

**« Contributions to the characterisation of risks
posed by marine biotoxins »**

Mémoire de

Habilitation à Diriger des Recherches

Présenté par :

Philipp Hess

29 Mars 2010

Contents

List of Abbreviations.....	5
Acknowledgements	7
1.Executive Summary.....	9
2.Preface	11
3.Curriculum Vitae.....	14
3.1. Professional Career	14
3.2. Qualifications.....	15
3.3. Courses and training periods	15
3.4. Publications.....	16
3.4.1. Peer reviewed publications.....	16
3.4.2. Book chapters	20
3.4.3. Scientific Reports	20
3.4.4. Conference proceedings	22
3.4.5. Oral presentations	24
3.4.6. Poster presentations	26
3.4.7. Application notes	28
3.5. Academic Supervision.....	29
3.5.1. Undergraduates	29
3.5.2. Postgraduates	31
3.5.3. Postdocs	32
3.6. PhD Examination and Science Performance Auditing.....	32
3.6.1. PhD Examinations	32
3.6.2. CEFAS Science Audit.....	32
3.7. Project Proposals, Management and Evaluation.....	33
3.7.1. Project Proposals and Management.....	33
3.7.2. Project Evaluation	33
3.8. Organisation of Conferences	34
3.9. Editorial and Refereeing Activities	34
3.9.1. ICMSS 2004, 2009	34
3.9.2. Marine Drugs.....	34
3.9.3. Peer Reviews.....	34
3.10. Expert Committees and Consultancy.....	35
4.Scientific part	36
4.1. Introduction.....	36
4.1.1. Shellfish poisoning and algal toxin groups.....	37

4.1.1.1.	Chemical diversity of algal toxin groups	37
4.1.1.2.	Algal toxins and bivalve molluscs	38
4.1.1.3.	Shellfish poisoning	56
4.1.1.4.	Biological targets and the mechanism of action of phycotoxins	58
4.1.2.	<i>Risk evaluation and management of shellfish toxins</i>	61
4.1.2.1.	Difficulties in protecting the consumer	61
4.1.2.2.	Challenges in the production of safe shellfish	62
4.1.2.3.	Risk analysis principles in Codex Alimentarius	68
4.1.2.4.	Risk Management	74
4.2.	<i>Method development for the analysis for phycotoxins</i>	76
4.2.1.	<i>Mass spectrometry and paralytic shellfish toxins</i>	77
4.2.1.1.	Ion exchange chromatography coupled to mass spectrometry for the detection of saxitoxin group toxins	78
4.2.1.2.	Hydrophilic interaction chromatography coupled to mass spectrometry for the detection of saxitoxin group toxins	80
4.2.2.	<i>Mass spectrometry and lipophilic toxins</i>	85
4.2.2.1.	Critical parameters in the analysis of lipophilic toxins by LC-MS	86
4.2.2.2.	Evaluation of matrix effects in mass spectrometry	91
4.2.2.3.	Clean-up techniques for the removal of matrix effects	95
4.3.	<i>Distribution of algal toxins in the marine environment</i>	102
4.3.1.	<i>Okadaic acid in America</i>	102
4.3.2.	<i>Azaspiracids not just in Ireland</i>	104
4.3.3.	<i>Species differences in the uptake of OA and AZA</i>	106
4.3.4.	<i>Domoic acid and scallops</i>	107
4.3.5.	<i>Environmental distribution of algal toxins</i>	113
4.4.	<i>Preparative isolation of natural products</i>	116
4.4.1.	<i>AZAs</i>	116
4.4.2.	<i>DTX2</i>	118
4.4.3.	<i>PTX2sa-fatty acid esters</i>	120
4.5.	<i>Quality control and the performance of methods</i>	121
4.5.1.	<i>Reference materials</i>	121
4.5.1.1.	Classification of reference materials	121
4.5.1.2.	Certified calibrants	122
4.5.1.3.	Matrix reference materials	124
4.5.2.	<i>Proficiency testing</i>	130
4.5.2.1.	Homogeneity and stability of test materials for proficiency testing	131
4.5.2.2.	Proficiency testing for domoic acid	132
4.5.2.3.	Proficiency testing for lipophilic toxins	133
4.5.2.4.	Proficiency testing for saxitoxin group toxins	134
4.5.3.	<i>Method comparison, evaluation and fitness for purpose</i>	136
4.5.3.1.	LC-UV, -MS and antibody-based methods for DA & OA	136
4.5.3.2.	LC-MS versus MBA for lipophilic toxins	142
4.6.	<i>Characterisation of hazards from marine biotoxins</i>	147
4.6.1.	<i>Physico-chemical characteristics and metabolic transformations of shellfish toxins</i>	147
4.6.1.1.	Lipophilicity of marine biotoxins	147
4.6.1.2.	Chemical stability of azaspiracids	152

4.6.1.3.	Transformation of azaspiracids in shellfish	155
4.6.1.4.	Chemical stability of okadaic acid and analogues	158
4.6.2.	<i>Influence of industrial processing and heat treatment procedures of shellfish on toxin concentration</i>	160
4.6.2.1.	Evisceration of scallops to reduce domoic acid content	160
4.6.2.2.	Influence of heat treatment on content of domoic acid in mussels.....	161
4.6.2.3.	Influence of heat treatment on content of okadaic acid in mussels	163
4.6.2.4.	Influence of heat treatment on content of azaspiracids in mussels.....	166
4.6.3.	<i>Interaction of toxins with biological systems</i>	167
4.6.3.1.	Relative toxicity of DTX2	167
4.6.3.2.	Toxicity of azaspiracids and mechanism of action.....	168
5.	Conclusions and outlook	175
5.1.	<i>Methods of analysis</i>	175
5.2.	<i>Distribution of toxins in the environment</i>	175
5.3.	<i>Preparative isolation of bioactive natural products</i>	176
5.4.	<i>Quality control and the performance of methods</i>	176
5.5.	<i>Characterisation of hazards from marine biotoxins</i>	177
5.6.	<i>Looking forward</i>	178
6.	References	180
7.	French Summary of “HDR”	198
7.1.	<i>Introduction</i>	198
7.2.	<i>Développement des méthodes</i>	203
7.3.	<i>Distribution des toxines dans l’environnement</i>	208
7.4.	<i>Isolement préparatif de composés bioactifs naturels</i>	212
7.5.	<i>Contrôle qualité</i>	214
7.6.	<i>Caractérisation des dangers</i>	220
7.7.	<i>Perspectives</i>	225
7.8.	<i>Résumé final</i>	227

List of Abbreviations

ASP	Amnesic shellfish poisoning
ASTOX	Azaspiracid Standards and Toxicology (IE-funded project 2003 – 2007)
ASTOX2	Azaspiracid Standards and Toxicology
AZA	Azaspiracid
AZP	Azaspiracid poisoning
BDS	Base-deactivated Silica (chromatographic phase)
BTX	Brevetoxin
CRL	Community Reference Laboratory
COSY	Correlation Spectroscopy
CRM	Certified reference material
CTX	Ciguatoxin
CV	Coefficient of Variation
DA	Domoic Acid
DCMNR	Department of Communications, Marine and Natural Resources
D/EMP	Département Environnement, Microbiologie et Phycotoxines
DiFMUP	6, 8-difluoro-4-methylumbelliferyl phosphate
DTX	dinophysis toxin
DSP	diarrhetic shellfish poisoning
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organisation
EGFR	Epidermal Growth Factor Receptor
EMP	epithelial membrane proteins
EQUIV	Equivalents
ER	Endoplasmic Reticulum
ERK	Extracellular Regulated Kinase
EtOAc	Ethyl Acetate
EU	European Union
FAPAS	Food Analysis Proficiency Assessment Scheme
FAO	Food and Agricultural Organisation
FSAI	Food Safety Authority Ireland
FLD	Fluorescence Detector
GABA	Gamma Aminobutyric Acid
GPC	Gel permeation Chromatography
GYM	Gymnodimine
HABs	Harmful Algal Blooms
HP	Hepatopancreas
HPLC	High Performance Liquid Chromatography
HYOU1	Hypoxia up-regulated 1
ICMSS	International Conference on Molluscan Shellfish Safety
IOC	Intergovernmental Oceanographic Commission
IUPAC	International Union of Pure and Applied Chemistry
IRMM	Institute for Reference Materials and Measurements
ISO	International Standards Organisation
K _{ow}	Octanol-water Partitioning Coefficient
LC-MS	Liquid Chromatography - Mass Spectrometry
LC-MS-MS	Liquid Chromatography – tandem Mass Spectrometry
LDLR	Low Density Lipoprotein Receptor
LFIC	Lateral Flow Immuno-Chromatography
LOAEL	Lowest observable adverse effect level

LPLC	Low Pressure Liquid Chromatography
LRM	Laboratory Reference Material
MAPK	Mitogen Activated Protein Kinase
MBA	Mouse Bioassay
MEA	Microelectrode Arrays
MI	Marine Institute
MeOH	Methanol
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Na ⁺	Sodium ion
NDP	National Development Plan
NIST	National Institute for Standards and Technology
NOAA	National Oceanic and Atmospheric Administration
NOAEL	No Observable Adverse Effect Level
NOESY	Nuclear Overhauser Effect Spectroscopy
NMR	Nuclear Magnetic Resonance
NRC-IMB	National Research Council – Institute of Marine Biosciences
NRL	National Reference Laboratory
NSP	Neurotoxic Shellfish Poisoning
NSVS	Norwegian School of Veterinary Science
NVI	Norwegian Veterinary Institute
OA	Okadaic acid
OECD	Organisation for Economic Co-operation and Development
PCR	Polymerase Chain Reaction
PMVK	Phosphomevalonate Kinase
PP1 / PP2a	Phosphoprotein Phosphatase 1 / 2a
PT	Proficiency Testing
PTX	Pectenotoxin
PSP	Paralytic Shellfish Poisoning
QC	Quality Control
QUASIMEME	Quality Assurance of Information for Marine Environmental Monitoring in Europe
RP	Reverse Phase
RM	Reference Material
ROESY	Rotating Frame Overhauser Effect Spectroscopy
RSD	Relative Standard Deviation
SD	Standard Deviation
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SOP	Standard Operating Procedure
SPX	Spirolide Toxin
STX	Saxitoxin
TDI	Tolerable Daily Intake
TOCSY	Total Correlated Spectroscopy
TEER	Transepithelial Electrical Resistance
UME	Uncooked Mussel Extract
UV	Ultra Violet (wavelength range in spectroscopic detection)
VEGF	vascular endothelial growth factor
VGSC	Voltage-gated Sodium Channel
VLC	Vacuum Liquid Chromatography
WHO	World Health Organisation
WP	Work Package
YTX	Yessotoxin

Acknowledgements

The work of a person is generally the product of the person's efforts multiplied by the efforts and the excellence of the person's teachers and by collaborators' input into the work. With this in mind, I am very grateful to a number of people who have helped me on my way, either through their teaching or through their direct collaboration.

First of all I would like to thank the many professors and their assistants encountered during my undergraduate studies in Germany and France who have helped us translate our fascination with chemistry into the understanding of basic principals. Particular thanks here also to Profs J.M. Leibenguth and M.J.F. Leroy in Strasbourg who exemplified the international dimension of science through the concept of multilingual teaching of chemistry. Prof. Leroy has also brought to life aspects of quality assurance in analytical chemistry through special courses by Eddie Maier and Ben Griepink, two of the founders of the "Bureau Communautaire de Référence", later transformed into the "Standards Measurement and Testing" programme; some of their work is now being continued by the "Institute for Reference Materials and Measurement" in Geel, Belgium. I had the opportunity to collaborate with Hendrik Emons and Håkan Emteborg (IRMM) in 2007, and would like to express my sincere thanks for their efficient yet collegial way of collaboration in the preparation of reference materials.

