pWG) that would work between the sessions to examine the report from the Joint FAO/WHO/IOC *ad hoc* Expert Consultation on Biotoxins in Bivalve Molluscs and prepare a discussion paper for consideration by the CCFFP with the following terms of reference:

- Assess how the CCFFP might use the expert advice and make recommendations with respect to approaches that the CCFFP could consider to integrating the advice into the Proposed Draft Standard for Live and [Raw] Molluscs and the section of the Code on Live and [Raw] Bivalve Molluscs;
- Identify new questions that the CCFFP may wish to pose to FAO/WHO;
- Identify areas in the report that may need further clarification;

Table 7.1 Summary data used in the derivation of the ARfD, as well as derived and current guidance levels, and Codex maximum level in the Standard for live and raw bivalve molluscs (Codex Stan 292-2008)

	LOAEL (μg/kg bw)	Safety factor	Provisional ARfD	Derived guidance level (mg/kg Shellfish Meat)	Level implemented in some countries in 2004 (mg/kg Shellfish Meat)	Codex maximum level/ kg of mollusc flesh
Azaspiracid	0.4	10 (human data)	0.04 μg/kg 2.4 μg/adult	0.024 (1) 0.0096 (2) 0.0063 (3)	0.16	≤0.16 mg
Brevetoxin	NA	NA	NA	NA	0.8 mg/kg SM as PbTx-2	≤200 mouse units or equivalent
Domoic acid	1,000	10 (human)	100 μg/kg 6 mg/adult	60 mg/kg SM (1) 24 mg/kg SM (2) 16 mg/kg SM(3)	20 mg/kg SM	≤20 mg domoic acid
Okadaic Acid	1	3 (human)	0.7 μg/kg 42 μg/adult	0.2 mg/kg SM (1) 0.08 mg/kg SM (2) 0.05 mg/kg SM (3)	0.16 mg/kg SM	≤0.16 mg of okadaic equivalent
Saxitoxin	2	3 (human)	0.33 μg/kg 21 μg/adult	0.42 mg/kg SM (1) 0.17 mg/kg SM (2) 0.11 mg/kg SM (3)	0.8 mg/kg SM	≤0.8 mg (2 HCL) of saxitoxin equivalent

In the Derived guidance level (mg/kg Shellfish Meat) column, (1), (2) and (3) level were derived based on the assumption of consumption of 100, 250, and 380 g molluscs per one meal, respectively

- As appropriate, make recommendations on the validation of methodology (e.g. such as identifying other international organisations that are working in this area);
- As appropriate, make recommendations on possible changes to the Proposed Draft Standard for Live and [Raw] Molluscs and the section of the Code on Live and [Raw] Bivalve Molluscs arising from the expert advice and other issues arising from the deliberations of the pWG.

The FAO/IOC/WHO expert consultation utilized existing chemical, single exposure based risk assessment approach, which is similar to risk assessments of pesticide residues, and they tried to establish a provisional acute reference dose (ARfD). Table 7.1 shows the outcomes of risk assessments, regulatory levels implemented in some countries in 2004, and established Codex maximum levels of each toxin per kg of mollusc flesh.

With regards to azaspiracid (AZA), the expert consultation established a provisional ARfD of 0.4 μg/kg body weight (bw), based on the Lowest Observed Adverse Effect Level (LOAEL) of 23 μg per person in humans and a bw of 60 kg, using a tenfold safety factor because of the small number of people involved. Insufficient data on the chronic effects of AZA prevented the establishment of a Tolerable Daily Intake (TDI). As shown in Table 7.2, the consumption of 100, 250 or 380 g shellfish meat by adults would result in a derived guidance level of 0.0096 mg/kg shellfish meat (SM) and 0.0063 mg/kg SM respectively.

At the pWG in Canada in 2006, the expert consultation report was reviewed. Given the data available, the existing history of regulatory programs and the level of consumer protection provided by those programs, the pWG agreed that the action level of 0.16 mg/kg implemented in 2001 in Europe, New Zealand and Norway should be maintained (Codex 2006b).

According to the pWG report, the basis of the European action level was based on a risk assessment carried out by the Food Safety Authority of Ireland, which suggested a regulatory limit of 0.12 mg/kg following the first recorded outbreak of food poisoning linked to AZAs in 1995. However, the sensitivity of the mouse bioassay was insufficient to detect the toxin at this level. It was subsequently determined that the mouse bioassay threshold for detecting AZA was 0.16 mg/kg. Consequently, the regulatory limit for this toxin group was set at this level. Finally the WG recommended that the Codex standard (section 1.5) should identify an action level for AZA of 0.16 mg/kg (Codex 2006b).

During the discussion at the 28th session of the CCFFP, a Reevaluation of AZA was requested from FAO/WHO because there was a large difference between the guidance level for AZAs recommended by the Expert Consultation and the limit in the Proposed Draft Standard (Codex 2006a).

With regards to the brevetoxin group, based on a reported incident in humans with a 60-kg body weight who consumed an estimated 100–150 g shellfish at 120 μg PbTx-3 equivalents/100 g, an exposure of 2–3 μg PbTx-3 equivalents/kg bw was estimated. However, uncertainty existed in the accuracy of this dose estimate because of a possible underestimation of the toxin levels actually present in shellfish, and because the metabolites were not reliably extracted by the method used for

regulatory monitoring. The WG decided that the data were insufficient to complete the risk assessment. The relevant brevetoxins and their metabolites need to be identified and estimates of their oral potencies are needed before an ARfD can be established (Toyofuku 2006).

At the pWG, the pWG concurred with the WG's decision that there was currently insufficient evidence to complete the risk assessment of brevetoxins. However, despite the WG's decision regarding the available evidence for a risk assessment, the WG recognized the body of knowledge resulting from the existing history of regulatory programs (e.g. in the US, Mexico and New Zealand) and the absence of human illness in commercially harvested shellfish, where these programs are implemented. Finally the WG recommended (Codex 2006b) that the Codex standard identified an action level for the brevetoxins of 20 Mouse Units or equivalent (conditional on the equivalence information becoming available). During the discussion at 28th CCFFP, a request was made to FAO/WHO to re-evaluate brevetoxins because there was no brevetoxin limit recommended by the WG (Codex 2006a).

Regarding domoic acid (DA), the results of the first outbreak of amnesic shellfish poisoning that occurred in 1987 in Canada provide the best basis for developing an acute reference dose (tolerable single day intake, acute TDI). During this outbreak, a dose- related increase in the severity of signs and symptoms was observed in patients consuming between 1 mg/kg bw (the LOAEL) and 5 mg/kg bw. Studies in rodents and cynomolgous monkeys have generally supported these findings. To cover the full spectrum of human susceptibility, and account for the fact that 1 mg/kg bw was a LOAEL, this value was divided by a safety factor of 10, to derive a provisional ARfD of 0.1 mg/kg bw. This value seems reasonable, as one person who consumed 0.33 mg/kg bw did not become ill. The provisional ARfD of 0.1 mg/kg bw provided the basis for the establishment of the maximum residue limit (MRL) for DA by Canadian authorities, which on the basis of an intake of 250 g shellfish and a body weight of 60 kg, was 24, rounded down to 20 μg DA/g shellfish. If instead of 250 g shellfish, a value of 300 g shellfish was used, the MRL would be exactly 20 μg DA/g shellfish.

Very few animal studies have been conducted on the subchronic and chronic toxicity of DA, and these limited data suggest that cumulative effects of low doses of DA are unlikely. In this regard, studies based on subacute mouse studies revealed no differences in behavioral toxicity scores upon re-exposure to DA compared to a single dose (i.e., behavioral equivalent of kindling). The available data indicate that chronic sequelae, such as epilepsy and memory deficit, were observed only in those patients who had suffered severe acute neurological effects (examined up to 3.5 years post-event) after they had ingested a single high dose of DA. It is therefore unlikely that people who habitually consume small amounts of DA (exposures less than 0.1 mg DA/kg bw) would experience any chronic effects. Thus, this ARfD also may be considered a provisional chronic TDI. As shown in Table 7.1, the consumption of 100, 250 or 380 g shellfish meat by adults would lead to a derived guidance level of 60, 24 or 16 mg DA/kg shellfish meat, respectively (Toyofuku 2006). The pWG noted that the action levels derived in the report support the current

level identified in the draft Codex Standard (20 mg/kg), and the WG agreed that the level of 20 mg/kg is appropriate (Codex 2006b). No further discussion was noted in the report of the 28th CCFFP (Codex 2006a).

With regards to the okadaic acid (OA) group of toxins, OA and dinophysistoxins (DTXs) possess tumour-promoting activity; OA also possesses genotoxic and immunotoxic activity. These effects raise questions as to the human health risks of (sub)-chronic exposure to low levels of these compounds. A pressing problem was the lack of sufficient quantities of purified toxins to perform sub-chronic animal toxicity studies. The pWG determined that no TDI could be established because of insufficient data on the chronic effects of OA, and established a provisional ARfD of 0.33 μg OA equ/kg bw, based on the LOAEL of 1.0 μg OA/kg bw. A safety factor of 3 was chosen because of documented human cases involving more than 40 people and because DSP symptoms are readily reversible. The consumption of 100, 250 or 380 g shellfish meat by adults would lead to a derived guidance level of 0.2, 0.08 or 0.05 mg OA equivalent/kg shellfish meat, respectively (Toyofuku 2006). The pWG discussed the action levels used in various countries and the level of consumer protection which they have provided to date. The current standard, its practical application and demonstrated results indicate that the level of 0.16 mg/kg provides adequate protection for consumers (Codex 2006b).

With regards to the saxitoxin group (STX) of toxins, the pWG established a provisional ARfD of 0.7 μg STX equivalents/kg bw, based on an LOAEL of 2 μg STX equ/kg bw. A safety factor of 3 was chosen because documented human cases included a wide spectrum of people (occupation, age, and sex) and mild illness is readily reversible. The Expert Consultation determined no TDI could be established because of insufficient data on the chronic effects of STX. As shown in Table 7.1, the consumption of 100, 250 or 380 g shellfish meat by adults would lead to a derived guidance level of 0.42, 0.17 or 0.11 mg STX equ/kg, respectively (Toyofuku 2006). The pWG considered that the long-standing enforced tolerance limit of 0.8 mg/kg STX.2HCl equiv., established for consumer protection, was considered to be successful (over nearly 50 years), with no human illnesses from commercially harvested product (Codex 2006b).

The CCFFP during the 28th session agreed to advance the Proposed Draft Standard for Live and Raw Bivalve Molluscs to Step 5 for adoption by the 30th Session of the Commission. The sections on hygiene (including biotoxin level) would be sent to the CCFH for endorsement. At the 38th session of the CCFH, the CCFH was of the opinion that these provisions on marine biotoxins should be considered under the section on contaminants in the draft Standard and that consideration of these issues were outside the competence of the CCFH. The CCFH was of the view that the matter of marine biotoxins should be sent to the Codex Committee on Contaminants in Food (CCCF) for their advice and endorsement, if necessary (Codex 2007a). At the 2nd session of the CCCF in 2008, the representative of WHO noted that the levels proposed by the CCFFP were different to those proposed by the expert consultation and expressed concern that the current proposed levels might exceed those of the ARfD for several marine biotoxins at normal consumption levels. The WHO representative also expressed

the view that further review of the proposed levels would be necessary, but that data may currently be limited. Some delegations were of the view that it would be difficult to endorse the levels without some understanding on how the levels had been reached in the CCFFP and supported the concern of the WHO with regard to potentially high exposure to biotoxins. Because the Expert Consultation had been unable to complete a risk assessment on brevetoxins due to the lack of sufficient data, one delegate requested clarification on how the CCFFP had set a level for this biotoxin. In reply, it was clarified that the CCFFP had agreed to the level of 200 mouse units/kg in view of the knowledge resulting from the existing history of regulatory programmes and the absence of human illness in commercially harvested shellfish where these programmes were implemented. After some discussion the CCCF agreed to provisionally endorse the proposed levels, with the recommendation that the levels would require complete review in the coming few years with a view to revising these levels where necessary, when more data became available (Codex 2008a).

Salmonella *in Bivalve Molluscs*

The CCFFP, at the 29th session, discussed the sampling plans for *Salmonella* in the draft Standard for Live and Raw Bivalve Molluscs. It was pointed out that the criteria proposed for *Salmonella* was not based on a risk assessment as required in the *Principles for the Establishment and Application on Microbiological Criteria for Foods* and general requirements for Codex food safety standards. As a result, it was proposed either to ask for a specific risk assessment in order to justify the use of these criteria, or transfer to the Code of Practice. It was also pointed out that when testing for *Salmonella* in the areas where the occurrence was known to be high, the number of samples should be much higher (50 or 60) but that for routine testing five samples could be used. A proposal was made to prepare a table with the different sampling levels that could be used according to the prevalence of *Salmonella*. The CCFFP agreed that the number of samples would depend on the incidence of *Salmonella* but recognized that it was not possible to develop such a table at this stage. After some further discussion, the CCFFP agreed to retain the text referring to five samples, as mentioned above, and to ask for scientific advice from FAO and WHO on the following question:

> In the context of harvesting area monitoring for faecal contamination and lot contamination, estimate the risk mitigation for *Salmonella* in bivalve molluscs when different sampling plans and microbiological criteria are applied (Codex 2008b).

At the 30th session of the CCFFP, the Representative of FAO informed the CCFFP that the estimation of risk mitigation required risk assessment and, since there were currently no national or international risk assessments available for *Salmonella* in bivalve molluscs, the FAO/WHO had requested Codex members to provide data on sampling plans and *Salmonella* detection from their shellfish

harvesting area monitoring programmes and epidemiological data linking outbreaks of *Salmonella* to bivalve molluscs. The data showed that most countries do not monitor shellfish harvesting areas for *Salmonella*, but rely on monitoring faecal contamination using indicator bacteria in shellfish meat or surrounding water.

Epidemiological data showed that bivalves have rarely been involved in outbreaks of salmonellosis, suggesting that there was no particular public health problem associated with *Salmonella* in bivalve molluscs. The FAO Representative indicated that most studies on *Salmonella* in bivalve harvesting areas have used single samples (n = 1) and there were very limited data using multiple samples and, therefore, data are inadequate for the evaluation of sampling plans. The CCFFP was further informed that the International Commission on Microbiological Specifications for Foods (ICMSF) did not recommend microbiological criteria for *Salmonella* in bivalve molluscs and that the Codex Guidelines on Sampling (CAC/GL 50-2004) recommends sampling plan classification according to the nature of the problem. Relating epidemiological data to these guidelines would suggest continuation of currently recommended (n = 5; c = 0, m = non dateable in 25 g) sampling plan, when there is a need for testing for *Salmonella*. In this two class sampling plan, n means the number of sampling units to be drawn independently and randomly from the lot, c means the maximum allowable number of sample units that yield unsatisfactory test results, and m separates good quality from non-acceptable or defective quality. Therefore, the current two-class sampling plan in the *Standard for Live and Raw Bivalve Molluscs* need not be changed.

Then a question was raised whether there was a need for criteria for *Salmonella* in the Standard, especially taking into account the guidance for the development of criteria given in the *Principles for the Establishment and Application of Microbiological Criteria for Food* (CAC/GL 21-1997) which stated that criteria should be developed only when there was a need for such criteria and that such criteria are meaningful for consumer protection.

The CCFFP agreed to request that FAO/WHO undertake a risk assessment to determine whether there was a significant public health risk of exposure to *Salmonella* associated with consumption of bivalves. FAO/WHO were also asked to evaluate whether criteria for *Salmonella* are meaningful in ensuring adequate consumer health protection. FAO/WHO agreed to retain the current criteria for *Salmonella* and the associated sampling plan as their recommended advice until the result of this assessment became available (Codex 2009).

At the 31st session of the CCFFP, the FAO representative presented the work done by FAO/WHO through an electronic Expert Group. The latter noted that the current Code of Practice for Fish and Fishery Products (CAC/RCP 52-2003) recommends control of harvesting areas by monitoring faecal coliforms and *E. coli* and does not recommend pathogen testing for routine monitoring of harvesting waters. Available data on the prevalence of *Salmonella* from bivalves indicate differences in levels of prevalence in areas which are controlled by monitoring faecal indicator bacteria (1 % prevalence in areas where bivalves go directly to

Table 7.2 The number of samples required, for a given level of confidence, that the frequency of contamination in a lot is below the specified level

	Confidence required that the result is correct				
	50 %	90 %	95 %	99.9 %	99.9 %
Acceptable proportion of contaminated samples	*Number of samples that must be tested:*				
≤1 in 10	7	22	29	44	66
≤1 in 100	69	229	299	459	688
≤1in 1,000	693	2,301	2,995	4,607	6,906
≤1 in 10,000	6,932	23,025	29,957	46,050	69,080
≤1 in one million	693,148	2,302,594	2,995,750	4,605,202	6,908,723

Adapted from (Codex 2011b)

market and 2–15 %, depending on geographical location and season, in areas where bivalves go for a purification step before marketing). Studies at market level show a *Salmonella* prevalence of <1–3.4 %. Diverse serovars have been observed at a few locations where *Salmonella* in bivalves was investigated. Many of the serovars were not commonly found in human outbreaks. Lack of quantitative data on levels of *Salmonella* in contaminated molluscs, and data on human consumption, such as serving size and the proportion of the population consuming live bivalves, limits the ability to make any realistic exposure assessment.

Epidemiological data indicates that outbreaks of salmonellosis associated with live bivalve molluscs are very rare and, even considering the underreporting factor in some countries, the current model over-estimates the risk. The work of the expert group on the performance of a sampling plan indicates that to detect *Salmonella* at a 1 % level of prevalence (seen in areas controlled by faecal indicator bacterial monitoring) with 95 % confidence level, 299 samples need to be tested. Testing of 60 samples would be able to detect only 45 % of a contaminated batch. Although testing of a lower number of samples may be adequate for areas with higher prevalence, molluscs from these areas would have higher levels of indicator bacteria and are unlikely to reach market without purification with the current practices. Thus, the present sampling plan would have little value in public health protection. Table 7.2 shows the number of samples required, when all samples are negative, to be able to detect different frequencies of contamination and for particular levels of confidence. (Codex 2011b).

Based on these findings, the CCFFP discussed whether or not to retain the current criteria for *Salmonella* in the Standard for Live and Raw Bivalve Molluscs, concluding that it might be necessary to remove the criteria for *Salmonella* from the Standard. It was agreed to discuss this issue further at the next session pending the availability of the final report. The CCFFP also agreed to request the CCFH to provide their advice on whether the criteria for *Salmonella* should be retained in the Standard for Live and Raw Bivalve Molluscs based on the final report of the Expert Group (Codex 2011a, c).

At the 43rd session of the CCFH, the CCFH considered this issue and again discussed whether the criteria for *Salmonella* should be retained in the Standard. Some delegations were of the view that the criteria should be removed from the Standard as it was inconsistent with the *Principles for the Establishment and Application of Microbiological Criteria for Food* (CAC/GL 21-1997) and that the criterion provided little or no added protection for salmonellosis, above that achieved by risk management strategies, such as sanitary surveys and faecal indicator monitoring. Other delegations were of the opinion that the criterion should be retained, as it was widely used in their jurisdiction, especially due to the high consumption of live and raw bivalve molluscs which were not submitted to any treatment to reduce the level of *Salmonella* contamination. The CCFH agreed to a proposal, which provided a level of flexibility to the application of the criterion by indicating that it could be implemented by competent authorities taking into account the epidemiological situation and based on environmental monitoring as well as other surveillance. Noting that this type of provision was more appropriate to a code of practice, the Committee agreed to recommend to the Committee on Fish and Fishery Products (CCFFP) to remove the criterion for *Salmonella* (Section I-6.5) from the Standard for Live and Raw Bivalve Molluscs (CODEX STAN 292-2008) and to include in the Code of Practice for Fish and Fishery Products (CAC/RCP 52-2003), Section 7.2.2.2, the following: "When appropriate, taking into account the epidemiological situation as indicated by the results of environmental monitoring and/or other surveillance, the competent authority may decide to implement a criterion for *Salmonella*." (Codex 2011d).

Vibrio parahaemolyticus *in Seafood*

At the 38th session of the CCFH, the CCFH agreed to request FAO and WHO to use the risk assessment on *Vibrio parahaemolyticus* in seafood, which they are developing to provide scientific guidance to the Codex Committee on Fish and Fishery Products, to follow up on the recommendations of the CCFH regarding the hygiene provisions in the Proposed Draft Standard for Live and Raw Bivalve Molluscs. The following risk management question is proposed:

- Estimate the risk reduction from *V. parahaemolyticus* when the total number of *V. parahaemolyticus* or the number of pathogenic *V. parahaemolyticus*, ranges from absence in 25 g to 1,000 cfu or MPN per gram (Codex 2007a).

At the 39th session of the CCFH, FAO and WHO presented the work which considered the impact of three different limits for *V. parahaemolyticus*: 100, 1,000 and 10,000 cfu/g. These limits were considered to be applied when the products were cooled after harvesting, or when the population of *V. parahaemolyticus* had stabilised (i.e., when the temperature becomes too low for further growth but not so low that die-off occurs).

Table 7.3 Reduction in illness, based on meeting specified target numbers of *V. parahaemolyticus*, together with commensurate rejection of product for raw consumption

	Reduction (%) in the number of predicted illnesses			Product (%) rejected to achieve these reductions in illness		
Specified target	Australia (summer)	New Zealand (summer)	Japan (autumn)	Australia (summer)	New Zealand (summer)	Japan (autumn)
100 cfu/g	99	96	99	67	53	16
1,000 cfu/g	87	66	97	21	10	5
10,000 cfu/g	52	20	90	2	1	1

Adapted from (Codex 2007b)

An estimation of the risk reduction associated with the implementation of such levels was developed based on information from three countries, Australia, New Zealand and Japan. However, where the appropriate data was not available, surrogate data from the US was used. The estimation is based on the assumption that all (100 %) harvested oysters meet a specified target limit compared with the baseline distribution of *V. parahaemolyticus* for each of these countries. The results presented include an estimation of both the reduction in human illness and the amount of product rejection that would occur if all market products were to meet the specified target (Codex 2007b).

The impact of three different limits for *V. parahaemolyticus*: 100, 1,000 and 10,000 cfu/g was evaluated by risk assessment methods. These limits were considered to be applied when the products were cooled after harvesting, when the population of *V. parahaemolyticus* had stabilized i.e. when the temperature became too low for further growth but not so low that die-off occurred. An estimation of the risk reduction associated with the implementation of such levels was developed, based on information from three countries, Australia, New Zealand and Japan. The variation in risk reduction and product rejection for each of the countries highlights the relationship between the specific target and baseline levels of *V. parahaemolyticus* in oysters of a particular country, and emphasizes the fact that the establishment of international limit for *V. parahaemolyticus* may have greater impact on product rejection in some countries. For example, the limit of 100 cfu/g implies rejection of 67 % of Australian oysters for consumption as raw product, but would have much less impact on Japanese oysters, while reduction percentages in the number of predicted illness remain the same. On the other hand, the limit of 10,000 cfu/g implies rejection of only 2 and 1 % of Australian and Japanese oysters, respectively, whilst the reduction in the number of predictive illnesses are 52 and 90 %, respectively.

As a result, both Guidelines on the Application of General Principles of Food Hygiene to the Control of *Vibrio* species in seafood, and Standard for Live and Raw Bivalve Molluscs contained no Microbiological Criteria (MC) on pathogenic *Vibrio* species in seafood.

Discussion and Conclusions

For biotoxins the CCFFP should use scientific methods to make risk management decisions and document the reasons why the CCFFP did not accept recommendations from the expert consultation group. Risk management decision should be based on science and other legitimate factors, if relevant. From the *Salmonella* example, the need for risk assessment was highlighted. Some hygienic provisions in the Standards developed by CCFFP need scientific information for decision making. So CCFFP as a risk manager should ask timely and cogent risk management questions, understand the outcomes of risk assessments together with associated uncertainties, and make risk management decisions in a transparent manner.

The *Vibrio* example might be considered as a good example of utilizing the risk analysis framework. CCFH asked a precise risk management question to FAO/WHO, and FAO/WHO replied to the question in a timely manner. As a result, CCFH made a decision which was not to establish microbiological criteria on pathogenic *Vibrio* spp. in seafood.

From these examples, the following policy lessons were drawn:

- Risk management should be based on the results of risk assessments, and decisions on risk management should be clearly documented.
- Risk assessment needs data, resources, expertise, time, etc. Sometimes large data gaps prevent risk assessments; however, even in those cases, risk managers need to make a decision. Therefore, continuous interaction between risk assessment and risk management is needed from the beginning to the end of the risk analysis process, in order to better use risk assessments in the decision making process.
- The risk analysis process should be well planned, coordinated, transparent, and documented.
- In converting the outcomes of risk assessments into mitigations, clear documentation of the process and the reasons for selecting an option are needed.
- Risk managers should be encouraged to understand risk assessment.
- The biggest concern is that risk managers use a hazard based risk management option because of the history of successful implementation of such an option. However, this does not guarantee future successes, because of possible lower sensitivities in previous surveillance systems and/or underreporting factors.
- As indicated in the Criteria for *Salmonella* in bivalve molluscs, some hazard based/microbiological criteria might not be meaningful depending on the prevalence of *Salmonella*. Establishing and implementing MC is a risk management tool; however, such MC should be based on sound science and the MC must protect public health of the consumers.

References

Codex (2002) Report of the twenty fifth session of the Codex Committee on Fish and Fishery Products. Alesund, Norway, 3–7 June 2002 (Alinorm 02/18)

Codex (2003) Report of the twenty sixth session of the Codex Committee on Fish and Fishery Products. Alesund, Norway, 13–17 October 2003

Codex (2005) Report of the twenty seventh session of the Codex Committee on Fish and Fishery Products. Cape Town, South Africa, 28 February–4 March 2005

Codex (2006a) Report of the twenty eighth session of the Codex Committee on Fish and Fishery Products. Beijing, China, 18–22 September 2006 (Alinorm 07/30/18)

Codex (2006b) Report of the working group meeting to assess the advice from the joint FAO/WHO/IOC *AD HOC* Expert Consultation on Biotoxins in Bivalve Molluscs, (CX/FFP 06/28/6-Add.1)

Codex (2007a) Report of the thirty eighth session of the Codex Committee on Food Hygiene. Houston, USA, 4–9 December 2006 (ALINORM 07/30/13)

Codex (2007b) Progress report on the joint FAO/WHO Expert Meeting on microbiological risk assessment (JEMRA) and related matters (CX/FH 06/39/3)

Codex (2008a) Report of the 2nd session of the Codex Committee on Contaminants in Food. The Hague, the Netherlands, 31 March–4 April 2008 (Alinorm 08/31/41)

Codex (2008b) Report of the twenty ninth session of the Codex Committee on Fish and Fishery Products. Trenheim, Norway, 18–23 February 2008 (Alinorm 08/31/18)

Codex (2009) Report of the thirties session of the Codex Committee on Fish and Fishery Products. Agadir, Morocco, 28 September–2 October 2009 (Alinorm 10/33/18)

Codex (2011a) Statements of principle concerning the role of science in the Codex decision making process and the extent to which other factors are taken into account, 209. Codex Procedural Manual 20th edition available from ftp://ftp.fao.org/codex/Publications/ProcManuals/Manual_19e.pdf

Codex (2011b) Interim Report of the electronic expert group on *Salmonella* in bivalve molluscs (CCFFP/31 CRD 12)

Codex (2011c) Report of the thirty first session of the Codex Committee on Fish and Fishery Products. Tremso, Norway, 11–16 April 2011 (REP 11/FFP)

Codex (2011d) Report of the forty third session of the Codex Committee on Food Hygiene. Miami, Florida, USA, 5–9 December 2011 (REP 12/FH)

FAO/WHO (2003) Assuring food safety and quality: guidelines for strengthening national food control systems, ANNEX 7. Introducing JECFA, JMPR, JEMRA and GM Food Risk Assessment, 58–62. Available from http://www.who.int/foodsafety/publications/capacity/en/Englsih_Guidelines_Food_control.pdf

Toyofuku H (2006) Joint FAO/WHO/IOC activities to provide scientific advice on marine biotoxins (research report). Mar Pollut Bull 52:1735–1745

World Health Organization (2011) Establishment of the Global Initiative for Food-related Scientific Advice (GIFSA) from http://www.who.int/foodsafety/codex/Gifsa.pdf

World Trade Organization (1995) Sanitary and phytosanitary agreement from http://www.wto.org/english/docs_e/legal_e/15-sps.pdf

Chapter 8
Defining a Sampling Strategy for Okadaic Acid (OA) Toxins in Shellfish to Determine the Human Health Status of a Growing Area

Nathalie Wesolek, François-Gilles Carpentier, Dominique Parent-Massin, and Alain-Claude Roudot

Introduction

Okadaic acid (OA) and its analogues, the dinophysis toxins (DTX-1, DTX-2, and DTX-3), form together the group of OA-toxins. They are produced by toxic dinoflagellates, which are part of the phytoplankton. As bivalve shellfish feed on phytoplankton, they become contaminated by the presence of these toxic dinoflagellates in seawater.

OA-group toxins cause Diarrhetic Shellfish Poisoning (DSP) in humans, which cause dose-dependent symptoms, including diarrhoea, abdominal cramps and sometimes nausea and vomiting. This illness is not fatal and recovery occurs within 3 days, with or without medical treatment. However, OA and DTX-1 have been shown to be potent tumour promoters, and considering that stomach, small intestine, and colon contain binding sites for OA, they could be implicated in the worldwide increase of gastrointestinal tumors (Suganua et al. 1988; Fujiki et al. 1988). DSP human poisoning is frequently confused with the illness caused by

N. Wesolek (✉) • F.-G. Carpentier • D. Parent-Massin • A.-C. Roudot
Food and cellular toxicology laboratory, Université de Bretagne Occidentale (UBO), Brest, France
e-mail: nathalie.wesolek@univ-brest.fr; Carpentier@univ-brest.fr;
dominique.parent-massin@univ-brest.fr; alain-claude.roudot@univ-brest.fr

D. Parent-Massin
Laboratoire de Toxicologie Alimentaire et Cellulaire (EA 3880), Université Européenne de Bretagne – Université de Bretagne Occidentale (UEB-UBO), 6 Av. Victor Le Gorgeu – CS93837, 29238 Brest, Cedex 3, France
e-mail: dominique.parent-massin@univ-brest.fr

A.-C. Roudot
Laboratoire de Toxicologie Alimentaire et Cellulaire (EA 3880),
UFR Sciences et Techniques, Université de Bretagne Occidentale,
6 Av. Victor Le Gorgeu – CS93837, 29238 Brest, Cedex 3, France
e-mail: alain-claude.roudot@univ-brest.fr

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_8,

the enterophathogens: *Vibrio parahaemoliticus* or *Bacillus cereus*, which are both routinely found in bivalve molluscs. Misdiagnosis is due to the fact that DSP toxins and enteropathogens have similar symptomatologies. Thus there is no accurate information linked to the annual DSP human poisoning episodes (Gestal-Otero 2000). However, some poisoning events have been reported in detail. Thus in France, in 1984 and 1985, cultured mussels caused DSP-like symptoms in 10,000 and 2,000 people respectively (Durborow 1999). Because of the extent of the problem, appropriate sampling plans are required in order to monitor bivalve production areas to check for the presence of these biotoxins, knowing that a species with the highest contamination rate can be used as an indicator species (Regulation 854/2004/EC). Because mussels have one of the highest accumulation rates (Vale and de Sampayo 2002; Suzuki and Mitsuya 2001), it is considered as the best indicator species. A sampling plan validation method, primarily developed by Whitaker (Whitaker et al. 1972) and widely applied (Whitaker et al. 2007a, b), is used to compute probabilities of acceptance. Knowing that the true contamination level of a lot is never known, as only sample analytical results can be obtained, the probability of acceptance is defined as the probability that the sample analysis results are lower than the food safety threshold concentration level for OA. The probabilities of acceptance, as determined by different sampling schemes, are plotted against mean OA lot concentrations. The curves obtained, which are referred to as Operating Characteristic (OC) curves, enable one to quantify consumer and producer risks. Consumer risk is the probability that a lot having a true concentration above the threshold (unsafe lot) is authorized for sale and consumption. Producer risk is the probability that a lot at a true concentration lower than the threshold (good lot) is rejected for sale. Then a best fit sampling plan can be proposed, taking into account the two risk types, as well as considering the practical feasibility of the sampling plan.

Material and Method

The sampling plan validation method developed by Whitaker consists of a series of calculations on contaminant concentration data from mussel samples taken from various mussel lots. The sample concentrations from a lot are adjusted to a theoretical distribution by a goodness of fit test. This process is repeated on a few lots. Furthermore, the variability between sample concentrations within a lot is studied, in order to predict this variability for the mean concentration of any lot, within a given range of concentrations. Both the theoretical distribution and the prediction of concentration variability between samples of the same lot is used to calculate the probabilities of acceptance of lots for the sampling plan tested. All these steps are further explained in the following sections and used to evaluate different sampling strategies designed to detect potentially harmful levels of okadaic acid in mussels.

For Whitaker's Method

Theoretical Distribution

Data Used

To examine okadaic acid in mussels, we used the data of Dr. Arne Duinker who has supplied us (personal communication) with raw data on individual mussels contaminated with okadaic acid toxin equivalents, obtained during field experiments that led to a publication (Duinker et al. 2007). These data consist of OA levels in mussels contaminated on collectors cultured at high density in a stratified fjord. Four different lots were sampled, and all the samples from a given lot were taken at the same sampling point, at the same time, knowing that each sampling point and sampling time was specific to each lot. For each lot: 29 or 30 samples were taken, each sample consisting of one mussel. Then each individual mussel was submitted to chemical analysis. Given the Regulation 853/2004/EC, the data, expressed in concentration in steamed mussels, must be converted to concentration in raw mussels. This conversion is done according to McCarron (McCarron et al. 2008). They published a theoretical conversion value: the concentration level in steamed meat must be divided by 1.2667 to obtain the concentration level in raw meat.

Theoretical Distribution and Goodness of Fit Tests

Probability density functions of the observed data were drawn. They suggested a possible skewness, orienting towards a theoretical distribution type. Once a theoretical distribution is identified, its parameters can be calculated by the method of moments. Then, the visual comparison between the observed and theoretical cumulative frequency distributions is achieved. Finally, the goodness of fit of the observed data to the theoretical distribution can be tested by the Kolmogorov-Smirnov statistical test, which is a goodness of fit test. This test measures the differences between the theoretical and observed probabilities for each contaminant concentration within one lot. It involves finding the maximum vertical distance between the cumulative frequency distributions.

The hypothesis tested by Kolmogorov-Smirnov goodness of fit are:

H_0: The observed distribution conforms to the theoretical distribution.
H_1: The observed distribution does not conform to the theoretical distribution.

At the desired risk level, H_0 can not be rejected if the test statistic (D_{calc}) is less than a critical value found in a table for the corresponding number of samples in the lot. This means that the adjustment of the observed data to the theoretical distribution test can not be rejected at the risk level chosen. The *p-value* is the probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis H_0 is true.

Variance

Data Used

Variance and mean concentration data were gathered from the literature. Variance data were compiled from 11 publications (Carmody et al. 1996; Duinker et al. 2007; Edebo et al. 1988; Godhe et al. 2002; Kacem et al. 2009; Klöpper et al. 2003; Lindegarth et al. 2009; Mak et al. 2005; Reizopoulou et al. 2008; Sidari et al. 1998; Svensson and Förlin 2004) from various countries (Sweden, Italy, Germany, Ireland, Hong Kong, Tunisia) and one thesis (Wrange 2008) from Sweden. The literature review revealed a much higher number of publications about okadaic acid levels in mussels, but all were not relevant for variance data gathering. Moreover, it was decided to take into account variance data for as many countries as possible, to ensure that the sampling plan validation would not be country specific, but would represent a global validation.

Equation of Variance as a Function of the Concentration

The variability, more precisely the total variance, between sample concentrations is due to sampling, sub-sampling and analytical errors. We know that total variance is the sum of variance components, due to the fact that variance components are additive because they are due to independent sources of random error. So, we assume, according to Whitaker et al., that total variance ($S^2{}_t$) is the sum of sampling variance ($S^2{}_s$), sub-sampling variance ($S^2{}_{ss}$) and analytical variance ($S^2{}_a$) in Eq. 8.1:

$$S^2{}_t = S^2{}_s + S^2{}_{ss} + S^2{}_a \tag{8.1}$$

In Whitaker's method, $S^2{}_t$, $S^2{}_{ss}$, and $S^2{}_a$ are accurately quantified. But, given the fact that, when working on experimental data, Whitaker et al. always found that $S^2{}_{ss}$, and $S^2{}_a$ were negligible in comparison with $S^2{}_t$ we decided not to calculate the negligible variances. This is a slight modification of Whitaker's method that makes the method easier to undertake, with little loss of accuracy. When assuming that $S^2{}_{ss}$ and $S^2{}_a$ are negligible, the following approximation can be made:

$$S^2{}_t = S^2{}_s \tag{8.2}$$

This approximation is now used to define sampling variance instead of total variance.

In order to determine which type of experimental data to use, further definition of the sampling variance is required. In two samples with different means, but which are drawn from the same population, then the difference between the means is simply due to sampling error. Two factors determine the magnitude of the sampling error: population variance, and the number of individuals in the sample:

1. For population variance: the larger the population variance, the larger the sampling error.
2. For the number of individuals in each sample: the larger the number of individuals sampled, the smaller the sampling error. This principle is called the law of large numbers.

The last factor requires further explanation:

Variability between sub-samples which consists of pools of individuals is the variability between means. Indeed, we can consider that the OA concentration of a pool is equal to the mean of the concentrations of the individuals in the pool. The standard error of the mean is the standard deviation of the sample mean estimate of a population mean. It is usually estimated by the sample estimate of the population standard deviation divided by the square root of the sample size (assuming statistical independence of the values in the sample).

Knowing that the standard deviation is the square root of the variance, we can deduce, in Eq. 8.3 that the sampling variance for pools (${S^2}_{s\,for\,pools}$) multiplied by the number n of individuals in each pool is equal to sampling variance (${S^2}_s$).

$$ {S^2}_{s\ for\ pools} * n = {S^2}_s \tag{8.3} $$

Variance data is plotted against the respective mean concentration level c (in µg/kg): ${S^2}_s = \mathrm{f}(c)$. Each point in the graph corresponds to data obtained for one lot with sampling variance and mean concentration calculated from the samples taken from the lot. Then, a regression curve is obtained which shows the variance as a function of the concentration. This equation is considered to be useful in computing the probabilities of acceptance of shellfish lots under various sampling schemes.

Published levels of OA concentration data were given for the hepatopancreas, and had to be re-calculated as whole flesh concentrations, because the European regulation 853/2004 states that the okadaic acid concentration must be given per kg of whole flesh. In order to achieve this, the concentration in the hepatopancreas was divided by 6 in order to obtain the concentration in whole flesh. This conversion value was calculated from concentration results reported in the publication of Duinker et al. (2007).

Probabilities of Acceptance

For a lot of a given mean concentration, the probability of acceptance is computed as the probability that a sample consisting of a pool of individuals taken from the lot, has got a concentration level less than, or equal to, the threshold. This probability is calculated from the theoretical distribution and the total variance equation obtained in the previous sections.

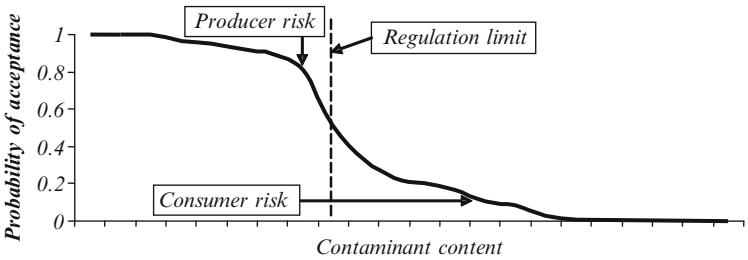


Fig. 8.1 Operating Characteristics (*OC*) curve principle for a contaminant

Operating Characteristics (OC) Curves Principle

Operating Characteristics (OC) curves enable one to calculate the probability of a mistake in determining the average contaminant concentration level by various sampling schemes (Fig. 8.1).

They show the risk of:

- accepting lots at a true concentration above the threshold (consumer risk);
- rejecting lots at a true concentration under the threshold (producer risk).

The shape of the OC curve, for a specific contaminant and food, is unique for a specific sampling plan design. More concretely, the shape varies along with many criteria stated in the sampling plan:

– the number of samples;
– the sample size and
– the concentration threshold chosen for acceptance or rejection of samples.

A sampling plan must be selected, considering both the technical and economic feasibility of the plan, as well as the consumer and producer risk levels. It is therefore important to consider both types of risks, in order to select an optimum sampling plan. An ideal sampling plan would lead to the acceptance of all the lots with contaminant content below the allowed limits, and to the rejection of those with contaminant content higher than these limits, thus reducing the risk to consumer and producer to zero. In reality however, these risks can not be totally eliminated, but only reduced as much as possible, bearing in mind the technical and economic constraints of a given plan. An optimum sampling plan is achieved when risks of OA poisoning are reduced as much as feasible and at modest cost, as well as when good lots are not inappropriately rejected.

Results with Interpretation

For Whitaker's Method

Theoretical Distribution

The probability density functions obtained for each of the four lots show distributions highly skewed to the right, which shows that the right tail is longer, the mass of the distribution is concentrated on the left of the figure. An example of a right-skewed distribution obtained is given in Fig. 8.2.

As it is used for continuous data and can simulate highly positively skewed probability density functions, the best distribution tested is the lognormal. Furthermore, the lognormal distribution parameters can be easily calculated by the method of moments. The comparison of the observed and lognormal cumulative frequency distributions for each lot shows a good visual fit. An illustration of cumulative distribution functions comparisons is given in Fig. 8.3.

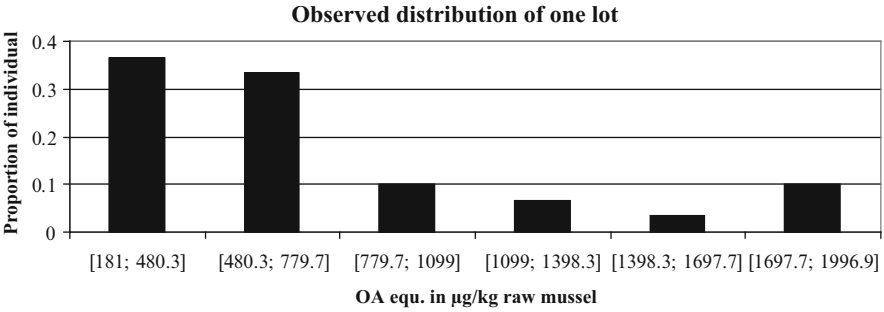


Fig. 8.2 Histogram of observed probability density function for a lot

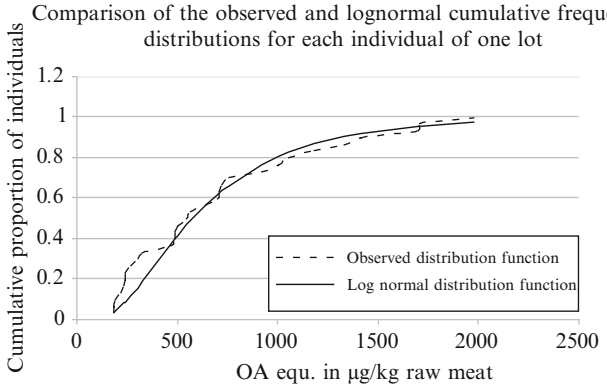


Fig. 8.3 Comparison of theoretical and observed cumulative distribution functions for a lot

Table 8.1 Goodness of fit test results

	D_{calc}	Critical value	*p-value* (%)
Lot n°1	0.1393	0.2457	57.91
Lot n°2	0.1829	0.2457	25.39
Lot n°3	0.2503	0.2417	3.84
Lot n°4	0.1547	0.2417	42.66

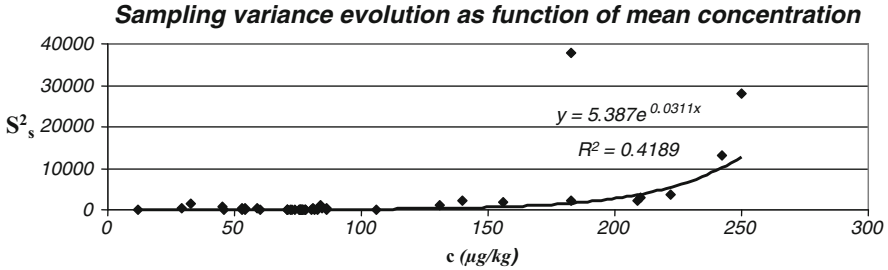


Fig. 8.4 Development of an exponential regression equation

The conformity of the observed distribution to the lognormal distribution is further tested by the Kolmogorov-Smirnov goodness of fit statistical test. The results of these tests are given in Table 8.1.

Critical values and *p-values* are obtained for a risk level of 5 %.

For lot numbers 1, 2 and 4, for both tests, the test statistics (D_{calc}) are less than the critical values, which means that the null hypothesis, that the observed distribution conforms to the lognormal, cannot be rejected at the 5 % level. The *p-values* are all >5 %, which suggests too that the null hypothesis cannot be rejected at the 5 % level. So, at a 5 % risk level, the lognormal distribution of the population cannot be rejected. The results for lot number 3, appears to be anomalous, with opposite values, which suggest that the null hypothesis can be rejected. We consider that the three lots validated out of four by the goodness of fit test is enough to consider that when samples from any lot are drawn, their OA levels fit the lognormal distribution. Moreover, the sample distribution is still considered to be lognormal, even if the samples taken are of a bigger size than one mussel per sample.

Variance

The data (Fig. 8.4) as discussed in the material and methods section, enables us to develop a regression model (Eq. 8.4) to predict $S^2{}_s$ as function of c (in µg/kg).

$$S^2{}_s = 5.387 * \exp(0.0311 * c) \tag{8.4}$$

Table 8.2 An example of calculation of the probabilities of acceptance

c (µg/kg)	S^2_s	$S^2_{s\ for\ pools}$ for a pool size of 30 shellfish	*mu*	*sigma*	Probability of acceptance
10	7.35211953	0.24507065	2.301361234	0.04947432	1
20	10.0340935	0.334469783	2.99531436	0.02891064	1
30	13.6944226	0.456480755	3.40094385	0.02251826	1

There is between mussels variability, even for mussels taken at the same sampling point and at the same time. This variability probably stems from food access variability and variability of response to the toxins. As regards food access, the variability is primarily due to the fact that toxic dinoflagellates are drifted by the currents. Moreover, accumulation and elimination rates of DSP toxins vary within a shellfish species after a contamination event (Duinker et al. 2007). After ingestion, a fraction of the toxins may be transformed by acylation. Acylation has been demonstrated in bivalves by Suzuki and Mitsuya (Suzuki et al. 1999; Suzuki and Mitsuya 2001).

Probabilities of Acceptance

Probabilities of acceptance depend on the sample size, and can be calculated for any sample size as shown in Table 8.2.

Here, the variable c is an input. S^2_s is calculated with Eq. 8.4. After defining the sample size (the number, n, of individuals in each pooled sample), $S^2_{s\ for\ pools}$ is calculated thanks to Eq. 8.3. The parameters of the lognormal distribution: *mu* and *sigma* are calculated by the method of moments. The probability of acceptance is computed as the ordinate of the lognormal theoretical cumulative frequency distribution at the regulatory threshold value, which is set at 160 µg/kg. These probabilities of acceptance correspond to a sampling plan in which a single sample is taken.

Tests and Selection of the Best Fit Sampling Plan Using OC Curves

The probabilities of acceptance as calculated above, correspond to a sampling plan in which a single sample is taken, and are obtained by computing the probability that this sample is less than or equal to the threshold concentration, for a lot of that mean concentration. In order to obtain an OC curve, the probabilities of acceptance, for a given sample size, must be plotted against c. In the following, various sampling strategies have been tested in order to observe their effect on the shape of the OC curve.

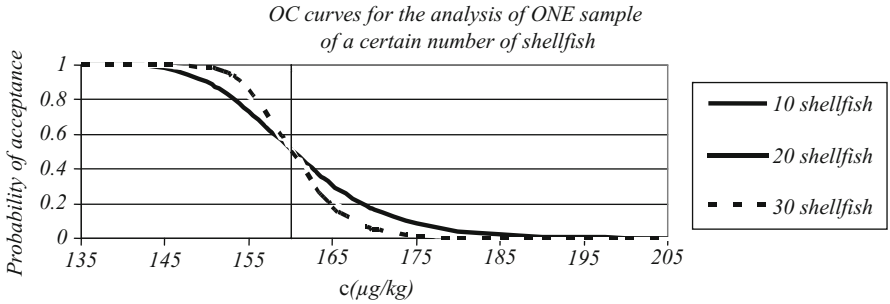


Fig. 8.5 OC curves for sampling plans with one sample taken of a sample size of 10, 20, or 30 shellfish

Single Sample Sampling Plans: Effect of Sample Size

When testing single samples, it was observed that increasing the sample size reduces uncertainty (Fig. 8.5). As sample size increases, the OC curves become steeper around the threshold concentration. As a result, consumer and producer risks become smaller. So, if the sample size is increased, the sampling results and decisions based on them, become more accurate.

In Fig. 8.5, the sampling strategy with the lowest consumer and producer risks is that in which a 30 shellfish sample was analyzed. Here, the probability of acceptance is at 95 % for a concentration of 152 µg/kg. Therefore the producer risk is at 5 % for this concentration. The probability of acceptance is at 5 % for a concentration of 169 µg/kg, so, this means that the consumer risk is at 5 % at this concentration.

Multiple Samples Strategy

The other strategy tested consists of taking a number of samples, q, from the same lot. This lot is only accepted if all q samples tested give results less than the threshold concentration, then:

For a given sample size, the probabilities of acceptance resulting from the multiple samples plan, correspond to the probabilities of acceptance of a single sample raised to the power of q. Comparison of the OC curves for a single and a double sample sampling plan are shown in Fig. 8.6, with a sample size of 30 shellfish.

From Fig. 8.6 one can see that the two sample strategy enables one to decrease the consumer risk (Probability of acceptance at 5 % for a concentration of 164 µg/kg) but increases slightly the producer risk (Probability of acceptance at 95 % for a concentration of 151 µg/kg).