During my PhD thesis at the Marine Laboratory in Aberdeen (MLA), Scotland, I was influenced by the contribution of two scientists in particular: Dr. David Wells, my PhD tutor, and Dr. Ian Davies. While David had led me in my doctoral studies on method development and validation of methods for planar aromatic compounds in the marine environment and their comparison to biological effects monitoring, he also reinforced my experience with quality control through familiarisation with proficiency testing. Ian had been a great colleague and had helped me tremendously during my thesis through technical guidance and constructive criticism of my scientific writing.

Susan Gallacher at MLA has not only given me my first opportunity of a job after my PhD, she has also allowed me to change from the vast field of environmental contaminants to the exciting area of natural toxins. I also owe her thanks for encouraging me to perfect my science writing skills in English, as well as the introduction to my next great tutor, Dr. Michael Quilliam. I would like to give special recognition to Leslie Stobo (Bates) and Nigel Brown who made teamwork at MLA enjoyable.

In 1999, Mike Quilliam hosted me for three months in his laboratory in Halifax and has truly lit my avid interest in the chemistry of marine biotoxins. While we were looking for STX-related compounds in bacterial isolates, he taught me a lot about the analytical chemistry of toxins, in particular using techniques related to liquid chromatography coupled to mass spectrometry (LC-MS). We have collaborated ever since and I hold his contributions to the field in very high esteem.

During my time in Ireland, I had the chance to meet many great characters, including one of the last Celts, Micheal O'Cinneide, who has contributed to many of my studies through his genuine interest and support, always translating political stakeholder pressure into funding of scientific projects. Terry McMahon is not only one of the few remaining

Irish smokers and revolutionists, he has also been a great guy for looking at all angles and refereeing papers before they go out, thanks for all your patience and advice !

I also had a great team working with me in Dublin and Galway, thanks to all for your enthusiasm and perseverance beyond cultural diversity. Particular thanks also to Jane Kilcoyne for her interest in and care of the Q-ToF, and for her smile which has made many of our rainy days sunny, and to Stephen Burrell for raising our spirits with his never-ending energy. My time in Ireland would not have been half as enjoyable if there hadn't been three of my post-graduate students worth mention here: Pearse McCarron, Elie Fux and Nils Rehmann. Thanks to Nils and Pearse for playing guinea pigs as my first PhD students. My gratitude goes to Pearse in particular for pursuing our many ideas and for his "grand" way of making the time together pleasurable. My regards also to Elie who has made best use of his time and who has persevered in areas where many others would have thrown the towel.

I also owe thanks to the Marine Institute for allowing me to spend time in our Japanese collaborators' laboratory in Sendai. Satake- and Ofuji-san were extremely helpful to me getting to grips with the concept of bioassay-guided fractionation in general and the purification of azaspiracids specifically; many thanks for your energy during our 16h days in the heat of the Japanese summer and your dedication to detail both of which were only matched by your hospitality. Special thanks are also due to Prof. Yasumoto who has altruistically taught me preparative isolation chemistry during several meetings and many communications.

I would also like to mention the fruitful discussions I had with many scientists of the "New Zealand contingent", amongst which Chris Miles has provided the strongest input in my work: in his low key way and through many travel efforts he has facilitated our interactions.

Over the past years I have become more and more interested in the interaction between chemicals and biological systems. This interest was greatly nurtured by Greg Doucette, Mike Twiner, Tore Aune and Gian Paolo Rossini: I express my deepest gratitude to you all for your patience and clarity in making toxicology more understandable to me in such a friendly way.

Many of my contributions in the international context would never have been possible if several people hadn't placed their trust in me to clarify scientific issues at stakeholder meetings. I would like to mention specifically Phil Busby from the New Zealand Food Safety Authority, Prof. Lahsen Ababouch at FAO (Rome) and Alan Reilly of the Food Safety Authority of Ireland.

In the category of pleasant collaborators, I would like to thank especially John Slater and John Aasen, there is many more friends and colleagues I should mention here but space has its limitations; suffice to say that I think I have not only enjoyed working in this field so much because it is scientifically interesting but mostly because of the great openness of many contributors and so many friendships made along the way.

I would like to thank heartedly the four people at Ifremer who have patiently and rapidly read over the final manuscript in English and French: Henri Loréal, Patrick Lassus, Véronique Séchet and Martial Catherine.

Finally, all my scientific work would never have been possible were it not for the support of my wife, Nathalie: thanks for your patience and for keeping both my feet on solid ground on many occasions.

1. Executive Summary

Toxic algae producing an array of bioactive compounds may accumulate in shellfish, which in turn cause poisoning, following human consumption of contaminated shellfish. The chemistry, occurrence and toxicity of the toxins involved was reviewed. Following this review, the risk analysis and management processes are outlined for the production of shellfish. Subsequent sections describe five elements of risk characterisation to which my studies have significantly contributed: methods of analysis, distribution of toxins in the marine environment, preparative isolation of toxins, quality control tools in the determination of the toxins and characterisation of the chemical hazard in terms of lipophilicity, stability, reactivity and toxicity.

The use of liquid chromatography coupled to mass spectrometry for the detection of both hydrophilic and lipophilic toxins is outlined, with emphasis on the contributions clarifying critical parameters. For STX-group toxins, hydrophilic-lipophilic interaction chromatography has been introduced and demonstrated to achieve similar detection limits to the current regulatory bioassay, while providing a wealth of additional information in terms of toxin profiles. The determination of lipophilic toxins was critically evaluated. Matrix effects are systematically described and recommendations for their elimination or reduction are given.

The geographical distribution of AZAs has been shown to extend all along the Atlantic coast of Europe, and the biogeographic differences between European and a North American strain of *Dinophysis* were elucidated. Mussels were shown to accumulate higher levels of AZA- and OA-group toxins than oysters through the introduction of routine monitoring of lipophilic shellfish toxins by LC-MS in Ireland. The contamination of scallops with DA and the causes of its variability were studied in Scotland and Ireland, with results contributing to legislation implemented. The distribution of shellfish toxins was also studied using passive sampling techniques. Thanks to these techniques, the existence of toxins previously unreported in Ireland was demonstrated (DTX1, YTX, SPX). The capability of predicting the occurrence of shellfish toxins using passive samplers was not confirmed in our studies.

Compounds from three different toxin groups were isolated in the studies described: AZA, OA and PTX-group. Particular efforts were dedicated to the isolation of AZAs; these efforts have culminated in the production of a certified calibrant for AZA1 and in the discovery of 8 previously unknown analogues of AZAs, bringing the total number of analogues known to 20. DTX2 has been isolated to satisfactory purity, thus allowing further toxicological evaluation as well as the production of a standard currently undergoing certification at NRCC. The isolation of PTX2sa-fatty acid esters has outlined the complexity of natural products in shellfish.

Shellfish tissue reference materials were prepared, and factors affecting their homogeneity and stability were clarified. The materials were fit for in-house validation of methods, interlaboratory method validation, proficiency testing and certification. Two materials have been prepared for certification: an AZA-contaminated wet mussel material and a multitoxin-contaminated freeze-dried mussel tissue. Proficiency testing was introduced for DA-, OA-, AZA- and STX-group toxins. The applicability of LC-UV and -MS was demonstrated for the determination of DA. Immunoassays were compared to LC-methods for both DA- and OA-group toxins. The MBA was compared to an in-house validated LC-MS method for AZA-group toxins demonstrating that both were applicable for the current regulatory limit. The MBA has grave limitations as it cannot detect lower levels and is thus not fit-for-purpose, considering the transformation of AZAs observed in mussels following heat-treatments.

The acidity and lipophilicity constants of lipophilic shellfish toxins have been determined chromatographically. AZAs have been shown to be sensitive to alkaline and acidic treatments. AZA3 was also shown to be more sensitive to heat treatments than AZA1 and -2. OA was shown to degrade rapidly under the influence of strong acids. The heat treatments of mussels appear to affect the concentrations of AZA- and OA-group toxins, suggesting that the increase in concentrations need to be taken into account by shellfish producers in their HACCP plans. The toxicity of DTX2 was shown to be approximately half that of OA. The toxicity of AZA1 was investigated at cellular and molecular level. Cytotoxicity, teratogenicity and effects on gene regulation were investigated. The mechanism of action has not been completely elucidated, however, indicative results of gene-chip experiments suggest that lipid-biosynthesis and wound-healing pathways are affected by this toxin group.

2. Preface

When I took the decision to write this compendium of my work so far, I had initially been tempted to bridge the entire range of chemical entities I have worked on, including the environmental contaminants I dealt with during my PhD. There are many similarities and many differences in the approaches analytical chemists use in the quantitative determination of man-made compounds, such as PCBs, and natural toxins, including phycotoxins. Among the similarities are the need for chromatographic separation in the analysis of environmental matrices, considerations on lipophilicity of the compounds in method development and the environmental distribution of the compounds, as well as the concept of toxic equivalence factors for the conversion of concentrations into units that are relevant to human health. Among the differences, I would foremost mention the need for preparative isolation in the area of natural toxins to be able to work on purified compounds, a restraint that is usually not necessary in the field of contaminants, as many of the environmental contaminants have been synthesized at commercial scale and are available in sufficient quantities to conduct both chemical and toxicological evaluation. After some reflection however, I decided to limit the thesis to the description of marine toxins, firstly because this is the area I have worked on since my PhD, and secondly as it is an area in which I would consider to have made a coherent contribution over the past 12 years.

The area of marine toxins is a fascinating field, in which many disciplines need to work together to advance the understanding of processes such as the generation of harmful algal blooms, the accumulation of toxins in fish and shellfish, and the risks these phenomena pose to the human consumer of seafood. Interestingly, the main phenomenon is entirely natural and the role of man's recent impact on the environment in magnifying the natural development of harmful algae remains heavily debated. A multitude of scenarios can be considered which reflect the complexity of the aquatic ecosystems: benthic dinoflagellates occurring in tropical reefs may produce compounds that accumulate in fish and shellfish whereas pelagic diatoms and dinoflagellates may occur also in temperate and cold oceans, and concentrate in filter-feeding bivalves, which may dwell in intertidal zones (close to the surface of the water) or in subtidal zones, sometimes at significant water depth (e.g. a hundred meter and more). Currents sometimes transport micro-algae dozens or hundreds of kilometers from the primary production zone to the place where they will cause nuisance. Aquacultural practices can change significantly from country to country, however, even within a country, differences in the ecological niche of the same shellfish species may result in significant differences in their uptake of potentially toxic algae. Shellfish consumption is different in many parts of the world and the risks posed may not be the same depending on portion sizes consumed. There is many different compounds involved and the nature of toxicity ranges from relatively harmless gastric disturbance for several days over long-lasting effects of sensitization to heat-cold reversed touch sense to very severe effects, including death.

Every discipline typically brings their own view to the problem; biologists are interested in the ecology of the organisms producing toxins and their interactions with fish and shellfish, while toxicologists will study the nature of the effects exerted by the toxins as well as the mechanism of action. My view is one of a chemist, and over a few years I have seen that chemical sciences are often called upon by the other disciplines to aid their understanding of pieces in the puzzle. For instance, a toxicologist will need the chemical understanding of different analogues of a toxin group that may contribute to the toxic effect caused, and in-depth toxicological studies rely on highly purified compounds to exclude interference from impurities in the assessment of the toxicity of a specific analogue. In the present collation of works, I have attempted to take a step

back, and to look at the bigger picture from a more societal viewpoint. To this aim, I have arranged chapters around some aspects of the risk analysis process.

In the introduction, I have tried to give an overview of the diversity of marine toxins, their causative organisms and effects. I couldn't help myself including a few cases of the accumulation of toxins in mussels as observed in the field to exemplify the difficulties in the production of safe shellfish as well as the challenges of the official control authorities in protecting consumers from the threat of phycotoxins in our complex coastal marine environment. I have completed my introduction by giving an overview of the risk analysis process to clarify my approach, and the contributions to different steps of risk assessment and management.

The following section summarises some of the contributions to analytical methodology, an area I started in when I came to the field of marine toxins. The systematic evaluation of a novel type of chromatography (hydrophilic interaction liquid chromatography, HILIC) is at the center of developments in the area of saxitoxins. Since our pioneering work in this area, HILIC has found many applications and I anticipate that it will play a major role in the reduction and replacement of bioassays for paralytic shellfish toxins in the medium term. In the area of lipophilic toxins, I had the opportunity to study – with the help of several students - many aspects of sample clean-up, chromatographic developments and mass spectrometric parameters for the quantitative determination using liquid chromatography coupled to mass spectrometry (LC-MS). Together with the next chapter on the distribution of phycotoxins, the quantitative determination of marine toxins constitutes one of the first steps in characterizing the hazards that are relevant to understanding the extent of the problem. Sound methodology provides the knowledge of what exactly is contaminating seafood and how much contamination is present.

The distribution of toxins in the marine environment gives us an insight into the environmental compartments affected, the shellfish species that are particularly prone to accumulate certain algae and how persistent the pollution is. Environmental studies also contribute to the understanding of transfers along the trophic web. The area of accumulation of domoic acid in scallops has been given special attention through studies in Scotland and Ireland where this resource is heavily affected, similar to areas such as Brittany and Galicia. Geographical differences such as those observed for azaspiracids in different European countries or the occurrence of okadaic acid in Europe versus America also allow for adjustment of monitoring programs to take into account local priorities. Finally, the distribution of toxins in the water column is an aspect that has recently been exploited to attempt prediction of toxins; our studies in this area were based on laboratory developments and examine the prediction capability in field studies at the example of okadaic acid.

As abovementioned, the preparative isolation of toxins is an aspect specific to the research of natural compounds (as opposed to man-made chemicals). The purified substance is required as calibrant in the quantitative determination of toxins and also for the characterisation of the hazard in toxicological terms. Preparative isolation is a particular challenge to the analytical chemist as it involves many different types of chromatography. Recoveries of a substance that would be perfectly acceptable in a simple analytical setting will lead to very poor overall yields in complicated multi-step procedures, e.g. processes with six or eight clean-up steps with 80 % recovery each result in yields of 26 % and 17 % respectively. As toxins are often present at concentration of mg/kg – or even µg/kg – levels in naturally contaminated shellfish, usually several kg of starting material are needed to achieve the mg-yields required to conduct toxicological studies or to produce certified reference calibrants. I describe here the efforts undertaken in the isolation of azaspiracids (AZAs) as well as dinophysistoxin-2 (DTX2) both of

which were carried out as part of the ASTOX project (Azaspiracid Standards and TOXicology). As often in natural sciences, we also found non-expected compounds when analyzing some of the crude extracts produced for the isolation of AZAs; the fatty acid esters of pectenotoxin-2 seco-acids have been isolated as a consequence of this discovery.

Once reliable, quantitative methods have been developed, the distributions of bioactive compounds are clarified and purified calibrants are available, a decision has to be made whether the compounds are sufficiently toxic to pose a threat to the consumer of contaminated seafood. If the compound is considered a serious danger posing severe risks, regulators will most certainly aim to establish limits which must not be exceeded for safe food consumption. In this case, the validation of methods and their implementation under well-controlled conditions is the next step required to ensure safe risk management programs can be implemented. In this chapter, I describe our efforts in the production of reference materials, and their application in proficiency testing and formal validation of methods. The evaluation of methods and their fitness for purpose is also described in the final subsection, allowing for a critical view of different techniques and methods available. In particular, the comparison of the mouse bioassay with LC-MS-MS based methodology will attract the attention of many readers.

Furthermore, I included a section on characterisation of marine toxins as chemical hazards. The stability of toxins is important for many of the analytical methods and preparative isolation and purification of the toxins. However, stability is also relevant to understanding the toxicology of phycotoxins and the fate of these compounds in the marine environment. Both chemical reactivity and thermal stability have been evaluated for a range of toxins in our studies, and I attempted to give an overview which should stimulate further studies filling the gaps in our systematic approach. Lipophilicity is another parameter in the overall characterisation of marine toxins. Lipophilicity will affect chromatographic separation and is therefore a critical parameter in method development; as such I am also closing a circle of studies in analytical sciences. Interestingly, lipophilicity is also a key factor in the absorption of toxins across biological membranes, and our interest in this area will surely trigger further studies in the area of pharmacokinetics. The thermal stability and the behaviour of toxins in the heat treatment of shellfish tissues is not only relevant to the preparation and characterisation of reference materials but also governs the phenomena observed in home cooking and commercial processing of shellfish. These aspects have wide-reaching consequences for the risk assessment of toxins, in particular the dose which may have been eaten by affected consumers and also the concentrations which shellfish processing companies may expect in their products after commercial processing. Even though I am no toxicologist, I have taken a more and more avid interest in the interaction of toxins with the biological targets. As I have had the opportunity to collaborate with several scientists in this area, and as I have formulated research projects with the aim of elucidating the molecular mechanism of action of azaspiracids, I have decided to also include a section on these studies. DTX2 has been a compound of particular importance to the Irish scene and has therefore been included. The most recent studies on the synergistic effects of toxins have been included thanks to their importance in future risk assessments and to allow other scientists access to our preliminary work in this area.

In the end, I hope that my collation of studies will raise the interest of the reader; I trust that you will see shortcomings and gaps in this work: more recently, this has become commonly accepted as being part of the iterative process of scientific progress, and my reward will be to see students and colleagues conduct the studies filling these gaps and overcoming the pitfalls.

Philipp Hess, Nantes, 29 March 2010.

3. Curriculum Vitae

Date of birth: 9th August 1968; marital status: married

Place of birth: Saarbrücken, Germany; fluent in French, English & German

3.1. Professional Career

- Oct 2008 - to date** **Head of Department Environment, Microbiology & Phycotoxins**, Ifremer, Nantes Atlantic Centre, France
- Management of department (36 permanent staff, 19 contractors & students)
 - Scientific & administrative direction of 2 research laboratories & the national reference laboratory for microbiology of shellfish
 - Conduct research & provide expertise in the areas of shellfish safety, natural products chemistry, marine biotoxins & micro-organisms, biodiscovery, risk characterization & evaluation
- Ma 2001- Sep2008** **Team Leader**, Marine Institute, Galway, Ireland
- « Technical Manager », specified role in formal quality control management (ISO 17025)
 - Accreditation of all marine biotoxin methods used in official control in Ireland (ISO 17025)
 - Management of routine analytical unit (6-8 technicians and attendants), over 4000 test per annum (DA, OA, AZA, PSPs)
 - Management of research unit (supervision of 5-7 students & a postdoc)
 - Responsible for all chemical activities of the Irish National Reference Laboratory for marine biotoxins, research projects and commercial services (see project management)
 - Responsible for scientific reports and publications (see list of publications, separate document)
- Ju 1998 – Fe 2001** **Team Leader** – Higher Scientific Officer, Marine Laboratory, Aberdeen, UK
- Management of routine analytical unit (2 technicians), over 2000 tests per annum (DA, OA, AZA, PSPs)
 - Responsible for all chemical activities of the UK National Reference Laboratory for marine biotoxins
- No 1993 - Ju 1998** **Research Assistant** Robert Gordon University, Aberdeen, UK
- Preparation of fish oil reference materials & characterisation of these materials for proficiency testing scheme QUASIMEME
 - Development and validation of analytical methods for PAHs, PCBs & PCDD/Fs in marine biological matrices & sediments, using HPLC & GC couple to low and high resolution mass spectrometry
 - Certification of a mussel reference material for PCBs

3.2. Qualifications

- 1998 : **PhD** "*Determination and environmental significance of planar aromatic compounds in the marine environment*" Robert Gordon University, Faculty of Science and Technology, Aberdeen and FRS - Marine Laboratory, Executive Agency of the Scottish Office, Aberdeen. Presentation 20 March 1998 (external examiner: Prof. Ballschmiter, Univ. Ulm, Germany)
- 1993 : **Ingénieur EHICS** à l'Ecole Européenne des Hautes Etudes des Industries Chimiques de Strasbourg, now ECPM (Ecole Chimie, Polymères, Matériaux, Strasbourg)
- 1990 : **Vordiplom (BSc) in chemistry** – faculty of sciences of the Saarland, Saarbrücken, Germany
- 1987 : **Abitur, (A-levels)** (specialisations : chemistry, maths, English, Politics), "Gymnasium am Schloss" – Saarbrücken, Germany

3.3. Courses and training periods

- Oct-Nov 2009 **Course on the taxonomy of recent Dinophyceae**, Dr. M. Elbrächter, Forschungs-Institut Senckenberg & Alfred-Wegener-Institut, Wattenmeer-Station List, Sylt, Germany.
- Sep 2007 **Intensive course in Medicinal Chemistry**, Prof. Michael Wiese, Pharmacy, University of Bonn, Germany
- July 2001 **Research visit to** the laboratory of Prof. Yasumoto / Dr. Satake, University of Tohoku, Sendai, Japan
Mission : Preparative isolation of azaspiracids from contaminated mussels
- Oct-Dec 1999 **Research visit to** Dr. Michael Quilliam, NRCC, Halifax, Nova Scotia, Canada
Mission : Method development for the analysis of marine biotoxins using LC-MS, and analysis of PSP toxins in bacteria
- Mar-June 1993 **Research training period** in laboratory of Prof. Wegscheider, Technische Universität Graz, Institut f. analytische, Mikro- und Radiochemie, Graz, Austria
Mission : Parameterstudien an einem kapazitiv gekoppelten Plasma als Detektor für die Gaschromatographie und Optimierung der Stickstoff- und Phosphor-bestimmung
- Oct-Dec 1992 **Research training period** in laboratory of Dr. Joseph Pfeiffer, CIBA-Geigy AG, Schweizerhalle, Suisse.
Mission : Synthesis of Leucocrystal violet, tris-(4-dimethylamino-phenyl)-methane

3.4. Publications

3.4.1. Peer reviewed publications

1. Hess P. (2010) Requirements for screening and confirmatory methods for the detection and quantification of marine biotoxins in end-product and official control. Accepted in Anal. Bioanal. Chem. Jan 2010; DOI 10.1007/s00216-009-3444-y.
2. Stewart L, Hess P, Connolly L, Elliott C. (2009) Development and single laboratory validation of a pseudo-functional biosensor immunoassay for the detection of the okadaic acid group of toxins. Anal. Chem. 81 10208-10214.
3. Hackett J. D., Tong M., Kulis D.M., Fux E., Hess P., Bire R., Anderson D.M. (2009) DSP toxin production de novo in cultures of *Dinophysis acuminata* (Dinophyceae) from North America. Harmful Algae 8, 873-879.
4. Gerssen A., McElhinney M.A., Mulder P.P.J., Bire R., Hess P., de Boer J. (2009) 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
5. Hess P., Butter T., Petersen A., Silke J., McMahon T. (2009) Performance of the EU harmonised mouse bioassay for lipophilic toxins for the detection of azaspiracids in naturally contaminated mussel (*Mytilus edulis*) hepatopancreas tissue homogenates characterised by liquid chromatography coupled to tandem mass spectrometry. Toxicon 53, 713-722.
6. McCarron P., Kilcoyne J., Miles C.O., Hess P. (2009) Formation of Azaspiracids-3, -4, -6, and -9 via decarboxylation of carboxy-azaspiracid metabolites from shellfish. J. Agric. Food Chem. 57, 160-169.
7. Fux E., Biré 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.
8. Alfonso C., Rehmann N., Hess P., Alfonso A., Wandscheer C., Abuin M., Vale C., Otero P., Vieytes M., Botana L.M. (2008) Evaluation of various pH and temperature conditions on the stability of azaspiracids, and their importance in preparative isolation and toxicological studies. Anal. Chem., 80 (24) 9672-9680. (IF 2007: 5.646; cité 0 fois, relevé sur ISI, 29/05/09)
9. Fux E., Rode D., Bire R., Hess P. (2008) Approaches to the evaluation of matrix effects in the liquid chromatography-mass spectrometry (LC-MS) analysis of three regulated lipophilic toxin groups in mussel matrix (*Mytilus edulis*). Food Addit. Contam. 25 (8), 1024-1032. (IF 2007: 1.780; cité 3 fois, relevé sur ISI, 29/05/09)
10. Vale P., Bire R., Hess P. (2008) Confirmation by LC-MS/MS of azaspiracids in shellfish from the Portuguese northwestern coast. Toxicon 51, 1449-1456. (IF 2007: 2.246; cité 3 fois, relevé sur ISI, 29/05/09)