Thus, for food safety reasons, the optimum strategy is a two samples plan with each sample consisting of 30 individual mussels.

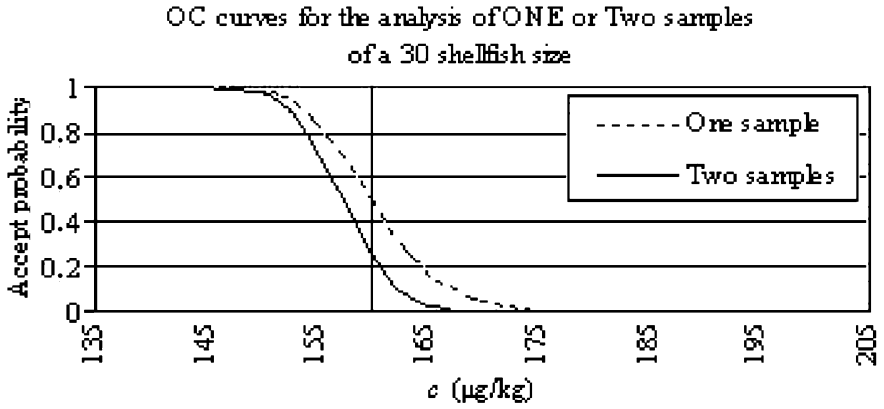


Fig. 8.6 OC curves for sampling plans with one sample taken, or two samples taken

Conclusions

We have demonstrated here that Whitaker's method is applicable to phycotoxins in shellfish. To our knowledge, this is the first time that a probabilistic evaluation of sampling plan designs for phycotoxins in shellfish has been undertaken. Sampling plan designs are of the utmost importance when determining, in a trustworthy way, the human health status of a particular shellfish growing area. This method has been validated on variance data that we had at our disposal, but it needs to be validated on further data. It is suggested that a Food Sanitary Agency, involved in phycotoxin monitoring, test this method on a larger data set.

Acknowledgements This project was financed by the BASELINE European research project, which is part of the 7th Framework Programme (grant agreement 222738). This project is titled "Selection and improving of fit-for-purpose sampling procedures for specific foods and risks" and spans from 2009 to 2013.

References

Carmody EP, James KJ, Kelly SS (1996) Dinophysistoxin-2: the predominant diarrhoetic shellfish toxin in Ireland. Toxicon 34:351–359

Duinker A, Bergslien M, Strand Ø, Olseng CD, Svardal A (2007) The effect of size and age on depuration rates of diarrhetic shellfish toxins (DST) in mussels (*Mytilus edulis* L.). Harmful Algae 6:288–300

Durborow RM (1999) Health and safety concerns in fisheries and aquaculture. Occup Med State Art Rev 14(2):373–406

Edebo L, Lange S, Li XP, Allenmark K, Lindgren K, Thompson R (1988) Seasonal, geographic and individual variation of okadaic acid content in cultivated mussels in Sweden. APMIS Acta Pathol Microbiol Immunol Scand 96(7–12):1036–1042

Fujiki H, Suganuma M, Suguri H, Yoshizawa S, Takagi K, Uda N, Wakamatsu K, Yamada K, Murata M (1988) Diarrheic shellfish toxin, dinophysistoxin-1, is a tumor promoter on mouse skin. Jpn J Cancer Res 79:1089–1093

Gestal-Otero JJ (2000) Non-neurotoxic toxins. In: Botana LM (ed) Seafood and freshwater toxins. CRC Press, Boca Raton, pp 45–64

Godhe A, Svensson S, Rehnstam-Holm A-S (2002) Oceanographic settings explain fluctuations in *Dinophysis* spp. and concentrations of diarrhetic shellfish toxin in the plankton community within a mussel farm area on the Swedish west coast. Mar Ecol Prog Ser 240:71–83

Kacem I, Hajjem B, Bouaïcha N (2009) First evidence of okadaic acid in *Mytilus galloprovincialis* mussels, collected in a Mediterranean lagoon, Tunisia. Bull Environ Contam Toxicol 82:660–664

Klöpper S, Scharek R, Gerdts G (2003) Diarrhetic shellfish toxicity in relation to the abundance of *Dinophysis* spp. in the German bight near Helgoland. Mar Ecol Prog Ser 259:93–102

Lindegarth S, Torgersen T, Lundve B, Sandvik M (2009) Differential retention of okadaic acid (OA) group toxins and pectenotoxins (PTX) in the blue mussel, *Mytilus edulis* (L.), and European flat oyster, *Ostrea edulis* (L.). J Shellfish Res 28:313–323

Mak CY, Yu H, Choi MC, Shen X, Lam MHW, Martin M, Wu RSS, Wong PS, Richardson BJ, Lam PKS (2005) Okadaic acid, a causative toxin of diarrhetic shellfish poisoning, in green-lipped mussels *Perna viridus* from Hong Kong fish culture zones: method development and monitoring. Mar Pollut Bull 51:1010–1017

McCarron P, Kilcoyne J, Hess P (2008) Effects of cooking and heat treatment on concentration and tissue distribution of okadaic acid and dinophysistoxin-2 in mussels (*Mytilus edulis*). Toxicon 51(6):1081–1089

Reizopoulou S, Strogyloudi E, Giannakourou A, Pagou K, Hatzianestis I, Pyrgaki C, Granéli E (2008) Okadaic acid accumulation in macrofilter feeders subjected to natural blooms of *Dinophysis acuminata*. Harmful Algae 7:228–234

Sidari L, Nichetto P, Cok S, Sosa S, Tubaro S, Honsell G, Della Loggia R (1998) Phytoplankton selection by mussels, and diarrhetic shellfish poisoning. Mar Biol 131:103–111

Suganua M, Fujuki H, Suguri H, Yoshizawa S, Hirota M, Nakayasu M, Ojika M, Wakamatzu K, Yamada K, Sugimura T (1988) Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. Proc Natl Acad Sci USA 85:1768–1771

Suzuki T, Mitsuya T (2001) Comparison of dinophysistoxin-1 and esterified dinophysistoxin-1 (dinophysistoxin-3) contents in the scallop *Patinopecten yessoensis* and the mussel *Mytilus galloprovincialis*. Toxicon 39:905–908

Suzuki T, Ota H, Yamasaki M (1999) Direct evidence of transformation of dinophysistoxin-1 to 7-O-acyl-dinophysistoxin-1 (dinophysistoxin-3) in the *scallop Patinopecten yessoensis*. Toxicon 37:187–198

Svensson S, Förlin L (2004) Analysis of the importance of lipid breakdown for elimination of okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis*: results from a field study and a laboratory experiment. Aquat Toxicol 66:405–418

Vale P, de M Sampayo M-A (2002) Esterification of DSP toxins by Portuguese bivalves from the Northwest coast determined by LC-MS – a widespread phenomenon. Toxicon 40:33–42

Whitaker TB, Dickens JW, Monroe RJ, Wiser EH (1972) Comparison of the observed distribution of aflatoxin in shelled peanuts to the negative binomial distribution. J Am Oil Chem Soc 49:590–593

Whitaker TB, Saltsman JJ, Ware GM, Slate AB (2007a) Evaluating the performance of sampling plans to detect Hypogycin A in ackee fruit shipments imported to the United States. J AOAC Int 90(4):1060–1072

Whitaker TB, Slate AB, Hurley JM, Giesbrecht FG (2007b) Sampling almonds for aflatoxin, Part II: estimating risks associated with various sampling plan designs. J AOAC Int 90(3):778–785

Wrange A-L (2008) Investigating mechanisms behind species-specific differences in uptake of diarrhetic shellfish toxins (DST) between oysters (*Ostre edulis*) and mussels (*Mytilus edulis*). Master thesis

Part II
Health Risk Assessment/Évaluation de risques à la santé

Chapter 9
Contribution of Shellfish Consumption to the Dietary Exposure of the French Population to Chemical Contaminants

Nathalie Arnich, Véronique Sirot, and Jean-Charles Leblanc

Introduction

In June 2011, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) published the final report of the second Total Diet Study (TDS) conducted in France between 2006 and 2009 (ANSES 2011). The first TDS was published in 2004 based on samples collected in 2000–2001 (Leblanc et al. 2005).

Regarding the scope of the ICMSS conferences, it was decided to estimate the contribution of shellfish consumption to the dietary exposure of the French population to the main chemical contaminants. This work has been specially performed for the 2011 conference in Charlottetown (Canada). It has not been published elsewhere.

Method

TDS Methodology

The French TDS used a standardised method recommended by the World Health Organization (WHO). The detailed methodology is available in the report (ANSES 2011) and in Sirot et al. (2009). The main points are briefly summarized below.

N. Arnich (✉) • V. Sirot • J.-C. Leblanc
French Agency for Food, Environmental and Occupational Health & Safety, Risk Assessment Department, ANSES, 27-31 avenue du General Leclerc, 94701 Maisons-Alfort, France
e-mail: nathalie.arnich@anses.fr; sirotv@gmail.com; jleblanc@inapg.inra.fr

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_9,

Food Sampling

Two main criteria were considered for food sampling: (i) the most heavily-consumed foods and (ii) foods not heavily consumed but likely to be highly contaminated. A total of 212 different food types were thus selected, covering around 90 % of dietary consumption in the adult and child populations. Out of these 212 food types, 116 were considered as having no or little inter-regional variability (composition or contamination). The other 96 foods were the subject of inter-regional lists in order to take into account potential variability in composition or contamination between regions (production and/or animal feeding methods, environmental pressure). Eight inter-regional food lists were thus drawn up.

For each of the 212 food types, a sampling plan was followed taking into account consumption habits in France, the flavour, the product's origin, claims such as 'low-fat' or 'organic' for example, points of purchase (hypermarket or supermarket, retail shops, markets), the storage method (fresh, deep-frozen, canned), the market shares of the various brands, and so on. Purchases were made year-round, from June 2007 to January 2009, thus covering seasonal variations in food supply. Lastly, each sample was purchased twice during the study, in order to cover potential seasonal variability in composition or contamination. In the end, approximately 20,000 foods were purchased in thirty-three large towns across mainland France.

Sample Analysis

For each food, only the edible part was used, and then the foods were prepared 'as consumed'. For example, fruits and vegetables were washed. Vegetables, meat and seafood products were cooked: braised, pan-fried, grilled, baked, deep-fried, etc. The foods were then combined into 1,319 composite samples representative of shopping baskets and consumer purchases for the eight surveyed inter-regions and analyzed by accredited laboratories. Each composite sample was composed of 15 subsamples of equal weight of the same food. These analyses led to the production of over 230,000 analytical results, after relevant substances were tested in the various samples: each substance was thus tested in those foods that were known or assumed to contain it according to the scientific literature. Food analyses were undertaken by around a dozen laboratories chosen for their capacities (national reference laboratories and accredited laboratories) for most of the tested substances in the targeted foods.

Exposure Assessment

Individual and population dietary exposure to each compound were assessed by combining contamination data and national consumption data, as described below:

$$E_{i,j} = \frac{\sum_{k=1}^{n} C_{i,k} \times L_{k,j}}{BW_i} \tag{9.1}$$

where

$E_{i,j}$ is the exposure to the contaminant j for the subject i,
$C_{i,k}$ is the consumption level of the food k by the subject i (k = 1 to n),
$L_{k,j}$ is the level of contaminant j in the food k,
BW_i is the body weight of the subject i.

The national consumption data come from a French survey called INCA-2 performed in 2006–2007. This survey was based on a representative sample of the French population. It included two types of questionnaires. On the one hand, there was an individual face-to-face questionnaire. During the interview, the weight and the height were measured. The questions deal with leisure-time and physical activity, the profession and education level of the head of the family, etc. On the other hand, there was a self-administered questionnaire, including a 7-day food record, based on a food portion-size manual with pictures. The questions also deal with health status and smoking habits. The respondents were also asked to record how food was kept and how it was consumed. The under-reporters were excluded. At the end, the survey included 1,918 adults aged between 18 and 79 years old (839 men and 1,079 women) and 1,444 children aged between 3 and 17 years old.

Shellfish and Contaminants

In the 2nd French TDS, contamination data were available for the following shellfish species: oysters, mussels, and scallops (*Pecten maximus*).

- oysters, n = 5 composite samples × (15 subsamples) = 75 food samples
- mussels, n = 10 composite samples × (15 subsamples) = 150 food samples
- scallops, n = 6 composite samples × (15 subsamples) = 90 food samples

All the compounds studied in the TDS were not relevant regarding shellfish contamination (for example, acrylamide, mycotoxins, phytoestrogens). The followings contaminants were selected for this specific study:

- 15 trace elements: Lead (Pb), Cadmium (Cd), Mercury (Hg), Arsenic (As), Aluminium (Al), Silver (Ag), Cobalt (Co), Chromium (Cr), Manganese (Mn), Molybden (Mo), Nickel (Ni), Antimony (Sb), Selenium (Se), Tin (Sn), Vanadium (V)
- Sum of 17 (PCDD/F) + 12 (DL-PCBs) according to Regulation EC 1881/2006 setting maximum levels for certain contaminants in foodstuffs
- Sum of 6 (NDL-PCBs) (PCB-28, 52, 101, 138, 153, 180) according to EFSA 2005
- 4 PAHs: benzo[a]pyrene, chrysene, benz[a]anthracene and benzo[b]fluoranthene
- 16 perfluoroalkylated substances: carboxylates (PFOA, PFBA, PFPA, PFHxA, PFHpA, PFNA, PFDA, PFUnA, PFDoA, PFTrDA, PFTeDA) and sulfonates (PFOS, PFBS, PFHxS, PFHpS, PFDS)
- 8 PBDEs (28, 47, 99, 100, 153, 154, 183, 209)

- 3 PBBs (52, 101, 153)
- 3 isomers of HBCDs (alpha, beta, gamma)
- and 54 pesticides: Aldicarb (sum), Azinphos-methyl, Camphechlor (Toxaphene), Carbaryl, Carbendazim (sum), Carbetamide, Carbofuran, Chlordane (sum), Chlorfenvinphos, Chlorothalonil, Chlorpyrifos-ethyl, Chlorpyrifos-methyl, Cyhexatin (sum), DDT (sum), Deltamethrin, Diazinon, Dichlorvos, Dicofol (sum), Dieldrin (sum), Dimethoate (sum), Endosulfan (sum), Endrin, Ethion, Fenbutatin oxide, Fenitrothion, Fenthion (sum), Fentin acetate, Fentin hydroxide, Folpet, HCH (sum), Heptachlor (sum), Hexachlorobenzene, Imazalil, Iprodione, Lindane (HCH γ), Malathion (sum), Methidathion, Methomyl (sum), Mevinphos, Monocrotophos, Ofurace, Oxydemeton-methyl (sum), Parathion (sum), Phorate (sum), Phosalone, Phosmet, Phosphamidon, Pirimiphos-methyl, Prochloraz, Quinalphos, Sulfotep, Thiometon, Tri-allate, Vinclozolin (sum).

Results and Discussion

The results for trace elements are presented in the Tables 9.1 and 9.2.

Regarding silver (Ag), shellfish consumption contributes to 16 % of overall dietary exposure for adults and 4.3 % for children.

For cadmium, shellfish consumption contributes to 5.2 % of overall dietary exposure for adults, and 1.5 % for children. For lead, the contributions are lower, respectively 2.5 % for adults and 1.1 % for children.

For total arsenic, shellfish contribute to 11.5 % of overall dietary exposure for adults and 3.7 % for children. Inorganic arsenic is far more toxic as compared to the organic arsenic. Making a number of assumptions for the part of inorganic arsenic in total arsenic for each type of food, the inorganic arsenic exposure and the contribution of shellfish is estimated to be far lower than in the case of total arsenic. In fact arsenic in shellfish is mainly present as arsenobetaine, widely assumed to be of no toxicological concern (EFSA 2009).

The contributions of the other inorganic contaminants investigated are summarized in the Table 9.2, from the highest to the lowest values (based on data for adults).

Table 9.1 Trace elements with the highest contribution of the shellfish consumption to the mean overall dietary exposure of the French population

Trace element		Ag	Cd	Pb	As (total)	As (inorganic)
Adults	Mean dietary exposure (μg/kg bw/day)	1.29	0.16	0.20	0.78	0.24
	Shellfish contribution (%)	16	5.2	2.5	11.5	0.7
Children	Mean dietary exposure (μg/kg bw/day)	1.60	0.24	0.27	1.21	0.30
	Shellfish contribution (%)	4.3	1.5	1.1	3.7	0.3

Table 9.2 Trace elements with low contributions of the shellfish consumption to the mean overall dietary exposure of the French population

Trace elements		Al	Mo	V	Se	Co	Mn	MeHg	Sb	Ni	Cr	Sn
Adults	Mean dietary exposure (μg/kg bw/day)	280	93.9	0.86	64.4	0.18	2,160	0.017	0.03	2.33	277	3.9
	Shellfish contribution (%)	1.6	1.1	1	0.9	0.8	0.8	0.3	0.2	0.2	0.2	0.02
Children	Mean dietary exposure (μg/kg bw/day)	440	74.7	1.06	41.5	0.31	1,460	0.022	0.04	3.83	223	7.3
	Shellfish contribution (%)	0.7	0.4	0.5	0.5	0.3	0.2	0.1	<0.1	<0.1	<0.1	<0.01

Table 9.3 Contribution of the shellfish consumption to the mean overall dietary exposure of the French population to dioxins, PCBs and PAHs

		Dioxins and DL-PCBs	Non DL-PCBs	PAHs (as sum of 4)
Adults	Mean dietary exposure	0.47 $pg_{TEQ\text{-}OMS}$/kg bw/day	1.83 ng/kg bw/day	1.5 ng/kg bw/day
	Shellfish contribution (%)	3.9	5.3	13
Children	Mean dietary exposure	0.76 $pg_{TEQ\text{-}OMS}$/kg bw/day	2.84 ng/ kg bw/day	2.3 ng/kg bw/day
	Shellfish contribution (%)	1.3	2.0	4.2

Table 9.4 Contribution of shellfish consumption to the mean overall dietary exposure of the French population to perfluoroalkylated substances (*PFAs*)

		PFOA	PFOS	Other PFAs
Adults	Mean dietary exposure (ng/kg bw/day)	0.01	0.04	0.34–3.23
	Shellfish contribution (%)	0.25	0.4	0.07–0.4
Children	Mean dietary exposure (ng/kg bw/day)	0.01	0.05	0.83–7.21
	Shellfish contribution (%)	<0.1	0.1	0.02–0.08

Mercury in seafood is mainly present as methylmercury, an organic form of the contaminant. But, even in this organic form, the contribution is very low (less than 1 % both for adults and children).

In all cases, the contribution to the overall dietary exposure is considered as negligible. In terms of risk assessment and risk management, this low contribution means that it is not relevant to set maximum regulatory levels for these contaminants in shellfish.

The results for dioxins, PCBs and PAHs are presented in the Table 9.3.

The results for dioxins and dioxin-like PCBs are expressed as the sum of 17 (PCDD/F) + 12 (DL-PCBs) according to the European Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. Non-dioxins like PCBs are expressed as the sum of 6 congeners (PCB-28, 52, 101, 138, 153, 180) according to EFSA (2005).

For dioxins and dioxin-like PCBs, shellfish consumption contributes 3.9 % of overall dietary exposure for adults, and 1.3 % for children. The values are 5.3 % for adults and 2 % for children for non dioxin-like PCBs.

Currently, maximum levels in food for polycyclic aromatic hydrocarbons in food are based on benzo(a)pyrene, used as a marker (European Regulation (EC) No 1881/2006). In 2008, EFSA recommended to use the sum of 4 PAHs: benzo[a]pyrene, chrysene, benz[a]anthracene and benzo[b]fluoranthene. Based on benzo(a)pyrene as a marker, the shellfish consumption contributes to 5.9 % of the overall dietary exposure for adults and 1.9 % for children. When the results are expressed as the sum of 4 compounds the contribution is 13 % for adults and 4.2 % for children.

The results for perfluoroalkylated substances (PFAs) are presented in Table 9.4.

Table 9.5 Contribution of the shellfish consumption to the mean overall dietary exposure of the French population to brominated flame retardants (*BFRs*)

		PBDEs	PBBs	HBCDs
Adults	Mean dietary exposure (ng/kg bw/day)	0.540	0.001	0.165
	Shellfish contribution (%)	0.88	0.92	2.6
Children	Mean dietary exposure (ng/kg bw/day)	1.008	0.001	0.237
	Shellfish contribution (%)	0.28	0.37	1.1

The samples were analyzed for 16 perfluoroalkylated substances: carboxylates (PFOA, PFBA, PFPA, PFHxA, PFHpA, PFNA, PFDA, PFUnA, PFDoA, PFTrDA, PFTeDA) and sulfonates (PFOS, PFBS, PFHxS, PFHpS, PFDS). The results show that the contribution of shellfish to the overall dietary exposure is negligible, far below 1 % for adults and children.

The results for brominated flame retardants (BFRs) are presented in the Table 9.5.

The samples were analyzed for 8 PBDEs (28, 47, 99, 100, 153, 154, 183, 209); 3 PBBs (52, 101, 153) and 3 isomers of HBCDs (alpha, beta, gamma). The results show that the contribution of the shellfish to the overall dietary exposure is negligible, below 1 % for adults and children except for HBCDs.

Among the 54 pesticides investigated, only 1 sample was above the limit of detection for 1 substance, namely Imazalyl (a fungicide) in oysters. Even with upper bound[1] assumptions, the contribution of shellfish consumption to the dietary exposure of the French population was below 0.1 % both for adults and children. It can be concluded regarding pesticides that the shellfish contribution was negligible.

Conclusion

The contribution of shellfish consumption to the overall mean dietary exposure of the French general population to chemical contaminants has been assessed, based on the data provided by the 2nd French Total Diet Study. For a large majority of contaminants, our study shows that the contribution is low. However, for some contaminants, shellfish consumption seems to contribute significantly. The highest contributions are observed for silver and PAHs (when we consider the sum of 4 compounds). Results can be summarized as follows:

– Silver (Ag): 16 % for adults, 4.3 % for children
– PAHs (sum of 4): 13 % for adults, 4 % for children

[1]Upper bound assumption: a non-detected substance is considered as present at the limit of detection, and a detected but non-quantified substance is considered as present at the limit of quantification. The upper bound assumption over estimates levels and thus exposure and is therefore conservative.

- Cadmium: 5 % for adults 1.5 % for children
- Lead: 2 % for adults: 1 % for children
- other inorganic contaminants: ≤1 %
- dioxins and PCBs: 4–5 % for adults 1–2 % for children
- PFAs and BFRs: <1 % except HBCD 3 % for adults 1 % for children
- Pesticides: <0.1 % for adults and children

However, it should be pointed out that total diet studies are representative of the general population. They are not representative of high consumers of shellfish and other seafood products (mostly along the coasts) who eat seafood products at least twice a week. For this subpopulation, a specific study has been conducted in France, called CALIPSO, and was published in 2006 (AFSSA 2006).

In case of risk assessment dealing with shellfish contamination, ANSES relies on the two sets of data, to have a complete picture of the French population.

References

AFSSA (2006) CALIPSO: fish and seafood consumption study and biomakers of exposure to trace elements, pollutants and Omega-3, 162 pp. Available online: www.anses.fr, in French and in English

ANSES (2011) Total Diet Study 2 – 2006–2010. Part 1: inorganic contaminants, minerals, persistent organic pollutants, mycotoxins. Part 2: pesticide residues, additives, acrylamide, polycyclic aromatic hydrocarbons). Available online: www.anses.fr, in French and in English

Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union L 364:5–24

EFSA (2005) Opinion of the Scientific Panel on contaminants in the food chain on a request from the commission related to the presence of non dioxin-like polychlorinated biphenyls (PCB) in feed and food (Question N° EFSA-Q-2003-114). EFSA J 284:1–137. Available online: www.efsa.europa.eu

EFSA (2008) Polycyclic aromatic hydrocarbons in food. Scientific opinion of the panel on contaminants in the food chain (Question N° EFSA-Q-2007-136). EFSA J 724:1–114

EFSA (2009) Scientific opinion on arsenic in food. EFSA J 7(10):1351, 198 pp. Available online: www.efsa.europa.eu

Leblanc JC, Guerin T, Noel L, Calamassi-Tran G, Volatier JL, Verger P (2005) Dietary exposure estimates of 18 elements from the 1st French Total Diet Study. Food Addit Contam 22:624–641

Sirot V, Volatier JL, Calamassi-Tran G, Dubuisson C, Menard C, Dufour A, Leblanc JC (2009) Core food of the French food supply: second Total Diet Study. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 26(5):623–39

Chapter 10
Probabilistic Exposure Assessment to Phycotoxins by Recreational Shellfish Harvesters: Results and Influence of Shellfish Species and the Cooking Process

Cyndie Picot, G. Limon, G. Durand, Dominique Parent-Massin, and Alain-Claude Roudot

Introduction

The past few decades have seen an increase in the frequency, concentrations, and geographic distribution of marine algal toxins (phycotoxins), secondary metabolites produced by marine microalgae (phytoplankton). These phycotoxins are considered as an important food safety issue because of their accumulation by shellfish until becoming unfit for human consumption (Van Dolah 2000). However quantitative exposure assessments to phycotoxins aimed at evaluating the likely intakes of phycotoxins *via* shellfish consumption are almost non existent (EFSA 2008, 2009, 2010; Picot et al. 2011a). Concerning phycotoxins, a total population exposure assessment has almost no interest because shellfish consumers are a minority group, and averaging the total population exposure will be low. So, it is of more interest to define and study an at-risk sub-population, which will give an upper threshold of the exposure. Recreational shellfish harvesters appear to be an at risk subpopulation because *a priori* they consumed a larger quantity of seafood than the general population, because their practice is both recreational and a cheap source of food

C. Picot • D. Parent-Massin
Laboratoire de Toxicologie Alimentaire et Cellulaire (EA 3880), Université Européenne de Bretagne – Université de Bretagne Occidentale (UEB-UBO), 6 Av. Victor Le Gorgeu – CS93837, 29238 Brest, Cedex 3, France
e-mail: cyndie.picot@univ-brest.fr; dominique.parent-massin@univ-brest.fr

G. Limon • G. Durand
IDHESA Bretagne Océane, Technopôle de Brest-Iroise, BP 52-120 Avenue de Rochon, 29280 Plouzané, France
e-mail: gwendolina.limon@idhesa.fr; gael.durand@idhesa.fr

A.-C. Roudot (✉)
Laboratoire de Toxicologie Alimentaire et Cellulaire (EA 3880), UFR Sciences et Techniques, Université de Bretagne Occidentale, 6 Av. Victor Le Gorgeu – CS93837, 29238 Brest, Cedex 3, France
e-mail: alain-claude.roudot@univ-brest.fr

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_10,

(Burger et al. 1998; Gagnon et al. 2004; Leblanc 2006; Picot et al. 2011b). Therefore it is critical to assess their exposure to phycotoxins *via* shellfish consumption. For that, the estimated individual phycotoxin concentrations in shellfish has to be combined with available consumption data (Kroes et al. 2002; WHO 1985). Thus, getting actual levels of phycotoxins in shellfish is a prerequisite but such data are missing because shellfish contamination is only analysed in cases of phytoplankton blooms (Ifremer 2011). Obtaining shellfish consumption data from recreational shellfish harvesters is also needed. These considerations led us to monitor monthly phycotoxins in shellfish and to conduct a 1-year survey of shellfish consumption by heavy consumers from the same area, i.e. recreational shellfish harvesters. In this study, the assessments were of three phycotoxins of interest to humans: Domoic Acid and analogues (DAs), Okadaic Acid and analogues (OAs) and Spirolides (SPXs). Our investigations were aimed at carrying out a probabilistic exposure assessment and comparing dietary phycotoxin intakes with toxicological reference doses to determine whether phycotoxin exposure is a matter of concern for human health or not. Acute and chronic exposures were assessed in both approaches.

Material and Methods

Input Data

Shellfish Consumption Data

The population studied was a group of recreational shellfish harvesters living along the coasts of Finistère (Western Brittany, France). Shellfish consumption was investigated from February 2008 to February 2009 through two complementary methods: a Food Frequency Questionnaire (FFQ) and a food diary. The FFQ was conducted through face-to-face interviews at the harvesting sites. As this tool provides long-term consumption data, but relied upon memory. This drawback was counteracted by using the records versus time of each shellfish meal (with quantities) kept in the food diary. The food diary gave additional information such as the origin of consumed shellfish (harvest, shop, restaurant . . .), consumption by different household members and the way shellfish had been prepared. Data were validated for bivalve and gastropod groups (for more details, see Picot et al. 2011b). Consumption data included five of the most heavily consumed bivalves expressed as frequency, portion size and daily shellfish consumption rates both with the mean and 95th percentile (P95 see Table 10.1).

Table 10.1 Descriptive statistics of shellfish consumption data according to shellfish species and origin (harvest or purchase)

Harvested bivalves						Purchased bivalves					
		Portion size (g/portion)		Daily consumption rate (g/day)			Portion size (g/portion)		Daily consumption rate (g/day)		
Bivalve species	% of consumers	Mean	P95	Mean	P95	% of consumers	Mean	P95	Mean	P95	% of raw consumption
Oyster	20.3	36.6	172.8	1.68	7.58	27.2	34.4	102.4	2.02	8.12	97.8
Mussel	27.3	69.4	396.0	1.65	10.1	33.6	80.0	264.0	4.04	11.6	0.00
Cockle	63.7	109	271.5	3.15	13.8	2.6	2.7	nc	0.09	0.26	2.38
Carpet shell clam	74.6	73.7	259.5	2.43	10.7	4.7	3.2	nc	0.14	0.47	31.7
Razor clam	23.4	27.6	167.8	0.57	3.31	0.0	0.0	nc	0.00	0.00	0.00
King scallop	0.40	0.13	nc	0.0012	nc	20.3	15.2	85.50	0.45	2.50	3.69

Consumption rates derived from the total population, including non consumers
nc not calculable (because of an insufficient number of consumers)

Shellfish Contamination Data

To counteract the lack of data about phycotoxin levels in shellfish, samples were harvested monthly, from June 2009 to June 2010, on beaches of Finistère selected by three criteria: (i) the presence of several bivalve species, (ii) regular shellfish harvesters and (iii) regular phycotoxin events (Picot 2010). OAs and SPXs were analysed after methanolic extraction from samples, purification by solid phase extraction and quantification by HPLC-MS/MS. DAs were extracted with a water/methanol mixture (50/50; v/v) and analysed by HPLC-MS/MS (Picot et al. 2012). Contamination data are often excluded because of the limits of detection (LOD) and quantification (LOQ) of analytical methods. The GEMS/Food–Euro framework proposed different treatments according to the prevalence of censored-data (WHO 1995):

– the quantity of censored data is less than or equal to 60 %, then, the censored data are replaced by the corresponding LOD or LOQ divided by 2 (T1);
– the number of censored data is greater than 60 % and then:
  - either the censored data are replaced by zero (T2a)
  - or they are replaced by the corresponding LOD or LOQ (T2b).

The contamination data are described in Table 10.2. As, for OAs and DAs, the censored data accounted for more 60 % of values, zero and LOD (or LOQ) values were used in two separate estimations of the distributions and calculations (mean, median, percentiles . . .). On the other hand, as the censored values for SPXs were less than 60 %, they were replaced by the half of the corresponding LOD or LOQ.

Exposure Modelling

General Exposure Model

An acute exposure corresponds to a short exposure to a harmful compound at high dose. Let us consider a phycotoxin denoted by m. The acute phycotoxin exposure is the amount of m ingested in a single meal. It is obtained by multiplying the edible portion size of one shellfish species by the concentration of m in this portion. For each phycotoxin, acute intakes were calculated individually for each shellfish species.

Chronic exposure is a repeated exposure to low, or very low, doses for a long time. The chronic phycotoxin exposure is the amount of m ingested daily from the daily consumption of all shellfish species. The general exposure model used, here, to assess individual phycotoxin intake from shellfish consumption can be expressed as follows:

$$E_m = \frac{\sum \left(C_{mj} * C R_j * P_j\right)}{BW} \tag{10.1}$$

Table 10.2 Descriptive statistics of domoic acid, okadaic acid and spirolide contamination data set by shellfish species (ng/g) according to the censored data treatment

Phycotoxin	Bivalve species	Number of analyses (n =)	<LOD (%)	<LOQ (%)	Mean			Median			Max		
					T1	T2a	T2b	T1	T2a	T2b	T1	T2a	T2b
DAs	Oysters	13	69.2	7.7	nc	264.4	274.1	nc	0.0	10.0	nc	1,730	1,730
DAs	Cockles	13	69.2	7.7	nc	562.3	571.2	nc	0.0	10.0	nc	3,707	3,707
OAs	Oysters	13	76.9	0	nc	31.0	38.7	nc	0.0	10.0	nc	200.6	200.6
OAs	Mussels	13	61.5	0	nc	203.6	209.7	nc	0.0	10.0	nc	1,423	1,423
SPX	Oysters	13	0	0	14.2	nc	nc		nc	nc		nc	nc
SPX	Mussels	13	15.4	0	15.5	nc	nc		nc	nc		nc	nc

nc non concerned, *LOD* limit of detection, *LOQ* limit of quantification, *T1* parameters are estimated after replacing values below LOD with LOD/2 and values lying between LOD and LOQ with (LOQ − LOD)/2, *T2a* parameters are estimated after replacing values below LOD by zero and values below LOQ by LOD, *T2b* parameters are estimated after replacing the censored data by the corresponding LOD or LOQ

where E_m is the individual exposure (mg/kg/day) to the phycotoxin, m, from the ingested shellfish species, j, C_{mj} is the concentration (mg/kg) of the same phycotoxin in the edible portion of the same species, CR_j is the daily consumption rate (kg/day) of this species, P_j is the proportion of a given shellfish species in a consumer diet (unitless), and BW is the consumer body weight (kg) assumed, in this study, to be 60 kg (USEPA 2000).

The difference in acute- and chronic-exposure assessments stands in the consumption parameter to be used: the former takes into account the portion size of a given shellfish species, whereas the latter considers the daily consumption rate of all shellfish species.

In this study, focus was on five of the most consumed bivalve species in the geographical area under study: oysters, mussels, cockles, carpet shell clams and razor clams; king scallops were also considered in assessments about DA.

The approach used for exposure calculation usually depends on the nature of the available data. In this study it was based on the probabilistic approach described in Kroes et al. (2002).

Probabilistic Approach

Given that a shellfish consumer will not eat, at each time, the same portion size and that the toxin level in the eaten portion will not be the same, the probabilistic calculation considers all of the combinations of occurrence and consumption data. Distributions for both the food consumption and the contamination data were used in the model to simulate dietary intakes by repeatedly drawing random values for each input distribution. The description of input variables in terms of distributions allows one to characterise their variability and/or uncertainty. Monte Carlo simulation techniques are used by the model to generate output distributions of dietary intakes liable to be ultimately considered in probabilistic risk characterisation. Output distributions (i) give several exposure data (mean, median, minimum, maximum and all percentiles) and (ii) include a comprehensive analysis of the sensitivities of the resulting exposure with respect to uncertainties in parameters (Counil et al. 2005; Kroes et al. 2002; Tressou et al. 2004). The @Risk package, version 4.5 (Palisade, USA) with the Microsoft Excel spreadsheet under XP (Microsoft, USA) was used to perform risk analysis from Monte Carlo simulations and probability distributions so as to develop the exposure model on taking into account uncertainty and variability. Each simulation was run for 10,000 iterations to mimic the inherent uncertainty in shellfish-contamination and -consumption as well as the uncertainty in the mathematical process.

As the phycotoxin levels are affected by the cooking process in use (temperature, dry cooking or not), this parameter has to be considered (McCarron and Hess 2006; Vidal et al. 2009). The analyses were made on raw bivalves. To take into account the cooking process impact, for each toxin, the ratio between the phycotoxin rates in raw samples and in cooked samples was determined in a preliminary study. The latter study was made using classical cooking conditions for an at home use of shellfish:

shellfish were cooked in a can on open fire, during 2–5 min until their natural opening. Mussels were analysed after cooking with and without their cooking juice (for more details, see Picot et al. 2012). Then, a normal distribution describing the impact of cooking was assigned to the contamination levels of raw bivalves.

Results and Discussion

Acute- and chronic-exposures to each of the phycotoxins under study were assessed through a probabilistic approach, producing a probability density distribution of dietary intakes from all the bivalves under study.

Exposure Assessment to Phycotoxins

Acute Exposure

Acute-exposure corresponds to the phycotoxin intake by an individual over a meal composed of a single portion of bivalves. For each bivalve species, the exposure is the quantity obtained by multiplying the portion size by the contamination data. Table 10.3 presents the main results about acute exposure from the probabilistic assessment.

Domoic Acid and Analogs

For DAs, the exposure distribution obtained by multiplying a portion size distribution by a contamination distribution for each of the five shellfish species under study led to: (i) a mean up to 0.74 $\mu g/kg \cdot bw$, (ii) a median value up to 0.29 $\mu g/kg \cdot bw$ and (iii) a 95th percentile value up to 3.02 $\mu g/kg \cdot bw$.

Okadaic Acid and Analogs

Concerning OAs, the exposure distribution led to a maximal (for harvested cockles) mean value, a median value and a 95th percentile value equal to 0.70, 0.24 and 2.81 $\mu g/kg \cdot bw$, respectively.

Spirolides

For the SPXs exposure distribution, the highest (for harvested cockles) mean value, median and 95th percentiles were equal to 78.5, 49.6 and 250 $ng/kg \cdot bw$, respectively.

Table 10.3 Acute dietary intakes of domoic acid, okadaic acid and spirolide obtained by a probabilistic approach and comparison with toxicological reference values for each bivalve species

	DA			OA			SPX		
	Exposure* (μg/kg · bw/portion)			Exposure* (ng/kg · bw/portion)			Exposure* (ng/kg · bw/portion)		
	Mean	Median	P95	Mean	Median	P95	Mean	Median	P95
Harvested oysters	0.16	0.06	0.65	19.4	7.62	78.1	8.60	5.46	27.4
Harvested mussels	0.52	0.20	2.09	456	151	1,912	26.0	13.3	94.8
Harvested cockles	0.70	0.27	2.75	702	243	2,808	78.4	49.6	250
Harvested carpet shell clams	0.74	0.29	3.02	378	149	1,466	19.8	12.3	60.00
Harvested razor clams	0.14	0.05	0.53	133	44.9	569	8.58	5.50	27.2
Purchased oysters	0.15	0.06	0.61	17.7	7.1	72	8.10	5.21	25.5
Purchased mussels	0.61	0.24	2.41	183	79	706	29.9	15.6	105
Purchased cockles	0.02	0.01	0.07	4.00	1.7	18	1.90	1.20	6.00
Purchased carpet shell clams	0.03	0.01	0.13	3.80	1.5	15	0.7	0.45	2.20
Purchased razor clams	0	0	0	0	0	0	0	0	0
ARfD		100[a]/30[b]			333[a]/300[b]		No ARfD allocated		

ARfD acute reference dose

*Assuming a body weight equals to 60 kg

[a]*JECFA* Joint FAO/WHO Expert Committee on Food Additives

[b]*EFSA* European Food Safety Authority

Chronic Exposure

The chronic exposure assessment corresponds to the level of exposure after a daily consumption of shellfish, thus the useful consumption data are the daily consumption rates. Table 10.4 illustrates the chronic-exposure levels issued from the probabilistic exposure approach for harvested-, purchased-bivalves and "all bivalves".

Domoic Acid and Analogs

For DAs, it shows that, for "all bivalves", the median value and the mean are unchanged between T2a and T2b scenarios and equal to 0.11 and 0.09 μg/kg · bw/day, respectively; the 95th percentile value is slightly different in T2a and T2b scenarios (0.26 and 0.28 μg/kg · bw/day, respectively).

Okadaic Acid and Analogs

Concerning OAs, the "all bivalves"-related exposure distribution presents maximal means of 54.1 and 56.2 ng/kg · bw/day for T2a and T2b scenarios, respectively, as well as median values of 39.0 and 41.1 ng/kg · bw/day and 95th percentiles of 149 and 155 ng/kg · bw/day. One should note that the censored value scenario (T2a or T2b) has a very limited effect upon the chronic dietary exposure to phycotoxins. Comparing the contribution by harvested bivalves to purchased bivalves, Table 10.4 shows clearly that, for OAs, the level derived from harvest are about five-fold greater than those derived from purchase, this is mainly because the OA levels of harvested bivalves takes into account levels above the regulatory limit.

Spirolides

Concerning SPX, the chronic distribution of exposure (for "all bivalves") leads to a mean value of 5.4 ng/kg · bw/day, a median of 4.6 ng/kg · bw/day, and a 95th percentile of 11.9 ng/kg · bw/day. For SPX, the amount derived from harvest are about two-fold those derived from purchase.

Species Contribution

Figure 10.1 illustrates the contribution of each bivalve shellfish to the daily ingestion of each of the phycotoxins under study. It evidences that the three greatest contributor species are the cockles and the carpet shell clams from harvest as well

Table 10.4 Chronic dietary intakes of domoic acid, okadaic acid and spirolide obtained by a probabilistic approach and comparison with toxicological reference values for harvested, purchased and all bivalves

		DA			OA			SPX		
		Exposure* (μg/kg · bw/day)			Exposure* (ng/kg · bw/day)			Exposure* (ng/kg · bw/day)		
Censored values treatment	Bivalve source	Mean	Median	P95	Mean	Median	P95	Mean	Median	P95
T1 (SPX) or T2a (DA and OA)	Harvested bivalves	0.07	0.05	0.18	44.4	29.3	134	3.5	2.8	8.3
	Purchased bivalves	0.04	0.03	0.13	9.70	5.10	34.5	1.9	1.3	5.70
	All bivalves	0.11	0.09	0.26	54.1	39.0	149	5.4	4.60	11.9
T2b	Harvested bivalves	0.07	0.05	0.20	45.9	30.9	137			
	Purchased bivalves	0.04	0.03	0.14	10.3	5.40	36.6			
	All bivalves	0.11	0.09	0.28	56.2	41.1	155			
Tolerable daily intake		No tolerable daily intake allocated								
ARfD			100[a]/30[b]			333[a]/300[b]		No ARfD allocated		

ARfD acute reference dose
*Assuming a body weight of 60 kg
[a]*JECFA* Joint FAO/WHO Expert Committee on Food Additives
[b]*EFSA* European Food Safety Authority

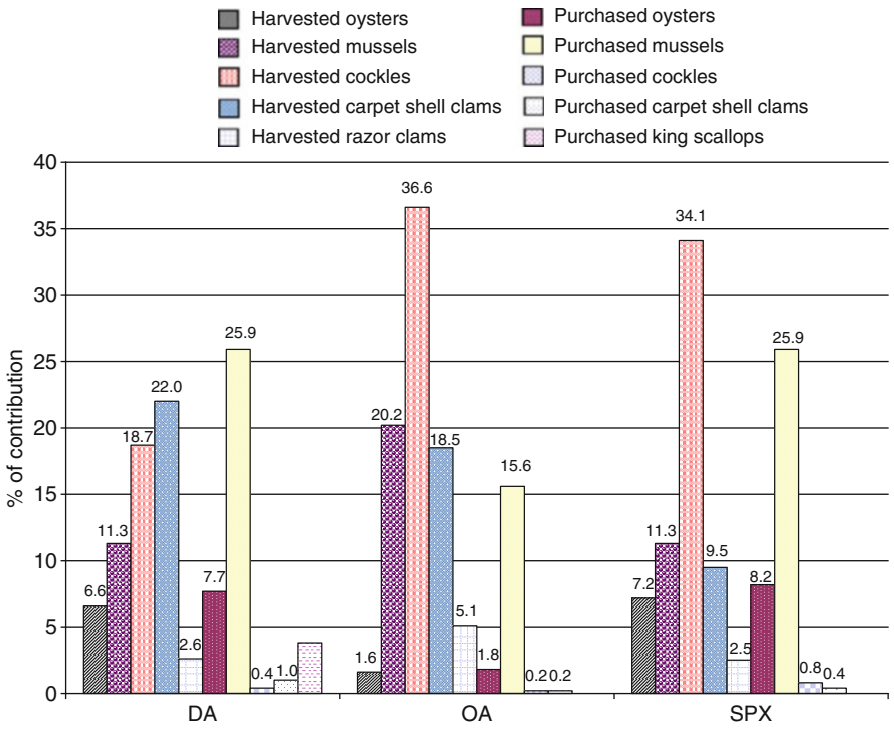


Fig. 10.1 Contribution of each bivalve species to the daily intakes of domoic acid, okadaic acid and spirolide determined through the probabilistic approach (T2a and T1 treatments of censored values)

as the mussels from both harvest and purchase. Their high contributions come from the high consumption and contamination rates for harvested-species and to the high consumption rate for purchased mussels.

Risk Characterization

Acute Risk Characterization

For acute-risk characterisation, probabilistic estimates of dietary exposure to phycotoxins have to be compared to the ARfD (Acute Reference Dose). The provisional ARfDs established by the JECFA (Joint FAO/WHO (Food and Agriculture Organization/World Health Organization) Expert Committee on Food Additives) are 100 and 0.33 $\mu g/kg \cdot bw$ for DAs and OAs, respectively, whereas

no ARfD has been allocated for SPX (Toyofuku 2006). In 2008 and 2009, the EFSA panel proposed ARfD values of 30 and 0.30 μg/kg · bw for DAs and OAs, respectively (EFSA 2008, 2009).

Domoic Acid and Analogs

In this study, all of the acute-exposure assessments for DA gave values less than the DA ARfD established by the JECFA and the EFSA.

Okadaic Acid and Analogs

Concerning OAs, the highest probabilistic assessment (for harvested cockles) led to a mean exposure and a 95th percentile of, respectively, about 2.5-fold and 9-fold the OA ARfD, but to a median exposure almost 1.25-fold less than the OA ARfD. One should note that, for purchased bivalves, all exposures (means, medians and 95th percentiles) were below the OA ARfD, and except for high consumers of mussels, they are above the acute reference value.

Spirolides

Characterizing the SPX acute risk by comparison of the acute exposure with the ARfD cannot be realized because of the unavailability of SPX ARfD value. This is explained by the lack of quantitative data for acute oral toxicity of this phycotoxin. However, only in order to have an idea of the order of magnitude of the margin of exposure, the acute exposure could be compared with the LD0 (Lethal Dose).[1] Munday (2008) studied acute oral exposure to SPX by mice. In the case of oral administration of SPX to mice, two were results LD0's equal to 53 and 400 μg/kg.bw, after for feeling and feeding with cheese cream containing SPX, respectively.

In the case of the most representative way of administration (mice feeding with cheese cream containing SPX), the highest probabilistic assessment (for harvested cockles) led to a mean exposure and a 95th percentile of, respectively, about 5,000-fold and 1,600-fold less than the SPX LD0. Comparing with the most protective LD0 (= 53 μg/kg · bw), the highest probabilistic assessment (for harvested cockles) led to a mean exposure and a 95th percentile of, respectively, about 675-fold and 210-fold less than the SPX LD0.

[1] LD_0 (Lethal Dose 0): the amount of a chemical that if administered to an animal will kill 0 % of the sample population.

Since the margin of exposure is higher than 100, it appears that acute SPX is not a matter of concern for human health. But this conclusion has to be confirmed with other relevant toxicological studies, after establishing an ARfD for SPX.

Chronic Risk Characterization

For chronic risk characterization, probabilistic estimates of dietary exposure to phycotoxins have to be compared to the TDI. But, as no TDI has been allocated to phycotoxins by international committees, we used two other methods, not satisfactory but the only ones possible: comparison with the corresponding ARfD and with Threshold of Toxicological Concern (TTC). The TTC is a principle, which refers to the establishment of a human exposure threshold value for all chemicals, below which there would be no appreciable risk to human health. This threshold value is equal to 2.5 ng/kg · bw/day for genotoxic substances and 25 ng/kg · bw/day for all other substances (Kroes et al. 2002; EFSA 2010).

Domoic Acid and Analogs

Comparison with TTC: concerning DAs, the levels of exposure are equal to 110; 90 and 260 ng/kg · bw/day, for the mean, median and 95th percentile, respectively. These levels of exposure are not below the TTC, thus it cannot be excluded that there would be a risk to human health.

Comparison with ARfD: according to the probabilistic approach, the mean and 95th percentile intakes for DA (0.11 and 0.26 μg/kg · bw/day, respectively) are about 100- and 250-fold less than the most conservative ARfD. Despite the lack of TDI allocated to DA by international committees, Mariën (1996) estimated that a value of 75 μg/kg · bw provides a sound basis for a TDI. The daily DA intakes found in this study are, at least, about 300-fold less than the proposal by Mariën (1996).

Okadaic Acid and Analogs

Comparison with TTC: concerning OAs, the levels of exposure are equal to 54; 39 and 149 ng/kg · bw/day, for the mean, median and 95th percentile, respectively. These levels of exposure are not below the TTC, thus it cannot be excluded that there would be a risk to human health.

Comparison with ARfD: for OAs, the values of the mean and 95th percentile intake issued from the probabilistic approach (0.054 and 0.15 μg/kg · bw/day, respectively) are only about five- and two-fold less than the most protective OA ARfD (0.30 μg/kg · bw). Thus chronic OA intakes were close to ARfD. TDI is, by definition, less than ARfD. The former is, derived from a NOAEL

value or a LOAEL determined from long-term toxicological studies, whereas the latter is determined from acute toxicological studies. Moreover, in addition to the traditional security factors employed for ARfD, the establishment of TDI requires the use of a few others such as an uncertainty factor of 10 to extrapolate subchronic to chronic exposure (Lewis 1995), leading to TDIs much lower than ARfD. The finding, in this study, of a chronic exposure to OA via shellfish consumption (only two- to five-fold below the ARfD) suggests that OA should be considered as a possible cause for concern for human health.

Spirolides

Comparison with TTC: for SPX, the values of the mean, the median and 95th percentile intake from the probabilistic approach (5.4; 4.6 and 11.9 ng/kg · bw/day, respectively) are higher than the TTC (2.5 ng/kg · bw/day). We made the comparison with the most protective TTC because no (sub)chronic and genotoxic data are available for SPX. Thus, the only exposure data do not allow us to reject a chronic risk due to SPX.