11. 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, 1081-1089. (IF 2007: 2.246; cité 1 fois, relevé sur ISI, 29/05/09)
12. Fux E., Marcaillou C., Mondeguer F., Bire R., Hess P. (2008a) 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. (IF 2007: 2.397; pas d'index de citation disponible sur ISI, 29/05/09)
13. Twiner M.J., Rehmann N., Hess P., Doucette G.J. (2008) Azaspiracid Shellfish Poisoning: A review on the ecology, chemistry, toxicology and human health impacts. *Marine Drugs* 6 (2) 39-74. (pas de IF disponible, cité 4 fois, relevé sur ISI, 29/05/09)
14. Rehmann N., Hess P., Quilliam M.A. (2008) Discovery of New Analogs of the Marine Biotxin Azaspiracid in Blue Mussels (*Mytilus edulis*) by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.*, 22, 4, 549-558. (IF 2007: 2.971; cité 14 fois, relevé sur ISI, 29/05/09)
15. Twiner M.J., Ryan J.C., Morey J.S., Van Dolah F.M, Hess P., McMahon T., Doucette G.J. (2008) Transcriptional profiling and inhibition of cholesterol biosynthesis in human T lymphocyte cells by the marine toxin azaspiracid. *Genomics* 91, 289-300. (IF 2007: 3.613; cité 3 fois, relevé sur ISI, 29/05/09)
16. Fux E., McMillan D., Bire R., Hess P., (2007) Development of an Ultra Performance Liquid Chromatography – Mass Spectrometry method for the detection of lipophilic marine toxins. *J. Chromatogr. A* 1157, 273-280. (IF 2006: 3.641; cité 15 fois, relevé sur ISI, 29/05/09)
17. Aune T, Larsen S., Aasen J., Rehmann N., Satake M. and Hess P. (2007) Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* 49 1-7. (IF 2006: 2.509; cité 11 fois, relevé sur ISI, 29/05/09)
18. Bogan, Y.M., Bender, K., Hervas, A., Kennedy, D.J., Slater, J.W., Hess, P. (2007) Spatial variability of domoic acid concentration in king scallops *Pecten maximus* off the southeast coast of Ireland. *Harmful Algae*, 6, 1-14. (IF 2006: 2.460; cité 4 fois, relevé sur ISI, 29/05/09)
19. Bogan, Y.M., Harkin, A.L., Gillespie, J., Kennedy, D., Hess, P., Slater, J.W. (2007) The influence of size on domoic acid concentration in king scallop, *Pecten maximus* (L.) *Harmful Algae*, 6, 15-28. (IF 2006: 2.460; cité 3 fois, relevé sur ISI, 29/05/09)
20. Bogan Y.M., Kennedy D., Harkin A.L., Gillespie J., Vause B.J., Beukers-Stewart B.D., Hess P., Slater J.W. (2007) Variation in domoic acid concentration in king scallop (*Pecten maximus*) from fishing grounds around the Isle of Man. *Harmful Algae*, 6, 81-92. (IF 2006: 2.460; cité 2 fois, relevé sur ISI, 29/05/09)

21. Hess P., McCarron P., Quilliam M.A. (2007) Fit-for-purpose Shellfish Reference Materials for Phycotoxins in Internal and External Quality Control. *Anal. Bioanal. Chem.* 387, 2463-2474. (IF 2006: 2.591; cité 3 fois, relevé sur ISI, 29/05/09)
22. Larsen K., Petersen D., Wilkins A.L., Samdal I.A., Sandvik M., Rundberget T., Goldstone D., Arcus V., Hovgaard P., Rise F, Rehmann N., Hess P., and Miles C.O. (2007) Clarification of the C-35 Stereochemistries of Dinophysistoxin-1 and Dinophysistoxin-2, and its consequences for binding to protein phosphatase. *Chem. Res. Toxicol.* 20 (6), 868–875. (IF 2006: 3.162; cité 10 fois, relevé sur ISI, 29/05/09)
23. McCarron P., Emteborg H., Hess P. (2007) Freeze-drying for the stabilisation of shellfish toxins in mussel tissue (*Mytilus edulis*) reference materials. *Anal. Bioanal. Chem.*, 387, 2475-2486. (IF 2006: 2.591; cité 6 fois, relevé sur ISI, 29/05/09)
24. McCarron P., Kotterman M., de Boer J., Rehmann N., Hess P. (2007) Feasibility of g-Irradiation as a Stabilisation Technique in the Preparation of Tissue Reference Materials for a Range of Shellfish Toxins. *Anal. Bioanal. Chem.*, 387, 2487-2493. (IF 2006: 2.591; cité 3 fois, relevé sur ISI, 29/05/09)
25. McCarron P., Burrell S., Hess P. (2007) Effect of Addition of Antibiotics and an Antioxidant, on the Stability of Tissue Reference Materials for Domoic Acid, the Amnesic Shellfish Poison. *Anal. Bioanal. Chem.* 387, 2495-2502. (IF 2006: 2.591; cité 4 fois, relevé sur ISI, 29/05/09)
26. Ronzitti G., Hess P., Rehmann N., Rossini G. P. (2007) Azaspiracid-1 alters the E-cadherin pool in epithelial cells. *Toxicol. Sci.* 95 (2) 427-435. (IF 2006: 3.598; cité 6 fois, relevé sur ISI, 29/05/09)
27. Bogan, Y.M., Kennedy, D., Harkin, A.L., Gillespie, J., Hess, P., Slater, J.W. (2006) Comparison of domoic acid concentration in king scallops, *Pecten maximus* from seabed and suspended culture systems. *J. Shellfish Res.* 25 (1), 129-135. (IF 2005: 0.479; cité 3 fois, relevé sur ISI, 29/05/09)
28. Hess P., Grune B., Anderson D.B., Aune T., Botana L.M., Caricato P., van Egmond H.P., Halder M., Hall S., Lawrence J.F., Moffat C., Poletti R., Richmond J., Rossini G.P., Seamer C. and Serratos Vilageliu J. (2006) Three Rs Approaches in Marine Biotxin Testing - The Report and Recommendations of a joint ECVAM/DG SANCO Workshop (ECVAM Workshop 55). *Altern. Labor. Anim. (ATLA)* 34, 193-224. (IF 2005: 0.670; cité 11 fois, relevé sur ISI, 29/05/09)
29. Kulagina, N.V., Twiner, M.J., Doucette, G.J., Ramsdell, J.S., Hess, P., McMahon, T., O'Shaughnessy, T.J., Ma, W. (2006). Effect of azaspiracid-1 on action potential generation and voltage-gated currents in cultured spinal cord neuronal networks. *Toxicol.* 47, 766-773. (IF 2005: 2.255; cité 10 fois, relevé sur ISI, 29/05/09)
30. McCarron P. and Hess P. (2006) Tissue distribution and effects of heat treatments on the content of domoic acid in blue mussels, *Mytilus edulis*. *Toxicol.* 47, 473-479. (IF 2005: 2.255; cité 7 fois, relevé sur ISI, 29/05/09)

31. Wilkins A.L., Rehmann N., Torgersen T., Rundberget T., Keogh M., Petersen D., Hess P., Rise F., Miles C. O. (2006) Identification of fatty acid esters of pectenotoxin-2 seco acid in blue mussels (*Mytilus edulis*) from Ireland. *J. Agric. Food Chem.* 54, 5672-5678. (IF 2005: 2.507; cité 14 fois, relevé sur ISI, 29/05/09)
32. Colman J.R., Twiner M.J., Hess P., McMahon T., Doucette G.J., and Ramsdell J.S. (2005) Teratogenic Effects of Azaspiracid-1 Identified by Microinjection of Japanese Medaka *Oryzias Latipes* Embryos. *Toxicon* 45, 881-890. (IF 2004: 1.862; cité 11 fois, relevé sur ISI, 29/05/09)
33. Dell'Aversano C., Hess P., Quilliam M.A. (2005) Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry for the Analysis of Paralytic Shellfish Poisoning (PSP) Toxins. *J. Chromatogr.* 1081, 190-201. (IF 2004: 3.359; cité 28 fois, relevé sur ISI, 29/05/09)
34. Hess P., Morris S., Stobo L.A., Brown N.A., McEvoy J.D.G., Kennedy G., Young P.B., Slattery D., McGovern E., McMahon T., Gallacher S. (2005) LC-UV and LC-MS methods for the determination of Domoic Acid. *TrAC*, 24, 4, 358-367. (IF 2004: 3.888; cité 6 fois, relevé sur ISI, 29/05/09)
35. Hess P., Nguyen L., Aasen J., Keogh M., Kilcoyne J., McCarron P., Aune T. (2005) Tissue distribution, effects of cooking and parameters affecting the extraction of azaspiracids from mussels, *Mytilus edulis*, prior to analysis by liquid chromatography coupled to mass spectrometry. *Toxicon* 46, 62-71. (IF 2004: 1.862; cité 23 fois, relevé sur ISI, 29/05/09)
36. Twiner M.J., Hess P., Bottein Dechraoui Y., McMahon T., Ramsdell J.S., Samsons M.S., and Doucette G.J. (2005) Cytotoxic and Cytoskeletal Effects of Azaspiracid-1 on Mammalian Cell Lines. *Toxicon* 45, 891-900. (IF 2004: 1.862; cité 25 fois, relevé sur ISI, 29/05/09)
37. Jaime E., Hummert C., Hess P., and Luckas B. (2001) Determination of paralytic shellfish poisoning toxins by high-performance ion-exchange chromatography. *J. Chromatogr. A.* 929, 43-49. (cité 13 fois, relevé sur ISI, pas facteur d'impact disponible le 29/05/09)
38. Campbell D.A.; Kelly M.S.; Busman M.; Bolch C.J.; Wiggins E.; Moeller P.D.R.; Morton S.L.; Hess P., and Shumway S.E. (2001) Amnesic shellfish poisoning in the King Scallop, *Pecten maximus*, from the west coast of Scotland. *J. Shellfish Res.* 20(1), 75-84. (pas d'index de citation disponible sur ISI le 29/05/09)
39. Hess P., Gallacher S., Bates L.A., Brown N., Quilliam M.A. (2001) Determination and confirmation of the amnesic shellfish poisoning toxin, domoic acid, in shellfish from Scotland by liquid chromatography and mass spectrometry; *J. Ass. Off. Anal. Chem.* 84 (5) 1657-1667. (cité 23 fois, relevé sur ISI, pas de facteur d'impact disponible le 29/05/09)
40. Hess P. and Wells D.E. (2001) Evaluation of dialysis as a technique for the removal of lipids prior to the GC analysis of ortho- and non-ortho chlorobiphenyls, using

14C-labelled congeners. *The Analyst* 126 (6), 829-834. (cite 6 fois, relevé sur ISI, pas de facteur d'impact disponible le 29/05/09)

41. Hess P., de Boer J., Cofino W.P., Leonards P.E.G., Wells D.E. (1995) Critical review of the analysis of non- and mono-ortho-chlorobiphenyls, *J. Chromatogr. A*, 703, 417-465. (cite 61 fois, relevé sur ISI, pas de facteur d'impact disponible le 29/05/09)

3.4.2. Book chapters

1. Rossini G.P. and Hess P. (2010) Phycotoxins: chemistry, mechanism of action and shellfish poisoning. Chapter 2.3 in "Clinical Toxicology", Vol. 2., pp. 65 – 122. Andreas Luch (ed.) "Experientia Supplementa" (EXS) book series, Birkhäuser Publishing, Basel.

2. Hess P. and Aasen J. A. B. (2007) Chemistry, origins and distribution of Yessotoxin and its analogues, chapter 10 in *Chemistry and Biochemistry of Marine Biotoxins*, ed. Luis Botana. Blackwell Publishing Ltd. Oxford, UK, ISBN-13: 978-0-8138-2700-1.

3. Wells D.E. and Hess P.; Separation, clean-up and recoveries of persistent trace organic contaminants from soils, sediment and biological matrices. Pages 73 - 113, Chapter 2 in "Sample Handling and Trace Analysis of Pollutants: Techniques, Applications and Quality Assurance", D. Barceló (Editor) Elsevier, 2000, ISBN: 0-444-82831-1; Volume 21, series "Techniques and Instrumentation in Analytical Chemistry.

4. Wells D.E. and Hess P.; Methods for the determination and evaluation of chlorinated biphenyls (CBs) in environmental matrices. Pages 239-285, Chapter 6 in "Sample Handling and Trace Analysis of Pollutants: Techniques, Applications and Quality Assurance", D. Barceló (Editor) ISBN: 0-444-82831-1. 2000; Volume 21, series "Techniques and Instrumentation in Analytical Chemistry.

5. Wells D.E., Hess P., Moffat C. Gas chromatography and mass spectrometry techniques. Chapter 2 in: *Environmental Contaminants in Food*. C.F. Moffat and K. Whittle (eds.) 1999; Sheffield Academic Press (ISBN: 1-85075-921-9), CRC Press (ISBN: 0-8493-9735-9).

3.4.3. Scientific Reports

1. EFSA (European Food Safety Authority), Marine biotoxins in shellfish – Summary Opinion, Scientific Opinion of the Panel on Contaminants in the Food chain; adopted on 13 August 2009, *EFSA Journal* (2009) 1306, 1 - 23.

2. EFSA (European Food Safety Authority), Marine biotoxins in shellfish – Domoic acid group, Scientific Opinion of the Panel on Contaminants in the Food chain; adopted on 2 July 2009, *EFSA Journal* (2009) 1181, 1 - 61.

3. EFSA (European Food Safety Authority), Marine biotoxins in shellfish – Pectenotoxin group, Scientific Opinion of the Panel on Contaminants in the Food chain; adopted on 27 May 2009, EFSA Journal (2009) 1109, 1-47.
4. EFSA (European Food Safety Authority), Marine biotoxins in shellfish – Yessotoxin group, Scientific Opinion of the Panel on Contaminants in the Food chain, adopted on the 2 December 2008, EFSA Journal (2009) 907, 1-62.
5. EFSA (European Food Safety Authority), Marine biotoxins in shellfish – Azaspiracid group, Scientific Opinion of the Panel on Contaminants in the Food chain, adopted on 9 June 2008, EFSA Journal (2008) 723, 1-52.
6. EFSA (European Food Safety Authority), Marine biotoxins in shellfish – Okadaic Acid and analogues, Scientific Opinion of the Panel on Contaminants in the Food chain, adopted on 27 November 2007, EFSA Journal (2008) 589, 1-62
7. BIOTOX (2008a) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. WP2, deliverable 7: Report on the MS detectors, MS and LC parameters, and performance characteristics, affecting chromatographic separation of lipophilic marine biotoxins, Biré R. and Hess P. 75 pp.
8. BIOTOX (2008b) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. WP2, deliverable 15: Report on improved extraction and clean-up procedures, Fux E., Gerssen A., Biré R., Mulder P., Rourke B., McElhinney M., Kotterman M., Hess P. 111 pp.
9. BIOTOX (2008c) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. WP2, deliverable 22 Report on matrix effects, Fux E., Biré R. and Hess P. 70 pp.
10. BIOTOX (2007) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. Report on transition study, Biré R. and Hess P. 43 pp.
11. Hess P., McCarron P., Rehmann N., Kilcoyne J., McMahon T., Ryan G., Ryan P.M., Twiner M.J., Doucette G.J., Satake M., Ito E., Yasumoto T. (2007c) Isolation and purification of azaspiracids from naturally contaminated materials, and evaluation of their toxicological effects– final project report ASTOX (ST/02/02). Marine Institute – Marine Environment & Health Series – No. 28, ISSN: 1649-0053; 129 pp.
<http://www.marine.ie/NR/rdonlyres/2B06863D-3366-47CD-9ABB-B3302629FE46/0/ASTOX.pdf>
12. Risk Assessment of Azaspiracids (AZAs) in Shellfish: A report of the Scientific Committee of the FSAI (Food Safety Authority of Ireland), 2006, 39 pages.
<http://www.fsai.ie>
13. Report of the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs, Oslo, Norway, Sept. 26-30, 2004 (advance pre-publication copy:

http://www.fao.org/es/ESN/food/risk_biotoxin_en.stm);
ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf

14. Hess P. "Methods for the detection of marine biotoxins - Difficulties in a regulatory context", paper presented at the joined WHO-FAO Expert Consultation on Phycotoxins for the Codex Committee on Fish and Fisheries Products. 22-24 March 2004. (copy available on request from author).

15. Hess P.; Brown N.A.; Bates L.A.; Turriff J.J.; Howard F.G., and Moffat C.F. A comparison of domoic acid concentrations in scallop (*Pecten maximus*) gonads processed by FRS MLA and those prepared by a commercial processor. Fisheries Research Services - Report No 03/00. 2000; 12pp.

162. Hess P. Isolation of azaspiracids from Irish shellfish – an introduction to natural product isolation. MI report on the study period at Sendai University July 2001, 35p. (copy available on request from author).

17. Hess P.; Bates L.A.; Brown N.; McEvoy J.D.G.; Kennedy D.G.; Young P.B.; Slattery D.; McGovern E.; McMahon T.; Morris S., and Gallacher S. Intercomparison of methods for the determination of domoic acid, the amnesic shellfish poisoning toxin, in standard solutions of domoic acid, extracts and homogenates of the scallop *Pecten maximus*. (UK National Reference Laboratory for Marine Biotoxins). Fisheries Research Services - Research Report. 2001 Mar: pp. 102 + appendices.

3.4.4. Conference proceedings

1. Hess P., Rehmann N. (2008) Pharmacological concepts and chemical studies relevant to evaluating the toxicity of azaspiracids. Pp 55 – 62 in "Proceedings of the 8th Irish Shellfish Safety Workshop", Marine Institute, Rinville, 7th December, 2007. (eds. McMahon T., Deegan B., Silke J., O'Cinneide M.) Marine Environment and Health Series Report No. 33, 2008.

2. Fux, E., Bire, R. & Hess, P. (2008) Comparison of the accumulation of lipophilic marine biotoxins in passive samplers, transplanted mussels and indigenous mussels on the west coast of Ireland. Pp 187-189, in "Proceedings of the 12th International Conference on Harmful Algae". International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, 2008 Copenhagen. pp 397. (Eds. : Moestrup, Ø., G. Doucette, H. Enevoldsen, A. Godhe, G. Hallegraeff, B. Luckas, N. Lundholm, J. Lewis, K. Rengefors, K. Sellner, K. Steidinger, P. Tester, A. Zingone).

3. Hess P. (2008) What's new in toxins? Pp 360-370, in "Proceedings of the 12th International Conference on Harmful Algae". International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, 2008 Copenhagen. pp 397. (Eds. : Moestrup, Ø., G. Doucette, H. Enevoldsen, A. Godhe, G. Hallegraeff, B. Luckas, N. Lundholm, J. Lewis, K. Rengefors, K. Sellner, K. Steidinger, P. Tester, A. Zingone).

4. Quilliam M.A., Hardstaff W.R., Chung S., Dell'Aversano C., and Hess P. "Analysis of PSP Toxins by Liquid Chromatography-Tandem Mass Spectrometry." Cawthron

Report 906, Proceedings of HAB Tech 2003, 26-30 November, Nelson, New Zealand; ISBN: 0-476-00622-8 (2004): 113-14.

5. Hess P. "Irish Monitoring Programme and LC-MS: Situation in EC." Cawthron Report 906, Proceedings of HAB Tech 2003, 26-30 November, Nelson, New Zealand; ISBN: 0-476-00622-8 (2004).

6. Hess P., McMahon T., Slattery D., Swords D., Dowling G., McCarron M., Clarke D., Gibbons W., Silke J., O'Cinneide M. "Use of LC-MS testing to identify lipophilic toxins, to establish local trends and interspecies differences and to test the comparability of LC-MS testing with the mouse bioassay: an example from the Irish biotoxin monitoring programme 2001". in : Molluscan Shellfish Safety; editors: A. Villalba, B. Reguera, J.L. Romalde, R. Beiras; publishers: Consellería de Pesca e Asuntos Marítimos da Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Santiago de Compostela, Spain. 2003 ISBN: 84-453-3638-X.

7. Hess P., McCarron P., Rehmann N., Ryan M.P., Ryan G. (2003) "Isolation and purification of azaspiracids from naturally contaminated materials and evaluation of their toxicological effects (ASTOX)" pp. 18-20 in Proc. 4th Irish Marine Biotoxin Science Workshop, Renvyle, November 7th 2003, Ireland. Download at: <http://www.marine.ie/scientific+services/monitoring/shellfish+safety/index.htm>

8. Clarke D., Devilly L., Gibbons B., Hess P., Keogh M., Kilcoyne J., Lyons J., McCarron M., Mulcahy N., Gallardo Salas R., Silke J., Slattery D., Swords D., White P. (2003) "A review of shellfish toxicity monitoring in Ireland for 2003" pp. 8-13 in Proc. 4th Irish Marine Biotoxin Science Workshop, Renvyle, November 7th 2003, Ireland.

9. Hess P., McMahon T., O'Cinneide M. (2003) "Monitoring strategies for DSP and related toxins in shellfish using animal testing and LC-MS techniques, at the example of shellfish monitoring in Ireland since 2001." pp 71-76 in Proc. 8th Canadian Workshop on Harmful Marine Algae. 28-30 May 2003, Moncton, Canada, 2003

10. Kryszewski S., Bernard C., Hess P. (2003) "Méthodologies d'analyse des phycotoxines et cyanotoxines" pp. 34-47 in Proceedings of the French Symposium "Journée scientifique d'échanges sur les biotoxines – Toxines de micro-algues et cyanobactéries", AFSSA Paris, 6 March 2003.

11. Quilliam M.A.; Hess P., and Dell'Aversano C. Recent developments in the analysis of phycotoxins by liquid chromatography - mass spectrometry. Chapter 11 in "Mycotoxins and Phycotoxins in Perspective at the Turn of the Millenium", Editors: Willem J. De Koe, Robert A. Samson, Hans P. Van Egmond, John Gilbert and Myrna Sabino. 2001; Proc. 10th IntL. IUPAC Symposium on Mycotoxins and Phycotoxins - 21-25 May, 2000 Guarujá (Brazil) pp. 383-391, ISBN: 90-9014801-9, published and distributed 2001 by W.J. de Koe (www.deKoe.nl).

12. Hess P.; McMahon T.; Slattery D.; Swords D.; Dowling D.; McCarron M.; Clarke D.; Devilly L.; Gibbons W.; Silke J., and O'Cinneide M. Biotoxin Chemical Monitoring in Ireland 2001. Proc. 2nd Irish Marine Biotoxin Science Workshop, Galway, October 11th 2001. Organised and published by the Marine Institute and Bord Iascaigh Mhara with the support of the Irish Shellfish Association. 2001:pp. 8-18.

13. Gallacher S.; Gillibrand P.A.; Heath M.R.; Hess P.; Howard F.G.; Kelly M.C.; Macdonald E.M., and Turrell W.R. (2000) The occurrence of amnesic shellfish poisons in shellfish from Scottish waters. 9th Conference on Harmful Algal Blooms, Tasmania 6 - 11 February 2000.