Comparison with ARfD: as neither ARfD nor TDI have been allocated to SPX by international committees, no comparison can be made. Though there is no toxicological reference value, the calculations made in this study highlighted the regular exposure of humans to low SPX doses. Thus, in the case where toxicological data indicate chronic impact by SPX on health, it would be worth taking into account exposure to SPX.

Impact of Cooking Process

The impact of cooking differs according to the phycotoxin. For lipophilic toxins, the cooking increased the exposure by a factor about 1.5–2. For DAs, considering the cooking process, the exposures decreased for cockles and razor clams but increased for mussels, donax and hard shell clams (Tables 10.5 and 10.6). Thus, the actual regulated level based on raw bivalves might over- or under-protect the consumers when they cooked shellfish.

Conclusion

Further to the increasing number of reports about phycotoxin-induced intoxications and deaths, these compounds have become a matter of concern for human health. But, phycotoxin exposure assessments are almost non-existent because related data about consumption and contamination are missing. This led us to study, in the same

Table 10.5 DA concentration (μg/g of whole flesh) for both raw and cooked bivalve species; percentages of variation between raw and cooked concentrations and (raw toxin concentration/cooked toxin concentration) ratio values

	Experiment 1				Experiment 2				Experiment 3				Mean	
Species	Raw (μg/g flesh)	Cooked (μg/g flesh)	% of variation	Ratio (raw/cooked)	Raw (μg/g flesh)	Cooked (μg/g flesh)	% of variation	Ratio (raw/cooked)	Raw (μg/g flesh)	Cooked (μg/g flesh)	% of variation	Ratio (raw/cooked)	% of variation	Ratio (raw/cooked)
Mussel[a]	84.95	81.02	−4.63	1.05	0.83	0.86	3.51	0.97	0.51	0.49	−5.76	1.06	−2.30	1.03
Mussel	1.29	1.87	44.76	0.69	0.69	1.02	47.84	0.68	nd	nd	nd	nd	46.30	0.68
Cockle	2.55	1.76	−30.90	1.45	2.53	1.43	−43.40	1.77	0.74	0.60	−19.28	1.24	−31.19	1.48
Carpet shell clam	0.94	2.27	140.40	0.42	1.89	3.69	95.00	0.51	0.59	0.93	58.41	0.63	97.94	0.52
Razor clam	2.01	1.57	−21.89	1.28	0.28	0.26	−5.41	1.06	nd	nd	nd	nd	−13.65	1.17
Donax	26.86	32.31	20.28	0.83	1.10	1.24	12.90	0.89	nd	nd	nd	nd	16.59	0.86

A ratio greater than one means a decrease in DA concentration in whole flesh due to the cooking process whereas a ratio of less than one means an increase
nd not determined
[a]With cooking juice; other bivalve species without cooking juice

Table 10.6 Comparison of (raw toxin concentration/cooked toxin concentration) ratio values for OAs and SPX toxins analysed in mussel matrix obtained for two or three different experiments (depending on bivalve species)

	Raw concentration/Cooked concentration) ratio			
Toxin in bivale species	Exp 1	Exp 2	Exp 3	Mean
OA in Mussel[a]	1.74	1.79	1.34	1.62
OA in Mussel[b]	0.39	0.78	nd	0.58
SPX in Mussel[b]	0.68	0.81	nd	0.75

A ratio greater than one means a decrease in OA or SPX concentration in whole flesh due to the cooking process whereas a ratio of less than one means an increase
nd not determined
[a]With cooking juice
[b]Without cooking juice

geographical area, shellfish consumption by humans and shellfish contamination by phycotoxins to assess exposure of humans to these compounds. The acute- and chronic-exposure assessments made through probabilistic approaches showed that: (i) in terms of acute risk, OAs appear to be a cause for concern for consumers in cases of high contamination levels that may exceed the OA ARfD. For instance, consumers could be exposed to an OA some intake up to nine-fold the ARfD; (ii) about chronic risk, the finding of daily OA intakes close to the ARfD, known to be, by definition, much greater than the TDI, suggests that, among the phycotoxins under study, OA is the most important one to be considered. Moreover, it should be noted that bivalves contain regularly SPX at low concentrations. Chronic and subchronic data on SPX are missing, but in case of (sub)chronic toxicity, SPX exposure should be taken into consideration.

These phycotoxin-exposure assessments were aimed at making a first realistic evaluation of human exposure to phycotoxins. Their interest is in that: (i) they were based on consumption- and contamination-data in the same subpopulation and area, (ii) the recreational shellfish harvesters under study constitute an at-risk sub population, (iii) inter-species variability in contamination and consumption data was taken into account, (iv) the impact of cooking process on phycotoxin levels was also considered.

To gain a comprehensive insight into this health issue, in the future, it would be worth: (i) increasing the number of shellfish species to be investigated, (ii) considering the contamination data relative to recorded cases of intoxication after ingestion by fish and crustaceans, (iii) extending the contamination database to several years and (iv) studying co-exposure to several phycotoxins.

References

Burger J, Sanchez J, Gochfeld M (1998) Fishing, consumption, and risk perception in fisherfolk along an east coast estuary. Environ Res 77:25–35

Counil E, Verger P, Volatier JL (2005) Handling of contamination variability in exposure assessment: a case study with ochratoxin A. Food Chem Toxicol 43:1541–1555

European Food Safety Authority (EFSA) (2008) Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – okadaic acid and analogues. EFSA J 589:1–62

European Food Safety Authority (EFSA) (2009) Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish – domoic acid. EFSA J 1181:1–61

European Food Safety Authority (EFSA) (2010) Scientific Opinion on marine biotoxins in shellfish – cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). EFSA J 2010(1628):1–39

Gagnon F, Tremblay T, Rouette J, Cartier J (2004) Chemical risks associated with consumption of shellfish harvested on the north shore of the St. Lawrence River's lower estuary. Environ Health Perspect 112:883–888

Ifremer (2011) Cahier de procédures et de programmation REPHY 2011. Ifremer, Brest

Kroes R,Müller D, Lambe J, Löwik MRH, Van Klaveren J, Kleiner J et al (2002) Assessment of intake from the diet. Food Chem Toxicol 40:327–385

Leblanc JC (2006) CALIPSO, Fish and seafood consumption study and biomakers of exposure to trace elements, pollutants and Omega-3. AFSSA (French food safety agency). Available: http://www.afssa.fr/Documents/PASER-Ra-Calipso.pdf. Accessed 24 Mar 2010

Lewis SC (1995) Subchronic to chronic exposure extrapolation: toxicologic evidence for a reduced uncertainty factor. Hum Ecol Risk Assess 1:516–526

Mariën K (1996) Establishing tolerable dungeness crab (Cancer magister) and razor clam (Siliqua patula) domoic acid contaminant levels. Environ Health Perspect 104:1230–1236

McCarron P, Hess P (2006) Tissue distribution and effects of heat treatments on the content of domoic acid in blue mussels, *Mytilus edulis*. Toxicon 47:473–479

Munday R (2008) Toxicology of cyclic imines: gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocentrolide, spiro-prorocentrimine, and symbioimines. In: Botana LM (ed) Seafood and freshwater toxins: pharmacology, physiology and detection, 2nd edn. CRC Press/Taylor & Francis Group, Boca Raton, pp 581–594

Picot C (2010) Evaluation du risque lié à l'exposition aux phycotoxines via la consommation de coquillages chez les pêcheurs à pied du Finistère. PhD thesis, Université de Bretagne Occidentale, Brest, France

Picot C, Limon G, Wesolek N, Parent-Massin D, Roudot AC (2012) Domoic acid, okadaic acid and spirolides: inter-species variability in contamination and cooking effects. Food Public Health 2(2):50–57

Picot C, Nguyen TA, Roudot AC, Parent-Massin D (2011a) A preliminary risk assessment of human exposure to phycotoxins in shellfish: a review. Hum Ecol Risk Assess 17:328–366

Picot C, Nguyen TA, Carpentier FG, Roudot AC, Parent-Massin D (2011b) Relevant shellfish consumption data for dietary exposure assessment among high shellfish consumers, Western Brittany, France. Int J Environ Health Res 21:86–105

Toyofuku H (2006) Joint FAO/WHO/IOC activities to provide scientific advice on marine biotoxins(research report). Mar Pollut Bull 52:1735–1745

Tressou J, Crépet A, Bertail P, Feinberg MH, Leblanc JC (2004) Probabilistic exposure assessment to food chemicals based on extreme value theory. Application to heavy metals from fish and sea products. Food Chem Toxicol 42:1349–1358

US Environmental Protection Agency (USEPA) (2000) Guidance for assessing chemical contaminant data for use in fish advisories, vol 2, 3rd edn, Risk Assessment and Fish Consumption Limits, EPA 823-B-94-008. US Environmental Protection Agency, Office of Water, Washington, DC

Van Dolah FM (2000) Marine algal toxins: origins, health effects, and their increased occurrence. Environ Health Perspect 108:S133–S141

Vidal A, Correa J, Blanco J (2009) Effect of some habitual cooking processes on the domoic acid concentration in the cockle (Cerastoderma edule) and Manila clam (Ruditapes philippinarum). Food Addit Contam 26:1089–1095

World Health Organization (WHO) (1985) Guidelines for the study of dietary intakes of chemical contaminants. WHO Offset Publication n° 87, 104p

World Health Organization (WHO) (1995) GEMS/Food-EURO second workshop on reliable evaluation of low-level contamination of food, Kulmbach, EUR/ICP/EHAZ.94.12/WS04-FSR/KULREP95

Chapter 11
Vibrio parahaemolyticus Risk Management in Japan

Hajime Toyofuku

Introduction

In Japan, raw consumption of fish and shellfish is common; therefore, the risk of gastroenteritis caused by *Vibrio parahaemolyticus* (*Vp*) is high. In the first half of the 1980s, *Vp* was identified as the causal agent of more than half of the reported cases and outbreaks of foodborne illnesses in Japan, and it remained the most common causal agent of foodborne illnesses in subsequent years with its incidence peaking in 1998 (839 outbreaks and 12,318 cases). Since 1998, however, the numbers of both outbreaks and cases of *Vp* infection have decreased, with 14 outbreaks and 280 cases reported in 2009.

Purpose

The purpose of this study is to review available data for foodborne illnesses caused by *Vp*, as published by the Ministry of Health, Labour and Welfare (MHLW), Japan, from 1990 to 2009 and to find out any factors that could be useful to understand the relationship between the decrease in *Vp* outbreaks and risk management tools that were implemented during the same period along with other potential factors.

H. Toyofuku (✉)
Department of International Health and Collaboration, National Institute of Public Health, 2-3-6-Minami, Wako, Saitama 351-0197, Japan
e-mail: toyofuku@niph.go.jp

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_11,

Methods

The number of cases and outbreaks of *Vp* infection were obtained from the Food Poisoning Investigation Report published by the MHLW, Japan (2000, 2001, 2002, 2003, 2005, 2006, 2007, 2008, 2009, 2010, 2011a, b).

Data on the number of outbreaks caused by different serotypes were obtained from the risk profile prepared by the Food Safety Commission (2012).

Food source attribution obtained through *Vp* outbreak investigations in 2000–2009 was reviewed based on the summary of food poisoning reports published by the MHLW.

The implemented risk management strategies were identified based on the regulations and directives published by the MHLW.

Results

Since Japanese people eat raw seafood, they are often exposed and are frequently infected by *Vp*. Therefore, *Vp*-related food poisoning has been the most frequently reported foodborne illness, particularly during the first half of the 1980s. Figure 11.1 shows the number of outbreaks and cases of *Vp* infection. Beginning in 1993, the numbers of cases and outbreaks of *Vp*-related food poisoning increased and peaked in 1998 (837 outbreaks and 12,318 cases); conversely, since 1999, both numbers have decreased significantly, and in 2009 only 14 outbreaks and 280 cases were reported. The number of outbreaks and cases of *Vp* infection in 2009 was only 1.6 and 2.3 % of those in 1998, respectively.

Table 11.1 shows the number of *Vp* outbreaks by serotype from 2000 to 2009. Unfortunately, serotype data are only available from 2000. The percentage of *Vp*-related outbreaks caused by the pandemic strain O3:K6 among serotype-identified *Vp*-related outbreaks ranged between 50 and 80 % (average, 66.7 %) from 2000

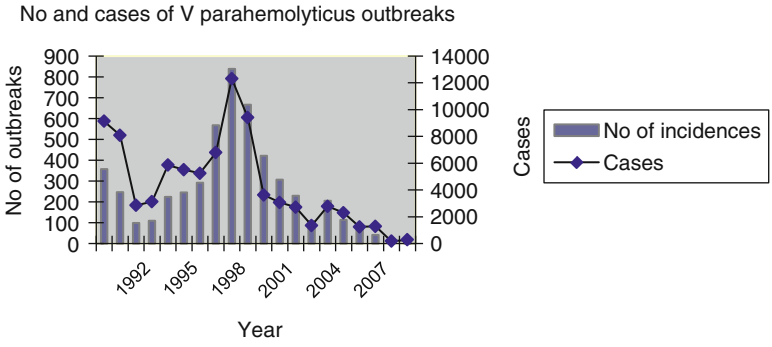


Fig. 11.1 The number of outbreaks and cases of *Vp* infection

Table 11.1 Number of *Vibrio parahaemolyticus* outbreaks by serotype

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	Total	%
No. of incidences	422	307	229	108	205	113	71	42	17	14	1,528	
No. of incidences that serotype identified	260	178	118	60	94	47	24	18	4	5	808	
Serotypes												
O3:K6	163	106	95	36	82	30	14	14	2	5	547	67.7
O1:K25	8	14	11	7	7	3	2	1	0	0	53	6.6
O4:K8	10	6	6	2	3	0	1	2	1	0	31	3.8
O4:K68	2	18	0	2	2	5	1	0	0	0	30	3.7
O3:K29	1	0	12	1	0	2	1	0	0	0	17	2.1
O1:K56	3	2	1	0	0	2	1	0	0	0	9	1.1
O6:K18	1	2	4	2	0	0	0	0	0	0	9	1.1
O1:KUT	2	1	2	1	0	1	0	0	0	0	7	0.9
O3:KUT	0	4	1	1	1	0	0	0	0	0	7	0.9
O3:K5	1	1	0	2	0	1	0	0	0	0	5	0.6
O4:K9	1	1	0	1	1	0	1	0	0	0	5	0.6
O5:K15	0	0	2	0	1	1	0	1	0	0	5	0.6

Adapted from Food Safety Commission (2012)

to 2008. In addition to O3:K6, the pandemic group included O3:K6 serovariants, such as O3:K6,O1:K25, O1:K56, O1:KUT, O3:K5, O3:KUT, O4:K8, O4:K68, and O6:K18 (Nair et al. 2007), and accounted for 86.4 % of all serotype-identified *Vp*-related outbreaks.

Table 11.2 presents the food sources responsible for *Vp*-related outbreaks, as identified in investigations from 2000 to 2009. Among 780 food source-identified *Vp*-related outbreaks, seafood accounted for 27.5 % of these outbreaks (206 outbreaks). Among seafood-related outbreaks, sashimi, including molluscan shellfish, cooked/processed molluscan shellfish, and cooked/processed crab meat accounted for 27.3, 25.6, and 7.1 % of outbreaks, respectively.

Table 11.3 presents the prevalence of *Vp* in seafood harvested in 2001, 2007, 2008, and 2009. The sampling areas in 2001 and 2007 covered similar areas in five regions in Japan. Sampling in 2008 and 2009 was from retail shops throughout Japan. The prevalence of total *Vp* and the tdh gene in fresh shellfish and fish samples collected and tested in 2001 were 95.4 and 10 % respectively, while those in 2007 were 75.7 and 6.5 %, respectively, and those in 2008 were 90.2 and 6.1 %, respectively (Table 11.3). Compared with the numbers of *Vp* outbreaks (307 in 2001, and 42, 17 in 2007, 2008, respectively), the contributions of both total *Vp* and the tdh gene in the decrease of *Vp* outbreaks were considered to be limited, and the strict temperature control throughout food chain and preventative measures to minimize contamination of seafood, e.g. use of pasteurized seawater, could be more important factors for the reduction of *Vp* outbreaks.

Table 11.4 shows the number of seafood samples in which TDH productivity was confirmed by serotype among the same samples presented in Table 11.3. In 2001, all TDH-expressing strains were O3:K6; however, only one-third of TDH-expressing strains in 2007–2009 were O3:K6.

Table 11.2 Food Source Attribution in *Vp* outbreaks in 2000–2009

Food sources identified by *Vp* outbreak investigations	Number	Outbreaks (%)	Percentage within seafood (%)
Sashimi including Molluscan Shellfish	65	30.50[a]	27.30
Cooked/processed Molluscan Shellfish	61		25.60
Cooked/processed Crab meat	17		7.10
Cooked/processed Fish	11		4.60
Cooked/processed Squids	11		4.60
Cooked/processed Sea Urchin	9		3.80
Sushi	7		2.90
Other seafood	57		23.90
Other food	542	69.5[b]	
Source identified	780	51.05[c]	
Total number of *Vp* outbreaks	1,528		

[a]Percentage of *Vp* outbreaks associated with seafood (238) out of *Vp* outbreaks which sources were identified (780)
[b]Percentage of *Vp* outbreaks associated with non-seafood (542) out of *Vp* outbreaks which sources were identified (780)
[c]Percentage of source identified *Vp* outbreaks (780) out of total number of *Vp* outbreaks (1,528)

Control measures taken by the MHLW were reviewed. In 2001, the MHLW established a labelling requirement which required seafood for raw consumption "shall be labeled for raw consumption purpose". In addition, standard for processing which required water for processing shall be potable, pasteurized seawater or artificial seawater made from potable water. In this case pasteurized seawater shall be *V. parahamolyticus* negative, by e.g. UV irradiation (more than 3 log reduction) (Tsurumi 2001) and storage standards which required fish and shellfish shall be stored at temperature of 10 °C or below (for frozen products: below −15 °C).

The following microbiological limits were also established:

- boiled octopus: *V. parahaemolyticus* negative /25 g
- boiled crab: *V. parahaemolyticus* negative /25 g
- fresh fish and shellfish for raw consumption: MPN 100/g or less
- shucked oyster for raw consumption: MPN 100/g or less
- frozen fish and shellfish for raw consumption purpose: MPN 100/g or less

Furthermore, the guidance for industries and consumers was published as a directive from the Director of the Food Safety Department. The guidance for industries is as follow:

- Seawater used for transportation of harvested fish, shellfish, live fish, and fish tanks and seawater used for washing unprocessed fish, shellfish, and shell-on bivalves should be pasteurized seawater (more than 3 log reduction) (Tsurumi 2001) or seawater which is not contaminated with *V. parahaemolyticus*. During this process, care should be taken to prevent contamination of other products.

Table 11.3 *Vp* isolation from fresh fish and shellfish

	Total *Vp*			*Vp* with *tdh* gene					
Samples	No. of samples	No. of samples from which *Vp* isolated	%	No. of samples	No. of samples from which tdh gene identified	%	No. of samples from which TDH producing *Vp* were isolated	Sampling year	Sampling month
Fresh shellfish and fish	173	165	95.4	329	33	10	11	2001	June–October
	247	187	75.7	247	16	6.5	5	2007	July–December
Bivalve and fresh fish	407	367	90.2	407	25	6.1	6	2008	June–October
Bivalve harvested in Japan	66	58	87.9	66	3	4.5	0	2009	July–December
Bivalve imported	123	106	86.2	123	21	17	7	2009	July–December

Adapted from Food Safety Commission (2012)

Table 11.4 Serotypes of TDH producing *Vp* isolated from fresh fish and shellfish

Sampling year	No. of samples from which TDH producing *Vp* were isolated	Serotype	Samples from which the serotype *Vp* were isolated
2001	11	O3:K6	11
2007	5	O4:K9	1
		O4:K37	1
		O4:K38	1
		O4:KUT	2
		OUT:K37	2
		OUT:K38	1
		OUT: KUT	2
		NT(O3: K6 negative)	1
		subtotal	11
2008	6	O3:K6	2
		O4:KUT	1
		O5:K17	1
		O10: K52	2
		O10: KUT	1
2009	7	O3:K6	4
		O1:KUT	1
		O3:K17	1
		O5: KUT	1
		O8: K21	2
		O10: KUT	1

Adapted from Food Safety Commission (2012)

- Pasteurized seawater and artificial seawater used for processing should be prepared immediately before use, and frequently changed. Re-use should be avoided.
- Fish and shellfish for raw consumption should be kept at a temperature of 4 °C or lower unless the products have a quality problem due to the temperature.
- The shelf-life of packed sushi, which is stored and sold at a temperature of 10 °C or above should be established based on scientific evidence.
- Sushi and sashimi served at restaurants should be served immediately after preparation, and should be consumed within 2 h after exposure to room temperature.

The guidance for consumer is as follow:

- Fresh fish and shellfish without a label 'for raw consumption purpose' should not be consumed raw. During shucking of bivalves for raw consumption, edible parts should be washed thoroughly with potable water.
- Sushi served at restaurants should be consumed as soon as possible.
- Fresh fish and shellfish for raw consumption should be kept at a temperature of 4 °C or lower at home, and should be consumed within 2 h after exposure to room temperature.

These strategies focused on minimizing contamination from environments including seawater and preventing the growth of *Vp* throughout the food chain from harvest to consumption.

Discussion

After 1998, the year in which the numbers of cases and outbreaks of *Vp* infection peaked (839 outbreaks, 12,318 cases), the numbers of both cases and outbreaks of *Vp* infection decreased continuously, reaching 14 outbreaks and 280 cases in 2009. The microbiological limit and processing and storage standards implemented as legal requirements from 2001 onwards, partially contributed to this decline. In addition, efforts to minimize the contamination and growth of *Vp* throughout the food chain could also have contributed to this decline. However, the prevalence of total *Vp* and *tdh*-positive *Vp* in seafood did not show the same drastic changes. At this moment, we do not have concrete data to explain this public health outcome, although it is possible that a new pandemic strain may appear in the future. Thus, we should continue to implement control measures and perform baseline surveillance, both of public health and of seafood to prevent outbreaks of *Vp* infection.

Conclusion

After the incidence of *Vp* foodborne outbreaks peaked in 1998, the numbers of cases and outbreaks of foodborne *Vp* infection decreased. The MHLW implemented several risk management strategies, including the establishment of a microbiological regulatory limit in 2001. Even though the factors responsible for this decrease are not completely understood it is hoped that this low level of public health burden is maintained by implementing risk management strategies throughout the food chain.

References

Food Safety Commission (2012) Risk profile on *Vibrio parahaemolyticus* in fresh seafood [in Japanese]. Food Safety Commission, Tokyo. http://www.fsc.go.jp/sonota/risk_profile/vibrioparahaemolyticus.pdf. Available on 11 Feb 2012

Ministry of Health, Labour and Welfare (MHLW) of Japan (2000) Summary of food poisoning reports in 1998. MHLW, Tokyo [in Japanese]

Ministry of Health, Labour and Welfare (MHLW) of Japan (2001) Summary of food poisoning reports in 1999. MHLW, Tokyo [in Japanese]

Ministry of Health, Labour and Welfare (MHLW) of Japan (2002) Summary of food poisoning reports in 2000. MHLW, Tokyo [in Japanese]

Ministry of Health, Labour and Welfare (MHLW) of Japan (2003) Summary of food poisoning reports in 2001. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2005) Summary of food poisoning reports in 2002. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2006) Summary of food poisoning reports in 2003. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2007) Summary of food poisoning reports in 2004. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2008) Summary of food poisoning reports in 2005. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2009) Summary of food poisoning reports in 2006. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2010) Summary of food poisoning reports in 2007. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2011a) Summary of food poisoning reports in 2008. MHLW, Tokyo [in Japanese]
Ministry of Health, Labour and Welfare (MHLW) of Japan (2011b) Summary of food poisoning reports in 2009. MHLW, Tokyo [in Japanese]
Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA (2007) Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin Microbiol Rev 20(1):39–48
Tsurumi K (2001) Establishment of standards and requirements for seafoods to prevent foodborne infections caused by *Vibrio parahamolyticus* under the Food Sanitation Law. Food Sanit Res 57(7):7–14 [in Japanese]

Chapter 12
Distribution of Cd, Pb, As and Hg in Oyster Tissue, Sediment and Water in Lingayen Gulf, Philippines

Reivin T. Vinarao, Gielenny M. Salem, and Rosario J. Ragaza

Introduction

Seafood safety has become a primary consideration in managing fish and other fish products in the Philippines. Being an archipelagic country, the majority of its fishery resources are derived in major coastal fishing grounds including bays and gulfs. The multitude of food and environmental hazards which may possibly harm living organisms, especially from human sources may come from microbiological and chemical contaminants, the latter including heavy metals. Caution should therefore be employed especially for heavy metals that are capable of being absorbed by fish, shellfish and other consumable aquatic resources which can be vectors of contamination.

Lingayen Gulf is a major traditional fishing ground in the Philippines (Silvestre and Hilomen 2004) that sustains subsistence and commercial fishing, as well as vast aquaculture activities using fish pens, cages and traps. The coastal communities of Lingayen Gulf also support the beach and resort tourism industry of Pangasinan Province. The Gulf is the major source of oysters in the Northern Philippines. Since the start of oyster industry in 1979, major production comes from the municipal farming grounds of Lingayen Gulf and are regularly distributed in Pangasinan and its neighboring provinces (BAS 2009). The oyster industry has continuously provided a cheap source of protein available to a large array of consumers. Hence it is imperative that food safety be one of the primary considerations in managing the industry and the coastal environment where these resources are derived.

R.T. Vinarao (✉) • G.M. Salem • R.J. Ragaza
Post Harvest Research and Development Division, National Fisheries Research and Development Institute, 101 Mother Ignacia Avenue, South Triangle, Quezon City, Philippines
e-mail: rtvinarao@uplb.edu.ph; taikyoku@yahoo.com

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_12,

The Gulf serves as a natural catchment receiving both organic and inorganic loads from farmlands, rural communities and commercial establishments. Two rivers, the Agno and Bued, drain into the Gulf. Although mining is not prevalent along Lingayen Gulf, mineral loads in the sediment are high enough to support a number of small-scale gold panning miners along the coast.

Past management efforts and environmental studies in Lingayen Gulf have concentrated on assessing water quality and productivity status of fisheries (Mines 1986; Calud et al. 1989; Silvestre et al. 1989; McManus and Chua 1990; Hilomen and Jimenez 2001; Hilomen et al. 2002; McGlone et al. 2004; Silvestre and Hilomen 2004); however, no attempts have been made to investigate heavy metal contamination, despite the existence of Philippine laws establishing acceptable limits for exposure and human consumption (DENR 1990; BFAR 2001b, c). Heavy metal contamination in the aquatic environment is of critical concern due to the toxicity and accumulation of metals in aquatic habitats (Ahmad et al. 2010). Once heavy metals are accumulated by aquatic organisms they can be transferred up the food chain (McGlone et al. 2004; Erdogrul and Ates 2006) and increase in concentration through bio-magnifications (Mance 1987; Langston 1990). Even at low levels, long term exposure of humans to Cd is known to cause renal dysfunction (McCluggage 1991; INECAR 2000; EU 2002; Young 2005). Ricklefts (2007) also reported that low concentrations of Hg, As and Pb are toxic to most forms of life. According to Lanphear et al. (2005), low levels of Pb have been reported as an anthropogenic neurotoxicant and are known to mostly affect children. An inverse relationship between blood lead concentrations and intelligent quotient (IQ) score was established; associating Pb with intellectual deficits. In a clinical study conducted by Riddell et al. (2007) in the Philippines, 21 % of 2,861 children living in rural areas had cases of elevated blood-lead levels. Metals in its ionic form such as Cd^{2+}, Pb^{2+}, $Hg^{2+}$and As^{3+} form very stable compounds within the body making them difficult to remove during medical detoxification therapy hence resulting in human poisoning over time (Duruibe et al. 2007).

The threats and hazards on food safety involving heavy metal contamination in the Gulf will always remain unnoticed unless a baseline information and responsive monitoring system is established.

Objective

The objective of the study was to determine the seasonal and temporal concentration of heavy metals Cadmium (Cd), Lead (Pb), Arsenic (As) and Mercury (Hg) in oyster tissues, as they relate to concentrations in the water column and sediments in Lingayen Gulf to establish baseline information for monitoring and designing responsive food safety and environmental management plans.

Method

Study Area

The study area was an embayment of the South China Sea on the northwestern coast of the island of Luzon and consists of coastal areas and tributary waters that are utilized for the aquaculture of milkfish and oysters. Geographical proximity of the oyster farming grounds to industrial and residential sites were considered to be significant as they may affect the amount of heavy metals present in the environment.

Sampling was done at 12 stations in Lingayen Gulf in the province of Pangasinan, Philippines (Fig. 12.1). Approximately 7.5–10 nautical miles distance in between sampling stations was employed to geographically represent the Gulf. Sampling points with commercial oyster growing structures were established using a Global Positioning System (GPS) instrument to accurately locate the sample collection area and to facilitate repetitive sampling (Table 12.1).

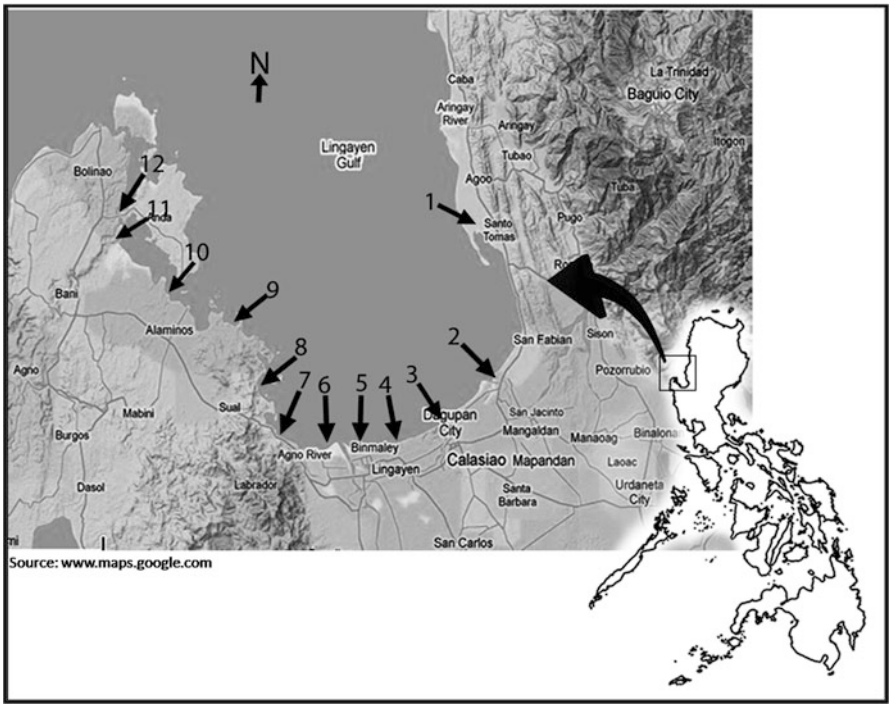


Fig. 12.1 Map of the study site and the 12 sampling stations along Lingayen Gulf

Table 12.1 Participating stations and their geographical location, May 2009 and April 2010

	Sampling area	
Station No.	Adjacent municipality	Geographic location
1	Raois, La Union	16°16′46N; 120°22′22E
2	Cayanga, San Fabian	16°06′73N; 120°23′83E
3	Sobol, San Fabian	16°07′89N; 120°24′45E
4	Binloc, Dagupan	16°06′25N; 120°22′60E
5	Lucao, Dagupan	16°17′52N; 119°54′02E
6	Manat, Binmaley	16°00′88N; 120°17′15E
7	Sagur, Labrador	16°03′08N; 120°07′07E
8	San Jose, Labrador	16°02′00N; 120°08′60E
9	Centro, Sual	16°03′54N; 120°05′52E
10	Mona, Alaminos	16°13′11N; 119°58′56E
11	Tambac, Bani	16°16′00N; 119°55′33E
12	Anda-Bolinao Boundary	16°17′52N; 119°54′02E

Sample Collection

The first sampling, representing the monsoon season, was conducted in May 2009, while the second, representing the pre-monsoon season, was completed in April 2010, prior to the onset of heavy rains.

Oyster Tissue

Cultivated oysters were handpicked (gloved hands) directly from the oyster growing strings and stake poles with the aid of knife. Samples were transported and preserved following the method described by BFAR (2001a). Twenty-five to thirty (25–30) pieces of shelled oysters, with nearly equal size per station, were packed in a 26.8×27.3 cm Ziploc^{TM} plastic bag and transported in an icebox to the laboratory for cleaning and shucking. Samples were brushed and initially rinsed with seawater (collected at the sampling site) until all attached particulate matter was removed, then finally with distilled water. The cleaned oysters were then shucked using shucking knives. Two hundred grams of soft tissue from the shucked oysters were double-packed (inner stomacher bag; outer Ziploc^{TM} bag) and preserved at 4 °C temperature until analyzed.

Sediment

Two hundred grams (200 g) of sediment samples were collected at the shallow waters near the banks of the sampling stations using a samples. All samples were taken from the top layer to a depth of over 30 cm, at stations where the flow rates

were low and sedimentation was assumed to occur (Sakai et al. 1986; Subramanian et al. 1987). Sampling tools were washed with fresh water and dried before the next sample was collected. Soil samples were packed in a 16.5 × 14.9 cm Ziploc^{TM} bag, air-dried in the laboratory at room temperature and ground to a fine mixture using a mortar and pestle before sieving with a 2 mm mesh to obtain the 0–75 μm sediment fraction (clay/silt fraction).

Water

A series of one liter (1.0 L) water samples was collected at each sampling site at 5–10 cm from below the surface. Collected water samples were kept in pre-labeled polyethylene plastic containers and maintained at 4 °C in coolers.

Determination of Heavy Metal Concentrations

Metal concentrations in water, digested oyster tissues, sediment extractions and digested sediment samples were determined using atomic absorption spectroscopy (Analytik Jena Vario 6 AAS, Germany) for analysis of Hg and As, while inductively coupled plasma-atomic emission spectrometry (Jobin Yvon Ultima 2 ICP, France) was used in the determination of Cd and Pb metals. All working standards and samples were quantified for As and Hg using Manual Hydride Generation Technique – AAS and Cold Vapor Technique Flow Injection Mercury System, respectively.

Heavy Metal Analysis in Oyster Tissues

For Pb, Cd and As analysis using USEPA 6010 method of preparation (USEPA 2000), oysters samples were homogenized and digested with nitric acid (HNO_3) then with hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl). The digestate was then filtered and diluted to 100 mL using distilled water. Mercury levels were analyzed according to AOAC method 977.15 (AOAC 2005). In Hg analysis, homogenized samples were digested with sulfuric acid (H_2SO_4) and HNO_3. The digestate was treated with H_2O_2 and diluted with H_2SO_4 and HNO_3 mixture.

Heavy Metal Analysis in Sediments

A portion of soil sample was digested with HNO_3, then with H_2O_2 and HCl. The digestate was filtered, diluted to 100 mL with distilled water following USEPA 6010 method (USEPA 2000) and injected into the appropriate instruments for Pb, Cd and As quantitation. In the determination of Hg, a portion of soil sample was

digested with aqua regia and potassium permanganate ($KMnO_4$). Distilled water was added to the digestates as stated in USEPA 7471 (USEPA 1994). Sodium chloride-hydroxylamine was added prior to quantification using the Cold Vapor Technique Flow Injection Mercury System.

Heavy Metal Analysis in Water

The analyses of metals in seawater was based on the American Public Health Association (APHA) method (APHA 1980). The water samples were digested with different acids and reagents: HNO_3 for Pb (APHA 3030E); HNO_3 and HCl for Cd (APHA 3030F); H_2SO_4 and potassium persulfate ($K_2S_2O_8$) for As (APHA 3114B); and H_2SO_4, HNO_3, $KMnO_4$ and $K_2S_2O_8$ for Hg (APHA 3112B). They were filtered using prewashed and pre-weighed membranes and diluted to 100 mL with distilled water. Water samples were analyzed following the same analytical technique as used for oyster tissues and sediments.

Quality Control

Standard stock solutions were prepared from ultra-high purity grade chemicals ($\geq$99.99 % pure). All working standards and samples were prepared in duplicates and average readings were taken from three instrument readings for all the heavy metals. Operational conditions such as limit of detection were adjusted according to the instrument and method requirements. The duplicate relative percentage difference (RPD) for all laboratory duplicates was $\leq$20 %. Concentrations were computed by the standard calibration method. Calibration curves were composed of at least three standard concentrations where computation for linearity was based. The instrument detection limits were determined from three standard deviations obtained from seven blanks. Percent recoveries of oysters, sediment and water samples are in the range of 80–120 %. Regular measurements of laboratory blanks and spikes for all metals and proficiency tests for water were done to assure quality control of the analysis. Blank digestion was also performed to quantify possible contamination during sample preparation and analysis.

Statistical Analysis

Within and between group differences were assessed using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (Bewick et al. 2004). Relationships between the heavy metal concentration in oyster tissues, sediments and water were evaluated by linear regression and by determination of Pearson correlation coefficients. All errors were calculated at 95 % confidence level.

Results and Discussions

Results of metal concentrations in oysters, water and sediments in Lingayen Gulf are presented in Table 12.2.

Heavy Metals in Oyster Tissues

Cultivated oysters have been used for studies on heavy metal determination in coastal ecosystems primarily due to their abundance, long life span and ease in collection (Frazier 1979; Schumacher and Domingo 1996; Al-Madfa et al. 1998; Ruelas-Inzanza and Paez-Osuna 1998). As a sedentary filter-feeding mollusc, oysters are good biological indicators for monitoring chemical concentrations at fixed locations (Phillips 1977; Rico and Ruiz 2001; de Astudillo et al. 2005). The accumulated metal concentrations in oyster provide integrated measures of the amount of bioavailable trace metals over time (Silva et al. 2001). They provide a truer indication of metal pollutants in aquatic habitats than do water and sediment measurements alone (Phillips and Rainbow 1994; Rainbow 1995).

In this study, traces of Cd, Pb and As were detected to have accumulated in oyster tissue samples (Table 12.2). Both Cd and As were consistently detected in both monsoon (2009) and pre-monsoon (2010) seasons while Pb was observed only during the pre-monsoon period. Among the metals detected in oyster tissues, concentrations of Cd (nd-1.50 mg/kg) were the highest, followed by Pb (0.55 mg/kg) and As (nd-0.015 mg/kg). Concentrations of Hg in oyster tissue in both seasons were below the limit of detection (0.04 mg/kg) and were reported as not detected (Fig. 12.2).

Cadmium accumulation in oyster tissue ranged from nd-0.749 mg/kg during the monsoon season (May 2009) and nd-1.50 mg/kg during the pre-monsoon season (April 2010). Six stations, two (stations 4 and 10) and four stations (stations 1–4), from monsoon and pre-monsoon exceeded the permissible Cd levels set by the Philippines for oyster (0.50 mg/kg) standards (BFAR 2001b, c). The high level of Cd concentration detected in oysters can be associated with the natural ability of molluscs to exhibit Cd accumulation (Frazier 1979; Ke and Wang 2001). That

Table 12.2 Summary of heavy metal traces in Lingayen Gulf, May 2009 and April 2010

	Oyster tissue, mg/kg		Sediment, mg/kg		Water, mg/L	
Metals	a	b	a	b	a	b
Cadmium	nd-0.749	nd-1.50	nd	nd	nd-0.092	nd
Arsenic	nd-0.007	nd-0.015	nd-6.50	nd-1.1	nd	nd
Mercury	nd	nd	nd	nd-0.30	nd	nd
Lead	nd	nd-0.55	nd-33.90	nd-17.10	nd	nd

nd not detected
[a]Sample collection done in May 2009 (monsoon)
[b]Sample collection done in April 2010 (pre-monsoon)

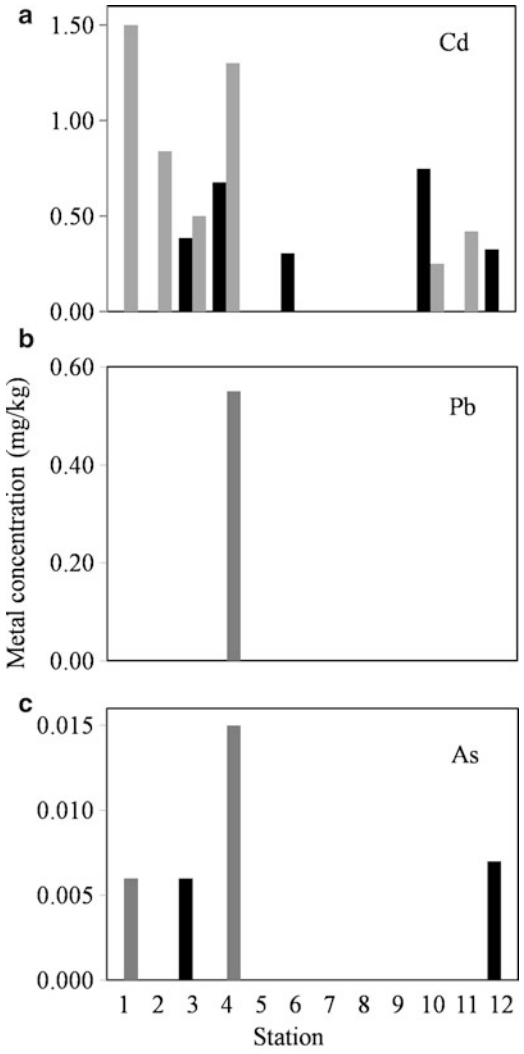


Fig. 12.2 Concentrations of (**a**) Cd, (**b**) Pb and (**c**) As in oyster tissue collected during monsoon (■ 2009) and pre-monsoon (□ 2010) seasons

levels of Cd in oyster tissue were higher during pre-monsoon may partially be related to higher temperature during the summer season. This observation was supported by Zaroogian and Cheer (1976) who reported that Cd accumulation from an experimentally contaminated environment didn't occur until temperatures reached greater than 15 °C. The notably higher concentration of Cd in oyster tissues observed during the pre-monsoon season may also be attributed to the magnitude of anthropogenic contribution of Cd in the study area. However, this hypothesis is yet to be verified through a follow-up study to identify the anthropogenic factors contributing to the observed results.

Data from the monsoon season revealed lower concentrations as compared to the pre-monsoon season which may be an effect of water influx during the sampling

period (Ferreira et al. 2005; Ahmad et al. 2010). It may be that some Cd metals present in the environment were dispersed due to intensive rains and strong water currents. Being a sedentary organism, oysters do not have the ability to move away and search for food. The strong water currents during monsoon season thus became a major contributory factor that hinders the availability and accumulation of any particulate matter to oysters. However, other research findings are completely opposite to the findings to this study, where Cd uptake increased significantly with decreasing salinity (Mackay et al. 1975; Ke and Wang 2001).

Although relatively low (upper limit: 0.007 mg/kg), mean As concentrations (0.01 mg/kg) were detected in oyster tissues. Results of this study were several hundred times lower than the average value obtained for the total arsenic concentration (15.17 mg/kg) in oysters in Taiwan during rainy seasons (Hsiung and Huang 2006). At present, the Philippines does not have standard limit set for As concentration in oyster tissues. However, when comparing this with the established standards of Australia and New Zealand (AUSAID 2007) which is 1.0 mg/kg for oysters, the values detected in the Philippines are comparatively lower. The concentration differences of As collected during the pre-monsoon and monsoon season were not significant ($p > 0.05$). The capacity of oysters to accumulate As in their system and the availability and abundance of As in oyster habitat can be among the factors that contributed to the levels detected.

Only one station (station 4) during the pre-monsoon season yielded a Pb value (0.55 mg/kg) for oyster tissue within our methodology's Limit of Detection (LOD) of 0.50 mg/kg. Incidentally, under the Philippine standards (BFAR 2001c), this value is just beyond the acceptable limit (0.50 mg/kg) that is fit for human consumption. This observation supports the fact that Pb does not usually bioaccumulate in most organisms except for filter-feeding organisms like mussels and oysters as they often possess special metal binding proteins that remove metals from general distribution in their organs (WHO 1989, 1995).

Mercury was not detected in oyster tissue samples within the machine's LOD (0.04 mg/kg). This basically implies that oysters in Lingayen Gulf are relatively safe from hazards due to Hg. However, caution should be employed in location specific areas (stations 2 and 12) with notably high concentration of Hg in the sediment because this could possibly be absorbed by oysters and retained in their system. As discussed by Shulkin et al. (2003), oysters have the capacity to retain low concentration of heavy metals making them suitable indicators of low to moderate contamination.

Heavy Metals in Sediments

Sediment samples were analyzed and found to be contaminated with Pb (nd-33.90 mg/kg), As (nd-6.50 mg/kg) and Hg (nd-0.30 mg/kg). Although Cd was not detected. The magnitude of distribution was described as Pb > As > Hg and was found to be significantly higher in the monsoon season than in the pre-monsoon season ($p < 0.05$) (Table 12.2 and Fig. 12.3).

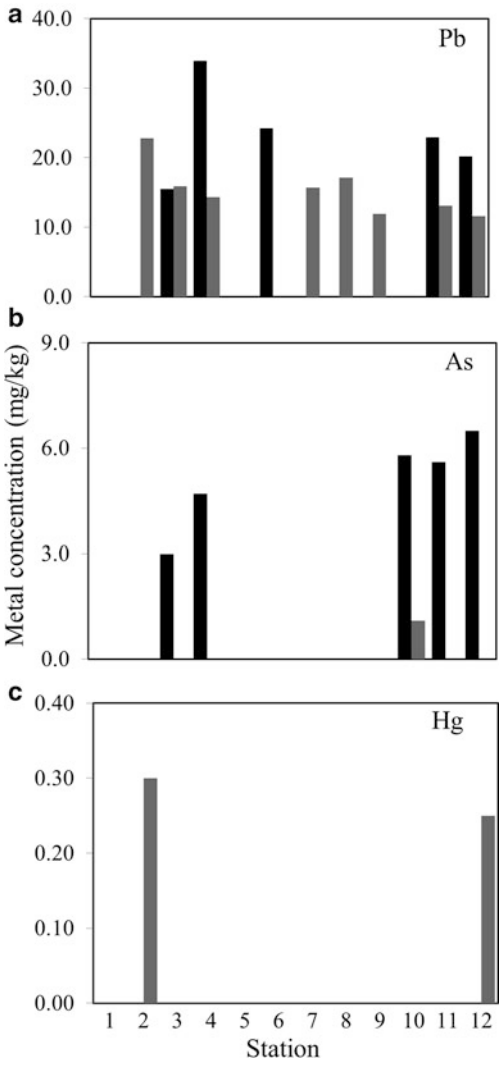


Fig. 12.3 Concentrations of (**a**) Pb, (**b**) As and (**c**) Hg in sediment during monsoon (■ 2009) and pre-monsoon (□ 2010) seasons

The significantly higher traces detected in the Gulf during the monsoon season may be brought about by run-off which transports metal contaminants from surrounding environment as a result of increased sedimentation. The strong water movement caused by increased run-offs may have also exposed the deposited heavy metals at the surface of the sea floor making more available during the sample collection period.

Results in Pb levels in this study are opposite to the study conducted by Ahmad et al. (2010) in Buriganga River, Bangladesh wherein higher Pb levels (upper limit: 77.13 mg/kg) in sediment samples were detected during the pre-monsoon season.

Heavy metals, either of natural or anthropogenic origin tend to be adsorbed to silt particles in sediments (Gonzalez et al. 1999; Millward et al. 1999; de Astudillo

et al. 2005) so they can act as a sink to pollutants in bodies of waters like rivers and creeks (USEPA 1992). The relatively high Pb level in stations 2, 4 and 6 in southeastern side and stations 11 and 12 in the northwestern side of Lingayen Gulf can be due to anthropogenic activities conducted in the coastal communities near the sampling station. The following sites are located closest to the center of aquaculture activities in the province of Pangasinan which covered the municipalities of Dagupan, Lingayen and Binmaley (southeastern side) and Bolinao and Anda (northwestern side). In a survey conducted by Palma (1989) on the patterns and aquaculture practices in the coastal municipalities adjoining Lingayen Gulf she documented that, of the 13, 452 total hectarage of brackish water fishponds in the Gulf, 69 % were located in these five municipalities. The site is also proximate to two (Dagupan and Alaminos) of the four cities of Pangasinan which are the province's centers of commerce and trade. Further, the Pb traces can be associated with lead-based gasoline residues from the fishermen's motorized boat, which is used for daily transportation and fishing operations. Tang et al. (2008) suggested that anthropogenic-sourced Pb can be derived from lead sulfide ore deposits and is released into the environment through the combustion of leaded gasoline and as a by-product of industrial lead use. It was also suspected that damaged and lost or unrecovered lead sinkers used in major fishing gears during fishing operations in the Gulf to have partially contributed to the traces detected.

Total As species were determined in the study and significant ($p < 0.05$) levels were detected in sediments for both monsoon and pre-monsoon seasons. With a rapid growth in population, several studies reported that most As contaminations were due to anthropogenic pollution (Lin and Hsieh 1999). Stations (3, 4, 10, 11, and 12) yielding high As traces comes mostly from first class or urbanized municipalities. These areas are also the center of aquaculture activities in Lingayen Gulf (Palma 1989). Population statistics show that 17.13 % (453,965) of the total population (2,645,395) from 48 municipalities in Pangasinan resides in these five municipalities (NSO 2007).

Traces of Hg on the other hand were only observed in sediment samples during pre-monsoon and contamination was found to be location specific suggesting that the values observed in the two distinctly separate stations (2 and 12) were anthropogenic in nature. The bulk of fish cages and fish pen aquaculture activities were concentrated within the vicinities of stations 2, 3 and 4 in southeastern and stations 10, 11 and 12 in northwestern side of Lingayen Gulf. Some of the Hg traces may come from impurities from pelleted commercial feeds that were used in the fish farms. Seasonal increase in fish cage and fish pen operations in the Gulf were evident during pre-monsoon season, thus more feed load inputs during these months are expected. Monsoon season has also the lean months for aquaculture activities in the Gulf since frequent flooding and strong water current during this period can destroy the fish farm structures. The observed presence of small-scale gold panning miners in the vicinities of stations 3 and 4 which are believed to be using Hg to extract gold from ore deposits can be an evidence of location specific anthropogenic activity accounting for the values detected.