3.4.5. Oral presentations

1. Hess P. Cyanotoxines et Phycotoxines - différences et similitudes dans les techniques de surveillance. Invited presentation at the 9th Annual Meeting of the French Scientific Interest Group on Cyanobacteria (GIS Cyanobactéries), 25-27th January 2010, Bourg-en-Bresse, France.
2. Hess P. Of mice and mussels: performance of the EU harmonized mouse bioassay for azaspiracides and case studies of atypical toxicities. Keynote presentation at the 7th Intl. Conf. Molluskan Shellfish Safety, 14 – 19th June 2009, Nantes, France.
3. Aasen J.A.B., Espenes A., Miles C.O., Rehmann N., Hess P., Aune T. Distribution of Azaspiracid-1 alone and in combination with Yessotoxin as measured by LC-MS/MS in different tissues of NMRI mice during an oral toxicity study. Oral presentation at the 7th Intl. Conf. Molluskan Shellfish Safety, 14 – 19th June 2009, Nantes, France.
4. Alexander J., Aune T, Benford D.*, Botana L., Eskola M., Fürst P., Heinemeyer G., Heppner C., Hess P., Krysz S., Rossini G.P., Preiss-Weigert A., van Egmond H.P., Verger P., Vernazza F., van Leeuwen R.F.X. European risk assessments of marine biotoxins. Oral presentation at the 7th Intl. Conf. Molluskan Shellfish Safety, 14 – 19th June 2009, Nantes, France.
5. McCarron P., Emteborg H., Giddings S., Nulty C., Rundberget T., Loader J.I., Miles C.O., Sandvik M., Emons H., Hess P., Quilliam M.A. A certified reference material (CRM) for the analysis of multiple groups of shellfish toxins. Oral presentation at the 7th Intl. Conf. Molluskan Shellfish Safety, 14 – 19th June 2009, Nantes, France.
6. Hess P. Azaspiracids and Yessotoxins: Target Discovery as Common Denominator between Drug Discovery and Food Safety. Invited oral presentation at the 122nd AOAC Annual Meeting and Exposition, 21 – 25 September 2008, Dallas, Texas, US.
7. Silke, J., Clarke, D., Gallardo-Salas, R., Hess, P., Mc Mahon, T., and O’Cinneide, M. A review of toxin producing algae and their control in Ireland. Oral presentation at the 13th Intl. Conference on Harmful Algal Blooms, 3 – 7 November 2008, Hong Kong, China.
8. Fux E., Gonzalez S., Lunven M., Gentien P. and Hess P. *Dinophysis acuta* and its toxins at low depths in the Celtic Sea. Oral presentation at the 13th Intl. Conference on Harmful Algal Blooms, 3 – 7 November 2008, Hong Kong, China.

9. Hess P. (2007) "Bioactive Compounds in Shellfish – Drug Discovery or Food Safety Management?". Invited Plenary Lecture at 5th European Conference on Marine Natural Products, Ischia, Italy, 16-21 Sept. 2007.
10. Philipp Hess, Pearse McCarron (2007) "Review of Proficiency Testing for Shellfish Toxins". Invited Plenary Lecture at 6th Intl. Conf. Molluscan Shellfish Safety (ICMSS), March 2007 Blenheim, New Zealand.
11. McCarron P., Cofino W., Hess P. Review of the QUASIMEME Proficiency Testing Schemes for Shellfish Toxins; Oral presentation at 11th Intl. Conf. Biological and Environmental Reference Materials (BERM-11) October 28th to November 2nd 2007, Tsukuba, Japan.
12. Hess P. (2006) Plenary Lecture: "What's new in toxins", 12th Intl. Conference on Harmful Algal Blooms, September 2006, Copenhagen, Denmark.
13. Hess P. Fit-for-purpose Shellfish Reference Materials for Phycotoxins in Internal and External Quality Control. Oral Presentation at 10th Intl. Conf. Biological and Environmental Reference Materials (BERM-10) April 30th to May 4th 2006, Charleston, South Carolina US.
14. Hess P., McCarron P., Rehmann N., Biré R, McElhinny M., van Loco J., Quilliam M.A. (2005) "LC-MS based methods for the determination of lipophilic phycotoxins in shellfish with a specific focus on the Okadaic Acid and Azaspiracid groups: single laboratory validation and collaborative study protocol." Invited lecture at the Wiley Symposium of the 119th AOAC Annual Conference, Orlando, Florida, 11-15 September 2005.
15. Philipp Hess, Barbara Grune, Paolo Caricato, Marlies Halder (2005) "Conclusions of the ECVAM – DG Sanco Workshop on the 3Rs' approaches in Marine Biotxin Testing" Invited oral presentation at 5th World Congress on Alternatives & Animal Use in the Life Sciences, Berlin, Germany, 21-25 August 2005 Berlin.
16. Hess P. (2005) "Recent developments in the area of LC-MS methodology and validation (CODEX, ECVAM, BIOTOX)." Oral presentation at the 1st Joint Symposium and AOAC Phycotoxin task force meeting. Baiona, Spain, 11-15 April 2005.
17. Hess P. (2004) "Measuring toxins or detecting phytoplankton? – a review of monitoring and surveillance techniques." Oral presentation at the 10th International Conference on Harmful Algae, Capetown, South Africa, 15-19 November 2004.
18. Hess P., Scurfield J., and Wells D.E. (2004) "Proficiency Testing for the Quantitative Determination of Domoic Acid, the Amnesic Shellfish Poisoning Toxin ." Oral Presentation at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.
19. Ryan G.E., Hess P., and Ryan M.P. (2004) "Development of a Functional in-Vitro Bioassay for Azaspiracids (AZA) Using Human Colonic Epithelial Cells." Oral Presentation at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland (2004).

20. Silke J., McMahon T., and Hess P. (2004) "Irish Shellfish Biotoxin Monitoring Programme." Oral Presentation at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland (2004).
21. Twiner M.J., Hess P., Bottein Dechraoui Y., McMahon T., Ramsdell J.S., Samsons M.S., and Doucette G.J. (2004) "Cytotoxic and Cytoskeletal Effects of Azaspiracid-1 on Seven Mammalian Cell Lines." Oral Presentation at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland (2004).
22. Hess P., McMahon T., Slattery D., Swords D., Dowling G., McCarron M., Clarke D., Gibbons W., Silke J., O'Cinneide M. "Use of LC-MS testing to identify lipophilic toxins, to establish local trends and interspecies differences and to test the comparability of LC-MS testing with the mouse bioassay: an example from the Irish biotoxin monitoring programme 2001". Oral Presentation at the 4th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2002, Santiago de Compostella, Spain (2002).
23. Hess P., Gallacher S., Quilliam M.A. "Determination and confirmation of the amnesic shellfish poisoning toxin, domoic acid, in shellfish from Scotland by liquid chromatography and mass spectrometry". Oral Presentation at the 113th AOAC Annual Meeting and Exposition, 14-18 September 1999, Houston, Texas, US (1999).

3.4.6. Poster presentations

1. Stewart L., Connolly L., Walker A., Hess P. Elliott C. A biosensor immunoassay for the rapid and reliable detection of okadaic acid and its analogues in shellfish extracts. Poster presentation at the 7th Intl. Conf. Molluscan Shellfish Safety, 14 – 19th June 2009, Nantes, France.
2. Hess P., Biré R., Sandvik M., Miles C.O., Kotterman M., van Loco J. BIOTOX intercomparisons on LC-MS analysis of lipophilic marine toxins from the OA-, AZA-, PTX- and YTX-groups. Poster presentation at the 122nd AOAC Annual Meeting and Exposition, 21 – 25 September 2008, Dallas, Texas, US.
3. McCarron P., Kilcoyne J., Miles C.O., Hess P. Formation of Azaspiracids-3, -4, -6 and -9 via decarboxylation of carboxyazaspiracid metabolites from shellfish. Poster presentation at the 13th Intl. Conference on Harmful Algal Blooms, 3 – 7 November 2008, Hong Kong, China.
4. McCarron P., Emteborg H, Nulty C., Rundberget T., Loader J.I., Miles C.O., Sandvik M., Emons H., Quilliam M.A., Hess P. Preparation of a Candidate CRM for the Analysis of Multiple Groups of Shellfish Toxins Poster presentation at 11th Intl. Conf. Biological and Environmental Reference Materials (BERM-11) October 28th to November 2nd 2007, Tsukuba, Japan.
5. McCarron P., Burrell S., Hess P. (2006) Effect of Addition of Antibiotics and an Antioxidant, on the Stability of Tissue Reference Materials for Domoic Acid, the Amnesic Shellfish Poison. Poster presentation at 10th Intl. Conf. Biological and Environmental Reference Materials (BERM-10) April 30th to May 4th 2006, Charleston, South Carolina US.

6. McCarron P., Kotterman M., de Boer J., Rehmann N., Hess P. (2006) Feasibility of Irradiation as a Stabilisation Technique in the Preparation of Tissue Reference Materials for a Range of Shellfish Toxins. Poster presentation at 10th Intl. Conf. Biological and Environmental Reference Materials (BERM-10) April 30th to May 4th 2006, Charleston, South Carolina US.
7. Bogan, Y., Bender, K., Hervas, A., Slater, J., Hess P. (2004). Spatial variability of domoic acid concentration in the king scallop, *Pecten maximus* off the south coast of Ireland. Poster presented at 11th Int. Conf. on Harmful Algae, Capetown, South Africa, 15 –19th Nov. 2004.
8. Bogan Y., Harkin A.L., Gillespie J., Vause B., Hess P., and Slater J. (2004) "Variation in Domoic Acid Concentration in Scallops From Fishing Grounds in the Isle of Man." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.
9. Bogan Y., Harkin A.L., Greenwood C., Gillespie J., Hess P., and Slater J. (2004) "The Influence of Scallop Size on Domoic Acid Concentration in the King Scallop, *Pecten Maximus*." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.
10. Colman J.R., Twiner M.J., Hess P., McMahon T., Doucette G.J., and Ramsdell J.S. (2004) "Teratogenic Effects of Azaspiracid-1 Identified by Microinjection of Japanese Medaka *Oryzias Latipes* Embryos." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.
11. Harkin A.L., Bogan Y., Gillespie J., Hess P., and Slater J. (2004) "The Efficiency of Extraction of Domoic Acid at Different Concentrations From a Range of Shellfish Species." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.
12. Hess P., Kilcoyne J., Swords D., Mulcahy N., McCarron M., Keogh M., Gibbons B., and Ronan J. (2004) "Impact of HPLC-UV Methods (Solid-Phase Extraction/UV Detection and Photodiode-Array Detection) for the Determination of Domoic Acid on Quality of Results and Sample Turn-Around Time." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.
13. Kulagina N.V., Twiner M.J., Doucette G.J., Ramsdell J.S., Hess P., McMahon T., O'Shaughnessy T.J., and Ma W. (2004) "Effect of Azaspiracid-1 on Action Potential Generation and Voltage-Gated Currents in Cultured Spinal Cord Neuronal Networks." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.
14. McCarron P., Kilcoyne J., and Hess P. (2004) "Preparation of Stable, Homogenous Shellfish Tissue Materials, Naturally Contaminated With Dinophysistoxins and Azaspiracids." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland.

15. Rehmann N., Kilcoyne J., Hess P., and Yasumoto T. (2004) "Isolation of Azaspiracid-1, -2 and 3." Poster Presented at the 5th Intl. Conf. Molluscan Shellfish Safety, 14-18 June 2004, Galway, Ireland (2004).

16. Gallacher S.; MacRae J.; Ferguson C.; Smith E.; Hess P.; Johnstone M.; Glover L.A.; Bates S.S., and Garthwaite I. (2000) Identity of bacteria isolated from the domoic-acid-producing diatom *Pseudo-nitzschia multiseriis* and investigations into mechanisms by which they influence toxin production. Poster presented at the 9th Conference on Harmful Algal Blooms, Tasmania 6 - 11 February 2000.

3.4.7. Application notes

1. Waters Application Note: A fast and sensitive UPLC/MS/MS method for the detection of lipophilic marine biotoxins in shellfish. Fux E., MacMillan D., Bire R., Hess P., 2006 (www.Waters.com).

3.5. Academic Supervision

3.5.1. Undergraduates

- 2009: **Fernanda de Freitas**, Master 2 “Bioproduction et Bioproduits des Écosystèmes marins», Université de Nantes (FR), 6 months at Ifremer: “Optimisation de culture en bio-réacteur et de la production toxinique de l’*Azadinium spinosum*, dinoflagellé producteur d’azaspiracides” ; co-supervised with V. Séchet.
- 2008: **Adela Keogh**, Galway Mayo Institute of Technology (IE), 6 months at Marine Institute: “Isolation of azaspiracids from mussel tissue (*Mytilus edulis*) and identification of analogs”, degree thesis; co-supervised with Jane Kilcoyne.
- Anne-Laure Prüfer**, Ecole Chimie, Polymères, Matériaux (FR), 3 months at Marine Institute for a study entitled: “Preparation of reference materials for QUASIMEME” in fulfilment of a thesis for a French degree; co-supervised with Stephen Burrell.
- Jennifer O,Donnell**, Galway Mayo Institute of Technology (IE), 6 months at Marine Institute: “Assessment of acidity constant and lipophilicity of regulated marine biotoxins by liquid-chromatography mass spectrometry”, degree thesis; co-supervised with Elie Fux.
- Oliver Stone**, Galway Mayo Institute of Technology (IE), 6 months at Marine Institute: “Characterisation of shellfish tissues for DSP/AZP and ASP LRM preparations”, work placement; co-supervised with Stephen Burrell.
- Nils Kristian Jordheim**, Galway Mayo Institute of Technology (IE), 6 months at Marine Institute: “Studies on AOAC method 2005.06 and evaluation of its performance for the characterisation of PSP shellfish tissue reference materials”, work placement; co-supervised with Stephen Burrell.
- 2007: **Daniela Rode**, Europa Fresenius Fachhochschule – University of Applied Sciences (DE), 6 months at Marine Institute for a study entitled: “Investigation of matrix effects in lipophilic toxins analysis by several liquid chromatography - mass spectrometry systems” in fulfilment of a thesis for a German degree; co-supervised with Elie Fux.
- Sandra Noel**, Ecole Chimie, Polymères, Matériaux (FR), 5 months at Marine Institute for a study entitled: “LRM characterisation for paralytic shellfish poisoning toxins using HPLC fluorescence and LC-MS methods” in fulfilment of a thesis for a French degree; co-supervised with Stephen Burrell.
- Anita Curley**, Galway Mayo Institute of Technology (IE), 8 weeks at Marine Institute: “Parameters influencing the variability of results obtained in the

analysis of okadaic acid following base hydrolysis of shellfish extracts”, degree thesis; co-supervised with Barry Rourke.

Denise Keon, Galway Mayo Institute of Technology (IE), 8 weeks at Marine Institute: “Lipids in shellfish tissues and their influence on matrix effects in LC-MS-MS analysis”, degree thesis; co-supervised with Mairead McElhinney.

2006: **Tiffanie Arnoult**, Ecole Chimie, Polymères, Matériaux (FR), 6 months of a sandwich year at Marine Institute, “Preparation of reference materials for shellfish toxins” ; co-supervised with Stephen Burrell and Jane Kilcoyne.

Rebecca Moffat, Dublin Institute of Technology (IE), 3 months for a study entitled: “Analysis of shellfish samples for lipophilic biotoxins and development of an LC-MS-MS method for the detection of spirolides”, degree thesis; co-supervised with Barry Rourke.

Sophie Cardot, Ecole Nationale Supérieure de Chimie de Mulhouse (FR), 3 months for a practical training period entitled “Preparation and analysis of passive sampling devices for the determination of marine biotoxins in seawater” ; co-supervised with Elie Fux.

2005 **Vanessa Fauregeors**, Ecole Chimie, Polymères, Matériaux (FR), 6 months at Marine Institute for a study entitled: “Preparation of shellfish homogenate materials, contaminated with diarrhetic shellfish toxins, and analytical characterization of these materials, using LC-MS techniques”, work placement.

Sarah O’Callaghan, Dublin Institute of Technology (IE), 6 months at Marine Institute: “Lipids in shellfish tissues and their influence on matrix effects in LC-MS-MS analysis”, degree thesis.

Laura Deedigan, Limerick Institute of Technology (IE), 3 months for a study entitled: “Preparation of a reference material for domoic acid and its characterisation by homogeneity and stability testing”, degree thesis.

2004 **Katrin Anna Bender**, Europa Fresenius Fachhochschule – University of Applied Sciences (DE), 4 months at Marine Institute for a study entitled: “Validation studies on a novel Enzyme- Linked Immuno Sorbent Assay for Domoic Acid and its application to the analysis and monitoring of Domoic Acid in king scallop (*Pecten maximus*)” in fulfilment of a thesis for a German degree.

2003 **Arnaud Osmont**, Ecole Chimie, Polymères, Matériaux (FR), 4 months at Marine Institute for a study entitled: “Preparation of shellfish homogenate materials, contaminated with diarrhetic shellfish toxins, and analytical characterization of these materials, using LC-MS techniques”, work placement.

Sarah Tulipani, Ecole Chimie, Polymères, Matériaux (FR), 3 months at Marine Institute for a study entitled: “Preparation of reference materials for Dinophysistoxin-2” in fulfilment of a thesis for a French degree.

Jeannette Devane, Dublin Institute of Technology (IE), 3 months for a study entitled: “Preparation of reference materials for proficiency testing of Domoic Acid”, degree thesis.

Nils Rehmman, Europa Fresenius Fachhochschule – University of Applied Sciences (DE), 4 months at Marine Institute for a study entitled: “Isolation and purification of azaspiracids from contaminated shellfish tissues” in fulfilment of a thesis for a German degree.

2002 **Sarah Ryan**, Dublin Institute of Technology (IE), 3 months for a study entitled: “Preparation and characterisation of reference materials for Domoic Acid”, degree thesis.

Frédérick Woodland, Ecole Chimie, Polymères, Matériaux (FR), 3 months at Marine Institute for a study entitled: “Preparation of reference materials for Azaspiracids” in fulfilment of a thesis for a French degree.

3.5.2. Postgraduates

2009-2012 Co-supervision of a PhD thesis at Ifremer (**Marie Geiger**, Université de Nantes, France): “Bio-activité de métabolites fongiques et micro-algaux dans le milieu marin environnant les coquillages”.

2009-2012 Co-supervision of a PhD thesis at Ifremer (**Thierry Jauffrais**, Université de Nantes, France): “Ecophysiologie des dinoflagellés du genre Azadinium – production toxinique et transfert envers les coquillages”.

2005-2008 Direct supervision of a PhD at the Marine Institute (**Elie Fux**, Dublin Institute of Technology, Ireland): “Development and evaluation of passive sampling and LC-MS based techniques for the detection and monitoring of lipophilic marine toxins in mesocosm and field studies”

2003-2008 Direct supervision of a PhD at the Marine Institute (**Nils Rehmman**, University College Dublin, Ireland): “Preparative isolation and purification of Azaspiracids and related toxins from blue mussels and characterisation of new toxin analogs”

2005-2007 Direct supervision of an MSc at the Marine Institute (**Mairead McElhinney**, Dublin Institute of Technology, Ireland): “Matrix effects, development of clean-up and LC techniques contributing towards a reference LCMS method for the analysis of lipophilic marine toxins”

2003-2007 Direct supervision of a PhD at the Marine Institute (**Pearse McCarron**, University College Dublin, Ireland): “Studies on the development of reference materials for phycotoxins, with a focus on azaspiracids”

2003-2006 Co-supervision of a PhD (**Yvonne Bogan**, Letterkenny Institute of Technology, Letterkenny, Ireland): “Factors affecting the concentration of domoic acid in scallop, *Pecten maximus*”

3.5.3. Postdocs

2005-2008 Direct supervision of Dr. **Ronel Biré** working at the Marine Institute as project leader of the EU-FP6 project BIOTOX

3.6. **PhD Examination and Science Performance Auditing**

3.6.1. **PhD Examinations**

Examination of a Norwegian PhD: John A.B. Aasen

Jun 2005 External examiner of a PhD thesis: “Identification and quantification of algal toxins in shellfish, and characterisation of toxin profiles in Norwegian mussels” (John A.B. Aasen, Norwegian School of Veterinary Science, Oslo, Norway; thesis supervisor: Prof. Tore Aune).

Examination of a Spanish PhD: Begonia Espina Barbeitos

Dec 2009 External examiner of the thesis: “Efectos de ficotoxinas sobre el metabolismo de carbohidratos y la estructura celular de hepatocitos de rata” (= “effects of phycotoxins on the metabolism of carbohydrates and the cellular structure of rat hepatocytes”; Begonia Espina, University of Santiago de Compostella, Lugo, Spain; thesis supervisor: Prof. Luis Botana).

Examination of a French PhD: Aurélie Ledreux

Mar 2010 External examiner of a PhD thesis: “Contribution à l’appréciation du risque pour l’homme lors de l’apparition de neurotoxines marines émergentes : analyse de réponses cellulaires et sélection de modèles expérimentaux de criblage”(= “Contribution to the characterisation of risks posed by emerging neurotoxins : analysis of cellular responses and selection of experimental models for screening”, Aurélie Ledreux, Musée National d’Histoire Naturelle, Paris, France; thesis supervisor : Prof. Cécile Bernard).

3.6.2. **CEFAS Science Audit**

Oct 2005 Expert for “DEFRA / CEFAS Science Audit (Environment & Food Safety)”. The aim of the Science Audit was to assess whether the science carried out by CEFAS is of a high and appropriate quality and is delivering to Defra’s requirements. I participated in one of four science assessment teams: “Environment and Food Safety”. The evaluation approach was based on the examination reports provided prior to the audit, presentations given by CEFAS staff and interviews with key personnel (at differing scientific levels including permanent and contract staff).

3.7. Project Proposals, Management and Evaluation

3.7.1. Project Proposals and Management

- 2009-2012 Manager of the 4-year national IFREMER project RISCAP “Risques Sanitaires Chimiques Associés aux Produits de la mer” (sanitary risks posed by chemical substances in seafood products), including five actions on chemical contaminants, toxic algae, shellfish, regulated and non-regulated phycotoxins (annual budget 2010: **€257 K**)
- 2009-2012 Proposal and leader of a 3-year international multidisciplinary project (funded by the “Région des Pays de la Loire”, a French regional funding agency, **€270 K**), COLNACOQ (Lipophilic natural compounds in shellfish)
- 2009-2012 Proposal of and participant in a 3.5-year international multidisciplinary project (natl. funds from National Development Plan **€1.1 Mio**), ASTOX 2 (Azaspiracid Standards and Toxicology)
- 2007-2013 Participation in the preparation of the « Beaufort Biodiscovery » initiative, funds obtained for 3 universities (NUIG, QUB, UCC) and the Marine Institute **€7.25 Mio**
- 2003-2010 Proposal and management of 3 projects from 1 to 3 years; service of reference material preparation for QUASIMEME Proficiency Testing (quality assurance in marine environmental matrices in Europe), total of **€120 K**
- 2005-2008 European project proposal (participant and leader of 4 out of 12 modules) STREP FOOD-CT-2004-514074 (BIOTOX, **€409 K** for Marine Institute)
- 2003-2007 Proposal and leader of a 3-year international multidisciplinary project (natl. funds from National Development Plan **€419K**), ASTOX (Azaspiracid Standards and Toxicology)
- 2001-2002 Proposal and leader of a 1-year pilot project 1 on the isolation of azaspiracids (national funds for the Marine Institute **€120K**)
- 2000 Proposal of a 3-year project in UK (national funds from FSA-UK for Marine Laboratory Aberdeen **£ 200K**)

3.7.2. Project Evaluation

- Jan 2009 Evaluation of a proposal for ANR (French National Research Agency): IsoBioMim - Chimiodiversité des substances naturelles marines : isolement, chimie biomimétique et biosynthèse pour de nouveaux candidats-médicaments

- Jan 2008 Evaluation of 3 proposals for the routine monitoring of marine biotoxins in Scotland (FSA-Scotland)
- Jul 2007 Evaluation of 3 proposals for a research project on method development and validation of marine biotoxins (FSA-UK)