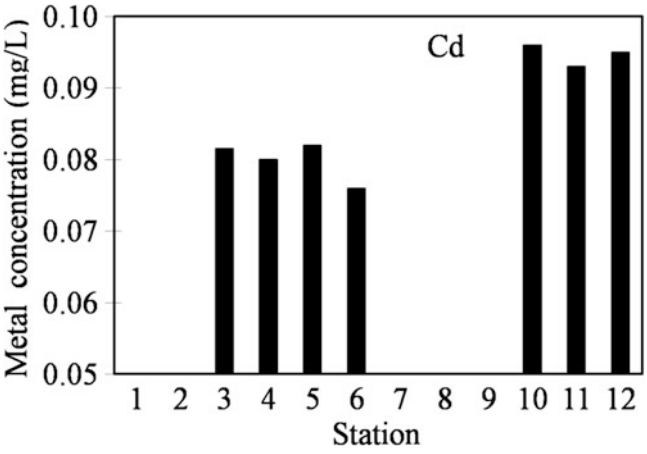


Fig. 12.4 Cadmium concentration in water collected during the monsoon (■ 2009) season. No traces of Cd were detected during the pre-monsoon (■ 2010) season

Heavy Metals in Water

The only contaminant found in the waters of Lingayen Gulf was Cd (Fig. 12.4). Most of these contaminations occurred during monsoon season where all stations yielding positive results exceeded the acceptable limits in Philippine standards (0.01 mg/L) that is suitable for propagation, survival and harvesting of shellfish for commercial purposes (DENR 1990).

This result paralleled the research conducted by Krissanakriangkrai et al. (2009) that reported significantly higher levels of Cd during rainy than dry seasons ($p < 0.001$). Cadmium levels detected in the water column might have been a result of the influx of heavy rains resulting in fluctuations of pH and salinity which, as discussed by Apeti et al. (2005), Goyer (1996) and Hamelink et al. (1994), usually render the metals bioavailable. It is speculated that some of the Cd traces may come from impurities in fertilizers that were used in land agriculture, mostly rice fields and other effluents from the environment containing Cd that are transported to the Gulf through run-off. In general, Cd levels in water during the pre-monsoon season were present with minimal variations.

Spatial distributions were not significantly different between sampling locations ($p > 0.05$). Cadmium is also released to the environment from point sources, such as industrial discharges.

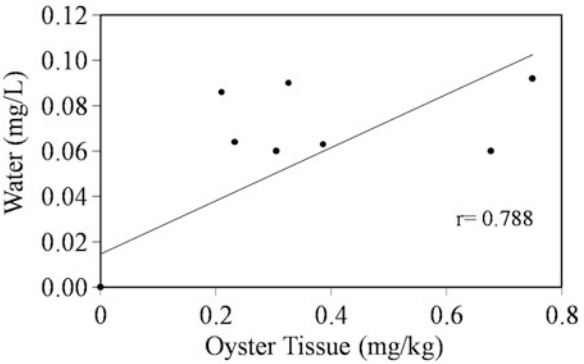


Fig. 12.5 Correlation between concentration of Cd in oyster tissue and water during monsoon season, May 2009

Heavy Metal Relative Abundance and Distribution

Significant levels ($p < 0.05$) of Cd were detected in oyster tissues but not in water ($p > 0.05$) and sediment ($p > 0.05$) samples. These high levels are attributable to the high Cd-binding capability of fish and oysters (Frazier 1979; Krissanakriangkai et al. 2009) in their muscle tissues which may have accumulated over time. The nil Cd levels in sediment and water in this study may be due to the strong water flow along the gulf and tributary waters where samples were derived during sample collection.

The relatively high concentrations of Pb and As in the sediment but low accumulation in oyster tissues study suggest that oysters have low Pb and As binding capacity.

Relationship Between Heavy Metals in Oyster Tissues to Sediment and Water Column

Cadmium was detected in oysters in 2009 and 2010 (Fig. 12.2a) but it was only during monsoon (2009) season that a strong and positive correlation ($r = 0.788$) of the metal concentrations between water and oysters (Fig. 12.5) was established. Higher average concentrations of Cd in oysters are evident during pre-monsoon season (0.80 mg/kg) than monsoon season (0.049 mg/kg) when waters are calm and water flow tends to be slow.

There was deposition of Pb in the sediment samples in both seasons but only one station showed Pb uptake (0.55 mg/kg) in oyster tissue during pre-monsoon season.

Strong correlation ($r = 0.807$) between As levels in sediments and oysters is evident in monsoon season (Fig. 12.6a) while a weak correlation ($r = -0.241$) during pre-monsoon season (Fig. 12.6b). This suggests that the uptake of As in oyster tissues varies depending on the season and can be assisted by water movement.

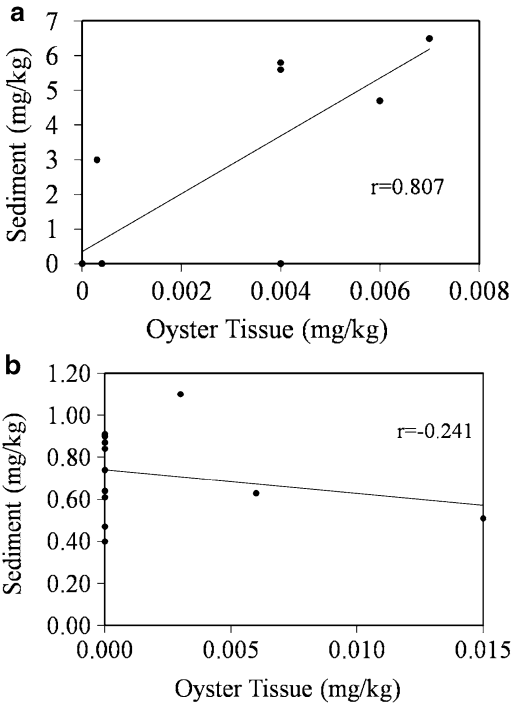


Fig. 12.6 (**a**) Correlation between concentration of As in oyster tissue and sediment during monsoon season, May 2009. (**b**) Correlation between concentration of As in oyster tissue and sediment during pre-monsoon season, April 2010

Conclusion

Levels of Cd detected in oyster tissues in monsoon and pre-monsoon seasons, but low levels in sediments and water, are evidence of the bio-accumulative capacity of oysters with Cd. This may be due to the high Cd-binding capability of molluscs, among which are oysters, as supported by earlier research. The significantly higher concentrations of Cd in oysters in selected stations had surpassed the acceptable limits (>0.50 mg/kg) for human consumption which imply a potential threat and hazard to public health.

Traces of Pb were also present in sediment and water column in two seasons; however, it did not easily accumulate in oysters regardless of season. Possible sources and factors of these contaminations are point sources like the use of motorized boats in fishing, Pb sinkers used in major fishing gears and other coastal community activities and non-point sources such as industrial run-offs and water movement. Although previous research has shown that sediment contamination, even at low concentration, is a prerequisite to oyster contamination, oyster samples in the Lingayen Gulf did not give significant levels of Hg and As. Lead content was found to be significant at only one station. In this study, some metals such as As and Hg are present in sediments but not in water and oyster tissues.

Movement (inflow and outflow) of seawater was not discussed and correlated in detail in this study. Hence further research correlating heavy metal concentrations and the dynamics of water flow is highly recommended. Drainage patterns and accessibility of bodies of water should also be studied to determine the point and non-point sources of heavily-contaminated areas. Studies to lower heavy metal levels in oysters can also be done to determine practices or methods to be employed so these organisms may be safely used for public consumption.

Acknowledgement The authors extend their sincerest gratitude to Dr. Demian Willette of the Old Dominion University ctPIRE Project for reviewing and giving invaluable comments to the paper. Our gratitude is also extended to Undersecretary Segfredo R. Serrano of the Policy, Planning, Research and Development and Regulation, Department of Agriculture (DA); Director Nicomedes P. Elleazar, DA-Bureau of Agricultural Research (BAR); Hon. Leopoldo N. Bataoil, House of Representative, 2nd District of Pangasinan and FAO for the partial travel grants to the first author's attendance to the 8th ICMSS held at Prince Edward Island, Canada. We also express our appreciation to Dr. Westly R. Rosario of the DA-Bureau of Fisheries and Aquatic Resources (BFAR), National Integrated Fisheries Technology Development Center (NIFTDC) for his initiatory efforts in conceptualizing this research; Ms. Adoracion V. Obinque and Ms. Virginia Delos Santos in assisting the researchers in sample collection and to Mr. Arvin Joseph M. Dimaano, DA-National Fisheries Research and Development Institute for doing some of the figures. This study was supported by the research fund of the National Fisheries Research and Development Institute, Philippines.

References

Ahmad MK, Islam S, Rahman S, Haque MR, Islam MM (2010) Heavy metals in water, sediment and some fishes of Buriganga River. Bangladesh Int J Environ Res 4(2):321–332

Al-Madfa H, Abdel-Moati MAR, Al-Gimaly FH (1998) *Pinctadaradiata* (Pearl oyster): a bioindicator for metal pollution monitoring in the Qatari Waters (Arabian Gulf). Bull Environ Contam Toxicol 60:245–251

American Public Health Association (APHA) (1980) Standard methods for the examination of water and wastewater, 15th edn. American Public Health Association, American Waterworks Association, and Water Pollution Control Federation, Washington, DC, p 1134

Apeti DA, Robinson L, Johnson E (2005) Relationship between heavy metal concentrations in the American oyster (*Crassostrea virginica*) and metal levels in the water column and sediment in Apachicola Bay. Fla Am J Environ Sci 1(3):179–186

Association of Official Analytical Chemists (AOAC) (2005) Method 977.15 for mercury analysis in fish using alternative flameless atomic absorption spectrophotometric method. In: Official methods of analysis of the AOAC International, 18th edn. AOAC International, Gaithersburg, p 36

Australian Aid for International Development (AUSAID) (2007) Fish and fish products. Asean guide. A guide to the identification of control of food safety hazards in the production of fish and fish products in ASEAN region. Australian Government, AUSAID, Asean Secretariat, Canberra

Bewick V, Cheek L, Ball J (2004) Statistics review 9: one-way analysis of variance. Crit Care 8(2):130–136. doi:10.1186/cc2836

Bureau of Agricultural Statistics (BAS) (2009) Fisheries statistics of the Philippines 2004–2006. Department of Agriculture-Bureau of Agricultural Statistics, Quezon City

Bureau of Fisheries and Aquatic Resources (BFAR) (2001a) Guidelines on the production, harvesting, handling and transportation of shellfish for implementation of the local government. Fisheries Administrative Order No. 209. S. 2001. DA-BFAR, Quezon City, Philippines

Bureau of Fisheries and Aquatic Resources (BFAR) (2001b) Rules and regulations on the exportation of fresh, chilled and frozen fish and fishery/aquatic products. Fisheries Administrative Order No. 210. S. 2001. DA-BFAR, Quezon City, Philippines

Bureau of Fisheries and Aquatic Resources (BFAR) (2001c) Requirement for pre-processing and processing plants, the SSOP thereof and the processing and quality requirement for shellfishes. Fisheries Administrative Order No. 210. S. 2001. DA-BFAR, Quezon City, Philippines

Calud A, Rodriguez G, Aruelo R, Aguilar G, Cinco E, Armada N, Silvestre G (1989) Preliminary results of a study of the municipal fisheries in Lingayen Gulf, pp 3–29. In: Silvestre G, Miclat E, Chua TE (eds) Toward sustainable development of the coastal resources of Lingayen Gulf, Philippines. ICLARM conference proceedings, Bauang, La Union, Philippines, 17, 200pp

de Astudillo LR, Yen IC, Bekele I (2005) Heavy metals in sediments, mussels and oysters from Trinidad and Venezuela. Int J Trop Biol 53(1):41–53

Department of Environment and Natural Resources (DENR) (1990) Revised water usage and classification/water quality criteria amending section nos. 68 and 69, chapter III of the 1978 NPCC rules and regulations. DAO 34, S. 1990. DENR, Quezon City, Philippines

Duruibe JO, Ogwuegbu MOC, Egwurugwu JN (2007) Heavy metal pollution and human biotoxic effects. J Phys Sci 2(5):112–118

Erdogrul O, Ates DA (2006) Determination of cadmium and copper in fish samples from Sir and Menzelet. Environ Monit Assess 117:281–290

European Union (2002) Heavy metals in wastes, European Commission on Environment. http://ec.europa.eu/environment/waste/studies/pdf/heavy_metalsreport.pdf. 14 June 2012

Ferreira AG, Machado AL, Zalmon IR (2005) Temporal and spatial variation on heavy metal concentrations in the oyster Ostreaequestris on the northern coast of Rio De Janeiro State, Brazil. Braz J Biol 65(1):67–76

Frazier JM (1979) Bioaccumulation of cadmium in marine organisms. Environ Health Perspect 28:75–79

Gonzalez H, Pomares M, Ramirez M, Torres I (1999) Heavy metals in organisms and sediments from the discharge zone of the submarine sewage outfall of Havana City Cuba. Mar Pollut Bull 38:1048–1105

Goyer RA (1996) Toxic effects of metals. In: Casarett & Doull's toxicology the basic science of poisons, 5th edn. McGraw-Hill, New York, pp 696–721

Hamelink JL, Landrum PF, Bergman HL, Benson WH (1994) Bioavailability: physical, chemical and biological interactions. Lewis Publishers/CRC Press, Boca Raton

Hilomen VV, Jimenez LF (2001) Status of fisheries in Lingayen Gulf (Appendix 1). In: McGlone M, Villanoy C (eds) Resource and social assessment of Lingayen Gulf. Project report submitted to the Fisheries Resource Management Project, Department of Agriculture. Marine Science Institute, University of the Philippines and the Marine Environment and Resources Foundation, Quezon City, Philippines

Hilomen VV, Licuanan W, Alino P, Jimenez L (2002) Status of the fisheries resources in Lingayen Gulf: erasing the pressure and enhancing the resources. Paper presented at the National Conference on Fisheries Resource and Social Assessments, Development Academy of the Philippines, Tagaytay City. Fisheries resource management project, Department of Agriculture, Quezon City, Philippines

Hsiung TM, Huang CW (2006) Quantitation of toxic arsenic species and arsenobetaine in Pacific oysters using an off-line process with hydride generation-atomic absorption spectroscopy. J Agric Food Chem 54(7):2470–2478, Study supported by the National Science Council of the Republic of China

Institute of Environmental Conservation and Research (INECAR) (2000) Position paper against mining in Rapu-Rapu. Published by INECAR, Ateneo de Naga University, Philippines. www.adnu.edu.ph/Institutes/Inecar/pospaper1.asp. 14 June 2012

Ke C, Wang W-X (2001) Bioaccumulation of Cd, Se and Zn in an estuarine oyster (*Crassostrea rivularis*) and coastal oyster (*Saccostrea glomerata*). Aquat Toxicol 56:33–51

Krissanakriangkai O, Supanpaiboon W, Juwa S, Chaiwong S, Swaddiwudhipong W, Anderson KA (2009) Bioavailable cadmium in water, sediment and fish in a highly contaminated area on the Thai-Myan May border. Thammasat Int J Sci Technol 14(4):60–68

Langston WJ (1990) Toxic effects of metals and the incidence of marine ecosystem. In: Furness RW, Rainbow PS (eds) Heavy metals in the marine environment. CRC Press, New York, p 256

Lanphear BP, Hornung R, Khoury J, Yolton K, Baghurst P, Bellinger DC, Canfield RL, Dietrich KN, Bornschein R, Greene T, Rothenberg SJ, Needleman HL, Schnaas L, Wasserman G, Graziano J, Roberts R (2005) Low-level environment lead exposure and children's intellectual function: an international pooled analysis. Environ Health Perspect 113(7):894–899

Lin S, Hsieh IJ (1999) Occurences of green oyster and heavy metals contaminant levels in the Sien-San area. Taiwan Mar Pollut Bull 38:960–965

Mackay NJ, Williams RJ, Kacprzac JL, Kazacos MN (1975) Heavy metals in cultivated oysters (*Crassostrea commercialis – Saccostrea cucullata*) from the estuaries of New South Wales. Aust J Mar Freshw Res 26:31–46

Mance G (1987) Pollution threat of heavy metals in aquatic environment. Elsevier, London, 363

McCluggage D (1991) Heavy metal poisoning. NCS Magazine, published by The Bird Hospital, St Lakewood. www.cockatiels.org/articles/Diseases/metals.html. 14 June 2012

McGlone MLSD, Jacinto G, Velasquez I, Padayao D (2004) Status of water quality in Philippine coastal and marine waters, p 96–108. In: DA-BFAR (Department of Agriculture-Bureau of Fisheries and Aquatic Resources). In: Turbulent seas: the status of Philippine Marine Fisheries. Coastal Resources Management Project of the Department of Environment and Natural Resources, Cebu City, Philippines, 378pp

McManus LT, Chua TE (eds) (1990) The coastal environmental profile of Lingayen Gulf, Philippines. ICLARM Tech Rep 22:69

Millward GE, Rowley C, Sands TK, Howland RJM, Pantiulin A (1999) Metals in the sediments and mussels of the Chupa Estuary (White Sea) Russia. Estuar Coast Shelf Sci 48:13–25

Mines A (1986) Assessment of the fisheries of Lingayen Gulf. Project report submitted to the Philippine Council for Agriculture and Resources Research and Development, National Science and Technology Authority. Institute of Fisheries Development and Research, College of Fisheries, University of the Philippines-Visayas, Quezon City, Philippines, 55pp

National Statistics Office (NSO) (2007) Index of population projection statistics. NSO, Quezon City

Palma A (1989) Patterns and levels of aquaculture practices in eight coastal municipalities adjoining Lingayen Gulf, p 71–82. In: Silvestre G, Miclat E, Chua TE (eds) Towards sustainable development of the coastal resources of Lingayen Gulf, Philippines. ICLARM Conference Proceedings 17, 200 p. Philippine Council for Aquatic and Marine Research and Development, Los Banos, Laguna, and International Center for Living Aquatic Resources Management, Makati, Metro Manila, Philippines

Phillips DJ (1977) Biological indicator organisms monitor metal pollution. Environ Pollut 13:281–317

Phillips DJH, Rainbow PS (1994) Biomonitoring of trace aquatic contaminants, 2nd edn. Chapman and Hall, London, 371

Rainbow PS (1995) Biomonitoring of heavy metals availability in the marine environment. Mar Pollut Bull 31:183–192

Ricklefts RE (2007) The economy of nature, 5th edn. W.H. Freeman and Company, New York

Rico LG, Ruiz RE (2001) Determination of total metals in cultivated oysters (*Crassostreagigas*) from the northwest coast of Mexico by microwave digestion and atomic absorption spectrometry. J AOAC Int 84(6):1909–1913

Riddell TJ, Solon O, Quimbo SA, Tan CMC, Butrick E, Peabody JW (2007) Elevated blood-lead levels among children in the rural Philippines. Bull World Health Organ 85:649–732

Ruelas-Inzunza J, Psez-Osuna F (1998) Barnacles as biomonitors of heavy metal pollution in the coastal waters of Mazatlan Harbor (Mexico). Bull Environ Contam Toxicol 61:608–615

Sakai H, Kojima Y, Saito K (1986) Distribution of metals in water and sieved sediments in the Toyohira river. Water Res 20:559–567

Schumacher M, Domingo J (1996) Concentrations of selected elements in oysters (*Crassostrea angulata*) from the Spanish Coast. Bull Environ Contam Toxicol 56:106–113

Shulkin VM, Presley BJ, Kavun VIA (2003) Metal concentrations in mussel *Crenomytilus grayanus* and oyster *Crasostrea gigas* in relation to contamination of ambient sediments. Environ Int 29:493–502

Silva CAR, Rainbow PS, Smith BD, Santos ZL (2001) Biomonitoring of trace metal contamination in the Potengi Estuary, Natal (Brazil), using the oyster, *Crassostrea rhizophorea*, a local food source. Water Res 35(17):4072–4078

Silvestre GT, Hilomen VV (2004) Status of Lingayen Gulf Fisheries – a brief update, p 285–291. In: DA-BFAR (Department of Agriculture-Bureau of Fisheries and Aquatic Resources). In turbulent seas: the status of Philippine Marine Fisheries. Coastal Resources Management Project of the Department of Environment and Natural Resources, Cebu City, Philippines, 378pp

Silvestre GT, Miclat E, Chua TE (eds) (1989) Towards sustainable development of the coastal resources of Lingayen Gulf, Philippines. ICLARM Conf Proc 17:200

Subramanian V, Grieken RV, Vant DL (1987) Heavy metal distribution in the sediments of Ganges and Brahmaputra rivers. Environ Geol Water Sci 9(2):93–103

Tang CW, Ip CC, Zhang G, Shin PK, Qian PY, Li XD (2008) The spatial and temporal distribution of heavy metals in sediments of Victoria Harbour, Hong Kong. Mar Pollut Bull 57:816–825

United States Environmental Protection Agency (USEPA) (1994) Method 7471A: mercury in solid or semisolid waste (Manual cold-vapor technique). http://www.epa.gov/epaoswer/hazwaste/test/pdfs/7471a.pdf. Accessed 20 Sept 2010

United States-Environmental Protection Agency (USEPA) (1992) National study of chemical residues in fish, vol 1. EPA 823-R-92-0089. Office of Science and Technology, Washington, DC

United States-Environmental Protection Agency (USEPA) (2000) Guide for assessing chemical contamination. Data for use in fish advisories. In: Fish sampling and analysis, 3rd edn, vol 1. EPA 826-B-00-007. Office of Science and Technology, Washington, DC

World Health Organization (WHO) (1989) Geographical distribution of arthropod-borne diseases and their principal vectors. World Health Organization, Geneva (unpublished document WHO/VBC/89.967)

World Health Organization (WHO) (1995) Inorganic lead. Environmental Health Criteria No. 165. World Health Organization, Geneva

Young RA (2005) Toxicity profiles: toxicity summary for cadmium. Risk Assessment Information System, RAIS, University of Tennessee. rais.ornl.gov/tox/profiles/cadmium.html. 14 June 2012

Zaroogian GE, Cheer S (1976) Accumulation of cadmium by the American oyster, *Crassostrea virginica*. Nature 261:408

Part III
Enteric Viruses/Virus entériques

Chapter 13
Proposed Draft Guidelines for the Application of General Principles of Food Hygiene to Control of Viruses in Food

Ingeborg L.A. Boxman

Introduction

Human viruses can be transmitted directly from person-to person, but also indirectly via virus-contaminated water, air, soil, surfaces or food. Some viruses are transmitted from animals to humans. Among others, the human enteric viruses most frequently reported as involved in foodborne outbreaks are norovirus (NoV) and hepatitis A virus (HAV). They infect via the gastrointestinal tract, enter living host cells in order to be able to multiply (replicate), and are excreted in faeces and/or vomit. High numbers of viral particles are shed in the stools or in vomit, whereas only a few viral/infectious particles (less than 100) are needed to cause infection that may lead to illness. Secondary spread of these viruses after primary introduction is common and often results in larger, prolonged outbreaks (Anon. 2008, 2011a, b).

These viruses can persist for months in food or in the environment and are more resistant than bacteria to commonly used control measures. In general, testing of foods for foodborne viruses is challenging and is based on detection of viral nucleic acids. Low levels of viruses may not be detected due to low extraction efficiency and/or the presence of PCR-interfering substances. Furthermore it is important to note that these methods cannot be used to distinguish between infectious and non-infectious viruses, which would allow an exact determination of whether the food poses a risk to human health (Anon. 2008, 2011a, b).

During the FAO/WHO Expert meeting on "Viruses in Food" (Anon. 2008), NoV and HAV were determined to be the viruses of greatest concern from a food safety perspective. Estimates of the proportion of viral illness attributed to food are in the range of around 5 % for HAV and 12–47 % for NoV. Furthermore, three major

I.L.A. Boxman (✉)
Food and Consumer Product Safety Authority (NVWA), P.O. Box 144, 6700 AC Wageningen, The Netherlands
e-mail: Ingeborg.boxman@vwa.nl

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_13,

sources of viral contamination of foods were identified: (1) human sewage/feces, (2) infected food handlers and (3) animals harboring zoonotic viruses. NoV and HAV in prepared (ready-to-eat) foods, bivalve molluscs, and fresh produce were selected as the virus-commodity combinations of greatest public health concern (Anon. 2008).

For bivalve molluscs, the major route of contamination is via human fecal contamination in growing or harvesting areas. Viruses have been observed to persist for 8–10 weeks in contaminated live bivalve molluscs and can be detected in the digestive tissue of bivalve molluscs (Atmar et al. 1995). Recent evidence has shown that some NoV genotypes bind specifically to bivalve molluscs' tissue receptor sites, which could explain why some viruses persist after depuration procedures as currently practiced in the industry (Le Guyader et al. 2006; Maalouf et al. 2010).

Effective control strategies need to focus on prevention of contamination as there are currently no effective, realistic and validated risk management options to eliminate viral contamination of both bivalve molluscs and fresh produce prior to consumption without changing the normally desired characteristics of the food. Therefore, the Joint FAO/WHO Food Standards Programme CCFH is working on guidelines entitled 'Guidelines on the application of the general principles on food hygiene to the control of viruses in food' (Anon. 2012).

FAO/WHO Proposed Guidelines to the Control of Viruses in Food

The primary purpose of the new guidelines is to give guidance on how to minimize the risk of illness arising from the presence of human enteric viruses in foods, and more specifically from NoV and HAV in foods. The guidelines are applicable to all foods throughout the food chain (Anon. 2012).

The guidelines should complement controls in place for any other pathogens and should therefore be used in conjunction with the Codex *Recommended International Code of Practice – General Principles of Food Hygiene*- (CAC/RCP 1-1969) and the *Code of Hygienic Practice for Precooked and Cooked Foods in Mass Catering* (CAC/RCP 39-1993), the *Code of Practice for Fish and Fishery Products* (CAC/RCP 52-2003) and the *Code of Hygienic Practice for Fresh Fruits and Vegetables* (CAC/RCP 53-2003).

The guidelines follow the format of the General Principles of Food Hygiene and the main document contains nine sections. Additional recommendations for specific virus-commodity combinations are presented in the annex on the *Control of Hepatitis A Virus* (*HAV*) *and Norovirus* (*NoV*) *in Bivalve Molluscs* (Annex I) and the annex on the *Control of Hepatitis A Virus* (*HAV*) *and Norovirus* (*NoV*) *in Fresh Produce* (Annex II) (Anon. 2012). This paper summarizes the most important issues of the draft proposed guidelines to the control of viruses in food dated December 2011 (Anon. 2011a, b) with a special focus on the control of viruses in bivalve molluscs.

Primary Production/Harvesting Area

Potential sources of viral contamination of the environment should be identified prior to production activities and food production should not be carried out in areas where the presence of viruses may lead to the viral contamination of food. In addition, hygiene and health requirements (see section on "Establishment: Personal Hygiene") should be followed to ensure that personnel who come directly into contact with food during production do not contaminate the product with fecal material or vomit. Water for primary production should be suitable for its intended use and not compromise food safety.

Bivalve Molluscs and Primary Production/Harvesting Area

It is important to ensure the seawater quality of growing areas for bivalve molluscs by improving sewage treatment efficiency for virus removal/inactivation and to avoid discharging inadequately treated sewage near the bivalve molluscs growing areas, e.g. from ships, recreational boats and of harvesting vessels. Sewage treatment plants should be designed to minimize storm overflows and leakage from private septic tanks or sewage collecting networks should be prevented.

A sanitary survey of harvesting and/or growing water should include an assessment of possible human fecal contamination sources and the intensity of the survey should be in agreement with the occurrence of viral diseases in the human domain and weather conditions, e.g. after heavy rain fall. *E. coli*/fecal coliforms/total coliforms are used as indicators for fecal contamination, however viruses may be present in the absence of bacterial indicators, e.g., a short-term depuration process commonly reduces low levels of bacterial contamination, but is inadequate for the elimination of viruses.

When there is a likelihood or evidence of virus contamination from epidemiological information, environmental events (e.g., heavy rainfall or sewage treatment overflows), or direct detection through virological analysis, closure of the area, destruction of contaminated bivalve molluscs, virucidal heat treatment before consumption or long term relaying for already harvested bivalve molluscs is recommended. Another option is a combination of depuration and relaying as determined by the competent authority. Viral testing of the bivalve molluscs or an equivalent approach to ensure safety should be used as part of the process of reopening the affected harvesting area depending on the requirements of the competent authority, using either standardized methods or alternative validated methods. Other conditions, including meeting the sanitary survey requirements, should also have been satisfied as a condition of reopening the area. In addition, suitable precautions should be taken to protect bivalve molluscs from being contaminated by human fecal material on board harvest vessels, and facilities and toilets on harvest vessels should be such to ensure that an appropriate degree of personal hygiene can be maintained.

Establishment: Design and Facilities

Sufficient numbers of personal hygiene facilities and toilets should be available and located in proximity to the production or processing areas (but not open directly to food handling areas), with adequate means for hygienically washing and drying hands before returning to food handling area to ensure that an appropriate and acceptable degree of personal hygiene can be maintained and to prevent food to become contaminated by fecal waste.

Control of Operation

Control of human enteric viruses such as NoV and HAV in food will typically require a stringent application of good hygienic practices, and other supportive programs. In the guidelines the (often limited) effects of specific processing procedures on virus infectivity in food are described. The effectiveness should be evaluated using virus infectivity assays where possible. In addition, preferably raw ingredients should be obtained only from suppliers or production plants with an adequate food safety management system. Based on the determined level of risk for the presence of viruses in a food product, a decision may be taken to recall the contaminated product from the market. The need for public information and communicated warnings should be considered.

Bivalve Molluscs and Control of Operation

Heat treatments of bivalve molluscs should be validated for their ability to inactivate viruses. An internal temperature of 90 °C for at least 90 s is considered to be a virucidal treatment (Anon. 1992). However, this degree of cooking would probably render specific bivalve molluscs, such as oysters, unpalatable to consumers. Even though cooking temperatures typically used by consumers may not achieve 90 °C for at least 90 s and thus ensure inactivation of viruses, any cooking would reduce viral levels and, depending on the initial level of contamination, could reduce the risk of causing foodborne infection. The possible inability of home or restaurant cooking to provide adequate assurance of consumer protection from consuming virally contaminated bivalve molluscs in certain circumstances or forms of consumption underlines the importance of harvesting bivalve molluscs from clean water growing areas.

High Hydrostatic Pressure (HHP) may also reduce virus titers in bivalve molluscs (Kingsley et al. 2009; Leon et al. 2011). The use of HHP alone or in combination with other inactivation procedures should be validated for the virus of concern in the specific bivalve mollusc species prior to its application.

Establishment: Maintenance and Sanitation

Specific guidance is given on preventive maintenance and especially sanitation procedures after an event of vomiting, diarrhea and/or notification of hepatitis. Disinfection should always be preceded by cleaning and should include all surfaces suspected to be contaminated with aerosols containing viruses, both in the hygiene facilities and toilets and as a preventive measure in food production areas. Food handling in the latter area(s) should be stopped and disposal of the implicated food should be considered. Infectious material should be dealt with immediately by a person trained in cleaning-up infectious material wearing gloves, facemasks and aprons or smocks. For surface disinfection, free chlorine solutions ($\geq$1,000 ppm for 5–10 min at RT), vaporized hydrogen peroxide treatment at >100 ppm for 1 h or UV irradiation at >40 mWs/cm^2 (= mJ/cm^2) can be used. Most other surface disinfectants lack efficacy against enteric viruses at manufacturer's recommended concentrations and exposure times.

Establishment: Personal Hygiene

There is a need for strict hygiene control by food handlers, particularly in relation to the prevention of NoV and/or HAV contamination, as the infectious dose of these viruses is very low. Persons with gastroenteritis or hepatitis should be excluded from handling food or from being present in the premises and should only be allowed to return to work after a period without symptoms of diarrhea and vomiting (e.g., period of 48 h). Persons, who have had hepatitis, should only be allowed to return to work after disappearance of jaundice. As shedding of viruses, such as NoV or HAV, may continue for several weeks after symptoms have subsided, and also asymptomatic shedding can occur, all food handlers should adhere to thorough hand washing instructions at all times, especially before handling food, after using the toilet or after being in contact with fecal matter (also after changing diapers/nappies, cleaning toilets).

Vaccination of food handlers against hepatitis A should be recommended where necessary to reduce the risk of viral contamination of the food, taking into account the epidemiological situation and/or immune status of the local population. The presence of non-authorized persons, such as children, to the extent possible, during food handling or on premises should be avoided.

Product Information and Consumer Awareness

Viruses can persist for a long time in food. As distribution of food between areas and countries complicates traceability, lot identity and integrity should be maintained to facilitate tracking. Countries should give consideration to educational programs to make consumers more aware of the risk of viruses in certain ready-to-eat foods.

Bivalve Molluscs, Product Information, and Consumer Awareness

It is recommended that growing areas for bivalve molluscs be registered for a 2 month period prior to harvest and that harvest areas also be registered. Furthermore, the competent authority should give consideration to labeling bivalve molluscs, so that the consumers are adequately informed with respect to their safety regarding possible viral contaminations; whether they are raw or treated.

Training

Food handlers engaged in food growing, harvesting or processing who come directly or indirectly in contact with foods should be trained or instructed to a level appropriate to the operations they are to perform. Recommendations are given in the document on the content of training programmes, which should be extensive and given to all new personnel and also to inspectors or other relevant authorities who inspect fields or premises. Also responsibilities of food business operators, managers and employers on their role in the control of viruses have been described. Incorporation of these instructions into the National Codes of Hygienic Practice would be advisable.

Bivalve Molluscs and Training

Personnel involved in the growing and harvesting of bivalve molluscs should have appropriate training in control measures to prevent fecal contamination of growing and harvesting areas, control measures to prevent bivalve molluscs from becoming contaminated by contagious food handlers and should be made aware of the lack of correlation between bacterial indicators and viral contamination.

Present Status of the Codex Document on Viruses

The proposed guidelines were distributed as document CX/FH 11/43/4 in October 2011 and was discussed at the 43rd Session of CCFH in Miami, United States of America, 5–9 December 2011 (Anon. 2011a, b). Suggestions to improve the text of the document before and during the meeting were discussed, and when agreed on, these were incorporated in the text. In brief these changes were: the insertion of a description of non-enveloped viruses (Introduction); editorial changes in the description of analytical methods and their limitations (Introduction), the omission

of total coliforms as an indicator for fecal contamination (Annex I and II), insertion of a definition for clean water (section on "FAO/WHO Proposed Guidelines to the Control of Viruses in Food" and Annex II) that differs from the clean water definition used in Annex I, insertion of an example of effective conditions of HHP for virus inactivation (section on "Control of Operation"), omission of gamma irradiation as a specific process step to inactivate viruses (section on "Control of Operation"), inclusion of the use of chlorine dioxide 200 ppm for surface disinfection (section on "Establishment: Maintenance and Sanitation"); redefining duration of NoV shedding and deletion of the example of the period without symptoms that food handlers should stay away from food handling (section on "Establishment: Personal Hygiene"), and finally inclusion of a description of factors that should be addressed in the sanitary survey of harvesting and/or growing water (Annex I). In addition to these changes, editorial amendments have been made, especially in Annex I. In December 2011, the CCFH meeting agreed on the revised document, which was subsequently submitted for adoption by the Codex Alimentarius Commission.

In July 2012, the document with editorial amendments was adopted by the Codex Alimentarius Commission and registered as CAC/GL 79–2012 (Anon. 2012). As the present paper was based on the proposed guidelines, the CAC/GL 79–2012 document should be the official document to use.

Acknowledgements I would like to thank all members of the Dutch Drafting Group, the Electronic Working Group (2011), the Physical Working Groups (2009–2010) and the WHO/FAO Expert Meeting (2008) for the expertise and efforts they have put in the development of these guidelines.

References

Anon. (1992) Council Decision of 11th December 1992 approving certain heat treatments to inhibit the development of pathogenic micro-organisms in bivalve molluscs and marine gastropods (93/25/EEC). Off J Eur Commun 16:22–23. Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31993D0025:en:NOT. Accessed 14 Nov 2012

Anon. EFSA [European Food Safety Authority] (2011a). Scientific Opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. EFSA J 9:2190, 96pp. doi:10.2903/j.efsa.2011.2190. Available from: http://www.efsa.europa.eu/en/efsajournal/doc/2190.pdf. Accessed 14 Nov 2012

Anon. FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization] (2008) Viruses in food: scientific advice to support risk management activities: meeting report. Microbiological risk assessment series no. 13. Available from: http://www.who.int/foodsafety/publications/micro/mra13/en/index.html. Accessed 14 Nov 2012

Anon. FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization] (2011b) Proposed draft guidelines on the application of the general principles on food hygiene to the control of viruses in food. Retrieved from: ftp://ftp.fao.org/codex/Meetings/CCFH/ccfh43/fh43_04e.pdf. Accessed 14 Nov 2012

Anon. FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization] (2012) Guidelines on the application of general principles of food hygiene to the control of viruses in food (CAC/GL 79-2012)

Atmar RL, Neill FH, Romalde JL, Le Guyader F, Woodley CM, Metcalf TG, Estes MK (1995) Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR. Appl Environ Microbiol 61:3014–3018

Kingsley DH, Calci K, Holliman S, Dancho B, Flick G (2009) High pressure inactivation of HAV within oysters: comparison of shucked oysters with whole-in-shell meats. Food Environ Virol 1:137–140. doi:10.1007/s12560-009-9018-5

Le Guyader F, Loisy F, Atmar RL, Hutson AM, Estes MK, Ruvoën-Clouet N, Pommepuy M, Le Pendu J (2006) Norwalk virus-specific binding to oyster digestive tissues. Emerg Infect Dis 12:931–936

Leon JS, Kingsley DH, Montes JS, Richards GP, Lyon GM, Abdulhafid GM, Seitz SR, Fernandez ML, Teunis PF, Flick GJ, Moe CL (2011) Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing. Appl Environ Microbiol 77:5476–5482

Maalouf H, Zakhour M, Le Pendu J, Le Saux JC, Atmar RL, Le Guyader FS (2010) Distribution in tissue and seasonal variation of norovirus genogroup I and II ligands in oysters. Appl Environ Microbiol 76:5621–5630

Chapter 14
Bioaccumulation and Removal Dynamics of Murine Norovirus in Manila Clams (*Venerupis philippinarum*) and Mussels (*Mytilus galloprovincialis*)

D. Polo, C. Álvarez, J. Díez, C.F. Manso, M. Angulo, M.L. Vilariño, S. Darriba, A. Longa, and J.L. Romalde

Introduction

Infectious diseases associated with shellfish consumption have been widely reported (Butt et al. 2004; Koopmans and Duizer 2004). Historically, the study of food-borne diseases associated with contaminated shellfish has mainly focused on bacterial pathogens. As a result, the development and design of shellfish sanitary controls and depuration practices has been strongly influenced and guided with the purpose to eliminate fecal coliforms. Compliance with the end-product fecal coliform standard is frequently mistakenly seen as a evidence of a satisfactory depuration process. However, due to their filter feeding nature, shellfish also provide a potential vehicle of transmission for a wide variety of others infectious agents, making the bivalve molluscs a high-risk food group (Butt et al. 2004; Polo et al. 2010; Vilariño et al. 2009). Human enteric viruses can be bioaccumulated and retained by shellfish. In fact, outbreaks after consumption of shellfish contaminated with enteric viruses like norovirus (NoV) and hepatitis A virus (HAV) have been widely reported (Koopmans and Duizer 2004; Lees 2000).

D. Polo • C.F. Manso • M.L. Vilariño • J.L. Romalde (✉)
Departamento de Microbiología y Parasitología, CIBUS- Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain
e-mail: david.polo@usc.es; carmenmaria.fernandez@usc.es; marialuz.vilarino@usc.es; jesus.romalde@usc.es

C. Álvarez • S. Darriba
INTECMAR (Instituto Tecnolóxico para o Control do Medio Mariño de Galicia), Consellería do Mar. Peirao de Vilaxoán, Vilagarcía de Arousa, Spain
e-mail: calvarez@intecmar.org; sdarriba@intecmar.org

J. Díez • A. Longa
Consello Regulador Denominación de Orixe Mexillón de Galicia, Vilagarcía de Arousa, Spain

M. Angulo
TRAGSATEC, Pontevedra, Spain

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_14,

Traditional bacterial indicators of fecal contamination, on which sanitary controls are based, and enteric viruses significantly differ in terms of transmission, resistance to sewage treatment, and persistence in the environment (Da Silva et al. 2007). Depuration allows the purging of gastrointestinal contents under controlled conditions in order to reduce the likelihood of transmitting infectious pathogens. The efficacy of depuration in the elimination of such agents is a critical issue for the development of improved shellfish sanitary controls. Although depuration can reduce bacterial levels from shellfish, the removal of viral particles is not as effective (Ueki et al. 2007) and therefore, depuration may not ensure the absence of viral contamination (Croci et al. 2007; Loisy et al. 2005; Schwabm et al. 1998). Periodic outbreaks of enteric diseases linked to shellfish which comply with legal standards and/or are subjected to depuration, indicate the inability of both bacterial indicators (Romalde et al. 2002; Umesha et al. 2008) and commercial depuration (Chalmers and McMillan1995; Heller et al. 1986; Le Guyader et al. 2003, 2006a) to predict the viral risk.

Depuration is a complex biological process, which varies according to bivalve species, pathogens (bacteria or virus) and possibly between viral species. Therefore, a better understanding of specific behavior of diverse viruses in different bivalve species is needed. Experimental depuration systems provide a useful tool to find the best operational parameters and to improve commercial depuration. This study evaluates and compares by reverse transcription-real time RT-PCR (qRT-PCR) the effectiveness of depuration in clams and mussels subjected to bioaccumulation with murine norovirus (MNV-1), as a surrogate of human norovirus (Wang et al. 2008) in an experimental depuration system.

Materials and Methods

Sample Collection and Depuration Facilities

A closed experimental depuration system (isothermal ASE M BINS system, 500 kg of capacity) (AdriaticSeaAquarium and Equipment SRL, San Clemente, Italy) with mechanical, biological and chemical static filter systems, thermal control and water sterilization by ozone and UV-C radiation was employed (Fig. 14.1). Ten depuration experiments were carried out with Manila clams (*Venerupis philippinarum*) and mussels (*Mytilus galloprovincialis*) (five with each species) after bioaccumulation with MNV-1. Each experiment was performed with 60 kg of mollusc.

Bioaccumulation of Virus by Molluscs

Molluscs were obtained from local producers, kept at 4 °C during shipment and arrived at the laboratory within the next 4 h. After 24 h of acclimatization in tanks with 100 l of seawater and continuous aeration, bioaccumulation was performed

Fig. 14.1 Experimental depuration system

by adding 10^2 pfu/ml (final concentration) of MNV-1 to the tanks (1 pfu equals to approximately 10^2 RNA copies (Baert et al. 2008)). Moreover, 500 ml of two species of phytoplankton (*Isochrysis* sp. and *Nanocloropsis* sp., 1:1 v/v) were added in order to induce the clams to filter. Five independent trials were carried out with each mollusc species.

After 24 h the efficacy of bioaccumulation (T_0) was determined and the molluscs were relocated in the experimental depuration system during 7 day, under exhaustive control of the following depuration parameters: dissolved oxygen (O_2), pH, water temperature (T°), ammonia (NH_3/NH_4), nitrites (NO_2), nitrates (NO_3), conductivity and salinity. Sampling was performed every 24 h (T_1–T_7) in order to evaluate the removal kinetics of viral loads. Each sample was composed of 20 clams or 10 mussels for each analysis.

Cell Culture and Viral Stocks

A mutant non-virulent infective strain of mengovirus (vMC_0) was employed as extraction control as previously described (Costafreda et al. 2006). MNV-1 was employed as a surrogate for modeling human NoV. Stocks of $vMC_0$and MNV-1 were generated by inoculation onto confluent monolayers of HeLa (Costafreda et al. 2006) and RAW 267.4 (Wobus et al. 2006) respectively.

Viral Recovery and RNA Extraction

For virological analysis, molluscs were shucked and the digestive tissue (DT) removed and homogenized as previously described (Wang et al. 2008). Viral RNA was extracted in duplicate from DTusing Nucleospin RNA Virus Kit (Macherey-Nagel; Germany) according to the manufacturer's protocol.

Extraction and qRT-PCR Controls

Prior to the viral RNA extraction, known amounts of mengovirus clone vMC_0 were spiked in sample homogenates (10 μl of mengovirus stock, 10^3 pfu) to be employed as a control for the process of nucleic acid extraction (Costafreda et al. 2006). To test the presence of RT-PCR inhibitors and calculate the qRT-PCR efficiency, an external control (MNV-1 RNA) was included in each reaction. The cycle threshold (C_t) value of a sample extracted RNA (2.5 μl) mixed with MNV-1 external control (2.5 μl) was compared to the C_t value of the external control mixed only with RNA-free sterile water. These steps allowed the identification of samples that required re-extraction and provided assurance that the samples with no amplification signal were negative (values below the limit of detection for the assays) and not simply inhibited. Negative controls containing no nucleic acid as well as positive controls, containing RNA of MNV-1, were introduced in each run.

Primers and Probes

qRT-PCR for viral detection and quantification was carried out with TaqMan probes using the Platinum Quantitative RT-PCR Thermoscript One-step System (Invitrogen, France) following the manufacturer's instructions in 25 μl of a reaction mixture containing 5 μl of extracted RNA. For MNV-1 detection 0.2 μM of reverse and forward primers as well as probe were added. Primer sets and probes employed were Fw-ORF1/ORF2, Rv-ORF1/ORF2 and probeMGB-ORF1/ORF2 (Baert et al. 2008).

Amplification Conditions

A Mx3005P QPCR System (Stratagene, USA) thermocycler was employed following amplification conditions described by Baert et al. (2008) with minor modifications. Briefly, after a RT step at 45 °C for 1 h, PCR amplification was carried out employing the following cycling conditions: an initial denaturation at 95 °C for 5 min, and 50 cycles of amplification with a denaturation at 95 °C for 15 s and annealing-extension step at 60 °C for 1 min.

Quantification Standards and Viral Quantification

Quantification of MNV-1 was estimated by standard curves constructed with serial dilutions of viral RNA, plotting the number of genome copies against the C_t. This quantification was corrected with the extraction and qRT-PCR efficiencies and expressed as number of RNA viral genome copies per gram of digestive tissue (RNA/g DT). A sample with a $C_t \leq 41$, with no evidence of amplification in the negative controls, was considered as positive. The number of RNA copies present in each positive sample was estimated by comparing the C_t value of the sample to the standard curve. All samples were tested in duplicate and with a ten-fold dilution. The final concentration was then adjusted based on the dilution factor used.

Statistics

ANOVA analysis compared the results of the number of copies of RNA/g DT obtained among the different depuration times (T_0–T_7) and between the two species of molluscs. Moreover, *post-hoc* tests were–employed to determine the statistical significance of bioaccumulation and the viral reduction detected for each depuration using the Student-Newman-Keuls (SNK) and Dunnett's tests. All statistical analyses were permormed using the statistical package IBM SPSS v20.0.0 software.

Results

Physicochemical parameters of water did not significantly differ for each depuration cycle, and showed normal levels for the depuration process. The average values were: conductivity (mS/cm) 49; salinity 30.6‰; pH 8.1; water temperature 14.5 °C and dissolved oxygen 7.6 ppm. In addition to these parameters, the concentration of ammonia, nitrites and nitrates were evaluated, showing no differences among trials. Ammonia and nitrite concentrations ranged between 0 and 0.3 mg/l except in one of the experiments where nitrite values reached to 0.8 mg/l. Nitrate concentrations were between 0 and 50 mg/l.

Experimental depuration trials carried out with mussels, showed a quantification range at the initial stage (T_0) between 4.7and 5.9 logRNA copies/g DT. However, in one of them (trial 3) higher contamination levels were achieved at T_1 (24 h of depuration) coinciding with the highest contamination value reached in this trial (5.6 log RNA copies/g DT) (Table 14.1). All experiments with mussels showed some reduction in viral quantification. At the end of the depuration (T_7), results showed contamination values between 4.1 and 4.9 log RNA copies/g DT (Table 14.1, Fig. 14.2). The average reduction for these trials was 0.8 log units (74 % of reduction in RNA copies/g DT), being statistically significant ($p < 0.05$) from 72 h (T_3) until the end of the depuration period (T_7).

Table 14.1 Depuration trials (designated as 1–5) on mussels contaminated with MNV-1

Trial	1	2	3	4	5	Av[a]
T_P	0	0	0	0	0	0 (±0.0)
T_0	5.44	5.88	4.66	5.72	5.11	5.36 (±0.5)
T_1	5.41	5.82	5.56	4.86	4.69	5.27 (±0.5)
T_2	4.85	5.22	5.01	5.94	4.57	5.12 (±0.5)
T_3	4.98	5.27	5.03	5.13	4.63	5.01 (±0.2)
T_4	4.54	5.15	4.93	4.20	4.32	4.63 (±0.4)
T_5	4.90	5.04	4.81	4.53	4.43	4.74 (±0.3)
T_6	5.02	4.83	4.59	4.86	3.86	4.63 (±0.5)
T_7	4.68	4.89	4.56	4.14	4.44	4.54 (±0.3)
vr[b]	0.76	0.99	0.10	1.59	0.67	0.82 (±0.54)

Results are as expressed as logRNA copies/g digestive tissues. T_P sampling at shellfish arrival, T_0 sampling at transfer of shellfish from bioaccumulation tanks to depuration system, T_1–T_7 day 1–7 samplings during purification process
[a]Average of the five-depuration trials and standard deviation
[b]Log virus reduction at the end of depuration

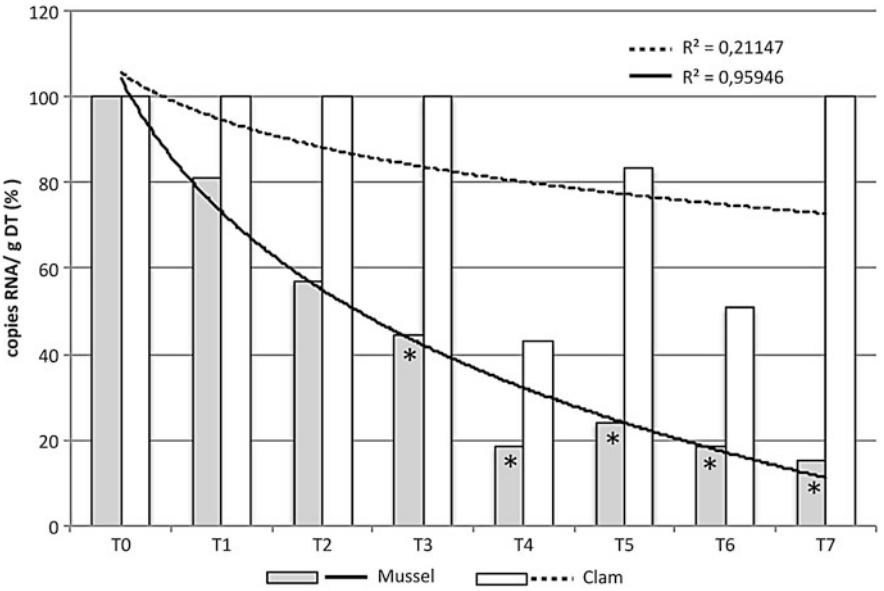


Fig. 14.2 Average removal kinetics for the five experimental depuration trials of mussels (*M. galloprovincialis*) and clams (*V. philippinarum*) artificially contaminated with MNV-1 (10^2 pfu/ml). Results are expressed as percentage (%) of RNA copies/g digestive tissues. T_P sampling at shellfish arrival, T_0 sampling at transfer of shellfish from bioaccumulation tanks to depuration system, T_1–T_7 day 1–7 samplings during purification process. *Significance at $p < 0.05$ with regard to the initial time of depuration

Table 14.2 Depuration trials (designated as 1–5) on clams contaminated with MNV-1

Trial	1	2	3	4	5	Av[a]
T_P	0.00	0.00	0.00	0.00	0.00	0.00 (±0)
T_0	6.49	5.54	6.27	6.04	6.57	6.18 (±0.4)
T_1	8.00	6.83	6.29	6.12	5.80	6.61 (±0.9)
T_2	7.63	7.24	5.27	5.51	5.77	6.28 (±1.1)
T_3	6.48	6.84	6.26	5.36	5.98	6.18 (±0.6)
T_4	6.07	5.81	5.85	5.65	5.70	5.82 (±0.2)
T_5	6.69	6.91	6.20	5.80	4.91	6.10 (±0.8)
T_6	6.41	6.94	5.54	5.52	5.03	5.89 (±0.8)
T_7	6.50	7.13	5.89	5.71	5.89	6.22 (±0.6)
vr[b]	−0.01[c]	−1.59[c]	0.38	0.33	0.68	−0.04 (±0.9)

Results are expressed as logRNA copies/g digestive tissues. T_P sampling at shellfish arrival, T_0 sampling at transfer of shellfish from the bioaccumulation tanks to the depuration system, T_1–T_7 day 1–7 samplings during purification process

[a]Average of the five-depuration trials and standard deviation

[b]Log units of viral removal at the end of the depuration

[c]Increase in the depuration rate between T_0 and T_7

In the five trials carried out with clams contaminated with MNV-1 (Table 14.2), quantification at T_0 ranged from 5.5 to 6.6 log RNA copies/g DT, however, in four out of five trials (1–4) the highest contamination levels were observed at T_1 (24 h of depuration), reaching values between 6.1 and 8.0 log RNA copies/g DT. In one of these trials (trial 2), the highest contamination value was reached at T_2 (7.2 log RNA copies/g DT). Only three out of five depuration trials (3, 4 and 5) showed some reduction in viral quantification (Table 14.2). The average reduction for these three experiments was 0.5 log units (41.4 % reduction in RNA copies/g DT). However, in trial 1 similar values were obtained at initial (T_0) and final (T_7) stage, and in trial 2 an increase in RNA copies/g DT of 1.6 log units was observed. At the end of the depuration (T_7), clams in the different experiments showed contamination values between 5.7 and 7.1 log RNA copies/g DT (Table 14.2). For the average of the five trials, no virus removal with regard to the initial depuration level (T_0) was achieved (Table 14.2, Fig. 14.2). No statistical significance was found for the average viral reduction among depuration times (T_0–T_7).

Significant differences ($p < 0.05$) in the viral uptake between molluscs were detected. Clams showed an average viral uptake of 5.4 log RNA copies/g DT, and mussels 6.2 log RNA copies/g DT. In addition, significant differences ($p < 0.05$) were also observed between clams and mussels for the average removal rate, being higher for mussels (74 % of reduction in RNA copies/g DT).

Discussion

The main objective of this study was to obtain a broad picture of the MNV-1 removal dynamics, as a model of human norovirus behavior, in clams and mussels subjected to depuration processes. The inadequacy of current European regulations to assess the sanitary quality of shellfish and classification of harvesting areas to prevent viral contamination is well known (Anonymous 2004; Le Guyader et al. 2003; Romalde et al. 2002).In addition, enteric viruses concentrated by shellfish can persist under depuration conditions that are sufficient for bacteria removal. How infectious viruses can persist within the shellfish and their removal kinetics under depuration conditions are important questions to be addressed.

The results of this study showed differences in the viral uptake and the removal rate for viruses between clams and mussels subjected to an artificial bioaccumulation process with MNV-1. Fluctuations of the viral loads in consecutive depuration stages were detected, which can be attributed to the heterogeneity distribution of viral load. After 24 h of bioaccumulation the average viral uptake in clams was 73.8 % higher than in mussels. However, only three out of five depuration experiments with clams showed some reduction in viral quantification. The average reduction in these three experiments was 0.5 log units (41.4 % reduction in RNA copies/g DT). Mussels showed viral reduction in all the depuration trials with an average reduction of 0.8 log units (74 % reduction in RNA copies/g DT).

Bioaccumulation rates and removal dynamics of MNV-1 seem to be different in manila clams and mussels. Higher bioaccumulation levels were reached in clams, but higher depuration rates have been observed in mussels.

These results indicate a different behavior ofMNV-1 in these two bivalve species. Nappier et al. (2008), also reported a statistically higher bioaccumulation and retention rate of MNV, NoV and HAV in *Crassostrea ariakensis* than in *C. virginica*. Other studies carried out in our laboratory have shown a different bioaccumulation and removal pattern in Manila clams artificially contaminated with HAV and MNV-1 (unpublished data).

Although there are other studies in which viral bioaccumulation and subsequent depuration of enteric viruses in bivalve molluscs is determined, to our knowledge, this is the first to compare the rate of bioaccumulation and subsequent removal dynamics of murine norovirus by qRT-PCR in these two molluscs.

The observed differences in MNV-1 uptake and removal dynamics could be related to viral properties and/or to the existence of specific ligands. The specific binding of NoV strains to digestive tract of shellfish through an A-like human histo-blood group antigens (HBGAs) has been demonstrated (Le Guyader et al. 2006b; Tian et al. 2006, 2007). Bivalve shellfish could, therefore, specifically concentrate different viruses or specific strains on the basis of these receptors/ligands, while other virus could be accumulated to a lesser extent by nonspecific mechanisms of attachment, like mechanical entrapment and ionic bonding (Burkhardt and Calci 2000; Di Girolamo et al. 1977; Schwabm et al. 1998), and consequently, be more rapidly depurated.

Previous studies have detected viral particles in the digestive tract lumen, inside gastrointestinal cells and in phagocytes (both in the epithelium and in connective tissue) (Le Guyader et al. 2006b; Mcleod et al. 2009; Wang et al. 2008). Viruses present in the digestive tract lumen could theoretically be removed relatively rapidly via defecation when shellfish are placed in clean water. The specific attachment and internalization of intact viral particles into gastrointestinal cells or captured by phagocytes in the main ducts could be a viral mechanism to avoid being degraded by the digestive system and may provide an explanation for the generally poor efficiency in removing viruses from shellfish (Le Guyader et al. 2006b; Mcleod et al. 2009). The relatively high levels of final viral loads in the depurated samples observed in this work support this hypothesis.

In terms of risk to the consumer, interpreting the real significance of these results is complicated. Although the final viral loads remain at relatively high levels in all samples, molecular techniques like qRT-PCR detect RNA copies rather than infective particles, and nucleic acids remain detectable for long periods. Assays are currently in progress to assess the infectivity of the virus detected after mollusc depuration.

Both the findings reported here, as in other studies mentioned above, suggest that virus concentration in shellfish is not a passive process. It may depend on many factors such as mucus production, glycogen content, water temperature, gonadal development (Maalouf et al. 2010), and possibly on the presence, amount and/or distribution of specific receptors in host tissues. In this way, not only a different virus or virus strain could behave differently in shellfish, but also one virus could behave differently in different bivalve species and/or in different stages of the mollusc life cycle.

Conclusions

Depuration can reduce viral levels in shellfish, but not sufficiently to consider them safe. The final viral load in samples remains in relatively high concentrations, and virus, unlike bacteria, can be infectious at very low doses. Thus, it is necessary to reduce virus to near negligible levels to improve the safety of shellfish but it is also necessary to understand that the depuration process is a method capable of reducing relatively low levels of contamination but not, at least for now, for highly contaminated shellfish. This study will provide the baseline for future studies focused on improving the efficacy of viral depuration of shellfish.

Acknowledgements This work was supported in part by Grant 10MMA200010PR from the Consellería de Economía e Industria, Xunta de Galicia (Spain) and contract 2008/CP776 from INTECMAR (Spain). The donation of viral stocks of $vMC_0$and MNV-1 kindly provided by Dr. Albert Bosch (University of Barcelona, Spain) and Dr. Herbert W. Virgin IV (University of Washington, USA), respectively, is appreciated.

References

Anonymous (2004) European Regulation (EC) Nº 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Off J Eur Union L226:83–127

Baert L, Wobus CE, Van Coillie E, Thackray LB, Debevere J, Uyttendaele M (2008) Detection of Murine Norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Appl Environ Microbiol 74:543–546

Burkhardt W, Calci KR (2000) Selective accumulation may account for shellfish-associated viral illness. Appl Environ Microbiol 66:1375–1378

Butt AA, Aldridge KE, Sanders CV (2004) Infections related to the ingestion of seafood. Part I: viral and bacterial infections. Lancet Infect Dis 4:201–212

Chalmers JWT, McMillan JH (1995) An outbreak of viral gastroenteritis associated with adequately prepared oysters. Epidemiol Infect 115:163–167

Costafreda MI, Bosch A, Pintó RM (2006) Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl Environ Microbiol 72:3846–3855

Croci L, Losio MN, Suffredini E, Pavoni E, Di Pasquale S, Fallacara F, Arcangeli G (2007) Assessment of human enteric viruses in shellfish from the northern Adriatic sea. Int J Food Microbiol 114:252–257

Da Silva AK, Le Saux JC, Parnaudeau S, Pommepuy M, Eimelech M, Le Saux JC, Le Guyader FS (2007) Evaluation of removal of noroviruses during wastewater treatment, using real time reverse transcription-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol 73:7891–7897

Di Girolamo R, Liston J, Matches J (1977) Ionic binding, the mechanism of viral uptake by shellfish mucus. Appl Environ Microbiol 33:19–25

Heller D, Gill ON, Raynham E, Kirkland T, Zadick PM, Stabwell-Smith R (1986) An outbreak of gastrointestinal illness associated with consumption of raw depurated oysters. Br Med J 292:1726–1727

Koopmans M, Duizer E (2004) Foodborne viruses: an emerging problem. Int J Food Microbiol 90:23–41

Le Guyader FS, Neil FH, Dubois E, Bon F, Loisy F, Kohli E, Pommepuy M, Atmar RL (2003) A semi-quantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak. Int J Food Microbiol 87:107–112

Le Guyader FS, Bon F, DeMedici D, Parnaudeau S, Bertone A, Crudeli S, Doyle A, Zidane M, Suffredini E, Kohli E, Maddalo E, Monini M, Gallay A, Pommepuy M, Pothier P, Ruggeri FM (2006a) Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. J Clin Microbiol 44:3878–3882

Le Guyader FS, Loisy F, Atmar RL, Hutson AM, Estes MK, Ruvoen-Clouet N, Pommepuy M, Le Pendu J (2006b) Norwalk virus-specific binding to oyster digestive tissues. Emerg Infect Dis 12:931–936

Lees DN (2000) Viruses and bivalve shellfish. Int J Food Microbiol 59:81–116

Loisy F, Atmar RL, LeSaux JC, Cohen J, Caprais MP, Pommepuy M (2005) Rotavirus virus like particles as surrogates to evaluate virus persistence in shellfish. Appl Environ Microbiol 71:6049–6053

Maalouf H, Pommepuy M, Le Guyader FS (2010) Environmental conditions leading to shellfish contamination and related outbreaks. Food Environ Virol 2:136–145

Mcleod C, Hay B, Grant C, Greening G, Day D (2009) Localization of norovirus and poliovirus in Pacific oysters. J Appl Microbiol 106:1220–1230

Nappier SP, Graczyk TK, Schwab KJ (2008) Bioaccumulation, retention, and depuration of enteric viruses by Crassostrea virginica and Crassostrea ariakensis oysters. Appl Environ Microbiol 74:6825–6831

Polo D, Vilariño ML, Manso CF, Romalde JL (2010) Imported molluscs and dissemination of human enteric viruses. Emerg Infect Dis 16:1036–1038

Romalde JL, Area E, Sánchez G, Ribao C, Torrado I, Abad X, Pintó RM, Barja JL, Bosch A (2002) Prevalence of enterovirus and hepatitis A virus in molluscs from Galicia (NW Spain). Inadequacy of the EU standards of microbiological quality. Int J Food Microbiol 74:119–130

Schwabm KJ, Neill FH, Estes MK, Metcalf TG, Atmar RL (1998) Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. J Food Protect 61:1674–1680

Tian P, Bates AH, Jensen HM, Mandrell RE (2006) Norovirus binds to blood group A-like antigens in oyster gastrointestinal cells. Lett Appl Microbiol 43:645–651

Tian P, Engelbrektson AL, Jiang X, Zhong W, Mandrell RE (2007) Norovirus recognizes histo-blood group antigens on gastrointestinal cells of clams, mussels, and oysters: a possible mechanism of bioaccumulation. J Food Protect 70:2140–2147

Ueki Y, Shoji M, Suto A, Tanabe T, Okimura Y, Kikuchi Y, Saito N, Sano D, Omura T (2007) Persistence of Caliciviruses in artificially contaminated oysters during depuration. Appl Environ Microbiol 73:5698–5701

Umesha KR, Bhavani NC, Venugopal MN, Karunasagar I, Krohne G, Karunasagar I (2008) Prevalence of human pathogenic entericviruses in bivalve molluscan shellfish and cultured shrimp in southwest coast of India. Int J Food Microbiol 122:279–286

Vilariño ML, Le Guyader FS, Polo D, Schaeffer J, Kröl J, Romalde JL (2009) Assessment of human enteric viruses in cultured and wild bivalve molluscs. Int Microbiol 12:145–151

Wang D, Wu Q, Kou X, Yao L, Zhang J (2008) Distribution of norovirus in oyster tissues. J Appl Microbiol 105:1966–1972

Wobus CE, Thackray LB, Virgin HW IV (2006) Murine Norovirus: a model system to study Norovirus biology and pathogenesis. J Virol 80:5104–5112

Chapter 15
Detection of Enteric Viruses in Shellfish

Gary P. Richards, Dean O. Cliver†, and Gail E. Greening

Introduction

Enteric viruses are major contaminants of foods, causing countless illnesses around the world. Contaminated molluscan shellfish represent an important host for enteric virus transmission to humans. Among the most significant foodborne enteric viruses are the noroviruses, which contribute to the vast majority of illnesses, and hepatitis A virus. The exceedingly small size of most enteric viruses and the inability to grow them in cell cultures make their detection difficult. Therefore, molecular methods, particularly reverse transcription-polymerase chain reaction (RT-PCR), has become the most common assay procedure. Before shellfish can be assayed, viruses must first be separated from the shellfish tissues, clarified, and concentrated. This paper describes some of the steps required for virus extraction and assay. A more comprehensive review of virus extraction and assay procedures will be published by the authors in the 5th edition of the American Public Health Association's Compendium of Methods for the Microbiological Examination of Foods (Richards et al. in press).

† Deceased. Dr. Dean Cliver, internationally recognized food virologist, approved the presentation of this paper at the ICMSS before his death on May 16, 2011. He will long be remembered for his significant contributions in this field for the past 50 years.

G.P. Richards (✉)
United States Department of Agriculture, Agricultural Research Service, James W.W. Baker Center, Delaware State University, Dover, DE 19901, USA
e-mail: gary.richards@ars.usda.gov

G.E. Greening
Food Group, Kenepuru Science Centre, Institute of Environmental Science and Research Ltd. (ESR), P.O. Box 50-348, Porirua 5240, New Zealand
e-mail: gail.greening@esr.cri.nz

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_15,

General Principles of Virus Extraction

Oysters, clams, mussels and cockles are filter-feeding bivalve molluscs which can concentrate viruses to high levels within their tissues. The first step in virus extraction is the collection and transport of samples from the environment, food distribution center, or from the consumer. Since enteric viruses are incapable of growth within shellfish, no increases in numbers will occur during transport; however, samples should be refrigerated or frozen to prevent product degradation. Viruses may be extracted from whole shellfish or from digestive tissues dissected from the shellfish. The latter has the benefit of reducing the volume of sample. Tissues may be diluted in a high salt and high pH buffer before homogenization for 2–3 min in a blender. The homogenized sample is centrifuged and the virus-containing supernatant is retained, while the pelleted tissues are discarded. Viruses may be precipitated using polyethylene glycol followed by centrifugation. Additional chemical treatments may be applied to remove potential assay inhibitors from the shellfish extracts, like the addition of cetyltrimethyl-ammonium bromide (CTAB), which can be used to reduce polysaccharides from the sample, and Freon TF (DuPont, Wilmington, DE), Vertrel XF (DuPont), or chloroform-butanol to remove lipids, as previously described (Richards et al. in press). Protein may also be removed using Pro-Cipitate (Biotech Support Group LLC, North Brunswick, NJ). At this point in the purification, intact viruses may be present. Intact viruses may also be separated from the mix by immunomagnetic capture of whole viruses on antibody-conjugated beads. A simpler method involves homogenization of shellfish digestive tissues followed by enzyme (protease K) digestion, centrifugation and subsequent RNA purification.

Practical assays to detect intact norovirus or hepatitis A virus are not available, primarily because there are no cell culture methods for the propagation of norovirus or most strains of hepatitis A virus. Virus detection requires that the RNA be extracted and tested by RT-PCR. To release viral RNA from within the viral capsid, capsids must be digested, usually by the addition of phenol and guanidinium isothiocyanate, in compounds like TRIzol® or TRI Reagent®, along with chloroform. The RNA may then be concentrated by binding it to a variety of commercially available silica or glass fiber matrix columns or filters, such as NucliSens (bioMérieux, Durham, NC), RNeasy Mini Kits or QIAamp Viral RNA Mini Kits (Qiagen, Valencia, CA), and Roche High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Indianapolis, IN). Viruses are also concentrated by immunomagnetic beads (López-Sabater et al. 1997) and poly dT magnetic beads (Kingsley and Richards 2001; Kingsley et al. 2002), porcine gastric mucin conjugated to magnetic beads (Tian et al. 2008), or histo-blood group antigens conjugated to magnetic beads (Harrington et al. 2004).

Limitations of Shellfish Extraction

The extraction of viruses from shellfish is typically a slow process which is labor intensive and costly. The procedure produces some hazardous chemical waste, and the virus yield and reproducibility are not always certain. The procedures are prone to contamination and can produce seasonal and shellfish species variability, depending on the composition of the shellfish. For instance, high glycogen and lipid levels in shellfish just before spawning may interfere with virus extraction or lead to the transfer of inhibitory substances in the final extract. Extracts often contain RT-PCR inhibitors which must be monitored. Extraction controls consist of a positive process control, such as added Mengovirus to monitor virus recovery efficiency during extraction. A negative process control of known non-contaminated shellfish should also be extracted to monitor for cross-contamination.

Virus Extraction Methods for Shellfish

One method for virus detection from shellfish is the United States Department of Agriculture (USDA) method developed by Kingsley and Richards (2001), which has been validated for use in Canada and can be used for regulatory purposes. Another procedure under evaluation for use in the European Union is known as the TAG4 method (Anon 2010; Lees and CEN-WG6-TAG4 2010). Other methods are also available. The USDA method, known as the GPTT method uses a glycine buffer elution of virus from shellfish tissues, PEG precipitation of virus, TRI-reagent extraction of viral RNA, and poly dT magnetic bead capture of viral RNA. This method was used to identify both hepatitis A virus and norovirus in clams imported from China, which were implicated in a restaurant-associated outbreak of illness in New York state (Kingsley et al. 2002). It was also used in identifying the source of contaminated shellfish traced to an outbreak of norovirus illness in Canada. The method was validated for use in Canada after a multi-laboratory evaluation sponsored by Health Canada. Labs participating in the validation included Health Canada, the Canadian Food Inspection Agency, Agriculture and Agri-Food Canada, British Columbia Centre for Disease Control, Centre québécois d'inspection des aliments et de santé animale, University of California at Davis, U.S. Food and Drug Administration, and the Alaska Environmental Health Laboratory. This method was published in Health Canada's Compendium of Analytical Methods (Trottier et al. 2010). Regulatory actions can be taken based on this method in the presence of other supporting information, such as epidemiological evidence. Noteworthy steps in this extraction procedure are pictured in Fig. 15.1. A more comprehensive overview of this procedure is shown in the diagram in Fig. 15.2.

In 2004, the European Committee on Standardization (CEN) established a Technical Advisory Group for Viruses (TAG4). Its purpose was to develop and publish standard virus extraction and assay procedures for food surfaces, soft fruit and salad vegetables, bottled water, and bivalve molluscan shellfish for the European

Fig. 15.1 Major steps in the GPTT procedure for virus extraction from shellfish

Union (Anon 2010; Lees and CEN-WG6-TAG4 2010). A schematic of the method for virus extraction from shellfish is shown in Fig. 15.3. The final method is in the validation stage and is expected to be approved by CEN and ISO in 2012. This is then expected to become the European Union's standard method for foods.

In New Zealand, a modified version of the CEN method for norovirus detection in bivalve shellfish was established and ISO 17025 accredited in 2007 (Greening and Hewitt 2008). This method has since been used to analyze over 650 shellfish samples from New Zealand and overseas for outbreak investigations, virus prevalence surveys, monitoring programs, commercial product clearances and regulatory purposes. In New Zealand, regulatory measures were introduced to manage contamination by viral and bacterial pathogens, including closure of harvest areas for at least 28 days after sewage contamination events and mandatory norovirus testing of five targeted samples before an implicated growing area can reopen.

Limitations of RT-PCR

For the noroviruses, hepatitis A virus, and others, detection must be accomplished by RT-PCR. Virus detection may be achieved using standard RT-PCR or the more recently developed real-time RT-PCR. Real-time methods may be quantitative if

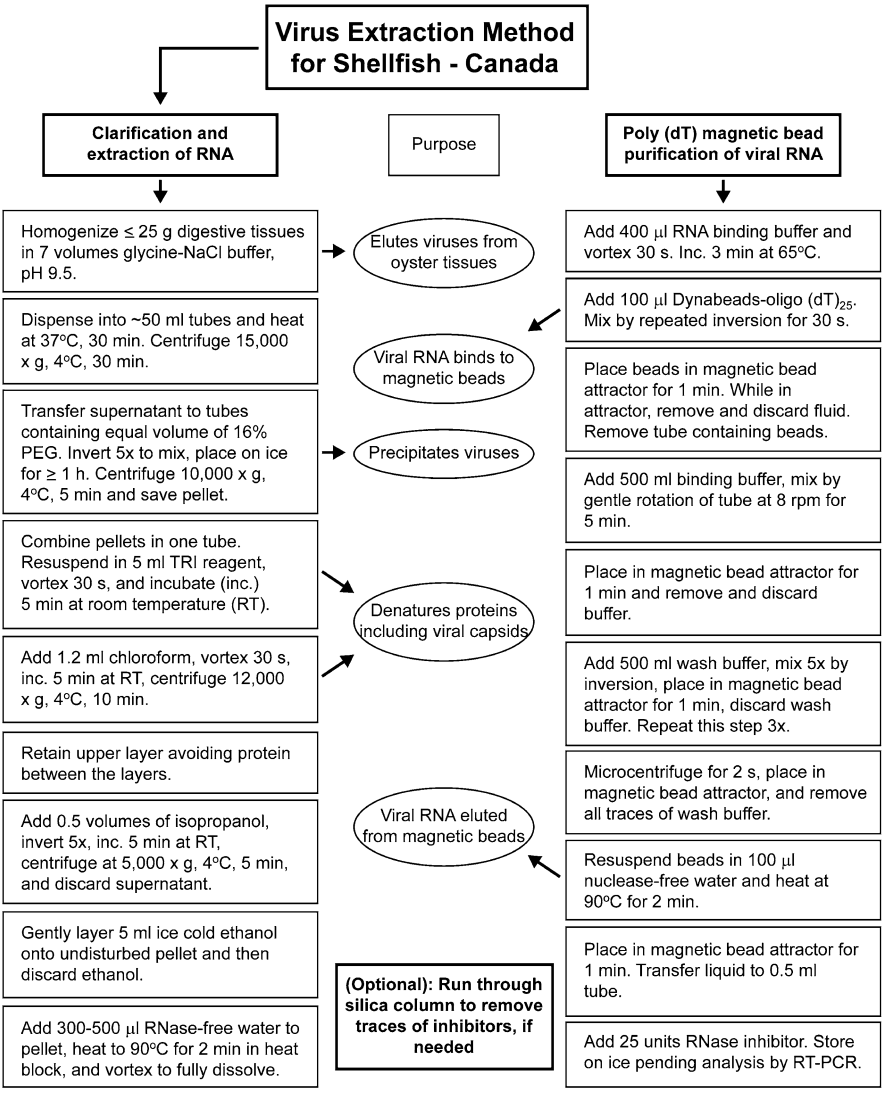


Fig. 15.2 Schematic diagram of steps in the GPTT procedure for virus extraction from shellfish

adequate controls are employed. In addition to the extraction controls mentioned above, controls are needed for RT-PCR and include an internal amplification control (e.g., plasmids, armored RNA, etc.); a positive RT-PCR control consisting of the virus being sought (norovirus, hepatitis A virus, etc.), and one or more negative RT-PCR controls. Together, the controls constitute an important part of the protocol. It should be recognized that RT-PCR has several important limitations which cannot be controlled for. Perhaps the greatest limitation is that RT-PCR detects total

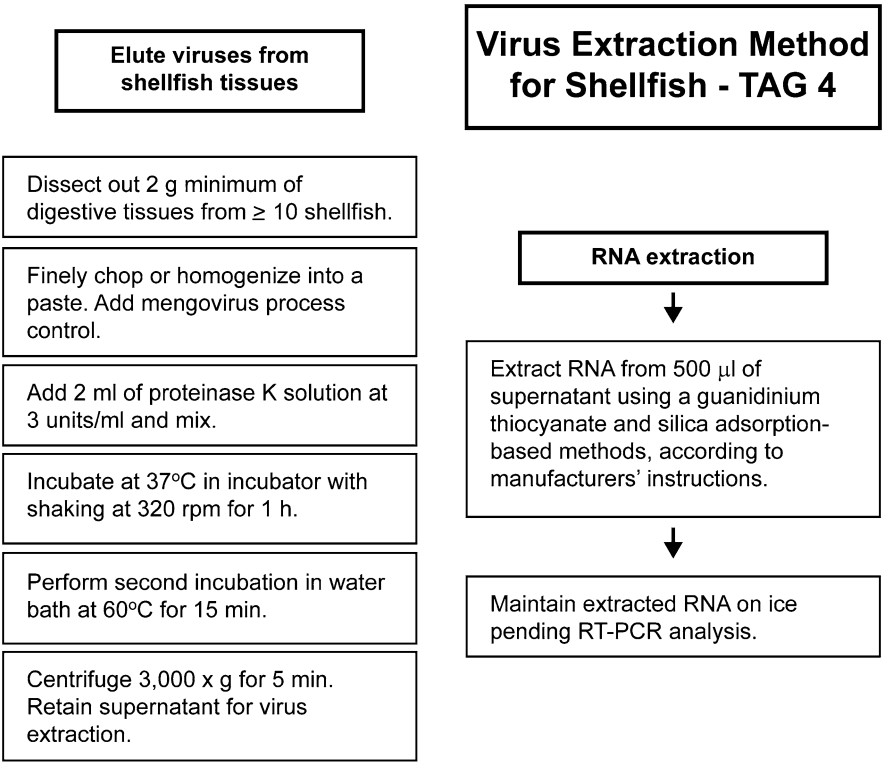


Fig. 15.3 Schematic representation of the European Committee on Standardization's Technical Advisory Group for Viruses (TAG4) method for virus extraction from shellfish, which is under evaluation within the European Union

viruses – both infectious, which are of public health concern, and non-infectious viruses, which are not a threat to public health (Richards 1999). Viruses inactivated by chlorine and other disinfectants, and by sunlight or other physical or chemical stressors are still detectable by RT-PCR, although they are not a public health problem. In addition, sensitivity is a concern because only a small volume of the total extract can be tested. Molecular assays are also technically complex, relatively expensive, subject to lab contamination (cross-contamination), and are not practical for testing large numbers of samples. In conclusion, the complexity in virus testing in shellfish is a deterrent to the establishment of routine virus monitoring programs. As has been the case in the past, virus analytical methods will likely be employed primarily in epidemiological investigations to track the sources of outbreaks. More simplified and practical methods are on the horizon to further improve the analysis of viruses in shellfish and other foods so that routine monitoring may be possible.

References

Anon (2010) CEN/TC275/TAG4 draft standards 2010: microbiology of food and animal feeding stuffs – Horizontal method for the detection of hepatitis A virus and norovirus in food using real-time RT-PCR – Part 1: method for quantitative determination, N501. 2010-05-07, version 8, Brussels, Belgium

Greening GE, Hewitt J (2008) Norovirus detection in shellfish using a rapid, sensitive virus recovery method and real-time RT-PCR detection protocol. Food Anal Method 1:109–118

Harrington PR, Vinje J, Moe CL, Baric RS (2004) Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions. J Virol 78:3035–3045

Kingsley DH, Richards GP (2001) Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish. Appl Environ Microbiol 67:4152–4157

Kingsley DH, Meade GK, Richards GP (2002) Detection of both hepatitis A virus and Norwalk-like virus in imported clams associated with food-borne illness. Appl Environ Microbiol 68:3914–3918

Lees D, CEN-WG6-TAG4 (2010) International standardisation of a method for detection of human pathogenic viruses in molluscan shellfish. Food Environ Virol 2:146–155

López-Sabater EI, Deng MY, Cliver DO (1997) Magnetic immunoseparation PCR assay (MIPA) for detection of hepatitis A virus (HAV) in American oyster (*Crassostrea virginica*). Lett Appl Microbiol 24:101–104

Richards GP (1999) Limitations of molecular biological techniques for assessing the virological safety of foods. J Food Prot 62:691–697

Richards GP, Cliver DO, Greening GE (in press) Foodborne viruses. Compendium of methods for the microbiological examination of foods, 5th edn. American Public Health Association, Washington, DC

Tian P, Engelbrektson A, Mandrell R (2008) Two-log increase in sensitivity for detection of norovirus in complex samples by concentration with porcine gastric mucin conjugated to magnetic beads. Appl Environ Microbiol 74:4271–4276

Trottier Y-L, Houde A, Buenaventura E et al (2010) Concentration of norovirus genogroups I and II from contaminated oysters and their detection using the reverse-transcriptase polymerase chain reaction. Available online at: http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume5/opflp_01-eng.php. Accessed 16 Mar 2011

Part IV
Chemical Analytical Methods/Méthodes d'analyse chimique

Chapter 16
Searching for Internal Standard for Chemical Routine Analysis of Lipophilic Shellfish Toxins

Trude S. Guldberg, T. Hatlen, and K. Aarstad

Introduction

Analytical methods based on liquid chromatography (LC) coupled to mass spectrometry (MS) have been used for several years to detect and quantify lipophilic marine biotoxins in crude methanol shellfish extracts. Identification and quantification in routine analysis relies on retention time and peak area response compared to certified standards. The analytical applications can be challenging as marine toxins are complex analytes which are present in complex biological matrices. Response drift and matrix effects (ion suppression or ion enhancement) in LC-MS analysis can lead to an over or underestimation of the toxin concentration.

Various approaches can be used to overcome these potential errors, including internal standards, standard addition, or by various clean-up techniques for removal of interfering matrix components. To our knowledge, no internal standards are available yet. Due to cost and limited availability of certified standards, the standard addition approach is generally not feasible in routine analysis. Clean-up applications by using solid phase extraction are rather time consuming and not necessarily successful with regards to eliminating matrix effects (Gerssen et al. 2009b; These et al. 2009). The lack of internal standards in LC-MS determinations makes the analysis vulnerable to analytical and instrumental variations. Correct quantification is dependent on accurate volumes during extraction and response stability during instrumental analysis.

An adequate internal standard requires non-interference with the masses specified for the toxin of interest and should be able to mimic the behavior of a particular analyte by being as similar as possible; ideally isotope labeled. Non-labeled internal standards are often homologs which elute close to the analyte thus appearing in the same vicinity with respect to any matrix effect.

T.S. Guldberg (✉) • T. Hatlen • K. Aarstad
Department of Clinical Pharmacology, St. Olav's University Hospital, Trondheim, Norway
e-mail: trude.guldberg@stolav.no; tone.hatlen@stolav.no; kjell.aarstad@live.no

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_16,

Purpose

The aim of this study was to improve the performance of LC-MS methods for lipohilic marine biotoxins by introducing internal standards as an analytical tool, which might compensate for response drift, matrix effects and unpredictable events during the analytical process.

Methods

Synthesized internal standards were designed from the skeletal structure of phenylbenzimidazole (PBI) by substitution reactions of different functional groups. Fifteen substances were tested in methods with negative and positive ionization mode. Two of the PBIs were further examined in the positive method; 1-pentyl-2-phenylbenzimidazole (Pent-PBI) and 1-heptyl-2-phenyl-benzimidazole (Hep-PBI). The monitored mass transitions were (precursor > fragment): Pent-PBI m/z 265.2 > 195.1 and Hep-PBI m/z 293.2 > 195.1.

In addition the polycyclic steroid deoxycholic acid (DHO) was tested for suitability in the method using negative ionization mode. DHO has comparable chemical and physical properties with the toxins in the diarrhetic shellfish toxins (DSP) group, and the monitored transition was m/z 391.1 > 345.2.

The impact of introducing an internal standard for reduced matrix effects was examined in naturally contaminated mussels (*Mytilus edulis*) and scallops (*Pectinidae*). Parallel extractions were carried out in which one group was extracted with methanol containing internal standard while the other was extracted using a published procedure (Stobo et al. 2005). Standard addition was performed by adding a known amount of multi-toxin solution into crude extracts. The quantified concentrations were compared with matrix-corrected values.

To monitor response drift with or without internal standard during analysis, calibration curves with at least five concentration levels were analyzed intermittently during analytical sequences. CRM[1] calibration standards were fortified in methanol. Calibration curve slopes, peak areas and response ratios were normalized to comparable levels, tested for significance and confidence intervals (CI) were determined. Analytical method precision, expressed as %RSD, was calculated based on CRM-DSP Mus-b (certified control sample) and naturally contaminated shellfish samples.

Chromatography was performed with two different systems; neutral and alkaline conditions. The methods used for analysis on single quadrupole MS was AOAC LC-MS method 88.5 2005 (modified) (Stobo et al. 2005), with a Zorbax Eclipse XDBC18, 4.6 × 50 mm, 1.8 μm column (Agilent), equipped with an in-line 0.2 μm filter (Agilent). For negative ionization mode mobile phase A was 100 % methanol

[1]Certified Reference Materials, NRC-CNRC. Exceptions: DTX-1 from Wako, Japan.

while mobile phase B was 100 % aqueous containing 50 mM ammonium acetate. A gradient from 30 to 90 % A was run over 2 min, and then held for 3 min. At 5 min the composition was reset to the initial conditions and 2 min equilibration time was allowed. The flow rate was set at 0.7 ml min^{-1} and 5 μl of each sample was injected onto the column at 50 °C.

For analysis in positive ionization mode, mobile phase A was 100 % methanol. Mobile phase B and C were 100 % aqueous, with 50 mM ammonium acetate and 25 mM formic acid, respectively. Starting with isocratic conditions of 12.5 % of eluent B and C for 2.5 min, a linear gradient from 75 to 85 % A was run over 3.5 min. At 6 min the composition was reset to the initial conditions and 2 min equilibration time was allowed. The flow rate was set at 1.0 ml min^{-1} and 10 μl of each sample was injected into the column at 30 °C. The autosampler maintained 4 °C, and the detector (Agilent MS-1956B) was equipped with an electrospray source (ESI).

For triple quadrupole analysis, alkaline conditions described in EU harmonised SOP LIPO LC-MS/MS (EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS & version 4. Retrieved July 2011; Gerssen et al. 2009a) were analysed with X-Bridge C18, 2.1×50 mm, 2.5 μm column (Waters), carried out on an Agilent MSMS-6460 (1200-series), electrospray ionization combined with positive and negative ionization modes. The mobile phases consisted of H_2O containing 0.05 % ammonia (pH 11) in channel A, and 95 % acetonitrile containing 0.05 % ammonia in channel B. Analysis were performed by running a linear gradient elution, starting from 10 to 90 % B from 1 to 6.7 min, followed by a 1.3 min hold at 90 % B, a 10 % A, decreasing to 10 % B over 2 min, and holding for 3 min until the next injection. The flow rate was 0.7 ml min^{-1}, and the injection volume was 10 μl at a column temperature of 40 °C. All results are, as far as possible, evaluated without distinction on instruments or methods.

Results and Discussion

Retention Times

PBI containing short aliphatic chains eluted too early in both ionization modes, or did not ionize in negative mode at all. Two of the PBI's; Hep-PBI and Pent-PBI, were tested further in the positive method, even though they differ chemically from the lipophilic toxins. Hep-PBI coeluted with azaspirazid-1 (Aza-1) while Pent-PBI, which is slightly more polar than Hep-PBI, eluted between pectenotoxin-2 (PTX-2) and Aza-1. Because of this, it is reasonable to assume that Hep-PBI will be appropriate as internal standard for Aza-1. The retention time of DHO was close to dinophysistoxin-1 (DTX-1), which may indicate DHO as a suitable internal standard for DTX-1.

Table 16.1 Average concentrations of DSP toxins (μg/kg) measured in three ways (Matrix corrected, ESTD and ISTD) and relative recovery (%) to matrix-corrected results

Toxin	Internal standard	LOQ ($\mu g\ kg^{-1}$)	n	Mean conc. ($\mu g\ kg^{-1}$)			Recovery (%)	
				Matrix corr.	ESTD	ISTD	ESTD	ISTD
OA	DHO	5.0	9	15.5	13.1	15.0	85.7	97.4
DTX-1	DHO	5.0	11	52.7	39.9	42.4	74.2	79.5
YTX	DHO	66.0	12	29,9	33.3	40.0	112.5	135.4
Aza-1	Pent-PBI	4.4	3	18.9	21.6	23.7	131.1	134.2
	Hep-PBI					21.3		114.5

Matrix Effects

The toxin concentrations were calculated in three ways to assess whether the use of internal standard compensate for the matrix effects (Table 16.1). Due to seasonal variations, naturally contaminated shellfish were in limited supply, and the survey was carried out on only 18 different samples; 14 mussels and 4 scallops, generally with low toxin amount. Samples with okadaic acid (OA) (n = 9), DTX-1 (n = 11), yessotoxin (YTX) (n = 12) and Aza-1 (n = 3) were extracted with a solvent-to-sample ratio (SSR) of five (Stobo et al. 2005).

Traditionally, toxin concentrations are measured from external calibration curves which are based on peak responses (ESTD). The calibration curves which are based on internal standards (ISTD) were generated from the ratio of peak responses between the toxin and the internal standard. Standard addition was performed in order to correct for individual matrix effects. As the standard addition method is the most widely accepted way to compensate for matrix effects, both the ISTD and ESTD concentrations were compared with those generated using standard addition. All concentration ranges stated in the results are matrix-corrected values determined by use of the formula suggested by Ito et al. (Ito and Tsukada 2001):

$$C_{ME-corr} = \frac{C_{theoretical} \times C_{unknown}}{(C_{std\ add} - C_{unknown})} \tag{16.1}$$

The concentration range of OA was from 5.3 to 31.3 $\mu g\ kg^{-1}$, and recovery for ISTD results was 97.4 % (95 % CI: 95.6–99.2 %), Table 16.1. All nine samples were mussels. It appears that using the DHO internal standard instead of standard addition can be helpful for quantification of OA, but the sample size is too low to draw any final conclusions. Statistical differences ($p < 0.05$) in toxin amount were detected by the use of paired t-test. However, the dissimilarity had no significance with regard to harvesting shutdown as all results were below the EU regulatory limit (160 $\mu g\ kg^{-1}$) (Regulation (EC) No 853/2004).

The toxin amount of Aza-1 ranged from 6.4 to 37.0 $\mu g\ kg^{-1}$, which was far below the EU regulatory levels (160 $\mu g\ kg^{-1}$) (Regulation (EC) No 853/2004). The recovery result of 114 % (95 % CI:110–119 %) was promising when using

Hep-PBIas an internal standard with regard to reducing the matrix effect. However, a larger number of samples are required before drawing a final conclusion. The sample size for Aza-1 results was too small to be handled statistically.

Discrepancies were observed between matrix-corrected and ISTD concentrations in relation to DTX-1 and YTX using DHO. ESTD results of DTX-1 were in agreement with the values from ISTD, in which no statistical significance was detected (paired t-test, $p < 0.05$). However, both ISTD and ESTD results had relative low recovery comparing to standard addition values, which indicated an underestimation of the toxin amount. The concentration range of DTX-1 was 9.9–225 $\mu g\ kg^{-1}$, and test samples in question were mussels only. One sample contained DTX-1 above the regulatory limit (160 $\mu g\ kg^{-1}$) (Regulation (EC) No 853/2004), and recovery relative to matrix-corrected result for ESTD and ISTD were acceptable; 82 and 87.4 %, respectively. The final toxin amount would cause harvesting shutdown no matter how the data was quantified. The idea of using DHO as an internal standard for DTX-1 because of the close retention time, is most likely not feasible, although a larger number of samples are required in order to draw a definitive conclusion.

Measurements of YTX ($n = 12$) ranged from 17.3 to 46.4 $\mu g\ kg^{-1}$, were low compared to the method limit of quantitation (LOQ) and EU regulatory limits (Regulation (EC) No 853/2004). Average recovery of ISTD was 135.4 % (95 % CI: 123–148 %). Quantified toxin amount of YTX were significantly higher when using internal standard than compared to standard addition, even though more samples are needed to draw a final conclusion. Unfortunately, no samples containing higher levels of YTX were available due to the seasonality of the sampling period.

In relation to PTX-2 ($n = 4$), two of four samples were scallops which clearly suffered from major matrix effect of more than 40 % enhancement by using both PBIs. The concentration range was wide, and extended from 8.9 to 155.7 $\mu g\ kg^{-1}$. For this reason, no comparisons based on the data were made. A high level of PTX-2 were measured in one of the samples (mussel), showing ESTD and matrix-corrected results of 147.4 and 155.7 $\mu g\ kg^{-1}$, respectively. ISTD values were measured to $<200\ \mu g\ kg^{-1}$. Calculations done by the use of internal standard did not seem to compensate for matrix effects affecting PTX-2, YTX or DTX-1, though more samples need to be analyzed.

Response Drift

Response drift during analytical sequences were monitored by analyzing calibration standards in the beginning, midway and the end of each analytical sequence. Each calibration curve consisted of five levels of CRMs fortified in methanol. The concentration levels of OA and DTX-1 ranged from 2 to 250 ng ml^{-1}, YTX; 10–500 ng ml^{-1}, and Aza-1 and PTX-2; 2–100 ng ml^{-1}.

Two sets of calibration curves were constructed each day; one set using the toxin peak areas (ESTD), and one set by the response ratios (ISTD). Four to five analytical

Table 16.2 Comparison of normalized calibration slopes of methanol standard curves

Toxin	Internal standard	No. of calibration curves	Mean slope variance (%)				P-value
			Area	95 % CI	Ratio	95 % CI	
OA	DHO	20	4.2	−1.2 to 9.7	1.7	−1.3 to 4.7	0.442
DTX-1	DHO	20	4.0	−3.0 to 11.0	2.4	−3.5 to 8.3	0.725
YTX	DHO	20	−2.3	−8.2 to 3.7	−3.8	−8.9 to 1.4	0.628
PTX-2	Pent-PBI	13	−4.5	−13.9 to 5.0	−1.0	−9.2 to 7.1	0.596
	Hep-PBI	13			3.6	−6.1 to 13.2	0.261
Aza-1	Pent-PBI	13	−6.4	−9.8 to −3.0	−2.7	−5.6 to 0.2	0.128
	Hep-PBI	13			1.8	−1.6 to 5.2	0.005

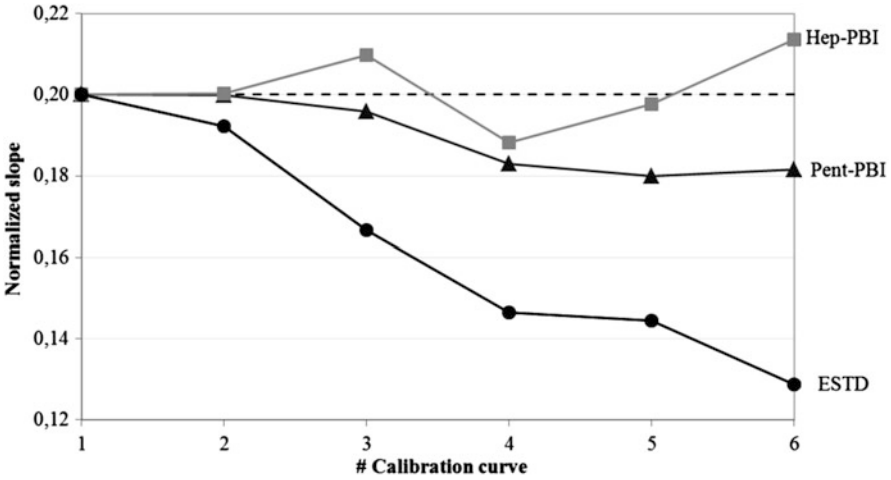


Fig. 16.1 Comparison of calibration slopes for Aza-1 in one analytical sequence, 145 injections

sequences were analyzed on subsequent days, and the determined calibration curve slopes were compared. When using internal standard to compensate for response drift, one would expect less slope variation if the internal standard drift is in the same direction at the same time as the toxin.

The average slope variances (Table 16.2) show the daily variation of the calibration curve slopes due to response drift in the system. The response drift increased with the length of the analytical sequence, both with respect to areas and ratios. Most calibration slopes based on ISTD partly smoothed out variations, shown by a tighter confidence interval. The ISTD calibration curves showed a slight improvement in the slope variations for all toxins except for YTX. Aza-1 was the only analyte with significantly less response drift using the ratio calculated from Hep-PBI versus peak area ($p = 0.005$), Table 16.2.

To illustrate response drift during a long analytical sequence, Fig. 16.1 shows the differences in slope values obtained for Aza-1 and how the internal standards level out the variations in the abundance. Six standard curves were generated from six

different calibration series spread out in one analytical sequence containing samples and controls, with a total number of 145 injections. All slopes were normalized to the slope obtained from the first calibration curve ($a = 0.20$). The normalized slope is shown in Fig. 16.1 as a dashed line.

The slopes of ESTD point toward a negative trend and deviate more from the normalized slope than the ISTD slopes. The slope variation using Hep-PBI differs in both positive and negative directions indicating that the internal standard response was not affected to the same extent as the toxin. Hence, Hep-PBI compensated for the negative trend of a decreasing slope which leads to overestimating of toxin concentrations.

Response Variation

The response stability for the analytes and internal standards during analytical sequences were tested in previously negative shellfish spiked with CRM (125 $\mu g\ kg^{-1}$). For Aza-1 and PTX-2 a total of 98 crude shellfish extracts were studied, of which 46 were mussels, 37 scallops and 15 cockles (*Cerastoderma edule*), analyzed by the neutral method (Stobo et al. 2005) over 4, 2 and 3 days, respectively. OA, DTX-1 and YTX were studied in 39 mussels and 37 scallops, analyzed over 2 days. The alkaline method (EU-Harmonised Standard Operating Procedure) was used during the first day of analysis whilst the neutral method (Stobo et al. 2005) was used during the second day. The determined peak areas were normalized to the first sample in each sequence before the comparison of day-to-day variations of response drift.

The results suggest that response variation depends on the species because their matrix behaves differently. For the negative ionization mode, DHO followed the response of OA ($p > 0.05$), and were not affected by different methods, instruments or species. Peak areas for DTX-1 and YTX drifted in opposite directions ($p < 0.05$) when considering the results without taking into account the different species (not shown). If the results were split by species the response variations of DTX-1 in mussels (Fig. 16.2) did not statistically differ from DHO. However, as seen in Fig. 16.2, there were lower DTX-1 values on day one than day two due to the difference in methodology and instrumentation, suggesting that a conclusion cannot be drawn based on this study. YTX behaved differently from DHO regardless of species, methodology and instrumentation.

The results for positive mode ionization demonstrated no statistical significance in the response variation between Hep-PBI and Aza-1 considering all three species together ($p = 0.14$) (not shown), or individually (example: scallops ($p < 0.05$), Fig. 16.3). In fact, using Pent-PBI for Aza-1 quantification seems promising in mussels (not shown) since their peak areas showed a linear relationship ($p = 0.4$). Pent-PBI was included at a later stage in the study, and that explains why no data is shown in Fig. 16.3 for the first and second day.

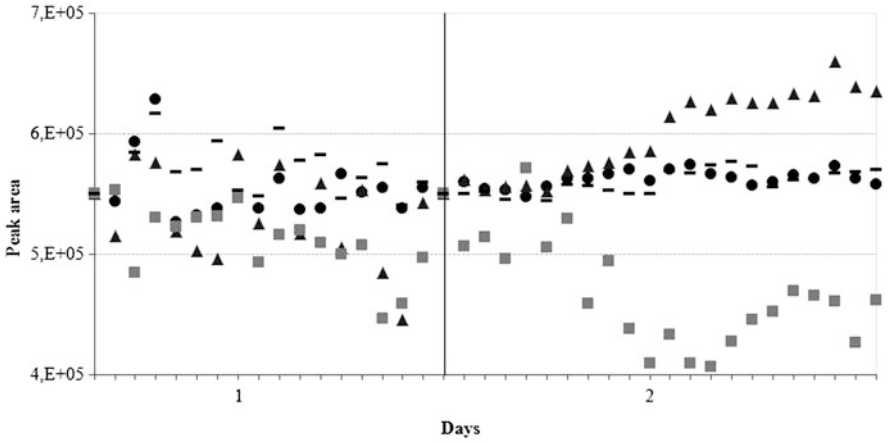


Fig. 16.2 Peak areas for OA (*black circles*), DTX-1 (*grey triangles*), YTX (*light grey squares*) and DHO (*black lines*) in mussels, analyzed during 2 days (n = 39)

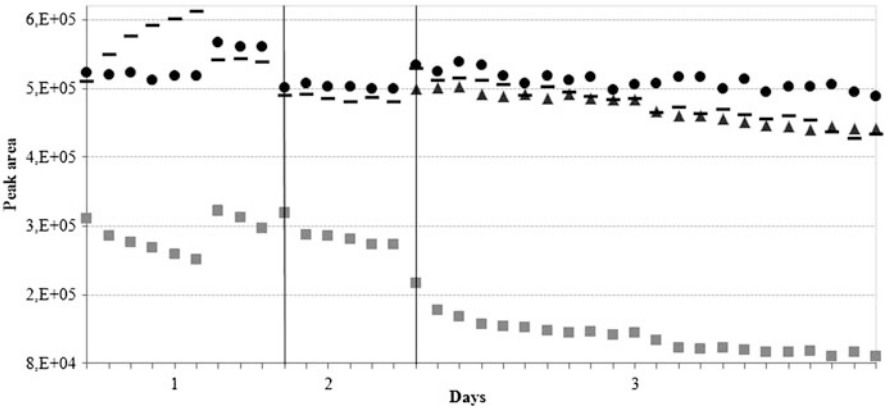


Fig. 16.3 Peak areas for Aza-1 (*black circles*), Pent-PBI (*grey triangles*), PTX-2 (*light grey squares*) and Hep-PBI (*black lines*) in scallops, analyzed during 3 days (n = 37)

The results for PTX-2 showed no correlation with Pent-PBI or Hep-PBI ($p < 0.05$) either when considering all species together or individually. However, by excluding data from scallops, PTX-2 peak area variation showed no significant differences from Pent-PBI ($p = 0.3$). Peak area response for PTX-2 in scallops differed from both mussels and cockles, which indicates substantial ion suppression, also shown by Ito and Tsukada (2001). The pronounced matrix effect observed for PTX-2 in scallops is illustrated in Fig. 16.3. None of the internal standard in this study was able to compensate for the ion suppression.

Table 16.3 Analytical precision by quantifying toxin concentrations by ISTD or ESTD with and without the use of internal standards

Toxin	Internal standard	Sample size	No. of replicate injections	% RSD	
				ESTD	ISTD
OA	DHO	10	89	2.7	3.5
DTX-1	DHO	12	117	3.1	3.9
YTX	DHO	6	46	8.8	8.8
PTX-2	Pent-PBI	4	40	8.5	9.2
	Hep-PBI				10.0
Aza-1	Pent-PBI	1	10	2.3	2.5
	Hep-PBI	10	52	2.7	4.8

An additional observation was made by subsequent injections after samples of scallop which were influenced by matrix deposition in the analytical system. The observed contamination means that the PTX-2 signal in the next sample or standard was affected by ion suppression from the previous scallop extract. This suggests that injections of organic solvent should be done after analysis of scallop extracts.

Precision

To demonstrate and compare the reproducibility of the two methods, naturally contaminated samples and CRM-DSP Mus-b were analyzed. The samples were run over three different days with multiple injections in each sequence. Table 16.3 shows analytical precision expressed as %RSD compared with (ISTD) and without internal standard (ESTD).

Calculation of analytical precision was slightly better using peak areas compared to internal standards. The precision results for most toxins were satisfactory.

Precision for YTX was acceptable for both calculation models bearing in mind the low toxin concentration due to the EU regulatory limit for intoxication in humans (Regulation (EC) No 853/2004). Sample size of four PTX-2 reflected the relatively high %RSD as the measured toxin amount was close to the LOQ (12 $\mu g\ kg^{-1}$) in three out of four samples.

Conclusions

This preliminary study illustrates the complexity of establishing appropriate internal standards for chromatographic and mass spectrometric determination of lipophilic shellfish toxins. Implementation of an internal standard can be a powerful analytical approach to improve the performance, as the analytical methods for marine toxins were clearly compromised by matrix effects.

The conclusions one can draw from the study are somewhat limited owing to the fact that the data set is small. However, the study undoubtedly represents a starting point to improve the performance of MS detection as a valuable tool for identification and quantification of marine biotoxins.

Calculations with the internal standards examined in this study are promising for OA and Aza-1. DHO seems to be more suitable as an internal standard for OA than for DTX-1, although DTX-1 elutes closer to DHO than OA.

The internal standard candidates examined in this initial work did not show improvements related to response drift or matrix effects for DTX-1, PTX-2 or YTX. The molecular structure and the chemical properties of YTX differ from the other marine toxins, and thus make it more challenging to find one suitable internal standard that matches all analytes in the methods. Further studies are required before any definite conclusions can be drawn.

References

EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS, version 4. Retrieved July 2011, from http://www.aesan.msps.es/en/CRLMB/web/procedimientos_crlmb/crlmb_standard_operating_procedures.shtml

Gerssen A, Mulder PPJ, McElhinney MA, de Boer J (2009a) Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. J Chromatogr A 1216:1421–1430

Gerssen A, McElhinney MA, Mulder PPJ, Bire R, Hess P, de Boer J (2009b) Solid phase extraction for removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 394:1213–1226

Ito S, Tsukada K (2001) Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins. J Chromatogr A 943:39–46

Regulation (EC) No 853/2004. Official Journal European Parliament and of the Council of 29 April 2004, p 99. Retrieved February 2011, from http://www.aesan.msps.es/en/CRLMB/web/legislacion_comunitaria/legislacion.shtml

Stobo LA, Lacaze JPCL, Scott AC, Gallacher S, Smith EA, Quilliam MA (2005) Liquid chromatography with mass spectrometry – detection of lipophilic shellfish toxins. J AOAC Int 88:1371–1382

These A, Scholz J, Preiss-Weigert A (2009) Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography-tandem mass spectrometry based on enrichment by solid-phase extraction. J Chromatogr A 1216:4529–4538

Chapter 17
Effect of Dilution Rate on *Azadinium spinosum* and Azaspiracid (AZA) Production in Pilot Scale Photobioreactors for the Harvest of AZA1 and -2

T. Jauffrais, V. Séchet, P. Truquet, Zouher Amzil, C. Herrenknecht, and P. Hess

Introduction

In 1995 the first azaspiracid (AZA) shellfish poisoning occurred in the Netherlands with symptoms similar to diarrhetic shellfish poisoning (McMahon and Silke 1996). A few years later, the toxin was identified and named azaspiracid (Satake et al. 1998) and then structurally revised (Nicolaou et al. 2004). Afterwards, a large number of analogues were identified in mussel tissues using biological assay and chemical analysis including liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), i.e. AZA2-32 (Diaz Sierra et al. 2003; James et al. 2003; McCarron et al. 2009; Ofuji et al. 1999, 2001; Rehmann et al. 2008). Nevertheless, since the first known poisoning event, it took 12 years until the discovery of a primary producer, the dinoflagellate *A. spinosum* (strain 3D9) (Krock et al. 2008, 2009; Tillmann et al. 2009). This small dinoflagellate (12–16 μm length and 7–11 μm width) produces AZA1 and -2 in culture (Tillmann et al. 2009). Since this recent discovery the organism has been encountered in different parts of the world: Ireland

T. Jauffrais (✉) • P. Truquet
Laboratoire Phycotoxines, IFREMER, Rue de l'Ile d'Yeu, 44311 Nantes, France
e-mail: thierry.jauffrais@ifremer.fr; philippe.truquet@ifremer.fr

V. Séchet • Z. Amzil
Laboratoire Phycotoxines, IFREMER, Rue de l'Ile d'Yeu, 44311 Nantes, France

IFREMER, Rue de l'Ile d'Yeu, BP 21105, 44311 Nantes, France
e-mail: veronique.sechet@ifremer.fr; Zouher.Amzil@ifremer.fr

C. Herrenknecht
Nantes Atlantique Université, MMS EA2160, 1 rue Gaston Veil, 44035 Nantes, France
e-mail: christine.herrenknecht@univ-nantes.fr

P. Hess
Laboratoire Phycotoxines, IFREMER, Rue de l'Ile d'Yeu, 44311 Nantes, France

Nantes Atlantique Université, MMS EA2160, 1 rue Gaston Veil, 44035 Nantes, France

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_17,

(Salas et al. 2011), France (Nezan et Siano, personal communication), Mexico (Hernandez-Becerril et al. 2010), Argentina (Akselman and Negri 2012), and AZA occurrences are now recognized as a worldwide phenomenon. Until now, AZAs were purified and isolated from contaminated bivalves, as it has been originally carried out with other marine biotoxins: okadaic acid group toxins, brevetoxins, saxitoxins, yessotoxins, domoic acid, cyclic imines and pectenotoxins (Rundberget et al. 2007). However, severe toxic events are required to obtain pure standards from contaminated bivalves, even though recovery has been improved recently and the number of purification steps required to purify AZAs from complex matrices reduced (Kilcoyne et al. 2012; Perez et al. 2010).

The primary AZA producer is now identified and adapted to culture, furthermore, natural occurring blooms are hard to predict and/or to find; preventing *in situ* direct extraction of AZA as developed by Rundberget et al. (2007). Thus, to avoid AZA1 and -2 scarcities it is important to have a sustainable production of toxins from *A. spinosum* culture for toxicological studies, and for instrument calibration in continuous monitoring programs.

The aim of this study was to evaluate the feasibility of AZA production from *A. spinosum* produced in a continuous series of pilot scale photobioreactors. We describe here how dilution rate influences cell concentration as well as toxin production in pilot scale chemostat bioreactors in series and the use of different solid phase extraction procedures to recover AZAs from large volumes of *A. spinosum* culture (200 L) after tangential flow filtrations.

Materials and Methods

Culture Conditions and Measurement

The strain (3D9) of *Azadinium spinosum* was the source of AZA1 and -2 for the experiment. The algae were produced in two chemostats of 100 L each, operated in series at different dilution rates (0.15, 0.2, 0.25 and 0.3 day^{-1}). Culture medium was a K modified medium (Keller et al. 1987), without NH_4Cl, tris buffer and with Na_2SeO_3 (10^{-8} M).

The photobioreactors were operating using the following conditions : the pH was maintained at 7.9 using CO_2 addition, T = 18 °C, a photon flux density of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on one side of the reactor, and a photoperiod of 16 h of light and 8 h of dark (Jauffrais et al. 2010). A Rushton turbine was homogenizing the algae at 40 rpm. Algae were collected in a harvesting tank (300 L), aerated and maintained at 18 °C (Fig. 17.1).

A particle counter (Beckman, Multisizer 3 Coulter counter) was used daily to assess cell concentrations ($cells \cdot mL^{-1}$), average size (μm) and cellular volume ($\mu m^3 \cdot mL^{-1}$). The bioreactors were considered at steady state after a minimum of 5 days at the same micro-algal concentration (±5 %).

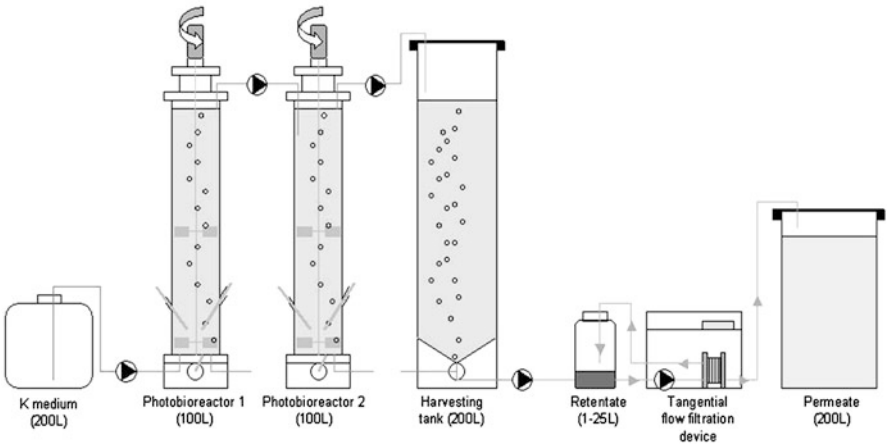


Fig. 17.1 Azaspiracids production system

Intra- and Extra-cellular Analysis of AZAs

At the different steady states studied daily over a week, triplicate samples of *A. spinosum* were taken from each bioreactor to assess toxin content, the same analyses were carried out from the 300 L harvesting tank before each tangential flow filtration for initial toxin content assessment.

The analytical procedure had been previously optimised (Jauffrais et al. 2012). Briefly, aliquots (10 mL) of *A. spinosum* cultures were collected and centrifuged (2,500 g, 20 min, 4 °C) in 15 mL tubes. The supernatant was collected (for extra-cellular toxin content) and the pellet was re-suspended with 0.5 mL of acetone/H_2O (9/1, v/v), transferred to an Eppendorf tube (1.5 mL) and bath sonicated (10 min). After sonication, the aliquot was centrifuged (15,000 g, 10 min, 4 °C). The supernatant was transferred to a 5 mL glass tube and gently evaporated under nitrogen on a heating block at 35 °C. This process was repeated so that the pellet was extracted three times in total. After evaporation of supernatants, the residue was reconstituted in 1 mL methanol. Subsequently, an aliquot was filtered with NANOSEP MF filter 0.2 μm (PALL) (15,000 g, 3 min, 4 °C), and transferred into a HPLC vial with a 250 μL insert for analysis.

After centrifugation of algal culture, the supernatant was transferred to a 15 mL glass tube and 5 mL of dichloromethane was added. The mixture was homogenized and centrifuged (2,500 g, 10 min, 4 °C). The organic phase was transferred to a 15 mL glass tube and gently evaporated under nitrogen on a heating block at 35 °C. The aqueous phase was extracted three times in this manner, and following evaporation, the residue was reconstituted and filtered as above.

AZAs Harvesting Procedures

Tangential flow filtration (Sartorius Stedim Biotech, Sortojet Pump with Sartocon Slice and 5×0.1 m^2 Hydrosart Open Channel Microfiltration Cassettes) was applied to separate the algae from the culture medium. Thus, 200 L of algal culture were divided into 1 L of algal concentrate (retentate) and almost 200 L of permeate (Fig. 17.1).

For toxin extraction from the retentate, the algal concentrate was sonicated (20 min in ice, Bioblock Scientific, Vibra-cell 75115), 25 g of activated Diaion HP20 polymeric resin was added, and gently agitated within the algal concentrate over 24 h, on a laboratory shaker (IKALABORTECHNIK, KS125basic). The resin was then washed with 1 L of Milli-Q water (Millipore, Integral 3 system), and placed in a glass column (3 by 60 cm). The toxin was eluted with three volumes of acetone (50 mL) at 1 $mL \cdot min^{-1}$. The extract was then evaporated using a rota-evaporator (Büchi, Rotavapor R-200) and the residue was reconstituted in 5 mL methanol.

For toxin extraction from the 200 L permeate, two procedures were tested:

1. Passive samplers were placed into the permeate as developed by MacKenzie et al. (2004) and Fux et al. (2008, 2009). Eight SPATT bags (solid phase adsorption toxin tracking) containing 3 g of activated Diaion® HP20 resin were added into the permeate and gently agitated within a submerged pump over 72 h. The resin was then extracted as above.
2. A submerged pump ($20\ L \cdot min^{-1}$) was placed into the permeate and connected to a column containing 25 g of activated Diaion® HP20 resin over 72 h. The resin was then extracted as above. This procedure was an adaptation of Rundberget et al. (2007) developed for large scale extraction of micro-algal biotoxin *in situ*.

LC-MS/MS Analysis

The samples were analyzed by LC/MS-MS using an Agilent 1100 model coupled to a triple quadrupole mass spectrometer (SCIEX-Applied Biosystems, API 2000) for quantification of AZAs. Five microlitre of each sample were injected into the LC-MS/MS, toxins were separated by reversed-phase chromatography with a silica-based column (Hypersil BDS C8 column, size 50*2 mm, 3 μm particle size; Phenomenex) The A and B mobile phases were 100 % water and acetonitrile/water (95/5, v/v) respectively, both containing 2 mM ammonium formate and 50 mM formic acid. The BDS-Hypersil column was eluted isocratically at a dilution rate of 250 $\mu L \cdot min^{-1}$ (75%B) at 20 °C for 10 min.

AZAs were quantified by comparison with a series of AZA-1 standard from the National Research Council, Canada (NRC). The two most intense product ions were selected with the following transitions: AZA1 m/z $842.5 > 824.5$ and $842.5 > 672.4$, and AZA2 $856.5 > 838.5$ and $856.5 > 672.4$

Results and Discussion

Cell concentration, mean diameter, cellular volume and toxin content remained constant at steady states when the dilution rate changed. However, differences were noticed between reactors in series (R1 and -2). At the different dilution rates studied, cell concentrations were equal to 190,000 and 215,000 cells $\cdot$ mL^{-1} in R1 and R2 respectively and results for mean diameter and cellular volume were comparable at all dilution rates but differed between R1 and R2 (Table 17.1). For each steady state studied, AZAs cell quota increased between bioreactor 1 and -2, showing a positive effect of bioreactors in series to enhance AZA cellular content. Interestingly, contrarily to cellular concentration, AZA cell content decreased as dilution rate increased, ranging from 67 to 24 fg $\cdot$ cell^{-1} for R1 and 98 to 63 fg $\cdot$ cell^{-1} for R2. Thus, the cell production increased as dilution rate increased whereas AZA production reached an optimum at 0.25 day^{-1} of 475 ± 17 μg $\cdot$ day^{-1} under the studied conditions.

Batch cultured *A. spinosum* (strain 3D9 or SM2) produced AZA1 and -2, with AZA1 as the predominant AZA and with a cell quota ranging from 5 to 40 fg $\cdot$ cell^{-1} (Jauffrais et al. 2010; Salas et al. 2011; Tillmann et al. 2009). In the present study, the same toxin profiles were found, however, AZAs cell quota of 24–98 fg $\cdot$ cell^{-1} were obtained depending on the dilution rate. There was a higher toxin concentration at low growth rate of *A. spinosum* than higher dilution rate (especially in R1), showing the necessity of chemostats in series at higher dilution rate to significantly increase toxin concentration.

As described above, continuous *A. spinosum* culture was shown to be valuable for production of AZAs using photobioreactors in series. Subsequently, AZA extractions were developed to optimise the recovery from bioreactors. Before filtration, 95 % of the toxin was intracellular, whereas after filtration, 50–70 % of the toxin was contained in the concentrate and 30–50 % released in the permeate. The observed variation was time dependent, with longer filtration times leading to higher proportions of toxin in the permeate.

Intracellular toxin content was recovered as algal paste after centrifugation of the retentate, however, this procedure caused the loss of some toxin from the supernatant ($\pm$10 %). To avoid this loss a solid phase adsorption was implemented using Diaion® HP20 resin as explained above, this procedure allowed for the recovery of up to 90 % of the total toxin from the retentate.

Extracellular toxin content was extracted using two procedures, the SPATT bags and a solid phase extraction procedure. SPATT bags were initially designed as a monitoring tool to follow and predict micro-algal toxic events around shellfish production areas (MacKenzie et al. 2004; MacKenzie 2010). The solid phase extraction procedure was implemented for biotoxin extraction from naturally occurring micro-algal blooms (Rundberget et al. 2007). These two methods allowed good recovery, however, recovery using SPATT bags showed more variability than the SPE procedure in the conditions tested. Even though, the procedures for AZAs extraction from the concentrate and permeate allowed for the recovery of 80 ± 5 % of toxins originally produced by *A. spinosum* pilot scale culture.

Table 17.1 *A. spinosum* concentration (cells · mL^{-1}), mean diameter (μm), cellular volume ($\mu m^3 \cdot mL^{-1}$), toxin content (fg · $cell^{-1}$), and cell and toxin productivity (cells · day^{-1} and μg · day^{-1} respectively) at the different dilution rate studied (0.15, 0.2, 0.25, 0.3 day^{-1}) in the two bioreactors in series (R1 and -2)

	0.15 day^{-1}		0.2 day^{-1}		0.25 day^{-1}		0.3 day^{-1}	
	R 1	R 2	R 1	R 2	R 1	R 2	R 1	R 2
***A. spinosum* concentration** (cells · mL^{-1})	193,000 ± 6,000	214,000 ± 3,000	194,000 ± 8,000	214,000 ± 7,000	190,000 ± 6,000	221,000 ± 5,000	187,000 ± 5,000	220,000 ± 4,000
***A. spinosum* mean diameter** (μm)	9.59 ± 0.15	9.90 ± 0.16	9.63 ± 0.23	10.11 ± 0.18	9.29 ± 0.09	9.93 ± 0.04	9.48 ± 0.12	10.02 ± 0.05
***A. spinosum* cellular volume** ($10^7 \times \mu m^3 \cdot mL^{-1}$)	9 ± 0.4	11 ± 0.4	9 ±0.6	12 ± 0.7	8 ± 0.3	12 ± 0.2	8 ± 0.1	12 ± 0.4
AZA1 (fg · $cell^{-1}$)	52 ± 6	74 ± 4	34 ± 12	76 ± 14	26 ± 2	61 ± 3	17 ± 1	45 ± 3
AZA2 (fg · $cell^{-1}$)	15 ± 1	24 ± 2	10 ± 2	19 ± 2	12 ± 2	25 ± 2	7 ± 1	18 ± 2
AZAs (fg · $cell^{-1}$)	67 ± 3	98 ± 5	44 ± 13	95 ± 16	38 ± 2	86 ± 3	24 ± 1	63 ± 5
Cell productivity ($10^9 \times$ cells · day^{-1})	2.90 ± 0.09	3.21 ± 0.05	3.90 ± 0.16	4.28 ± 0.14	4.75 ± 0.15	5.53 ± 0.13	5.61 ± 0.15	6.60 ± 0.12
Toxin productivity AZA1 + AZA2 (μg · day^{-1})	193 ± 9	314 ± 15	170 ± 50	406 ± 64	180 ± 10	475 ± 17	134 ± 5	415 ± 33

Conclusion

At a rate of 0.25 day^{-1}, we obtained about 3 mg of AZAs in crude extracts over 12 days (8 days of culture, 1 day of filtration and 3 days of extractions). The optimisation of the procedure demonstrated the feasibility of producing AZAs from *A. spinosum* cultured in photobioreactors in series. The AZAs obtained are suitable amounts for purification and production of certified standards for further toxicological study and for instrument calibration in monitoring programs.

Acknowledgment This study was carried out under the Sea Change strategy with the support of the Marine Institute and the Marine Research Sub-Programme of the National Development Plan 2007–2013 (ASTOX2 project PBA/AF/08/001(01)), co-financed by the European Regional Development Fund. Further funding was obtained through Ifremer from the French Ministry of Education, Research and Technology through Programme 187 of the National Finance Law. The authors would like to thank all the members of the laboratory EMP/PHYC at the Atlantic Centre of Ifremer for their help and technical advice during this study.

References

Akselman R, Negri RM (2012) Blooms of Azadinium cf. spinosum Elbrächter et Tillmann (Dinophyceae) in northern shelf waters of Argentina, Southwestern Atlantic. Harmful Algae 19:30–38. doi:10.1016/j.hal.2012.05.004

Diaz Sierra M, Furey A, Hamilton B, Lehane M, James KJ (2003) Elucidation of the fragmentation pathways of azaspiracids, using electrospray ionisation, hydrogen/deuterium exchange, and multiple-stage mass spectrometry. J Mass Spectrom 38:1178–1186

Fux E, Marcaillou C, Mondeguer F, Bire R, Hess P (2008) Field and mesocosm trials on passive sampling for the study of adsorption and desorption behaviour of lipophilic toxins with a focus on OA and DTX1. Harmful Algae 7:574–583

Fux E, Bire R, Hess P (2009) Comparative accumulation and composition of lipophilic marine biotoxins in passive samplers and in mussels (*M. edulis*) on the West Coast of Ireland. Harmful Algae 8:523–537

Hernandez-Becerril DU, Escobae-Morales S, Morreno-Gutiérez SP, Baron-Campis SA (2010) Two new records of potentially toxic phytoplankton species from the Mexican Pacific. Poster presented at the 14th international conference on harmful algae, Hersonissos, Nov 2010

James KJ, Sierra MD, Lehane M, Magdalena AB, Furey A (2003) Detection of five new hydroxyl analogues of azaspiracids in shellfish using multiple tandem mass spectrometry. Toxicon 41:277–283

Jauffrais T, Séchet V, Herrenknecht C, Tillmann U, Krock B, Amzil Z, Hess P (2010) Growth and toxin production of *Azadinium spinosum* in batch and continuous culture. Poster presented at the 14th international conference on harmful algae, Hersonissos, Nov 2010

Jauffrais T, Herrenknecht C, Séchet V, Sibat M, Tillmann U, Krock B, Kilcoyne J, Miles CO, McCarron P, Amzil Z, Hess P (2012) Quantitative analysis of azaspiracids in *Azadinium spinosum* cultures. Anal Bioanal Chem 403:833–846

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol 23:633–638

Kilcoyne J, Keogh A, Clancy G, Leblanc P, Burton I, Quilliam MA, Hess P, Miles CO (2012) Improved isolation procedure for azaspiracids from shellfish, structural elucidation of azaspiracid-6, and stability studies. J Agric Food Chem 60:2447–2455

Krock B, Tillmann U, John U, Cembella A (2008) LC-MS-MS aboard ship: tandem mass spectrometry in the search for phycotoxins and novel toxigenic plankton from the North Sea. Anal Bioanal Chem 392:797–803

Krock B, Tillmann U, John U, Cembella AD (2009) Characterization of azaspiracids in plankton size-fractions and isolation of an azaspiracid-producing dinoflagellate from the North Sea. Harmful Algae 8:254–263

MacKenzie LA (2010) In situ passive solid-phase adsorption of micro-algal biotoxins as a monitoring tool. Curr Opin Biotechnol 21:326–331

MacKenzie L, Beuzenberg V, Holland P, McNabb P, Selwood A (2004) Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. Toxicon 44:901–918

McCarron P, Kilcoyne J, Miles CO, Hess P (2009) Formation of azaspiracids-3, -4, -6, and -9 via decarboxylation of carboxyazaspiracid metabolites from shellfish. J Agric Food Chem 57:160–169

McMahon T, Silke J (1996) West coast of Ireland winter toxicity of unknown aetiology in mussels. Harmful Algae News 14:2

Nicolaou KC, Koftis TV, Vyskocil S, Petrovic G, Ling TT, Yamada YMA, Tang WJ, Frederick MO (2004) Structural revision and total synthesis of azaspiracid-1, part 2: definition of the ABCD domain and total synthesis. Angew Chem Int Ed 43:4318–4324

Ofuji K, Satake M, McMahon T, Silke J, James KJ, Naoki H, Oshima Y, Yasumoto T (1999) Two analogs of azaspiracid isolated from mussels, *Mytilus edulis*, involved in human intoxication in Ireland. Nat Toxins 7:99–102

Ofuji K, Satake M, McMahon T, James KJ, Naoki H, Oshima Y, Yasumoto T (2001) Structures of azaspiracid analogs, azaspiracid-4 and azaspiracid-5, causative toxins of azaspiracid poisoning in Europe. Biosci Biotechnol Biochem 65:740–742

Perez RA, Rehmann N, Crain S, LeBlanc P, Craft C, MacKinnon S, Reeves K, Burton IW, Walter JA, Hess P, Quilliam MA, Melanson JE (2010) The preparation of certified calibration solutions for azaspiracid-1,-2, and -3, potent marine biotoxins found in shellfish. Anal Bioanal Chem 398:2243–2252

Rehmann N, Hess P, Quilliam MA (2008) Discovery of new analogs of the marine biotoxin azaspiracid in blue mussels (*Mytilus edulis*) by ultra-performance liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 22:549–558

Rundberget T, Sandvik M, Larsen K, Pizarro GM, Reguera B, Castberg T, Gustad E, Loader JI, Rise F, Wilkins AL, Miles CO (2007) Extraction of microalgal toxins by large-scale pumping of seawater in Spain and Norway, and isolation of okadaic acid and dinophysistoxin-2. Toxicon 50:960–970

Salas R, Tillmann U, John U, Kilcoyne J, Burson A, Cantwell C, Hess P, Jauffrais T, Silke J (2011) The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels. Harmful Algae 10:774–783

Satake M, Ofuji K, Naoki H, James KJ, Furey A, McMahon T, Silke J, Yasumoto T (1998) Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. J Am Chem Soc 120:9967–9968

Tillmann U, Elbrachter M, Krock B, John U, Cembella A (2009) *Azadinium spinosum* gen. et sp. nov. (Dinophyceae) identified as a primary producer of azaspiracid toxins. Eur J Phycol 44:63–79

Chapter 18
A Comparison of Assay Techniques for the Analysis of Diarrhetic Shellfish Poisoning Toxins in Shellfish

R. Raine, A.M. Wilson, G. Hermann, and J.P. Lacaze

Introduction

Contamination of shellfish with Diarrhetic Shellfish Poisoning (DSP) toxins principally derived from *Dinophysis* spp. is the biggest problem for shellfish producers with respect to algal biotoxins along the west of the European Atlantic seaboard (Raine et al. 2010). The onset of these harmful algal events can occur in a matter of days due to rapid transport of toxic cells into an enclosed area by oceanographic processes. Rapid analysis of biotoxins in shellfish is therefore paramount. The standard method within Europe for the analysis of DSP toxins has been the mouse bioassay (MBA) (Yasumoto et al. 1978), which is often used in tandem with chemical methods such as liquid chromatography with mass spectrometry (LC-MS) (EC reg. 2074/2005). These methods have numerous limitations including their expense and use in a restricted number of laboratories which can cause long lag times, often exceeding that of the onset of a harmful algal event. These issues are particularly prevalent in geographically remote and peripheral regions, and have prompted the requirement of new analytical technologies for the analysis of algal biotoxins in shellfish to be performed rapidly, inexpensively, and locally which has particular relevance for local end product testing. This study investigates the use of two rapid techniques; an immunoassay and a functional assay, taking advantage of a national monitoring programme where results can be compared with MBA and LC-MS analysis in order to assess the accuracy, reliability and ease of use and applicability of these methods.

R. Raine (✉) • A.M. Wilson
The Ryan Institute, National University of Ireland, Galway, Ireland
e-mail: robin.raine@nuigalway.ie; annette.wilson@nuigalway.ie

G. Hermann • J.P. Lacaze
Marine Scotland Science, Aberdeen, Scotland, UK
e-mail: Guillaume.hermann@scotland.gsi.gov.uk; Jean-pierre.lacaze@scotland.gsi.gov.uk

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_18,

Methods and Materials

Sample Collection

Edible blue mussels (*Mytulis edulis*) were collected fortnightly during the period June–September 2009 and weekly from May–September 2010 from Killary Harbour (53° N 37′ W, 09° 48′ W) Connemara, Co. Galway, Ireland (Fig. 18.1). Approximately 40 individuals were collected as sub-samples of those collected under the Irish National Biotoxin Monitoring Programme (NMP) operated by the Marine Institute (MI), from three stations; inner: GY-KH-KI, middle: GY-KH-KM and outer: GY-KH-KO, covering the length of the fjord (16 km). Environmental parameters were recorded on each sampling occasion. Samples for phytoplankton analysis were collected using a 12 mm i.d. tube to achieve an integrated water sample over the depth range 0–10 m (Lindahl 1986). Discrete water samples at various depths (2, 5 and 10 m) dependent on the sample site were taken in addition to integrated samples in 2010. All samples were preserved with Lugol's iodine before analysis using an inverted microscope (McDermott and Raine 2010).

Mussels collected during 2009 were stored whole at −20 °C. For analysis, the mussels were thawed, cleaned and the shellfish removed by cutting the abductor muscles. At least 100 g of flesh from each sample was rinsed with deionised water and homogenised using a hand held blender for approximately 2 min. Homogenates were stored in graded polypropylene centrifuge tubes (50 ml) at −20 °C. Mussels collected during the 2010 period were prepared immediately to eliminate any suspected freeze-thaw storage effects. Samples were thawed and refrozen as required.

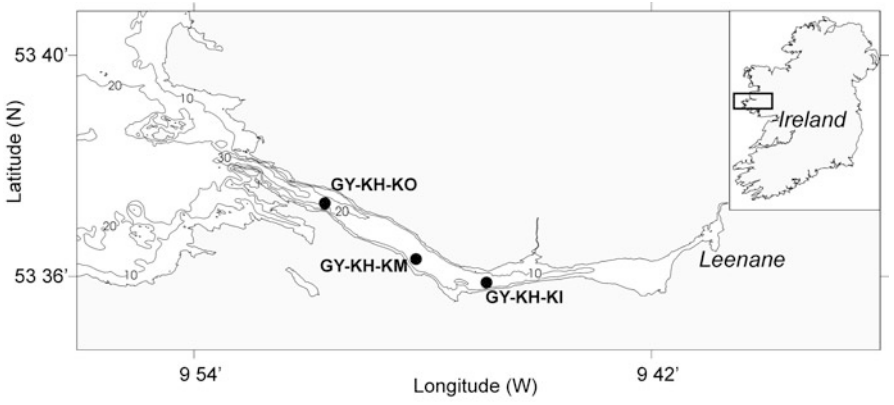


Fig. 18.1 Map of Killary Harbour, Co. Galway, Ireland showing the location of sampling sites and location of sampling stations in the outer (GY-KH-KO), middle (GY-KH-KM) and inner (GY-KH-KI) sections of the fjord

Toxin Extraction and Analysis

Toxins were analysed using commercially available kits. Both immunoassay kit (DSP ELISA, Abraxis) and an enzymatic protein phosphatase (PP2A) kit (OKAT-EST, ZEU-Inmunotec, Spain) were used for the detection of DSP toxins in the mussel extracts. The toxins were extracted from the shellfish homogenates using the manufacturer's instructions supplied with each kit. Briefly, DSP ELISA extracts were prepared by vortex mixing 1 g of mussel flesh with 9 ml 80 % (v/v) methanol followed by centrifugation (3,000 g for 10 min). Cleaned methanolic shellfish extracts were used for toxin analysis after filtration through 25 mm 3 μm pore size filter (Whatman, GF/C). PP2A (OKATEST) extracts were prepared in a similar manner by vortex mixing 5 g mussel flesh with 25 ml 100 % (v/v) methanol in a 50 ml centrifuge tube followed by centrifugation (2,000 g for 10 min at 4 °C). In 2009 the performance of the DSP ELISA kit only was used on relatively fresh extracts, a decision made on logistical grounds in the initial investigative period. Both methods were applied in 2010.

Both protocols were modified using an additional hydrolysis step in order to quantify the total DSP toxin content including esters and DTX-3. Extracts were diluted using sample dilution buffer supplied. All extracts were hydrolysed as part of the procedure and diluted accordingly.

Assays were carried out in 96-well microtitre plates supplied with the kits and incubated according to the manufacturers' instructions. Both assays operate on a colour reaction, the intensity being inversely proportional to the concentration of toxin present in the sample. Absorbance readings of the test mixtures and calibration standards were performed at 450 nm for the DSP ELISA and 405 nm for the DSP OKATEST using a plate reader (Biotek) with Gen5 software. Results were expressed as the concentration of okadaic acid and its equivalents, i.e. okadaic acid (OA) and its derivative dinophysistoxins DTX-1, DTX-2 and 7-O-acyl ester derivatives (DTX-3). Toxin concentrations were determined by external calibration using OA standards of known concentrations supplied with each kit.

Results

Levels of DSP toxins in mussel flesh from three monitoring sites in Killary Harbour through the summer of 2009 are summarised in Fig. 18.2a. These results were derived from LC-MS analysis as part of the Irish National Biotoxin Monitoring Programme. Contamination of mussel flesh with DSP toxins appeared in mid June and lasted through July until early August. DSP toxins levels rose to values exceeding the EU Maximum Permitted Level (MPL) of 0.16 μg OA eq $\cdot g^{-1}$ on 22 June at the outer and middle sites and on 29 June at the inner site. DSP toxin levels subsequently rapidly increased at all three sites to ca. 1.2 μg OA eq $\cdot g^{-1}$ on 5 July, with toxicity increasing faster at the outer and middle sites than the inner site

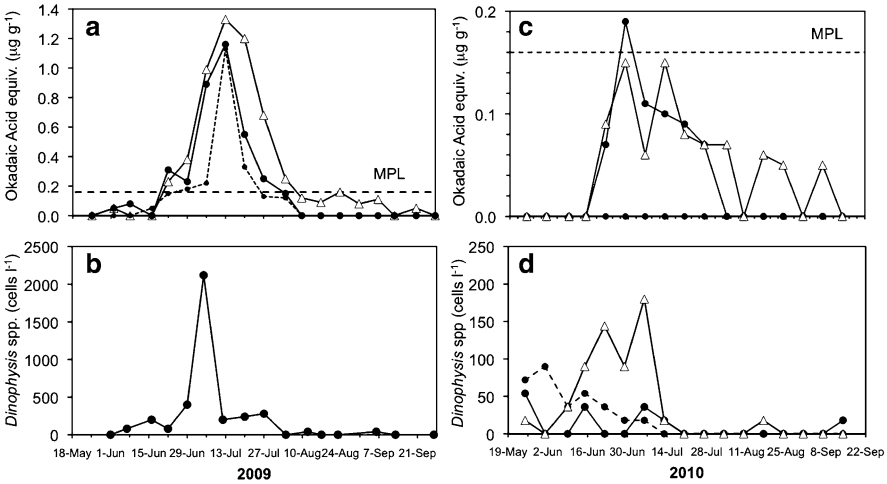


Fig. 18.2 Diarrhetic Shellfish Poisoning toxin levels in mussel flesh analysed by LC-MS and *Dinophysis* spp. levels in depth integrated water samples taken from the outer (*open triangles*) middle (*closed circles*, *solid line*) and inner (*closed circles*, *dotted line*) sampling sites in Killary Harbour, Ireland. (**a**) DSP toxin levels in 2009; (**b**) *Dinophysis* spp. levels at the middle site during 2009; (**c**) DSP toxin levels in 2010; (**d**) *Dinophysis* spp. levels during the summer of 2010. Note that no measurable levels of toxins were observed in samples taken from the inner site during 2010

suggesting that contamination was being transported into the harbour from outside. DSP toxin levels then decreased to levels below the MPL after mid-July at the inner and middle sites and from 10 August at the outer site. These dates co-incided with positive MBA results and enforced the closure of harvesting sites over a period of 7 weeks.

The contamination of mussel tissue with DSP biotoxins coincided with an increase in *Dinophysis acuminata* and *D. acuta* numbers in the water column (Fig. 18.2b). *Dinophysis* spp. cell densities in integrated samples increased to 2,100 cells $\cdot l^{-1}$ on 5 July corresponding to the initial sharp increase in DSP toxin levels in mussel flesh at this time. This clearly indicated that the DSP event in the fjord resulted from the influx of cells of *Dinophysis* spp., which is a known DSP producer.

Figure 18.2c shows DSP toxin levels in mussel flesh detected by LC-MS during the summer of 2010, during which a DSP contamination event also occurred. The event began in late June with a low, steady increase in toxin levels in mussel flesh. Relative to 2009, this DSP event was much smaller but still resulted in the closure of harvesting sites. DSP toxin concentrations in mussels detected by LC-MS reached the EU MPL on 28 June with levels of 0.19 µg OA eq $\cdot g^{-1}$ at the middle site and 0.15 µg OA eq $\cdot g^{-1}$ at the outer site. Co-incident positive MBA results on this date resulted in harvest closures. After 28 June at the middle site, DSP levels fell and remained below the MPL. However, levels reached 0.16 µg OA eq $\cdot g^{-1}$ on 12 July at the outer site, and a positive MBA resulted in further closure. Subsequently, toxin levels fell and remained within the range of 0.05–0.06 µg OA eq $\cdot g^{-1}$ in August

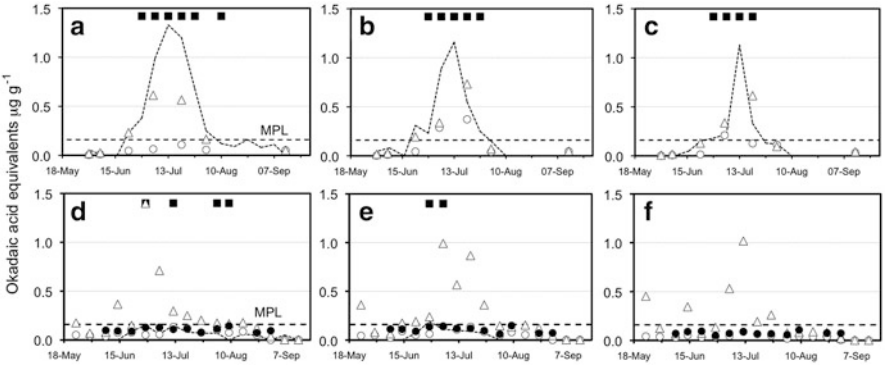


Fig. 18.3 Comparison of DSP toxin levels results using rapid assay techniques on mussel samples taken from Killary Harbour, Ireland in 2009 and 2010. Samples were taken from (**a**) outer site, (**b**) middle site and (**c**) inner site during 2009 and (**d**) outer site, (**e**) middle site and (**f**) inner site during 2010. *Open symbols* are results obtained before (*circles*) and after (*triangles*) hydrolysis using the DSP ELISA kit; *closed circles* for 2010 are results obtained using the protein phosphatase assay. Data from LC-MS are shown by comparison (*dashed line*) and can be referenced to Fig. 18.2a, c. The maximum permissible level (*MPL*) of DSP toxin in shellfish flesh is indicated by the *horizontal dashed line*, and the dates when positive mouse bioassay results were obtained are shown with *filled squares*. Sampling site locations are shown in Fig. 18.1

and September. Positive MBA on 3 and 9 August at the outer site resulted in a short closure. DSP levels remained below the limit of detection (LOD) at the inner site throughout the summer.

Dinophysis cell densities recorded in discrete and integrated water samples during this 2010 event again confirmed that it was caused by an influx of *Dinophysis* spp. (Fig. 18.2d). Higher cell densities were recorded in discrete samples compared with the integrated tube water samples. This is not unusual, as the organism can exist in sub-surface thin layers at high density (Farrell et al. 2012). Relatively low cell densities were recorded during the event compared to 2009. However, *Dinophysis* spp. are known to cause toxicity problems in shellfish at cell densities as low as 100–200 cells $\cdot l^{-1}$ (Botana et al. 1996). At the outer site, cell densities between 90 and 180 cells $\cdot l^{-1}$ were recorded in integrated water samples through June and the start of July. Lower densities (0–90 cells $\cdot l^{-1}$) were recorded at the middle and inner sites (Fig. 18.2d). Peak cell densities observed in water bottle samples were 300 cells $\cdot l^{-1}$ on 21 June at the outer site (10 m depth), 125 cells $\cdot l^{-1}$ on 28 July at the middle site (5 m depth), and 70 cells $\cdot l^{-1}$ on 2 August (2 m depth) at the inner site.

Comparative results of DSP toxin analysis using rapid techniques during 2009 and 2010 are shown in Fig. 18.3, where data are compared with those derived from LC_MS. In 2009, hydrolysed and non-hydrolysed ELISA samples and LC-MS data showed generally good agreement (Fig. 18.3a–c). Both data sets showed the same general trend; an initial non-toxic phase followed by a steady increase exceeding the MPL, followed by a steady decline. All three sites gave similar results using the immunoassay. Hydrolysed samples analysed by ELISA mimicked the LC-MS

results. However, the non-hydrolysed samples appeared to underestimate levels. All hydrolysed samples analysed by ELISA during the closure period produced positive results; no 'false positives' were found in hydrolysed samples determined by the ELISA. However, most non-hydrolysed samples gave results below the MPL during the closure period. Nevertheless, all positive results (i.e. >EU MPL of 0.16 μg OA eq $\cdot g^{-1}$) determined by LC-MS (and the MBA) were also positive using the ELISA method when the hydrolysis step was employed.

Figure 18.3d–f shows a comparison of DSP toxin levels in mussels flesh collected during summer 2010 when analysed by DSP ELISA (ABRAXIS), OKATEST and LC-MS methods. All the data sets show a similar pattern, with the notable exception of samples analysed by the DSP ELISA kit after the hydrolysis step. During the sampling period, only one sample from the middle sample site (28 June) gave a positive result by LC-MS (i.e. >MPL) whereas six positive results by MBA were recorded. During the 2010 sampling period, 35 samples analysed by LC-MS were below the limit of detection and/or quantification. However, both the immunoassay and enzymatic assay were able to detect DSP toxins at levels below the LC-MS LOD. Non-hydrolysed samples analysed by the ELISA method did not detect any positive DSP (>MPL) samples. DSP levels recorded by the enzymatic assay were more similar to the LC-MS data. Although no positive samples were detected during the closure period, high levels of DSP were detected by the OKATEST kit which were higher than those detected by the ELISA non-hydrolysed, and on two occasions slightly higher than the samples analysed by LC-MS. Hydrolysed samples analysed by ELISA gave significant overestimations of DSP levels in all samples. This was most likely caused by matrix effects resulting from the hydrolysis. These matrix effects were evident in samples with high and low concentrations of toxins, with 23 false positives found.

Table 18.1 shows a comparison of DSP toxin data from samples taken in 2009, stored and re-analysed using both the Protein Phosphatase (PP2A, Okatest) enzyme assay and LC-MS methods on non-hydrolysed and hydrolysed extracts from mussel flesh. Data from 2010 is also included. Good agreement is seen between the two methods in the 19 samples that were re-analysed. All but two extracts were in agreement and on both occasions the two errant results were borderline. On a sample originally taken on 20 July at the middle site, non-hydrolysed extract analysed by LC-MS gave a negative toxicity result, but when the hydrolysed sample was analysed a positive result was obtained, agreeing with the original MBA analysis, and also with the PP2A re-analysis on both hydrolysed and non-hydrolysed extracts.

Discussion

The DSP toxin group consists of the lipophilic toxin okadaic acid and its analogues dinophysistoxin-1 and -2 (DTX-1, DTX-2) and dinophysistoxin-3, a complex mixture of 7-O-acyl ester derivatives of OA, DTX-1,-2 (Suzuki and Quilliam 2011). Until 2011, detection of DSP toxins in shellfish was carried out by the MBA, as the

Table 18.1 A comparison of data on DSP toxins in mussel flesh derived from analysis using a protein phosphatase assay (PP2A) and LC-MS

	Method:	PP2A	PP2A	LC-MS	LC-MS
	Treatment:	Non-hydrolysed	Hydrolysed	Non-hydrolysed	Hydrolysed
Site	Date	μg OA eq g^{-1}	μg OA eq g^{-1}	μg OA eq g^{-1}	μg OA eq g^{-1}
Inner	07 June 2009	<0.06	0.08	0.02	0.03
Middle	07 June 2009	<0.06	0.12	0.04	0.07
Outer	07 June 2009	<0.06	0.10	0.03	0.04
Inner	05 July 2009	0.19	>0.38	0.18	0.67
Middle	05 July 2009	0.27	>0.38	0.23	0.68
Outer	05 July 2009	0.08	0.26	0.05	0.16
Inner	20 July 2009	0.13	0.30	0.08	0.17
Middle	20 July 2009	0.29	0.37	0.15	0.35
Outer	20 July 2009	0.12	0.28	0.07	0.19
Middle	02 Aug 2009	0.10	0.23	0.05	0.13
Inner	13 Sept 2009	<0.06	0.20	0.01	0.02
Middle	13 Sept 2009	<0.06	0.16	0.00	0.03
Outer	13 Sept 2009	0.09	0.18	0.03	0.05
Inner	08 June 2010	<0.06	0.07	0.02	0.01
Middle	08 June 2010	0.08	0.11	0.01	0.02
Outer	08 June 2010	0.08	0.10	0.02	0.02
Inner	14 June 2010	0.07	0.09	0.00	0.01
Middle	14 June 2010	0.08	0.11	0.01	0.03
Outer	14 June 2010	0.08	0.09	0.03	0.02
Inner	21 June 2010	0.07	0.09	0.00	0.01
Middle	21 June 2010	<0.06	0.09	0.03	0.05
Outer	21 June 2010	<0.06	0.09	0.03	0.05
Inner	28 June 2010	<0.06	<0.06	0.02	0.02
Middle	28 June 2010	0.07	0.14	0.07	0.11
Outer	28 June 2010	0.08	0.13	0.06	0.09
Middle	05 July 2010	0.10	0.14	0.09	0.11
Inner	05 July 2010	<0.06	0.07	0.04	0.04
Outer	05 July 2010	0.09	0.13	0.06	0.09
Inner	09 Aug 2010	<0.06	0.11	0.00	0.01
Middle	09 Aug 2010	0.08	0.15	0.02	0.03
Outer	09 Aug 2010	0.09	0.14	0.05	0.07

Mussel samples were taken from the outer, middle, and inner sites of Killary Harbour indicated in Fig. 18.1 on various dates in 2009 and 2010. Samples taken in 2009 had been stored frozen for 18 months prior to analysis using both methods. Analysis was carried out on both hydrolysed and non-hydrolysed extracts

EU official testing method. Commission Regulation (EU) No 15/2011 amending Regulation (EC) No 2074/2005, established the EU RL LC-MS/MS method as the reference method for the detection of lipophilic toxins in shellfish for the purposes of official controls. However, this analytical technique requires expensive equipment and maintenance as well as highly trained staff to perform routine

shellfish monitoring analyses. Alternative methods, cheaper to run and easier to use, are required by food business operators who are expected to perform end-product testing. Commercially available to research laboratories and the industry, the DSP ELISA (Abraxis) immunoassay and the OKATEST PP2A assay are designed for the detection in shellfish of OA, DTX-1,-2 and DTX-3, with the application of the important hydrolysis step. Although the immunoassay performed initially well in 2009, serious matrix effects can be seen when the kit was used to analyse hydrolysed samples. These matrix effects were apparent when mussel flesh samples containing both high and low levels of DSP toxins were analysed.

The DSP OKATEST performed well in detecting both high and low concentrations of DSP toxins in mussel samples. There were no effects similar to the matrix effects seen with the immunoassay data, and the data sets agreed with LC-MS on both fresh and stored samples. The PP2A assay is a functional assay based on the inhibition of the phosphatase enzyme by the OA-toxin group, which has the ability to hydrolyse a specific substrate, yielding a product that can be detected colorimetrically. Samples containing toxins from the okadaic acid group inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. Based on the data achieved in this study, the enzymatic based assay (PP2A) would be recommended in preference to the Abraxis immunoassay for rapid analysis, screening and end product testing of DSP toxins in shellfish. It is however important to bear in mind that the DSP OKATEST is a specific assay and therefore will not detect other regulated lipophilic toxins such as pectenotoxins, azaspiracids and yessotoxins. This limitation implies that the OKATEST cannot replace the multi-toxin LC-MS/MS method, but could confidently be used as an end-product testing technique by the industry in the case of shellfish solely contaminated with DSP toxins.

Acknowledgements The authors acknowledge the assistance of Simon Kennedy (Killary Fjord Shellfish), H. Kleivdal (Biosense Ltd.) and Sarah Cosgrove, Gary McCoy, Evelyn Keady and Nicolas Touzet. This work could not have been possible without access to the data obtained during the national biotoxin monitoring programme, carried out by the Marine Institute, for which the authors are very grateful. This work is a contribution to the project WATER and was part-funded through the INTERREG IVB Northern Periphery Programme.

References

Botana LM, Rodriguez-Vieytes M, Alfonso A, Louzao MC (1996) Phycotoxins: paralytic shellfish poisoning and diarrhetic shellfish poisoning. In: Nollet LML (ed) Handbook of food analysis, vol 2, Residues and other food component analysis. Marcel Dekker, New York, pp 1147–1169

Farrell H, Gentien P, Fernand L, Lunven M, Reguera B, Gonzalez-Gil S, Raine R (2012) Scales characterising a high density thin layer of *Dinophysis acuta* Ehrenberg and its transport within a coastal jet. Harmful Algae 15:36–46

Lindahl O (1986) Report of the ICES Working Group on exceptional algae blooms. ICES, C.M. 1986/L:26, Hirtshals, 17–19 Mar 1986

McDermott G, Raine R (2010) The settlement bottle method for quantitative phytoplankton analysis. In: Karlson B, Cusack C, Bresnan E (eds) Microscopic and molecular methods for quantitative phytoplankton analysis, Intergovernmental Oceanographic Commission manuals and guides no. 55. IOC/UNESCO, Paris, pp 21–24

Raine R, Mc Dermott G, Silke J, Lyons K, Nolan G, Cusack C (2010) A simple short range model for the prediction of harmful algal events in the bays of southwestern Ireland. J Mar Syst 83:150–157

Suzuki T, Quilliam MA (2011) LC-MS/MS analysis of diarrhetic shellfish poisoning (DSP) toxins, okadaic acid and dinophysistoxins analogues, and other lipophilic toxins. Anal Sci 27:571

Yasumoto T, Oshima Y, Yamaguchi M (1978) Occurrence of a new type of shellfish poisoning in the Tohoku district. Bull Jpn Soc Sci Fish 44:1249–1255

Part V
Metabolism of Toxins in Molluscan Shellfish/Métabolisme des toxines dans les coquillages

Chapter 19
Accumulation of Okadaic Acid and Detoxifying Enzymes in the Digestive Gland of *Mytilus galloprovincialis* During Exposure to DSP

A. Vidal, Y. Ruiz, P. Suárez, Ana Alonso Martinez, A.E. Rossignoli, J. Blanco, O. Garcia, and F. San Juan

Introduction

In the Galician Rias, *Dinophysis acuminata* and *Dinophysis acuta* are the main species of toxic microalgae, and have been shown to produce okadaic acid and dinophysistoxin-2 (Fernández et al. 1998). Okadaic acid, diniphysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) have been identified in most Diarrhetic Shellfish Poisoning (DSP) episodes in Galician mussels (Gago-Martinez et al. 1996), as well as being found in Irish (Carmody et al. 1996) and Portuguese (Vale and Sampayo 2002) shellfish.

OA and Dinophysistoxins (DTX's) are the principal toxic compounds causing DSP in humans (Yasumoto et al. 1985). These compounds are lipophilic polyether molecules produced by dinoflagellates *Dinophysis* sp. and *Prorocentrum* sp. genera (Yasumoto et al. 1978; Murata et al. 1982).

A. Vidal • Y. Ruiz • P. Suárez • F. San Juan (✉)
Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain
e-mail: adrianavidal@uvigo.es; yruiz@uvigo.es; psuarez@uvigo.es; fsanjuan@uvigo.es

A.E. Rossignoli • J. Blanco
Centro de Investigacións Mariñas (Xunta de Galicia), Pontevedra, Spain
e-mail: ara@cimacoron.org; juan.blanco@cimacoron.org

O. Garcia
Department of Biochemistry and Molecular Biology, University of Santiago de Compostela, Santiago de Compostela, Spain

A. Alonso Martinez
Department of Biochemistry, Genetics and Immunology, Faculty of Sciences, University of Vigo, Lagoas-Marcosende s/n, 36310 Vigo, Spain
e-mail: amam@uvigo.es

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_19,

Mussels, like other bivalves, can accumulate DSP toxins during algal blooms of these microalgal species. Accumulation takes place mainly in the digestive gland (Blanco et al. 2007) and causes a negative economic impact to the shellfishing industry in the Galician rias on an annual basis. Following ingestion of contaminated mussels by humans the DSP toxins can cause a gastrointestinal disease and result in a serious public health problem.

The genetic selection of molluscs with a greater capacity for detoxification, in order to reduce depuration times, could help to reduce the resultant significant economic and health problems.

There are few studies concerning the metabolism of biotoxins in bivalves, although some authors have suggested involvement of detoxification enzymes (Kodama and Sato 2002; FRS Marine Laboratory 2004) and described the induction of some antioxidant enzymes in crustaceans and scallops in the presence of toxic dinoflagellates (Campa-Córdova et al. 2009).

This work is a preliminary study of the metabolism of okadaic acid in *Mytilus galloprovincialis*. We analysed a diverse range of enzymes potentially involved in xenobiotic metabolism and follow enzyme activity variation in relation to OA accumulation during three different toxic blooms of *D. acuminata* and *D. acuta* in the Ria de Vigo.

Material and Methods

Samples

Mussels (*Mytillus galloprovincialis*) were sampled fortnightly from floating rafts of the Vigo estuary (NW Spain) from June 2001 until August 2002. For each sampling, 80 adult individuals of 6–8 cm in length were randomly collected. The digestive glands of 30 mussels were dissected, immediately frozen in liquid nitrogen, pooled and stored at −80 °C until the enzymatic assays. A section of mantle tissue was also dissected, fixed in Bouin's solution and histologically processed using routine histological techniques (paraffin embedded, 5 μm sectioning, stained with Harris' haematoxylin-eosin solution and analysed microscopically) to determine the sex of each mussel.

The soft tissues of the other subsample (50 mussels) were pooled, homogenized, lyophilized and stored for drying until toxin analysis. In *Mytilus*, enzymatic activity varies with the reproductive status, sex and environmental parameters (Borkovic et al. 2005; Bochetti and Regoli 2006; Monserrat et al. 2007; Verlecar et al. 2008; Cravo et al. 2009). In this study, the histological analysis showed that most of the mussels collected on the same date were at the same gametogenic stage. Because of this and because we separated males and females following collection, we felt justified in pooling each sample from the same sampling location.

Enzymatic Analysis

Following sex determination the digestive glands were pooled for each sampling date and by sex and then homogenized in 20 mM Tris-HCl buffer pH7.6 (1:4, w:v) containing 0.5 M sucrose, 0.15 M potassium chloride (KCl), 1 mM ethylene-diaminetetraacetic acid (EDTA), 1 mM â-mercaptoethanol and 0.1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 4 °C at 500 × g for 30 min and the resulting supernatant further centrifuged at 12,000 × g for 45 min. This last supernatant was considered to be the post-mitochondrial fraction, on which all enzymatic activities were assayed by spectrophotometric methods at 25 °C. Assays were performed in triplicate for each sample.

Cytochrome P450 reductase (CPR) was determined as described by Livingstone and Farrar (1984) using NADPH and NADH as electron donor and cytochrome c as substrate. 7-ethoxi-resorufine-O-deetilase (EROD) was assayed by the method of Burke and Mayer (1974). Xanthine oxidoreductase (XDH) was determinated following the method described by Lallier and Walsh (1991). DT-diaphorase activity (DTD) was determined as described by Ernster (1967) and Livingstone et al. (1992). Glutathione-S-transferase (GST) was determined as the conjugation enzyme by the method of Habig and Jakoby (1981) Among antioxidant enzymes, superoxide dismutase (SOD), total and selenium-dependent glutathione peroxidase (GPx-tot; GPX-Se); glyoxalases I and II (GLO I, GLO II) and Catalase (CAT) were assayed respectively, by the methods described by Ewing and Janero (1995), Lawrence and Burk (1976), Principato et al. (1983) and Aebi (1984). Glutathione reductase (GR) was assayed by the Ramos-Martínez et al. method (1983), as an enzyme involved in the redox cycle of glutathione, which yields an adequate concentration of reduced glutathione for GPX and GLO I activities. Activities were expressed in UI or mUI per gram of tissue.

Extraction and Analysis of Toxins

Toxin extraction for each sample date was carried out with 0.4 g of lyophilized mussel (equivalent to 2 g of wet weight). Three extractions were carried out adding 80 % MeOH at a ratio of 1:4 (weight: volume) according to Quilliam 1995. Extracts were clarified by centrifugation (10,000 × g for 10 min at 20 °C) and transferred into a volumetric flask through syringe filters of 0.22 μm. The extracts were evaporated in a rotavapor, resuspended freshly in MeOH 80 %, and then filtered through ultrafree centrifugal filters of 0.45 μm.

Aliquots of each extract were hydrolysed at 75 °C for 40 min. with 2.5 M NaOH, and the reaction stopped by adding 2.5 M HCl.

The presence of okadaic acid in the samples was determined by HPLC–MS/MS with a Surveyor MS HPLC system, coupled to a Deca XP plus ion trap mass

spectrometer (Thermo Fisher Scientific) with an electrospray interface, following the method of Gerssen et al. (2009). The chromatographic separation was carried out using a Gemini NX C18 column. The mobile phase consisted of 100 % water (A) and acetonitrile: water (95:5 %) (B), both containing 2 mM ammonium formate and 50 mM formic acid (pH 2.6). An isocratic elution consisting of a 10 % mobile phase B and 90 % mobile phase A, was run. The mobile phase flow was 0.4 mL/min and the injection volume was 20 μL.

OA was quantified by comparison with reference materials from NRC-CRM, Canada.

Results and Discussion

During our study three toxic episodes, caused by *D. acuminata* and *D. acuta*, occurred between June 18, 2001 and August 6, 2002 (INTECMAR: www.intecmar.org), during which our results showed that OA was accumulated by mussels (Fig. 19.1). DTXs were not detected in any samples.

Considering all enzymatic activities evaluated, only CPR, GST, GR, GLO I, GPXtot and CAT showed significant variations, which were related to the accumulation of OA in mussels.

The CPR activity showed a significant increase, mainly in males, inversely related to decrease of OA accumulated (r: 0.375, $p < 0.05$) (Fig. 19.1a; Table 19.1). This enzyme provides electrons to different oxygenases from the endoplasmic reticulum, between them to the oxygenase- dependant cytochrome P450, which is

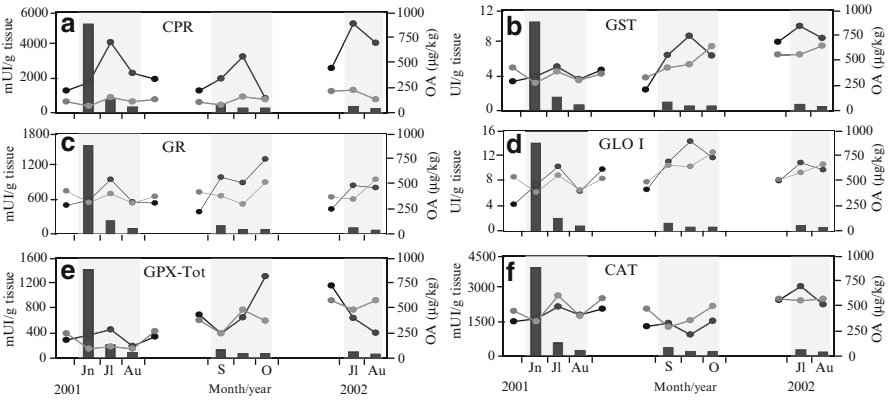


Fig. 19.1 OA accumulation and variation of CPR, GST, GR, GPX, CAT and GLO I activities from *M. galloprovincialis* digestive gland during three toxic episodes of *D. acuminata*. *Bars*: concentration of OA accumulated in mussel soft tissues. Enzymatic activities in females (○) and in males (●)

involved in xenobiotic metabolism, steroids and lipid signalling synthesis, sterol synthesis or the metabolism of desaturation or elongation of fatty acids, establishing a microsomal electron transport sequence known as "mixed function oxidase system". The, oxidation-reduction reactions of this system allow hydroxylation of multiple molecules and converts them into polar molecules in order to facilitate their elimination (Guengerich 1988). The relationship between CPR and OA in mussels is consistent with the involvement of some microsomal monooxygenases in the metabolism of OA. Other authors have described similar results in hepatocyte cultures from vertebrates (Tamaki et al. 2005; Guo et al. 2010).

The metabolites produced by the mixed function oxidase system are not easily eliminated and are usually more toxic than the initial compounds. Such metabolites can be conjugated with endogenous reduced glutathione to increase its polarity and hence make it easier to eliminate them. GST catalyzes the conjugation of a variety of endogenous and xenobiotic substrates with reduced glutathione (GSH) (Mannervik 1985; Listowsky et al. 1988). GST has an important role in preventing peroxidation and detoxification of toxic substances. In this work we observed an increase of this activity, parallel to decrease of OA and which was significantly correlated with CPR activity (r: 0.516, 0.544 in males and females, respectively; $p < 0.01$) (Fig. 19.1b; Table 19.1), suggesting its possible participation in OA metabolism.

The GST activity depends on the presence of reduced glutathione. The redox balance of cellular glutathione is maintained by glutathione reductase (GR). This enzyme catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Ulusu and Tandogan 2007), which is also necessary for other enzymatic activities, such as glutathione peroxidase and glyoxalase I. In our study, GR activity showed a different behaviour in relation to OA accumulation, which was dependant on the sex of the mussel. In males we observed an increase of GR activity at the beginning of intoxication and then a decrease parallel to GST activity, when the OA began decreasing. Thus our results show a significant correlation between GR and GST activity (r: 0.498, $p < 0.01$) and between GR and OA accumulated (r: 0.483, $p < 0.01$). In female mussels there are no notable differences with males, although our results shows a GR activity inhibition at the beginning of the toxic episodes (Fig. 19.1c), which is negatively correlated with OA accumulation (r: −0.514, $p < 0.01$) (Table 19.1).

The oxidative reactions catalyzed by the mixed function oxidase system generate large amounts of reactive species of O_2 and other molecules, such as α oxaldeydes, which are highly toxic. Such molecules can be eliminated by the activity of other enzymes concerned with oxidative defense. Some authors have also described the induction by OA of these enzymes in vertebrate cell cultures (Fujii et al. 1994; Matias et al. 1999) and mussels (Auriemma and Battistella 2004).

Among the other enzymes of oxidative defense, the glioxilases I and II catalyze the coordinated detoxification of reactive α oxaldeydes with mutagenic and cytotoxic activity, converting them into their corresponding α -hydroxy acids (thiol ester intermediaries) (Regoli et al. 1996).

The glioxalase I is also a GSH-dependent enzyme. α oxaldeydes and glutathione form spontaneously an intermediary hemithioaceatal, which is transformed

Table 19.1 Correlation factors between diverse enzyme activities related to xenobiotic metabolism from mussel digestive gland and accumulation of OA

	Males						Females					
	CPR	GR	GST	CAT	GPX-tot	GLO I	CPR	GR	GST	CAT	GPX-tot	GLO I
GR	–											
GST	0.516**	0.498**					0.544**	–				
CAT	0.338*	–	0.416**				–	–				
GPX-tot	–	–	0.430**	–			0.440**	–	0.608**	0.317*		
GLO I	–	0.771**	0.737**	–	0.412**		0.334*	0.557**			0.312**	
OA	0.375*	0.483**	–	–	–	0.370*	–	−0.514**	–	–	−0.366**	–

Spearman coefficient: *$p < 0.05$; **$p < 0.01$

to S-D-lactoilglutathione by the GLO I activity. This S-D-lactoilglutathione is subsequently hydrolyzed to D-lactate and glutathione (GSH) by GLO II enzyme. Our results show an increase of GLO I activity with OA intoxication in both sexes (Fig. 19.1d) with a statistically significant correlation in males (r: 0.370, $p < 0.05$) (Table 19.1). This enzyme also shows a significant correlation with GR (r: 0.771, $p < 0.01$) and GST (r: 0.737, $p < 0.01$) activities in males, and with CPR (r: 0.334, $p < 0.05$) and GR (r: 0.557, $p < 0.01$) activities in females (Table 19.1). These results are consistent with induction by OA of GLO I in *Mytilus* as also obtained by Auriemma and Battistella (2004).

Other oxidative defence enzymes that seem to be related to the episodes of intoxication by OA in mussels are glutathione peroxidase (GPXtot) and catalase (CAT). GPXtot activity increases in both sexes during intoxication, showing in females a negative correlation with accumulated OA (r: −0.366, $p < 0.01$) (Fig. 19.1e; Table 19.1) and positive one with CPR (r: 0.440, $p < 0.01$), GST (r: 0.608, $p < 0.01$) and with GLO I (r: 0.312, $p < 0.0$) activities. In males its activity is only correlated with GST (r: 0.430, $p < 0.01$) and GLO I (r: 0.412, $p < 0.01$) (Table 19.1). On the other hand, CAT activity has no correlation with accumulated OA (Fig. 19.1f), but shows significant correlation with CPR (r: 0.338, $p < 0.0\ 5$) and GST (r: 0.416, $p < 0.01$) in males and with GPXtot (r: 0.317, $p < 0.05$) in females.

Despite the preliminary nature of this work, the results obtained suggest the involvement of the microsomal monooxygenase enzymatic system dependent on cytochrome P450 in the okadaic acid biotransformation in *Mytilus galloprovincialis*. Moreover, the different enzymatic correlations in males and females seem to indicate sexual differences in the metabolic pathways followed. However, to confirm this and to define other possible enzymes and pathways involved in OA degradation and elimination in mussels further studies will be required.

Acknowledgment The authors thank Antonio Antepazos and the fishermen of the "Antepazos I", who kindly provided the mussels used in this work. This work was supported by a grant from Autonomous Galician Government (Ref. 2008/cp390) within the Strategic Action: EPITOX.

References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Auriemma R, Battistella S (2004) Biochemical and histological alterations of *Mytilus galloprovincialis* digestive gland after exposure to okadaic acid and derivatives. Invertebr Survive J 1:66–71

Blanco J, Mariño C, Martín H, Acosta CP (2007) Anatomical distribution of diarrhetic shellfish poisoning (DSP) toxins in the mussel *Mytilus galloprovinciallis*. Toxicon 50:1011–1018

Bochetti R, Regoli F (2006) Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic. Chemosphere 65:913–921

Borkovic SS, Saponjic JS, Pavlovic SZ, Blagojevic DP, Milosevic SM, Kovacevic TB, Radojicic RM, Spasic MB, Zikic RV, Saicic ZS (2005) The activity of antioxidant defense enzymes in the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. Comp Biochem Physiol 138C:411–427

Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab Dispos 6(2):583–588

Campa-Córdova AI, Núñez-Vázquez EJ, Luna-González A, Romero-Geraldo MJ, Ascencio F (2009) Superoxide dismutase activity in juvenile *Litopenaeus vannamei* and *Nodipecten subnodosus* exposed to the toxic dinoflagellate *Prorocentrum lima*. Comp Biochem Physiol C Toxicol Pharmacol 149(3):317–322

Carmody EP, James KJ, Kelly SS (1996) Dinophysistoxin-2: the predominant diarrhetic shellfish toxin in Ireland. Toxicon 34:351–359

Cravo A, Lopes B, Serafim A, Company R, Barreira L, Gomes T, Bebianno MJ (2009) A multi-biomarker approach in Mytilus galloprovincialis to assess environmental quality. J Environ Monit 11:1673–1686

Ernster L (1967) DT-diaphorase. Methods Enzymol 10:309–317

Ewing JF, Janero DR (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal Biochem 232:243–248

Fernández ML, Míguez A, Moroño A, Cacho E, Martínez A, Blanco J (1998) Detoxification of low polarity toxins (DTX3) from mussels *Mytilus galloprovincialis* in Spain. In: Reguera B, Blanco J, Fernández ML, Wyatt T (eds) Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Spain

FRS Marine Laboratory (2004) Marine biotoxins. Fisheries Research Services. Report AE 14/08, Aberdeen

Fujii J, Nakata T, Miyoshi E, Ikeda Y, Taniguchi N (1994) Induction of manganese superoxide dismutase mRNA by okadaic acid and protein synthesis inhibitors. Biochem J 301:31–34

Gago-Martinez A, Rodriguez-Vazquez JA, Thibault P, Quilliam MA (1996) Simultaneous occurrence of diarrhetic and paralytic shellfish poisoning toxins in Spanish mussels in 1993. Nat Toxins 4:72–79

Gerssen A, Mulder PPJ, McElhinney MA, de Boer J (2009) Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. J Chromatogr A 1216:1421–1430

Guengerich FP (1988) Cytochromes P-450. Comp Biochem Physiol 89C:1–4

Guo F, An T, Rein KS (2010) The algal hepatoxoxin okadaic acid is a substrate for human cytochromes CYP3A4 and CYP3A5. Toxicon 55:325–332

Habig WH, Jakoby WB (1981) Assays for differentiation of glutathione S-transferases. Methods Enzymol 77:398–405

Kodama M, Sato S (2002) Metabolism of the toxins responsible for paralytic shellfish poisoning. In: Proceedings of coastal management and sustainable development. UNU-Iwate-UNESCO joint international conference on conserving our coastal environment, Tokyo, Japan

Lallier FH, Walsh PJ (1991) Activities of uricase, xanthine oxidase, and xanthine dehydrogenase in the hepatopancreas of aquatic and terrestrial crabs. J Crustac Biol 11:506–512

Lawrence RA, Burk RF (1976) Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 71:952–958

Listowsky I, Abramovitz M, Homma H, Niitu Y (1988) Intracellular binding and transport of hormones and xenobiotics by glutathione-S-transferases. Drug Metab Rev 19:305–318

Livingstone DR, Farrar SV (1984) Tissue and subcellular distribution of enzyme activities of mixed-function oxygenase and benzo(a) pyrene metabolism in the common mussel *Mytilus edulis L*. Sci Total Environ 39:209–235

Livingstone DR, Archibal S, Chipman K, Marsh JW (1992) Antioxidant enzymes in liver of dab, Limanda limanda, from the North Sea. Mar Biol 112:265–276

Mannervik B (1985) The isoenzymes of glutathione transferase. Adv Enzymol Relat Areas Mol Biol 57:357–417

Matias WG, Traore A, Bonini M, Sanni A, Creppy EE (1999) Oxygen reactive radicals production in cell culture by okadaic acid and their implication in protein synthesis inhibition. Hum Exp Toxicol 18:634–639

Monserrat JM, Martínez PE, Geracitano LA, Lund Amado L, Martínez Gaspar Martins C, Lopes Leaes Pinho G, Soares Chaves I, Ferreira-Cravo M, Ventura-Lima J, Bianchini A (2007) Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp Biochem Physiol 146C:221–234

Murata M, Shimatani M, Sugitani H, Oshima Y, Yasumoto T (1982) Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull Jpn Soc Fish 48:549–552

Principato GB, Locci P, Rosi G, Talesa V, Giovannini E (1983) Activity changes of glyoxalase I-II and glutathione reductase in regenerating rat liver. Biochem Int 6:249–255

Quilliam MA (1995) Analysis of diarrhetic shellfish poisoning toxins in shellfish tissue by liquid chromatography with fluorometric and mass spectrometric detection. J AOAC Int 78(2):555–569

Ramos-Martínez JI, Bartolomé TR, Pernas RV (1983) Purification and properties of glutathione reductase from hepatopancreas of *Mytilus edulis L*. Comp Biochem Physiol 75B:689–692

Regoli F, Saccucci F, Principato G (1996) Mussel glyoxilase I as a possible marker for eco-toxicological studies: purification and preliminary characterization. Comp Biochem Physiol 113C:313–317

Tamaki H, Samuka T, Uchida Y, Jaruchotikamol A, Nemoto N (2005) Activation of CYP1A1 gene expression during primary culture of mouse hepatocytes. Toxicology 216:224–231

Ulusu NN, Tandogan B (2007) Purification and kinetic properties of glutathione reductase from bovine liver. Mol Cell Biochem 303(1–2):45–51

Vale P, Sampayo MAM (2002) Esterification of DSP toxins by Portuguese bivalves from the Northwest coast determinated by LC_MS-a widespread phenomenon. Toxicon 40:33–42

Verlecar X, Jena K, Chainy G (2008) Seasonal variation of oxidative biomarkers in gills and digestive gland of green-lipped mussel *Perna viridis* from Arabian Sea. Estuar Coast Shelf Sci 76:745–752

Yasumoto T, Oshima Y, Yamaguchi M (1978) Occurrence of a new type shellfish poisoning in the Tohoku district. Bull Jpn Soc Sci Fish 44:1249–1255

Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto GK, Clardy J (1985) Diarrhetic shellfish toxins. Tetrahedron 41:1019–1025

Part VI
Chemical Contaminants/Polluants chimiques

Chapter 20
In Vitro Toxicity and Histopathological Effects Induced in the Mantle Tissue of Males of *Mytilus galloprovincialis* During Short-Term Exposure to a Tar Mixture

Ana Alonso Martinez, P. Suárez, Y. Ruiz, A. Vidal, and F. San Juan

Introduction

Despite international legislation, environmental pollution has increased, mainly in coastal and estuarine areas, due to increases in population density, industrialization, ship traffic, port activities, fishing, and tourism in these areas (Srogi 2007). Polycyclic aromatic hydrocarbons (PAHs) are considered to be amongst the most ubiquitous marine pollutants. Marine organisms acquire these compounds by ingestion and diffusion across the epithelium, and then accumulate them due to their lipophilic nature.

The acute and chronic toxicity of PAHs presents a risk for organisms, as some chemical species and their intermediate biotransformation products are highly toxic, mutagenic and carcinogenic. Because of this, PAHs are under constant investigation and review by international organizations (IARC 1983, 1996; ATSDR 1995; WHO 1998; Boström et al. 2002; EPA 2010). The toxic effects of these pollutants, as well as its toxic mechanism are well understood in vertebrates, but fewer studies have been conducted in invertebrates. However, although much remains to be discovered about biotransformation and toxic action of PAHs in invertebrates, the toxic mechanism seems to follow those of vertebrates.

Several neoplastic diseases as well as endocrine and metabolic disruptions have been described among the main toxic effects of PAHs. These pathologies are related with mutagenicity and carcinogenicity of hydrocarbons and their capacity to bind estrogen receptors and to induce P450-dependent enzymes, leading to reproductive

A.A. Martinez (✉)
Department of Biochemistry, Genetics and Immunology, Faculty of Sciences, University of Vigo, Lagoas-Marcosende s/n., 36310 Vigo, Spain
e-mail: amam@uvigo.es

P. Suárez • Y. Ruiz • A. Vidal • F. San Juan
Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain
e-mail: psuarez@uvigo.es; yruiz@uvigo.es; adrianavidal@uvigo.es; fsanjuan@uvigo.es

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_20,

and growth alterations (Santodonato 1997; Morton 1988; Boström et al. 2002; Pufulete et al. 2004; Ono et al. 2008; Veeramachaneni 2008; Sanders et al. 2009). In marine invertebrates, histopathological and endocrine alterations have also been related to exposure by PAHs and others chemicals (Alonso et al. 2001; Aarab et al. 2011; Au 2004; Lavado et al. 2006; Ortiz-Zarragoitia and Cajaraville 2006; Jing-Jing et al. 2009; Schäfer and Köhler 2009; Ruiz et al. 2011). However, few studies have been undertaken to demonstrate these alterations in the laboratory.

Purpose

Taking into account the concentrations of accumulated hydrocarbons measured in mussels cultured in the Ría of Vigo by Ruiz et al. (2011), we conducted a study, under laboratory controlled conditions, to determine the gonadal histopathologies induced by a tar mixture, commonly used in cleaning mussel culture rafts.

Method

Analytical Composition of Tar

The chemical analysis of the tar mixture was contracted to the Spanish Institute of Oceanography (IEO, Center of Vigo), where analytical quality control is supported by the international intercalibration exercises from Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME 2003) or International Atomic Energy Agency (IAEA) (Villeneuve et al. 2004). The separation and quantification of PAHs was carried out by high-performance liquid chromatography (HPLC) with fluorimetric detection according to Viñas Diéguez (2002). A total of 13 PAHs were quantified: phenanthrene (Phe), anthracene (An), fluoranthene (Flt), pyrene (Py), benzo(a)anthracene (BaA), chrysene (Chry), benzo(e)pyrene (BeP), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenzo(a,h)anthracene (DBA), benzo(g,h,i)perylene (Bper) and indeno(1,2,3,-c,d)pyrene (IP). Results were expressed as mg/kg of mussel tissue.

Experimental Design

A total of 200 mussels (*Mytilus galloprovincialis)*, between 6 and 8 cm in valve length were randomly collected from floating rafts in the Ría of Vigo (NW Spain) in April of 2009. In the laboratory, mussels were placed in five 30 L polyethylene tanks in an open system of 20 μm filtered seawater, with a flow rate of 2 mL/min.

During each experiment, the mussels were maintained under constant aeration and temperature (20 °C), with natural photoperiod. They were fed a daily microalgae diet (0.0224 g microalgae/mussel) comprised of *Isochrysis galbana*, *Tetraselmis suecica* and *Chatoceros gracilis* in a 50:30:20 proportions.

After 15 days acclimatization to the described conditions, the mussels from each tank were exposed to different concentrations of the tar mixture. Previous studies by our research team regarding total hydrocarbon accumulation in mussels from the Ría of Vigo, had shown mean PAH concentrations of 400 μg/kg (Ruiz et al. 2011). This value was the lowest concentration measured in mussel tissues after laboratory exposure to 10 ppm of the tar mixture, applied for 17 days. We also employed three higher concentrations: 40, 60 and 80 ppm. Control mussels from the fifth tank were not treated with tar. The tar mixture was heated to 200 °C and added in 5 L of seawater, together with the microalgal diet, at a 2 mL/min flow rate. Treatment was continued for 17 days and samplings made at days 0, 10 and 17.

Tissue Preparation for Histology

In species of the genus *Mytilus*, the proximity of the genital papilla to the mantle tissue is used for the expansion of gonad and the gametogenic development taking place mainly in this tissue. For each sampling, the mantles from 10 individuals from each assay tank were dissected, fixed in 10 % neutral buffered formalin, dehydrated and processed in paraffin. Sections of 5 μm thickness were mounted on microscope slides and stained with Carazzi haematoxilin and eosin. Slides were examined by light microscopy (Nikon 90i Eclipse). The images were captured by an 8Mpx high resolution Nikon camera using Nis- Elements BR 2.30 version software.

The gametogenic stage of each mussel was determined according to Lubet (1959) and Suárez et al. (2005). Histological preparations were examined comparatively with those of the control mussels as a reference.

Statistical Analysis

A statistical survival analysis with a PROBIT regression test was performed (statistical package SPSS 17.0, Microsoft Co) in order to calculate the median lethal concentration (LC_{50}) of the tar mixture.

Results

The concentration of total hydrocarbons analyzed in the tar mixture (Σ13 parent PAH species) was 40,364 mg/kg. Therefore, the dilutions of 10, 40, 60 and 80 ppm employed in this experiment represent exposure concentrations of 400, 1,600, 2,400

Table 20.1 Tar composition and percentage of each PAH species

PAHs	mg/kg	%
Phenanthrene	13358.0	33.09
Anthracene	22070.0	5.62
Fluoranthene	7015.0	17.38
Pyrene	3817.3	9.46
Crysene	3210.0	7.95
Benzo(a)anthracene	3243.0	8.03
Benzo(b)fluoranthene	1774.6	4.40
Benzo(k)fluranthene	874.3	2.17
Benzo(e)pyrene	1404.2	3.48
Benzo(a)pyrene	1569.1	3.89
Dibenzo(a,h)anthracene	217.0	0.54
Benzo(g,h,i)pyrelene	576.5	1.43
Indene (1,2,3-c,d)pyrene	1033.0	2.56
∑PAHs	**40364.0**	

PAHs species with high mutagenic and carcinogenic potency

and 3,200 mg/kg, respectively. The concentrations of each PAH specie present in the mixture are summarized in Table 20.1. Among these, Chry, BaA, BbF, BkF, BaP, DBA and IP are considered to be highly mutagenic and carcinogenic (EPA 2010) and constitute 29.5 % of the total mixture.

The experiment was carried out in April, which is the main reproductive period of *Mytilus* in the Ría of Vigo. At the beginning of the experiment, mussels were in an advanced IIIC stage, or gonadal restoration stage of Suárez et al. (2005). Histologically, this stage is characterized by the presence of large follicles filled with gametes, and scarce reserve tissue, mainly constituted by vesicular cells (Fig. 20.1a). At the end of the experiment, the reference mussels were at an incipient spawning stage (IIIB), with larger follicles and scarce reserve tissue (Fig. 20.1b).

An increase in mortality and cellular disorders was observed in the mantle tissue from mussels, which was dependant on dose and exposure time to the tar mixture.

Survival of control mussels at the end of experiment was 100 %. However, after 12 days of tar exposure, accumulated mortality of mussels treated with 10, 40 and 60 ppm of mixture was 25.2, 26.8 and 48.0 %, respectively. Acute exposure to 80 ppm of tar mixture caused death of 40 % of mussels after 5 days and of 95 % after 10 days of treatment (Fig. 20.2a). These results suggest a toxic accumulative effect of tar. The median lethal concentration (LC_{50}) calculated was 67.90 ppm (Fig. 20.2b).

After 10 days exposure to 10 and 40 ppm of tar, an intense desquamation of germinal cells was observed histologically (Fig. 20.1c). This phenomenon has also been described in testicular vertebrate pathologies where it is considered as an untimely spawning induction (Aarab et al. 2011). Moreover, mussels exposed to 60 and 80 ppm of tar showed an interstitial tissue increase and an abnormal development of follicles leading to a clear follicular hypoplasia and a loss of their radial architecture. The basal membrane of these follicles was thickened and hyalinised and inside them a low mitotic activity of germinal cells was seen.

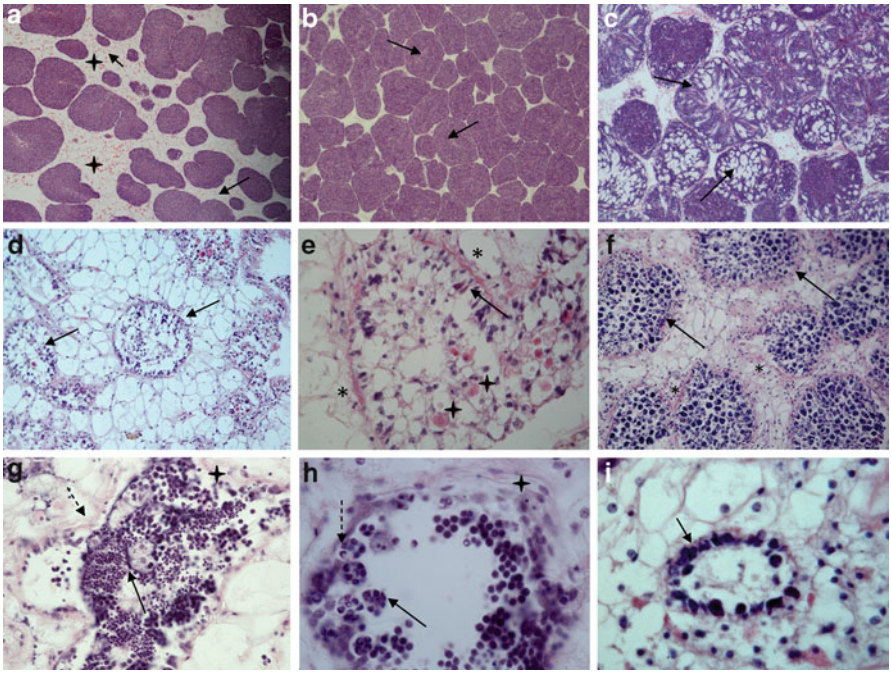


Fig. 20.1 Microphotographs of histopathological changes induced in male mantle tissue of *Mytilus galloprovincialis* exposed to a tar mixture: Control organisms: (**a**) Normal histology of mantle showing gonadal follicles, VCs (*arrow*) and ADGs (*star*) (10×). (**b**) Follicles showing evidence of spawning (IIIB stage) (10×). Treated mussels: (**c**) Spawn induction (*arrow*) (20×). (**d**) Hypoplasia follicular (*arrow*) (40×). (**e**) Auxiliary cells with hyperchromatic nucleus (*arrow*), eosinophilic deposits (*stars*) and thickened basal membrane showing different degrees of hyalinization (*asterisk*) (60×). (**f**) Aggregation of immature germinal cells (*arrow*) and hyalinization of membrane follicular (*asterisk*) (20×). (**g**) Basal membrane rupture (*star*), infiltration of the abnormal cells in the connective tissue (*arrow*) and fibrosis (*discontinue arrow*) (60×). (**h**) Aggregation of spermatocytes with picnotic and karyorhesic nucleus (*discontinue arrow*) and disintegrated basal membrane (*star*) (60×). (**i**) Atypical hypercromatic and hypertrophic germinal cells (*arrow*) (60×)

Many large auxiliary cells, with hyperchromatic nucleus and clear cytoplasm, with eosinophilic deposits could be observed (Fig. 20.1d, e). Similar effects have been related with seminomas in vertebrates (Boekelheide 2005).

After 17 days of exposure to 40 and 60 ppm of tar, similar effects to those caused by 10 days exposure to 80 ppm of tar could be observed. Moreover, the following was also observed (Fig. 20.1f–i): aggregation of immature spermatocytes; the occurrence of large and multinucleated germinal cells with picnotic nucleus, karyorrhexis and vacuolated cytoplasm; the breaking of follicular basal membrane; infiltration of abnormal cells in the connective tissue and fibrosis. All of these alterations could be considered as pre-neoplastic lesions of a carcinoma *in situ* affecting germinal cells. Along with these neoplastic disorders both hyperplasia and hypertrophy of vesicular cells with clear signs of edema occurred (Fig. 20.1g–i).

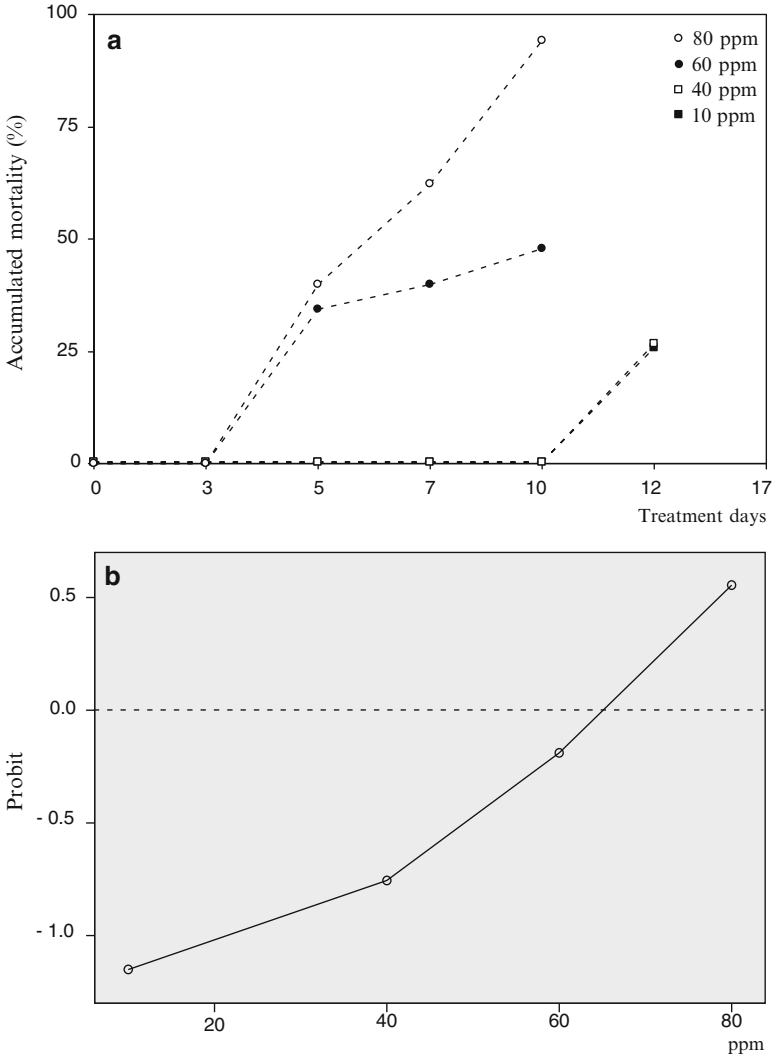


Fig. 20.2 (**a**) Cumulative lethality (percentage) as effect of short term tar exposure at different tar concentrations. (**b**) Median lethal concentration of tar. Probit test

Discussion

Toxic and mutagenic effects of several PAHs species, as well as their relation with the development of neoplastic diseases are reported in the literature (EPA 2010). However, few studies have been conducted to demonstrate induction of these pathologies in the laboratory. *Mytilus* spp. is considered suitable as a sentinel organism in marine environmental monitoring (Goldberg et al. 1978; Farrington

et al. 1987; Nelson 1990; O'Connor 2002; Monirith et al. 2003). Due to the commercial importance of mussel culture, the toxic effects of PAHs identified here could constitute a significant risk for mussel production, as well as a risk to human health following mussel consumption.

The chemical analysis of tar mixture used for cleaning culture mussel rafts suggests its potential toxicity. The lethal and histopathological effects induced in mussels, during this laboratory study, confirm its harmful effects at concentrations higher than 10 ppm.

Mortality and histopathological effects caused by tar mixtures in the laboratory depend on dose and time exposure, suggesting a cumulative toxic effect as described in vertebrates (Richburg et al. 2002; Strmac and Braunbeck 2002). However, lethality caused by high concentrations of tar (60, 80 ppm) could be enhanced by asphyxia of different organs as described by Iniesta y Blanco (2005).

Histopathological effects follow a progressive sequence of malignancy until the development of carcinoma *in situ,* affecting germinal cells. This sequence starts with the desquamation of germinal cells by untimely spawning induction, as described in testicular pathologies from vertebrates, or related to pollution in invertebrates (Cajaraville 1991; Cajaraville et al. 1992; Tay et al. 2003; Jing-Jing et al. 2009; Aarab et al. 2011). Next, the atrophy and hypoplasia of follicles occurs, accompanied by scarce proliferation and differentiation of germinal cells, as well as by many, large and vacuolated auxiliary cells within them. Similar histopathologies with abnormal follicles, consisting of abnormal auxiliary cells with a clear cytoplasm and deposits of eosinophilic material, have been reported in seminomas from vertebrates, caused by toxic damage (Nistal 1973; Nistal et al. 1998; Chapin et al. 1984; Boekelheide 2005). Membrane hyalinization of gonadal follicles and fibrosis have also been described in gonadal neoplasias of vertebrates in individuals exposed to chemicals, where it may become an irreversible process (Nistal 1973; Nistal et al. 1998). The sea urchin is the only invertebrate exposed to phenanthrene, where an increase of collagen fibers around the gonadal follicles, has been described (Schäfer and Köhler 2009). Other effects observed in the latter work, like aggregation of spermatocytes and spermatids, or existence of large and multinucleated germinal cells, are similar to the spermatogenic alterations reported after prolonged exposure to pollutants (Ono et al. 2008). Hyperplasia and hypertrophy of vesicular cells observed in this study are similar to those described in interfollicular connective tissue cells of gonadal carcinomas *in situ* from vertebrates (Dieckmann and Skakkebaek 1999; Ulbright 2005) and of gonadoblastomas from marine invertebrates (Elston et al. 1992; Peters et al. 1994; Ford et al. 1997). All of these chemically mediated effects suggest a possible endocrine disruption in mussels as described in vertebrates (Irvine 2000; Swan et al. 2003), resulting from the xenoestrogenic activity of several PAHs species from the tar mixture used.

Our results show that the tar mixture tested is lethal for mussels at high concentrations, but at sublethal doses can cause serious pathologies, sufficient to affect their reproduction. Moreover, the mussels' capacity to accumulate lipophilic compounds such as hydrocarbons could constitute a risk to human health. So, use of

this tar mixture for cleaning the culture mussels rafts, should be replaced by a less toxic chemical in order to ensure continued production and sustainability of mussel farms, as well as to produce a cultured mussel which is safe for human consumption.

Conclusions

The exposure of mussels to PAHs in a tar mixture used for cleaning purposes, causes histopathological effects, possibly due to endocrine disruption, which lead to the maturation arrest of germinal cells and to the development of germinal carcinoma, which could decrease fertility and reproductive capacity of cultured mussels.

Acknowledgements The authors wish to thank Antonio Antepazos and the fishermen of the "Antepazos I", who kindly provided the mussels used in this work. We also thank the Spanish Institute of Oceanography who completed the chemical analysis of the tar used in this project. This work was supported by a grant from Autonomous Galician Government (PGIDT10MDS312030PR).

References

Aarab N, Godal B, Bechmann R (2011) Seasonal variation of histopathological and histochemical markers of PAH exposure in blue mussel (*Mytilus edulis* L.). Mar Environ Res 71:213–217

Alonso A, Suárez P, Álvarez C, San Juan F, Molist P (2001) Structural study of a possible neoplasia detected in *Mytilus galloprovincialis* collected from the Ria of Vigo (NW Spain). Dis Aquat Organ 47:73–79

ATSDR (Agency for Toxic Substances and Disease Registry) (1995) Toxicological profile for polycyclic aromatic hydrocarbons (PAHs). U.S. Department of Health and Human Services, Public Health Service, Atlanta

Au DWT (2004) The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar Pollut Bull 48:817–834

Boekelheide K (2005) Mechanisms of toxic damage to spermatogenesis. J Natl Cancer Inst Monogr 34:1–3

Boström C, Gerde P, Hanberg A, Jernström B, Johansson C, Kyrklund T, Rannug A, Törnqvist M, Victorin K, Westerholm R (2002) Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. Environ Health Perspect 110(Suppl 3): 451–488

Cajaraville MP (1991) Efectos histopatológicos y citotóxicos de los hidrocarburos derivados del petróleo, y su cuantificación en el mejillón *Mytilus galloprovincialis* (Lmk.). Thesis doctoral, Universidad del País Vasco, España

Cajaraville MP, Marigómez JA, Angulo E (1992) Comparative effects of the water accommodated fraction of three oils on mussels – 1. Survival, growth and gonad development. Comp Biochem Physiol C Comp Pharmacol 102:103–112

Chapin RE, Ross MD, Lamb JC (1984) Immersion fixation methods for glicol methacrylate-embedded testes. Toxicol Pathol 12:221–227

Dieckmann KP, Skakkebaek NE (1999) Carcinoma *in situ* of the testis: review of biological and clinical features. Int J Cancer 83:815–822

Elston RA, Moore JD, Brooks K (1992) Disseminated neoplasia of bivalve molluscs. Rev Aquat Sci 6:405–466

EPA (Environmental Protection Agency) (2010) Development of a Relative Potency Factor (RPF). Approach for Polycyclic Aromatic Hydrocarbon (PAH) mixtures (External review draft), EPA/635/R-08/012A. U.S. Environmental Protection Agency, Washington, DC

Farrington JW, Davis AC, Tripp BW, Phelps DK, Galloway WB (1987) "MusselWatch"-Measurements of chemical pollutants in bivalves as one indicator of coastal environment quality. In: Boyle TP (ed) New approaches to monitoring aquatic ecosystems, ASTM STP 940. American Society for Testing and Material, Philadelphia, pp 125–139

Ford SE, Barber RD, Marks E (1997) Disseminated neoplasia in juvenile eastern oyster *Crassostrea virginica*, and its relationship to the reproductive cycle. Dis Aquat Organ 28:73–77

Goldberg ED, Bowen VT, Farrington JW, Harvey G, Martin JH, Parker PL, Risebrough RW, Roberston W, Schneider E, Gamble E (1978) The mussel watch. Environ Conserv 5:101–125

IARC (International Agency for Research on Cancer) (1983) Polynuclear aromatic compounds. Part 1. Chemical, environmental, and experimental data. In: IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, vol 32. IARC, Lyon

IARC (International Agency for Research on Cancer) (1996) Printing processes and printing inks, carbon black and some nitro compounds. In: IARC monographs on the evaluation of carcinogenic risks to humans, vol 65. IARC, Lyon

Iniesta y Blanco (2005) Bioacumulación de hidrocarburos y metales asociados a vertidos accidentales en especies de interés comerciales de Galicia. Revista dos Recursos Mariños (Monog.):2. 200 pp. ISSN 1885–6802

Irvine DS (2000) Male reproductive health: cause for concern? Andrologia 17:195–208

Jing-jing M, Lu-qing P, Jing L, Lin Z (2009) Effects of benzo[a]pyrene on DNA damage and histological alterations in gonad of scallop *Chlamys farreri*. Mar Environ Res 67:47–52

Lavado R, Janer G, Porte C (2006) Steroid levels and steroid metabolism in the mussel *Mytilus edulis*: the modulating effect of dispersed crude oil and alkyphenols. Aquat Toxicol 78(Suppl):S63–S72

Lubet P (1959) Recherches sur le cycle sexuel et l'émission des gamètes chez les Mytilidés et las Pectinidés. Revue des Travaux de l'Institut de Pêches Maritimes 23(4):389–545

Monirith I, Ueno D, Takahashi S, Nakata H, Sudaryanto A, Subramanian A, Karuppiah S, Ismail A, Muchtar M, Zheng J, Richardson BJ, Prudente M, Hue ND, Tana TS, Tkalin AV, Tanabe S (2003) Asia-Pacific mussel watch: monitoring contamination of persistent organochlorine compounds in coastal waters of Asian countries. Mar Pollut Bull 46:281–300

Morton D (1988) The use of rabbits in male reproductive toxicology. Environ Health Perspect 77:5–9

Nelson WG (1990) Use of the blue mussel, *Mytilus edulis*, in water quality toxicity testing and *in situ* marine biological monitoring. In: Landis WG, van der Schalie WH (eds) Aquatic toxicology and risk assessment, ASTM STP 1096, vol 13. American Society for Testing and Materials, Philadelphia, pp 167–175

Nistal M (1973) Testículo humano. Hipoplasia tubular intersticial difusa (hipogonadismo hipogonadotrópico). Arch Esp Urol 3:283–296

Nistal M, De Mora JC, Paniagua R (1998) Classification of several types of maturational arrest of spermatogonia according Sertoli cell morphology: an approach to aetiology. Int J Androl 21:317–326

O'Connor TP (2002) National distribution of chemical concentrations in mussels and oysters in the USA. Mar Environ Res 53:117–143

Ono N, Oshio S, Niwata Y, Yoshid S, Tsukue N, Sugawara I, Takano H, Takeda K (2008) Detrimental effects of prenatal exposure to filtered diesel exhaust on mouse spermatogenesis. Arch Toxicol 82:851–859

Ortiz-Zarragoitia M, Cajaraville MP (2006) Biomarkers of exposure and reproduction related effects in mussels exposed to endocrine disruptors. Arch Environ Contam Toxicol 50:361–369

Peters EC, Yevich JC, Harshbarger JC, Zaroogian GE (1994) Comparative histopathology of gonadal neoplasms in marine bivalve molluscs. Dis Aquat Organ 20:59–76

Pufulete M, Battershill J, Boobis A, Fielder R (2004) Approaches to carcinogenic risk assessment for polycyclic aromatic hydrocarbons: a UK perspective. Toxicol Pharmacol 40:54–66

QUASIMEME (2003) QUASIMEME laboratory performance studies. FRS Marine Laboratory, Scotland, July–Oct 2003

Richburg J, Johnson K, Heidi A, Schoenfeld M, Meistrich D (2002) Defining the cellular and molecular mechanisms of toxicant action in the testis. Toxicol Lett 135:167–183

Ruiz Y, Suarez P, Alonso A, Longo E, Villaverde C, San Juan F (2011) Environmental quality of mussel farms in the Vigo estuary: pollution by PAHs, origin and effects on reproduction. Environ Pollut 159:250–265

Sanders JM, Bucher JR, Peckham JC, Kissling GE, Hejtmancik RS, Chabra RS (2009) Carcinogenesis studies of cresols in rats and mice. Toxicology 257:33–39

Santodonato J (1997) Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: relationship to carcinigenity. Chemosphere 34:835–848

Schäfer S, Köhler A (2009) Gonadal lesions of female sea urchin (*Psammechinus miliaris*) after exposure to the polycyclic aromatic hydrocarbon phenanthrene. Mar Environ Res 68:128–136

Srogi K (2007) To polycyclic aromatic hydrocarbons: review. Environ Chem Lett 5:169–195

Strmac M, Braunbeck T (2002) Cytological and biochemical effects of a mixture of 20 pollutants on isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Ecotoxicol Environ Saf 53(2):293–304

Suárez MP, Álvarez C, Molist P, San Juan F (2005) Particular aspects of gonadal cycle and seasonal distribution of gametogenic stages of *Mytilus galloprovincialis* cultured in the Estuary of Vigo. J Shellfish Res 24(2):531–540

Swan SH, Kruse RL, Liu F, Barr DB, Drobnis EZ, Redmon JB, Wang C, Brazil C, Overrstreet JW (2003) Semen quality in relation biomarkers of pesticide exposure. Environ Health Perspect 111:1478–1484

Tay KL, Teh SJ, Doe K, Lee K, Jackman P (2003) Histopathology and histochemical biomarkers response of Baltic clan *Macoma balthica*, to contaminated Sydney harbor sediment, Nova Scotia, Canada. Environ Health Perspect 111:273–280

Ulbright T (2005) Germ cell tumors of the gonads: a selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. Mod Pathol 18:S61–S79

Veeramachaneni DN (2008) Impact of environmental pollutants on the male: effects on germ cell differentiation. Anim Reprod Sci 105:144–157

Villeneuve JP, De Mora SJ, Cattini C (2004) Report on the world-wide and regional intercomparison for the determination of organochlorine compounds, and petroleum hydrocarbons in mussels tissue, IAEA-432. IAEA-MEL 74, Vienna, 137pp

Viñas Diéguez LE (2002) Evaluación de hidrocarburos aromáticos policíclicos (HAPs) por cromatografía líquida de alta efeicacia (CLAE) en el entorno marino gallego. Tesis Doctoral. Universidad de Vigo. España, 266 p

WHO (World Health Organization) (1998) International Programme on Chemical Safety (IPCS), Environmental health criteria 202: selected non-heterocyclic polycyclic aromatic hydrocarbons. WHO, Geneve

Chapter 21
Histopathological Alterations of the Mantle of *Mytilus galloprovincialis* from the Ria of Vigo (NW Spain): Effect of Persistent Chemicals?

Y. Ruiz, P. Suárez, Ana Alonso Martinez, A. Vidal, and F. San Juan

Introduction

In marine environments, coastal and estuarine areas are the main productive zones, but also the most seriously affected by pollution impacts. These areas are characterized by a high biodiversity and variability in their environmental parameters as well as receiving organic matter from urban, industrial, agricultural and forest effluents and along with these also xenobiotics of diverse origin.

Galician Rías (NW Spain) are estuaries with special hydrodynamic characteristics, which favour nutrient upwelling, allowing a high biological production. This allows them to be important aquaculture areas, mainly mussel farming. However the Rías have undergone a rapid industrialization and an increase in population density, simultaneously increasing their pollution levels.

Pollutants are quickly accumulated by biota and some of them, such as PAHs and PCBs are highly persistent and toxic (Facchinelli et al. 2001; Perugini et al. 2004). Several studies relate the appearance of neoplastic histopathological alterations in bivalves with the presence of pollutants (Alonso et al. 2001; Thiriot-Quiévreux and Wolowicz 2001; Smolarz et al. 2005; Wolowicz et al. 2005), suggesting a toxic etiology for these pathologies.

Y. Ruiz (✉) • P. Suárez • A. Vidal • F. San Juan
Department of Biochemistry, Genetics and Immunology, University of Vigo, Vigo, Spain
e-mail: yruiz@uvigo.es; psuarez@uvigo.es; adrianavidal@uvigo.es; fsanjuan@uvigo.es

A.A. Martinez
Department of Biochemistry, Genetics and Immunology, Faculty of Sciences, University of Vigo, Lagoas-Marcosende s/n., 36310 Vigo, Spain
e-mail: amam@uvigo.es

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7_21,

Purpose

The aim of this study is to describe the neoplastic disorders observed in the gonad (mantle tissue) of *M. galloprovincialis* in the Ría of Vigo, discussing their possible relationship with the accumulation of persistent pollutants. This would allow one to use these pathologies as indicators of the pollution risks on mussel farms as well as an indicator of human food safety.

Material and Methods

Mussels (*M. galloprovincialis* Lmk.) were collected fortnightly between February 2001 and August 2002 from the floating rafts where are cultured in the farms of the Ría of Vigo (Galicia, NW Spain). For each sampling, 30 adult individuals from 6 to 8 cm in length were randomly collected at depths of 5 m. A small section (0.5 × 1 cm) of the central part of the gonad (mantle tissue) was fixed in Bouin's solution, dehydrated and embedded in paraffin. Sections 5 μm thick were stained with Harris' haematoxylin and eosin for histological analysis. The remaining tissues were pooled, homogenized in a Virtis microprocessor of tissues, immediately frozen and lyophilized for chemicals analysis.

For determination of the gametogenic stage the model proposed by Lubet (1959) and Suárez et al. (2005) were followed. The comparative microscopical analysis of histological slides allowed the observation of several histopathological alterations.

From lyophilized samples, PAHs, organochlorine compounds (PCBs and OCPs) and trace metals were extracted and quantificated according to Viñas Diéguez et al. (2002), De Boer (1988), González-Quijano and Fumega (1996), and Besada et al. (2002), respectively. A total of 13 PAHs were analyzed: phenantrene, anthracene, pyrene, fluoranthene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene; 16 organochlorine compounds were measured: 10 PCBs (CB:28, 31, 52, 101, 105, 118, 138, 153, 156 and 180) and 6 OCPs (αHCH, γHCH, p,p′-DDD, p,p′-DDE, o,p′-DDT, p,p′-DDT); and Cd, Cu, Hg, As, Pb and Zn as trace metals.

Statistical analysis was performed using SPSS Package 15.0 (SPSS Inc., Microsoft Co.). The variation of each variable was assessed by one-way analysis of variance (ANOVA). The level of significance was at $p < 0.05$. Correlations among variables were computed from Pearson coefficient (r). Multivariate analyses of principal component (PCA) and aggregation analyses (cluster) were carried out in order to determine the relationship between the analyzed variables and their distributions. Finally, an overlaying of the cluster analyses to PCA was performed using PRIMER 6.0 program (Clarke and Gorley 2006).

Results

Although macroscopically anomalies were not found, from comparative histological study, multiple cellular and tissular abnormalities were observed which can be considered as neoplastic disorders, affecting mainly germ cells.

The gonadal lesions observed were mainly found close to the gills and exhibited different degrees of malignancy. Histologically we observed a loss of tissue organization and of the gonadal follicles architecture (Fig. 21.1a, f). At cellular level we found larger hyperchromatic spermatocytes in males and pleomorphic oocytes in females (Fig. 21.1a, c, h); multinucleated germinal cells of great size, with clumped chromatin and irregularly shapes; pyknotic nuclei, karyorrhexis and karyolysis; vacuolizated cytoplasm; hyperchromatic nucleolus of large size; mitotic figures that suggest a high rate of cell division and enucleated cells with thin cytoplasm (ghost cells) (Fig. 21.1b, c, g–i). In many cases, the basal membrane surrounding follicles was breached and tumour cells invaded the adjacent connective tissue, sinusoids and haemolymphatic vessels. Intra- and extra-follicular fibrosis was present in the affected areas (Fig. 21.1d, i).

Along with these alterations an immune response is likely to take place, showing a proliferation of haemocytes and phagocytes around the affected follicles. Often, such haemocytes and phagocytes also show neoplastic features: numerous and atypical mitotic figures; a considerable increase of the nucleus/cytoplasm ratio; large haemocytes, with hyperchromatic, multiple or fragmented nuclei, prominent nucleolus, and often cytoplasmatic inclusions of lipofucsin or degradation products (Fig. 21.1e, j).

The prevalence of these histopathological alterations was 18 %, being higher in males (62.5 %) than in females (37.5 %). Moreover, it was during periods of active gametogenesis when the neoplastic incidence was highest: 61.5 % during spawn and gonadal restoration periods and 23.4 % during early stages of gametogenesis (Fig. 21.2).

The analyzed pollutants accumulated in *Mytilus* (PAHs, organochlorine compounds and trace metals) showed a statistically significant seasonal variation ($p < 0.01$). Total PAHs concentration ($\sum$13PAHs) ranged from 52.26 to 739 $\mu g\ kg^{-1}$ dw. Among them, the most toxic and carcinogenic species (Chry, BkF, BaA, BbF, BaP, DBA, IP) ranged between 8.86 and 310.70 $\mu g\ kg^{-1}$ dw, showing also a significant seasonal variation ($p < 0.01$). Accumulation of organochlorines was between 3 and 11 times lower than hydrocarbon concentrations. PCBs ($\sum$10 congeners PCBs) ranged between 12.25 and 58 $\mu g\ kg^{-1}$ dw, and penta- and hexachlorinated congeners including some dioxin-like, varied from 10.12 to 53.01 $\mu g\ kg^{-1}$ dw. Among OCPs analyzed, DDTs and HCHs showed significant seasonal variations ($p < 0.01$) but within a narrow range of values between 2.21 and 10.80 $\mu g\ kg^{-1}$ dw and 0.50 and 2.69 $\mu g\ kg^{-1}$ dw, respectively. Finally, the total trace elements accumulated varied between 99.25 and 375.73 mg kg^{-1} dw (Fig. 21.3).

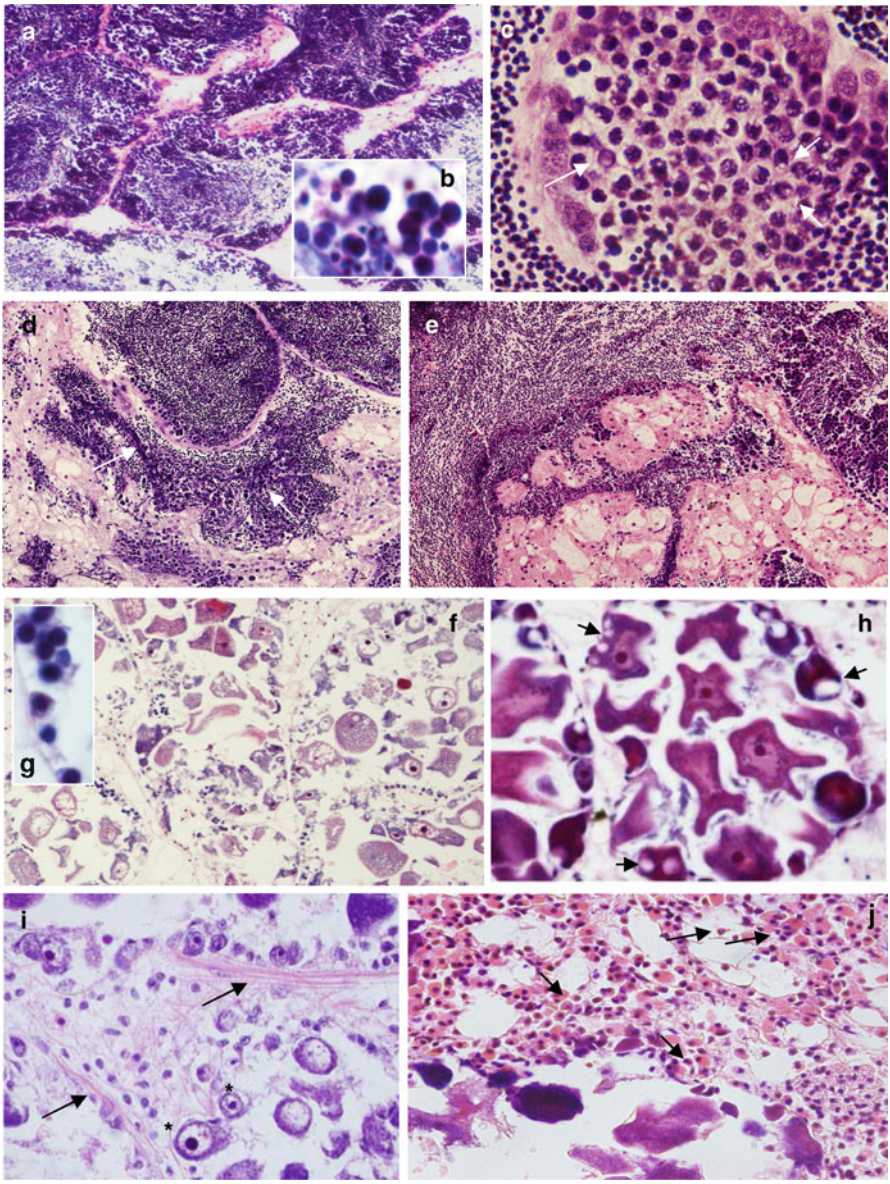


Fig. 21.1 Histological section of mantle tissue of *Mytilus galloprovincialis* with neoplasias. Males: (**a**) Loss of follicular structure (10×); (**b**) Multinucleated and hyperchromatic cells (100×); (**c**) Karyorrhexis and karyolisis (*arrows*; 100×); (**d**) Breach of follicular membrane and infiltration of tumour cells by connective tissue and vessels. Fibrosis (*arrows*) (20×); (**e**) Proliferation of haemocytes around the affected follicles (20×). Females: (**f**) Loss of follicular structure (10×); (**g**) Hyperplasia and hyperchromatic cells (100×); (**h**) Vacuolizated cytoplasm (*arrows*; 40×); (**i**) Fibrosis (*arrows*) and karyrrhesis (*asterisk*) (60×); (**j**) Altered haemocytes (*arrows*; 40×)

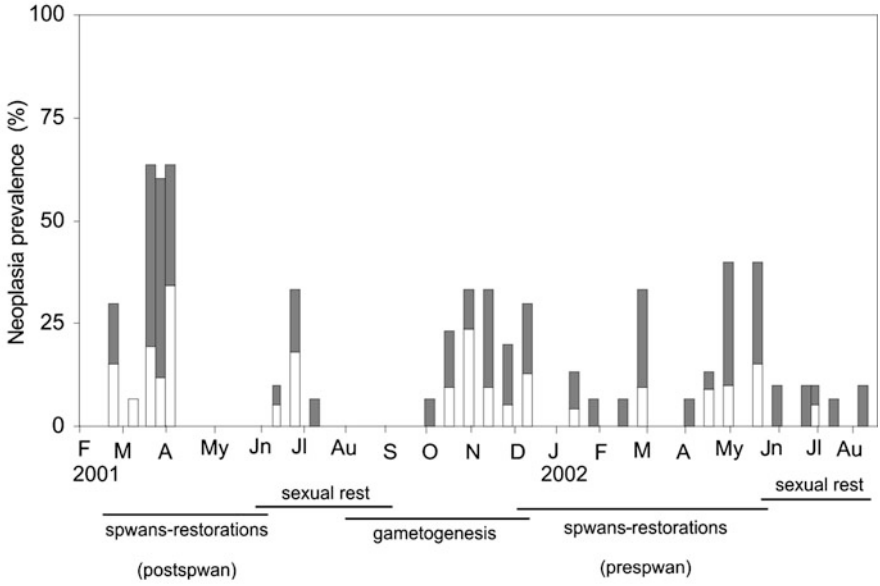


Fig. 21.2 Prevalence of neoplastic disorders (%) in the mantle of *Mytilus galloprovincialis* from the Ría of Vigo during different reproductive stages. Males (▒); Females (□)

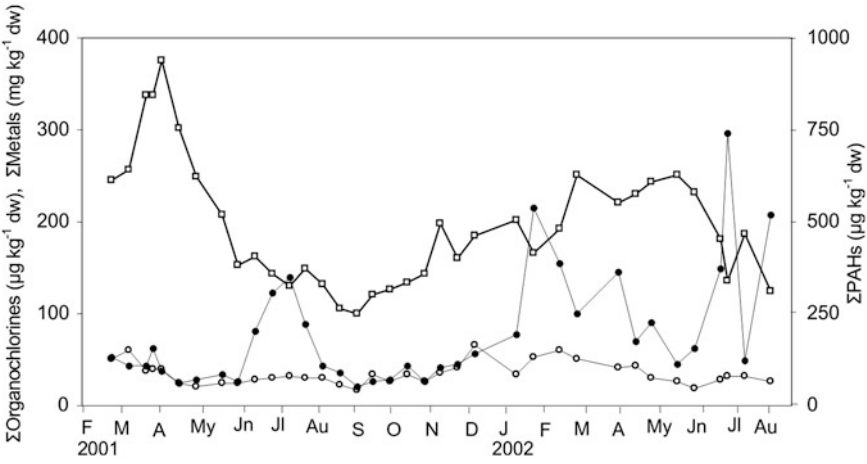


Fig. 21.3 Seasonal variation of PAHs (●), organochlorine compounds (○) and trace metals (□) accumulated in *Mytilus galloprovincialis* from the Ría of Vigo

The Pearson coefficient reveals a significant correlation of the neoplastic prevalence with mutagenic PAH's ($r = 0.605$; $p < 0.01$), PCB's of high molecular weight and chlorination degree ($r = 0.549$; $p < 0.01$), Cd ($r = 0.743$; $p < 0.01$) and Zn ($r = 0.570$; $p < 0.05$).

Figure 21.4a shows the multivariate analysis of principal components (PCA), with mutagenic species of PAHs, penta- and hexa-chlorinated congeners, Cd, Zn and neoplastic prevalence as variables, and the samplings as trials. The data matrix consisted of 180 elements (5 variables × 36 samples). The number of factors extracted from the variables was determined according to Kaiser's rule, which only retains factors with eigenvalues that exceed one. Most of the variance (87.5 %) was explained by the first two main components: PC1 explained 61.1 % of the total variance and PC2 accounted for 26.4 %. Three other factors were extracted as PC3, PC4 and PC5 which accounted for 9.9, 1.8 and 0.8 % of the total variance, respectively. PC1 was characterized by a high influence of neoplasia ($r < -0.91$), whereas PC2 was characterized by high correlation with Zn and PAHs ($r < -0.78$ and $r > 0.59$, respectively). PC3 was also corelated with Zn and PAHs ($r > 0.57$ and $r > 0.68$, respectively), PC4 by PCBs ($r < -0.94$) and PC5 by Cd ($r > 0.99$).

The score plot of the first two components allows the characterization of the samples. This plot shows how the samples are inter-related depending on the variables chosen. In this context, the distances between the samples were measured by multivariate cluster analysis (Fig. 21.4b), which can be overlapped to PCA analysis. The resulting cluster identified three main groups of samples. The first group corresponded to samples without neoplasia and minimal concentrations of pollutants. The second group included samples with high neoplasia prevalence (6.7–63.4 %), medium levels of PAHs and high levels of PCBs and metals. Finally, the third group corresponded to samples with maximum levels of PAHs, PCBs and metals, but low neoplasia prevalence (6.7–10%), suggesting a lethal effect.

Discussion

There are few references to gonadal neoplasms in bivalve molluscs. Most report effects on the germinal cells (germinoma), but in some cases both germinal cells and connective tissue cells (gonadoblastoma) (Pauley and Sayce 1968; Peters et al. 1994). These pathologies have been related to age, size, sex, season and reproductive cycle, and usually have a negative impact on reproduction (Yevich and Barszcz 1976; Hesselman et al. 1988; Bert et al. 1993; Peters et al. 1994; Ford et al. 1997; Alonso et al. 2001; Darriba et al. 2006).

Similar neoplastic alterations to those described as germinoma by Alonso et al. (2001) were observed in this work. Initially, the proliferation of germinal neoplastic cells within gonadal follicles suggests a tumor *in situ*. However, breaking of follicular membrane and invasion of the connective tissue by neoplastic cells demonstrates its infiltrating capacity. The presence of karyorrhexis, karyolysis and cytoplasm vacuolization of neoplastic cells are also indicative of necrotic processes. All these anomalies support the diagnosis of a germinoma in Stage 3, according to Bert et al. (1993) and Barber (2004).

Proliferation of haemocytes and phagocytes in tumor areas can be considered as a defensive response of organisms. Frequently, phagocytes contain degradation

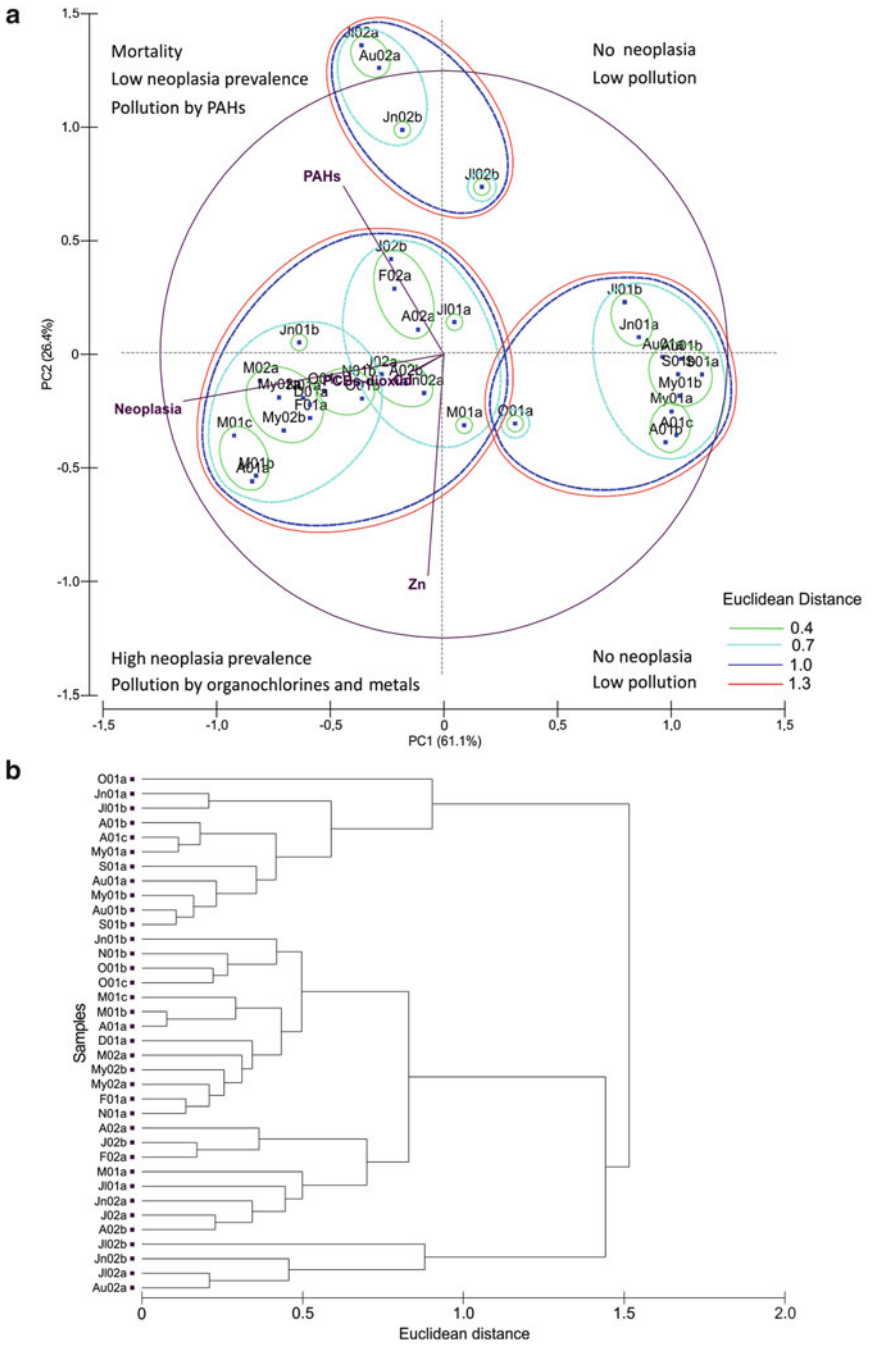


Fig. 21.4 Multivariate analysis. (**a**) PCA analysis of neoplastic disorders and pollutants (PAHs, dioxin-like PCBs, Zn and Cd), overlaying a cluster aggregation of samples. (**b**) Cluster aggregation and distances between samples

products of the abnormal cells, that because of their colour, are named by several authors "brown cells" (Cheng 1984; Zaroogian et al. 1989). However, haemocyte proliferation may becoming uncontrolled and malignant, resulting in what has been described as disseminated haemocitary neoplasia (Yevich and Barszcz 1976; Twomey and Mulcahy 1988; Peters et al. 1994).

A feature associated in vertebrates with malignancy of tumours is the desmoplastic reaction. This reaction is considered a diagnostic of metastasis and involves the formation of fibrous tissue around tumour, forming a more or less dense stroma. Fibrosis is generated by collagen production by myofibroblasts from the interstitial tissue and is activated by growth factors released by tumour cells (Walker 2001; Hauptmann et al. 2003). In this work, like in others molluscs (Yevich and Barszcz 1976; Peters et al. 1994; Alonso et al. 2001), we found evidences of a desmoplastic reaction associated with both the germinal and haemocitary neoplasias described.

Like other authors we observed certain seasonality in gonadal neoplasia prevalence related to reproductive status (Hesselman et al. 1988; Elston et al. 1992; Ford et al. 1997; Alonso et al. 2001). However most of these authors cite a higher prevalence in females (Yevich and Barszcz 1976; Hesselman et al. 1988; Gardner et al. 1991; Bert et al. 1993; Barber 2004), whereas in this work it was higher in males, as is also described by other authors (Pekkarinen 1993; Alonso et al. 2001; Darriba et al. 2006; Carella et al. 2009).

Several authors have reported an increase of neoplastic pathologies in bivalves related to the increase of environmental pollution (Thiriot-Quiévreux and Wolowicz 2001; Smolarz et al. 2005; Wolowicz et al. 2005). Other authors have shown histological alterations caused by exposure to specific chemicals in controlled laboratory conditions (Snedeker 2001; Huertas Peña 2005; Wolowicz et al. 2005). However, in the natural environment, the establishment of cause-effect relationship is not easy. In this work, the multivariate analysis carried out shows a significant correlation between levels of mutagenic PAH's, dioxin-like PCB's and certain trace metals in tissues and neoplastic disease development in the gonad of *Mytilus*, as well as the lethal effects at high concentrations of these persistent chemicals.

Conclusions

We discovered a significant correlation between levels of persistent pollutants accumulated in tissues and neoplastic disease development in the gonad (mantle tissue) of *Mytilus galloprovincialis*, as shown by histopathological changes in this tissue. The persistent chemicals measured in mussel tissues included mutagenic PAH's and dioxin-like PCB's. Other studies (Au et al. 2003; Aarab et al. 2004; Schäfer and Köhler 2009) have suggested that persistent chemicals, such as these, may result in decreased fertility in invertebrates. Our results suggest that further experimental studies are warranted to establish cause-effect relationships between exposure to persistent chemicals and the reproductive capacity of cultured mussels.

Acknowledgment The authors thank Antonio Antepazos and the fishermen of the "Antepazos I", who kindly provided the mussels used in this work. Furthermore, we would like to thank to the Spanish Institute of Oceanography from Vigo (IEO), contracted for the determination of contaminants. This work was supported by a grant from Autonomous Galician Government (PGIDT10MDS312030PR).

References

Aarab N, Minier C, Lemaire S, Unruh E, Hansen PD, Larsen BK, Andersen OK, Narbonne JF (2004) Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. Mar Environ Res 58:437–441

Alonso A, Suárez P, Álvarez C, San Juan F, Molist P (2001) Structural study of a possible neoplasia detected in *Mytilus galloprovincialis* collected from the Ria of Vigo (NW Spain). Dis Aquat Organ 47:73–79

Au DWT, Yurchenko OV, Reunov AA (2003) Sublethal effects of phenol on spermatogenesis in sea urchins (*Anthocidaris crassispina*). Environ Res 93:92–98

Barber BJ (2004) Neoplastic diseases of commercially important marine bivalves. Aquat Living Resour 17:449–466

Bert TM, Hesselman DM, Arnold WS, Moore WS, Cruz-López H, Marelli DC (1993) High frequency of gonadal neoplasia in hard clam (*Mercenaria* spp.) hybrid zone. Mar Biol 117:97–104

Besada V, Fumega J, Vaamonde A (2002) Temporal trends of Cd, Cu, Hg, Pb and Zn in mussel (*Mytilus galloprovincialis*) from the Spanish North-Atlantic coast 1991–1999. Sci Total Environ 288(3):239–253

Carella F, Restucci B, Maiolino P, De Vico G (2009) A case of germinoma in a limpet (*Patella coerulea*) (Patellogastropoda). J Invertebr Pathol 101:154–156

Cheng TC (1984) A classification of molluscan hemocytes based on functional evidences. In: Cheng TC (ed) Comparative pathobiology, invertebrate blood cells and serum factors, vol 6. Plenum, New York, pp 111–146

Clarke KR, Gorley RN (2006) PRIMER (Plymouth Routines in Multivariate Ecological Research) v6: user manual/tutorial. PRIMER-E Ltd, Plymouth Marine Laboratory, Plymouth

Darriba S, Iglesias D, Harshbarger JC, López C (2006) Germinoma in razor clam *Ensis arcuatus* (Jeffreys, 1865) in Galicia (NW Spain). J Invertebr Pathol 93:140–142

De Boer J (1988) Chlorobiphenyls in bound and non-bound lipid of fishes, comparison of different extraction methods. Chemosphere 17(9):1811–1819

Elston RA, Moore JD, Brooks K (1992) Disseminated neoplasia of bivalve molluscs. Rev Aquat Sci 6:405–466

Facchinelli A, Sacchi E, Mallen L (2001) Multivariate statistical and GIS-based approach to identify heavy metal source in soils. Environ Pollut 114:313–324

Ford SE, Barber RD, Marks E (1997) Disseminated neoplasia in juvenile eastern oyster *Crassostrea virginica*, and its relationship to the reproductive cycle. Dis Aquat Organ 28:73–77

Gardner GR, Yevich PP, Hurst P, Theyer P, Benyi S, Harshbarger JC, Pruell RJ (1991) Germinomas and teratoid siphon anomalies in softshell clams, *Mya arenaria*, environmentally exposed to herbicides. J Environ Health Perspect 90:43–51

González-Quijano A, Fumega J (1996) Determinación de congéneres individuales de bifenilos policlorados en organismos marinos. Boletín del Instituto Español de Oceanografía 160:27pp

Hauptmann S, Budianto D, Denkert C, Kobel M, Borsi L, Siri A (2003) Adhesion and migration of HRT-18 coloreetal carcinoma cells on extracellular matrix components typical for the desmoplastic stroma of colorectal adenocarcinomas. Oncology 65(2):174–181

Hesselman DM, Blake NJ, Peters EC (1988) Gonadal neoplasms in hard shell clams *Mercenaria* spp., from the Indian River lagoon Florida: occurrence, prevalence and histopathology. J Invertebr Pathol 52:436–446

Huertas Peña FJ (2005) Estimación de la exposición a xenoestrógenos en el cáncer de mama y seguimiento de las pacientes tras el tratamiento instaurado. Doctoral thesis, University of Granada, Granada, 356pp

Lubet P (1959) Recherches sur le cycle sexuel et l´émission des gamètes chez les Mytilidés et las Pectinidés. Rev Trav Inst Pêch Marit 23(4):389–545

Pauley GB, Sayce CS (1968) An internal fibrous tumor in a Pacific Oyster *Crassostrea gigas*. J Invertebr Pathol 10:1–8

Pekkarinen M (1993) Neoplastic diseases in the Baltic *Macoma balthica* (Bivalvia) off the Finnish coast. J Invertebr Pathol 61:138–146

Perugini M, Cavaliere M, Giammarino A, Mazzone P, Olivieri V, Amorena M (2004) Levels of polychlorinated biphenyls and organoclorine pesticides in some edible marine organisms from the Central Adriatic Sea. Chemosphere 57:391–400

Peters EC, Yevich JC, Harshbarger JC, Zaroogian GE (1994) Comparative histopathology of gonadal neoplasms in marine bivalve molluscs. Dis Aquat Organ 20:59–76

Schäfer S, Köhler A (2009) Gonad lesions of female sea urchin (*Psammechinus miliaris*) after exposure to the polycyclic aromatic hydrocarbon phenanthrene. Mar Environ Res 68:128–136

Smolarz K, Thiriot-Quiévreux C, Wolowicz M (2005) Recent trends in the prevalence of neoplasia in the Baltic clam *Macoma balthica* (L.) from the Gulf of Gdansk (Baltic Sea). Oceanologia 47(1):61–74

Snedeker SM (2001) Pesticides and breast cancer risk: a review of DDT, DDE and dieldrin. Environ Health Perspect 109(1):35–47

Suárez MP, Álvarez C, Molist P, San Juan F (2005) Particular aspects of gonadal cycle and seasonal distribution of gametogenic stages of *Mytilus galloprovincialis* cultured in the Estuary of Vigo. J Shellfish Res 24(2):531–540

Thiriot-Quiévreux C, Wolowicz M (2001) Chromosomal study of spatial variation of the prevalence of a gill neoplasia in *Macoma balthica* (L.) from the Gulf of Gdansk (Baltic Sea). Ophelia 54(1):75–81

Twomey E, Mulcahy MF (1988) Epizootiological aspects of a sarcoma in the cockle *Cerastoderma edule*. Dis Aquat Organ 5:225–238

Viñas Diéguez L, Franco Hernández A, González Fernández JJ (2002) Distribution of polycyclic aromatic hydrocarbons in superficial sediments of the Vigo estuary, Spain, central axis and adjacent shelf. Polycycl Aromat Compd 22:161–173

Walker RMA (2001) The complexities of breast cancer desmoplasia. Breast Cancer Res 3:143–145

Wolowicz M, Smolarz K, Sokolowski A (2005) Neoplasia in estuarine bivalves: effect of feeding behaviour and increase environmental pollution. In: Dame RF, Olenin S (eds) The comparative roles of suspension-feeders in ecosystems, vol 47, NATO advanced science. Springer, Dordrecht, pp 165–182

Yevich PP, Barszcz CA (1976) Gonadal and haematopoietic neoplasms in *Mya arenaria*. Mar Fish Rev 38:42–43

Zaroogian G, Yevich P, Pavignano S (1989) The role of the red gland in *Mercenaria mercenaria* in detoxification. Mar Environ Res 28:447–450

Appendices – Round Table Summaries

Ensuring Safety and Quality of Bivalves

Chair: Dorothy Leonard, Ocean Equities LLC, United States
The topics included depuration and improvements in current post-harvest treatments such as high pressure processing. These approaches have the potential to reduce the risk posed by pathogens while maintaining product quality and safety. The session opened with presentations from experts of France, Italy, Uruguay, Canada and the United States.

Dinorah Medina, Uruguay DINARA Molluscan Shellfish Safety in South America: Dr. Medina gave an overview of the importance of product safety to the Latin American countries which export their products to the European Union. Since 1980 Argentina has carried out monitoring of biotoxins in shellfish and in 2006 started the sanitary classifications of harvest areas. Since 1994 Brazil is conducting studies of phytoplankton mostly on the south coast. Chile has implemented the NSSP since 1989 and currently exports frozen mollusks to the EU. Uruguay has been implementing a coastal monitoring program since 1980 for domestic markets and a control program for the export of wild clams, frozen or live.

Cyr Couturier, Marine Institute, Memorial University, Newfoundland-Farmed Molluscan Shellfish Safety and Aquaculture Supply Chain in Canada. The Canadian aquaculture industry (150,000 tons valued at $2 billion) implements BMPs in farm-based HACCP programs with full traceability and in some cases, third party certification. Standards are science-based and validated regularly; no illnesses related to farmed seafood.

Patrick Lassus, IFREMER, France Storage and Detoxification of Bivalve Mollusks as a Tool in Marketing Strategy. He covered a study that was conducted to determine the best approach in post harvest treatment to allow harvest and marketing of oysters and mussels during toxic blooms of *Dinophysis acuminata* (DSP) and *Alexandrium minutum* (PSP producer). The Lassus paper can be found within this document.

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7,

Giuseppe Arcangeli, Istituto Zooprofilattico delle Venezie, Italy Effect of High Hydrostatic Pressure on Murine Norovirus in Manila Clams. The study investigated the ability of high hydrostatic pressure (HHP) to inactivate murine norovirus (MNV-1), a surrogate for noroviruses in experimentally infected Manila clams. Virus vitality post-treatment was assessed showing that HHPat 500 MPa for 1 min was effective in inactivating MNV-1.

Gordon Neal, Aqualife North America, Inc. Aqualife technology for Maintaining Safety and Quality. The Cleantech process allows long haul transport of live aquatic animals (fish, crustaceans, mollusks) by providing short-term storage, cooling and acclimation of live seafood before loading and after arrival through the use of Aquaports. Aquaports purify water using ozone, ultraviolet and membrane filtration to remove marine biotoxins, invasive species and other contaminants.

The Industry Panel was expanded to include discussions by Lea Murphy of Prince Edward Aqua Farms in Canada; Chris Roberts of Environment Canada, Bill Watkins of the US Food and Drug Administration, David Kingsley of the US Department of Agriculture Research Service; and Tom Howell, Spinney Creek Shellfish in Maine, USA. Discussions focused on post-harvest treatment, particularly high hydrostatic pressure (HHP) and depuration.

Depuration. Prince Edward Aqua Farms in New London, PEI, processes mussels, softshell clams, quahogs and oysters. In business since 1989, the company has used an existing saltwater well for their licensed depuration facility (1,000 gallons/min) for oysters and clams, sometimes using the facility to wet store mussels. There can be an elevation in temperature or salinity mitigated by ambient water. The question arose regarding high iron content in saltwater wells (France) but the well in New London has not had iron problems. The Spinney Creek facility in Maine processes soft-shell clams and oysters sourced from Canada south to the Mid-Atlantic states. Currently they are modernizing the depuration process focused on reducing noroviruses. They work closely with the ISSC Lab Methods Review Committee to validate the use of male-specific coliphage as an indicator.

High hydrostaticpressure (HHP). There is considerable interest in the use of HHP to reduce vibrios and viruses, the latter work being done by the USDA Agricultural Research Service in Delaware. HHP is used in Newfoundland (the plant cost was >1 million USD) to process crabs and in Maine to remove lobster meat from the shell. In Newfoundland, shellfish is processed, cooked, sometimes using modified atmosphere packaging (not acceptable for export to the US). Concern was expressed that molluscs processed by HHP are no longer alive, affecting shelf-life. The industry is very concerned about the consumer acceptance and additional cost. In a recent study in Florida it was found that consumers prefer the standard product and are not willing to pay extra for post-harvest processed oysters. Shelf life is equivalent to refrigerated fresh product.

Additional discussion focused on the costs of Aqualife's new transport system. Costs are reduced by less packaging, reduction of carbon footprint and much lower transport costs than air freight. Oysters and clams are preferred as mussels tend to clump up and adhere to the containers. The EU produces a large quantity of

mussels so Canadian product would not be competitive. The industry expressed concerns about the management of information by the regulatory agencies. Both Environment Canada and USFDA responded in support of careful handling of information that could affect the industry. The time it takes to develop improved procedures (source tracking, dilution studies at wastewater treatment plants, new methods and indicators) seems to take much more time than the industry feels it can afford. When the audience raised the question of how many human illnesses are a result of viruses in shellfish there were no solid figures. There have been no outbreaks in Canada and only one Hepatitis-A outbreak over 20 years ago in the US. However, naturally occurring vibrios transmitted through the consumption of shellfish remains a problem worldwide.

Risk Management and Impacts

Chair: Robyn Edwards, Canadian Food Inspection Agency

Panel members: I. Boxman, T. Howell, R. Lee, H. Toyofuku, A. Zammit
The discussion was held after a session with four presentations related to the use of risk management process and how it influences molluscan shellfish control systems in different countries or at international advisory bodies:

Boxman – Codex CCFH guidelines on control of viruses
Toyofuku – Regulator Perspective on translating science into policy
Lee – Salmonella in bivalves: public health considerations (Codex)
Howell – Industry perspective on translating Science into Policy

Concerns about the presence of viruses in bivalve molluscs are gaining prominence with epidemiologic evidence building up and the lack of solid scientific proof of prevalence. Questions were raised regarding the fact that the Codex Committee on Food Hygiene (CCFH) has issued a Proposed Draft Guidelines on the Application of General Principles of Food Hygiene to the Control of Viruses in Food[1] that has an annex specific to the control of hepatitis A virus (HAV) and norovirus in bivalves, while there has been no formal risk assessment produced by any CODEX group of experts.

Comments were made about the validity of a proposed heat treatment (90 °C for 90 s.) to control HAV and the possible understanding it is an acceptable post-harvest treatment for bivalves. In fact, the guidelines should clearly exclude the latter as an acceptable post-harvest treatment for bivalves harvested in a knowingly HAV

[1] ftp://ftp.fao.org/codex/Meetings/CCFH/ccfh43/fh43_04e.pdf [Accessed 14 November 2012]. This Codex GUIDELINES ON THE APPLICATION OF GENERAL PRINCIPLES OF FOOD HYGIENE TO THE CONTROL OF VIRUSES IN FOOD (CAC/GL 79-2012) was finalized and adopted in July 2012. Available from http://www.codexalimentarius.org/input/download/standards/13215/CXG_079e.pdf

exposed or potentially HAV contaminated growing area. No post-harvest treatment should prevail over a strict growing area classification program and contamination management measures.

A few more thoughts were expressed with regards to future discussions on guidelines and risk assessment:

- There should be more collaboration between WHO water experts and FAO mollusc experts in order to develop a better guidance document. We must not forget the waters studied by one group are the medium for growing the molluscs followed by other group.
- Virus risk assessment might take seasonal/water temperature variations into account where those conditions exist.
- Market surveys might be included among possible risk management tools. It has been pointed out that the comparison of surveys' results between countries or even regions would be problematic due to the compounding effect of varying natural contamination levels, local consumption patterns, seasonal conditions and many other factors.

In reference to risk assessment, it has been reported that the molluscan shellfish industry members lack a clear understanding of the meaning/ uses of/ differences between risk assessment and risk management. In consequence, they do not realise how those may affect their businesses. As a result, the industry might attempt to influence regulators that may introduce new control measures, request additional information (to satisfy consumer response), or directly question the measures put in place to guarantee safety. It has been suggested to put emphasis on risk communication.

Although risk management appears theoretical to many, some stakeholders have already turned viral risk perception to their advantage by making it part of their marketing tools. Some mollusc dealers use pathogen and virus testing (even though rapid commercial virus testing methods are not readily available) as one criterion to select providers and advertise "safety tested" products. Similarly, environmental lobbies may expose sewage treatment plants for using inefficient methods and subsequent contamination of growing areas with viruses.

Risk Assessment for Enteric Viruses

Chair: Gary P. Richards, USDA, United States

Panel members: William Burkhardt, Angelo DePaola, Rachael Hartnell, and William Ross

This roundtable was held after a session where the following four presentations related to risk assessment of noroviruses were presented:

Ross – Risk Calculation Framework
Burkhardt – US Perspective – Viral Risk

Hartnell – EU Perspective – Viral Risk
DePaola – What We Can Learn from the *Vibrio* Risk Assessment and Apply to Viruses

Discussion during the roundtable questioned whether it was appropriate to conduct a risk assessment before methodologies for virus detection were validated and standards were put in place. Although the EU and US are getting closer to that point, one panelist felt that we could not wait another 3–5 years to perform a risk assessment on norovirus and that it was critical to know what levels of norovirus are going into the water. The US and Canada have a timeline for the risk assessment to be completed in 1 year. In the EU, legislation based on virus criteria is expected to be put in place shortly.

When asked how data generated by RT-PCR can be used in performing a risk assessment when RT-PCR is known to detect both infectious and non-infectious virus particles, it was pointed out that there was no other method available and that any level of virus detection, whether infectious virus or not, indicates that the shellfish were exposed to norovirus. It was generally recognized that inadequacies of RT-PCR are the greatest contributors to uncertainty in a risk assessment. Systemic problems can occur in risk models when inactive viruses are counted and can throw the entire risk assessment into an unmanageable state. It is important to have information on the impact of active versus non-infectious viruses as part of the risk assessment. It was recognized that detection of non-infectious viruses could also lead to over regulation of the industry.

Another question was on how the illegal practice of dumping boat wastes or vomiting overboard in shellfish harvesting areas can be taken into account when performing a risk assessment. It was acknowledged that dumping of boat wastes is illegal, but that it does occur. There is a need to separate contamination by boat waste (misbehavior) from contamination by other means (e.g., storm water runoff, failure of sewage treatment plants and septic tanks, etc.). Different risk management approaches are needed to handle illegal dumping, which is a random act, versus contamination by other means (which are often more predictable). Separate analyses need to be performed and some information on the incidence of illegal dumping would be needed. One needs to know the magnitude of the problem as it can have a high impact. These occasional misbehaviors are consequential and can have a high impact compared to the failure of a waste water treatment plant, which has controls in place for such occasions. It doesn't matter how good waste water treatment is or what the dilution is if someone is vomiting in that harvest area.

Factors required for a risk assessment include knowing the attack rate and the number of infectious particles in the leftover food, so that one can determine a distribution as part of a Monte Carlo simulation. In such a case, one would know the number of exposed individuals (based on illnesses) and the number of viruses consumed to provide some sense of the level of risk. A third component which has been used in other contexts, but might be useful in a norovirus risk assessment deals with sporadic illnesses not typically measured [or recorded]. These three pieces of information need to be looked at together. This information must be combined

to bracket the relationship between the amount consumed and how likely you are to become ill.

When asked whether the risk assessment for norovirus takes into account variability in virulence levels among the different genogroups and strains of norovirus, the consensus was that there was currently no plan to address that. Both genogroups will be treated the same way and from a model perspective, both genogroups will be treated as equally infectious.

A point was made that *E. coli* or fecal coliforms are indicators but are not good for enteric viruses followed by the questions, will bacterial indicators be eliminated in the future? Are there acceptable levels established for noroviruses in shellfish? From a European perspective, on a sample-to-sample basis, *E. coli* or fecal coliforms may not be good predictors of NoV but on a time series bacterial data set basis gives a good relationship between noroviruses and *E. coli*. It seems unimaginable that any norovirus standard would ever replace the *E. coli* requirements in the EU. In North America, regulators classify growing areas based on sanitary surveys, identification of pollution sources, and mitigation strategies, as needed. Fecal coliforms are important in sanitary surveys.

Index

G. Sauvé (ed.), *Molluscan Shellfish Safety*, DOI 10.1007/978-94-007-6588-7,