3.8. Organisation of Conferences

- 2009 **7th Intl. Conf. Molluscan Shellfish Safety (ICMSS)**, 14-18/06/2009 Nantes, France. Member of the organizing committee (from Oct '08).
- 2009 **Annual National Shellfish Safety Workshop** (Journées REPHY), 1-3/04/2009 Ifremer Nantes, France. Chair of 2nd day on sanitary aspects of shellfish safety
- 2008 **11th Intl. Conf. Phycology (ISAP)**, 21-28/06/2008, Galway, Ireland. Member of the organizing committee, chair of 2 sessions
- 2008 **122nd Annual Meeting AOAC Intl.**, 21-24/09/2008, Dallas, Texas, chair of session: « Looking for the unknown – parallels in drug discovery and toxin detection »
- 2001→'08 Active participation on the organisation of all « **Irish Annual Shellfish Safety Science Workshops** »
- 2004 **Intl. Conf. Molluscan Shellfish Safety (ICMSS)**, 14-18/06/2004 Galway, Ireland. Member of the organizing committee, chair of session

3.9. Editorial and Refereeing Activities

3.9.1. ICMSS 2004, 2009

Member of the editorial board (organising and participating in the review of papers submitted to the ICMSS 2004 and 2009 proceedings)

3.9.2. Marine Drugs

Member of the editorial board

3.9.3. Peer Reviews

Anal. Bioanal. Chem. (6)
 Aquat. Liv. Res. (1)
 Altern. Techn. Labor. Anim. (1)
 Chem. Res. Toxicol. (1)
 Food Additives and Contaminants (1)
 J. Agric. Food Chem. (1)

J. Chromatogr. A (3)
J. Mass Spectrom. (1)
J. AOAC Int. (1)
Toxicon (8)

3.10. Expert Committees and Consultancy

AFSSA	Specialised Expert Committee on Contaminants in Food, French Food Safety Agency (2009 to date)
EFSA	Marine Biotoxin Working Group of the Contam Panel, risk evaluation and drafting of 8 opinions (2006 to 2010)
AOAC	Member of the Presidential Task Force on Methods of Analysis for Phycotoxins, topic advisor on okadaiates to General Referee (2005 – to date)
QUASIMEME-SAG	Scientific Advisory Group of the Proficiency Testing scheme Quality Assurance in Marine Environmental Matrices in Europe (QUASIMEME) (2003 – 2008)
MSSC	Irish Molluscan Shellfish Safety Committee (2001 – 2008), communication of scientific research and risk management
FSAI	Member of Scientific Committee, of Food Additives and Contaminants sub-committee and of Marine Biotoxin Working Group, Food Safety Authority of Ireland (2005 – 2008)
ECVAM	Chaired ECVAM/DG-SANCO workshop Jan 2005 (Feb 2005 – Nov '06, ECVAM-report 55), scientific feasibility of non-animal alternatives to toxicity testing
FAO/IOC/WHO	Chaired Analytical Working Group of the <i>ad hoc</i> expert consultation on shellfish toxins (2 workshops in 2004, summary report published on FAO website 2005)
UK-COT	<i>Ad hoc</i> advice to the UK Committee on Toxicology (Dec 2005)
FAO	Consultancy: Editorial work on the background report of the 2004 <i>ad hoc</i> expert consultation on shellfish toxins (12 days, 2009).

4. Scientific part

4.1. Introduction

The term phycotoxin indicates natural metabolites produced by unicellular micro-algae (protists). Most phycotoxins are produced by dinoflagellates although cyanobacteria have also been reported to produce saxitoxin, and domoic acid is produced by diatoms. Some of the toxins have initially been identified in associated organisms, e.g. okadaic acid which was initially identified in the sponge *Halichondria okadaei* (Tachibana et al, 1981), domoic acid in the red macroalga *Chondria armata* (Daigo et al., 1959a and b; Takemoto and Daigo, 1958 and 1960) or palytoxin in the soft coral *Palythoa toxica* (Moore and Scheuer, 1971).

Through accumulation in the food chain, these toxins may concentrate in a variety of marine organisms including filter-feeding bivalves, burrowing and grazing organisms (tunicates and gastropods) as well as herbivorous and predatory fish. All marine biotoxins described in this introduction have been selected because they are found in seafood and have been identified as bioactive compounds potentially causing seafood poisoning, even if some compounds have simply been identified through their bioactivity in animal trials and have not been proven to cause acute illness in humans.

Human poisoning due to ingestion of seafood contaminated by phycotoxins has occurred in the past, and historical records as well as the habits of some populations in coastal and tropical areas, show that harmful algal blooms (HAB) are naturally occurring events (Hallegraeff, 2004). In the last thirty years, HABs have attracted increasing attention from the scientific community and the society. The occurrence of episodes of human poisoning due to ingestion of toxic seafood involving tens or hundreds of people in several areas of the world (Hallegraeff, 2004) has certainly called for more attention onto HABs and their consequences on human health. The increased awareness has then supported more research efforts in the area, thus contributing to a better understanding of HABs and contamination of seafood by algal toxins, as well as the chemistry, mechanisms of action and toxicity of phycotoxins.

The accumulation of information in this field has led to the conclusion that we are witnessing a global increase in HABs and seafood contamination, and more effective and complex measures to prevent human intoxications have been developed and implemented worldwide. The increased recording of occurrence of toxic algae and HABs in coastal waters in several areas in the world is certainly a result of a deeper attention paid to the phenomenon. Other factors, however, are being recognized as contributing to the increasing frequency of HAB outbreaks, their appearance in areas of the world where they had not been recorded in the past, as well as the intensity and duration of HABs, with their possible consequences on seafood contamination and human intoxications/poisoning (Hallegraeff, 2004). The ongoing changes can be exemplified by the trend of recording of *Ostreopsis* species in the Mediterranean Sea, that has been essentially anecdotic in the past century, in keeping with the mainly tropical distribution of these algae. In these last five years, in turn, blooms of *Ostreopsis* in several parts of the Mediterranean Sea have been recorded, and in some cases these have been accompanied by human intoxications involving up to two hundred people, as it has occurred in Italy in 2005 (Gallitelli et al., 2005; Durando et al., 2007).

The factors proposed to be involved in the global increase in HABs, include the eutrophication of coastal waters as a consequence of increased aquaculture and fertilizers runoff from agriculture, as well as other economic activities linked to urbanization, the changes in climatic conditions, the transportation of toxic algae and their cysts from one coastal area to another as a consequence of their presence in the ballast water of ships or through the movement of shellfish stocks (Hallegraeff, 2004). Furthermore, a recent meta-analysis of published data and historical records

provided indications that the regional loss of species diversity and ecosystem services in coastal oceans increases the occurrence of algal blooms (Worm et al., 2006).

HABs and the contaminations of seafood, undoubtedly represent relevant social issues, because of the problems they pose to human health, economic activities, recreation and tourism. The many facets of the phenomenon and their complexity represent a powerful drive for a better understanding of the chemistry and biology of phycotoxins, as a basis for a more effective protection of human health and the support to several human activities.

The complexity of the subject approached here, and the vast literature devoted to it, will constraint the account to major issues. I apologize to the many scientists whose contributions will not be directly quoted in this section, and will point the interested reader to some excellent reviews devoted to specific topics, whenever appropriate.

4.1.1. Shellfish poisoning and algal toxin groups

4.1.1.1. Chemical diversity of algal toxin groups

Firstly, it should be noted that marine biotoxins are naturally produced compounds and therefore, many enzymatic systems in nature are capable of metabolising them. This characteristic puts them in contrast to man-made compounds such as polychlorinated biphenyls (PCBs) and certain pesticides many of which are extremely stable compounds for which nature has no metabolic processes foreseen. Similar to PCBs, dioxins or polycyclic hydrocarbons, most groups of marine toxins have also many analogues. Thus, between naturally produced analogues and metabolites of these, marine biotoxins constitute a vast array of bioactive chemicals. This section describes the characteristics of a selected range of marine biotoxins to demonstrate the wide-ranging chemical diversity of these compounds. From a natural products or biosynthesis point of view, the compounds described in this section belong to several classes including amino acids (domoic acid), alkaloids (saxitoxin and tetrodotoxin) and polyketides (all others). Therefore, algal toxins are often referred to as small molecules. The selection of toxins excludes all compounds that are typically referred to as natural polymers (proteins, carbohydrates, nucleic acids). Indeed the molecular weight of phycotoxins typically ranges between 300 to 1500 dalton; nevertheless, some compound groups such as palytoxins (PITXs) and maitotoxins (MTXs) are very sizeable molecules of 2677 and 3422 dalton, respectively. Maitotoxin has been reported as the largest non-proteinaceous natural toxin. The chemical nature and molecular size classification distinguish phycotoxins from the very large group of venoms from snakes, spiders or cone snails which are typically very potent mixtures of proteinaceous toxins. Table 1 gives an overview of some characteristics of the compound groups discussed in the specific sections.

In addition to the abovementioned difficulties in isolation of a toxin for its initial identification, it should be noted that one of the problems with natural compounds is the possible co-occurrence of isomers (compounds with the same molecular weight but slightly different structural arrangements) and analogues (compounds that derive from the same structural skeleton but have some structural difference leading to a different molecular weight). The term “analogue” is often used synonymously with the terms “metabolite” or “derivative”. All toxin groups have 10 or more analogues, often up to 30 or more.

Table 1.1.1. Characteristics of marine biotoxins, arranged by increasing molecular weight. Chemical class and formula, molecular weights, UV-absorption maxima, acidity constants and lipophilicity. Note, for the smaller toxins (DA, STX) the acidic or basic character outweighs their few lipophilic functions, resulting in overall hydrophilic compounds. The largest molecules have significant polar functions (2 sulphate groups for YTX, multiple OH-groups for PITX and MTX, 2 amide and a primary amine function for PITX, and 2 sulphate groups for MTX) giving them an amphiphilic character despite their long carbon chains (lipophilic part).

TOXIN	CHEMICAL CLASS	FORMULA	MOLAR WEIGHT	UV [NM]	PKA _{1,2,3,4}	LIPO-PHILICITY
SAXITOXIN	TETRAHYDRO-PURINE ALKALOID	C ₁₀ H ₁₇ N ₇ O ₄	299	N/A	8.1, 11.5	HYDROPHILIC
DOMOIC ACID	CYCLIC AMINO ACID, 3 CARBOXY GROUPS	C ₁₅ H ₂₁ NO ₆	311	242	2.1, 3.7, 5.0, 9.8	HYDROPHILIC
GYMNODIMINE	CYCLIC IMINE, MACROCYCLE	C ₃₂ H ₄₅ NO ₄	507	N/A	N/REP	LIPOPHILIC
13DM-SPIROLIDE C	CYCLIC IMINE, MACROCYCLE	C ₄₁ H ₆₁ NO ₇	691	N/A	N/REP	LIPOPHILIC
GAMBIEROL	LADDERSHAPED POLYETHER	C ₄₃ H ₆₄ O ₁₁	757	N/REP	N/A	LIPOPHILIC
OKADAIC ACID	POLYETHER, SPIRO-KETO ASSEMBLY	C ₄₄ H ₆₈ O ₁₃	804	N/A	4.9 [§]	LIPOPHILIC
AZASPIRACID	POLYETHER, SECOND AMINE, 3-SPIRO-RING	C ₄₇ H ₇₁ NO ₁₂	841	N/A	5.8 [§]	LIPOPHILIC
PECTENOTOXIN-2	POLYETHER, ESTER MACROCYCLE	C ₄₇ H ₇₀ O ₁₄	858	235	N/A [§]	LIPOPHILIC
BREVETOXIN-B	LADDERSHAPED POLYETHER	C ₅₀ H ₇₀ O ₁₄	894	208	N/A	LIPOPHILIC
PROROCENTROLIDE	CYCLIC IMINE, LACTONE MACROCYCLE	C ₅₆ H ₈₅ NO ₁₃	979	N/REP	N/REP	LIPOPHILIC
P-CIGUATOXIN-4B	LADDERSHAPED POLYETHER	C ₆₀ H ₈₅ O ₁₆	1061	223	N/A	LIPOPHILIC
YESSOTOXIN	LADDERSHAPED POLYETHER	C ₅₅ H ₈₂ O ₂₁ S ₂	1140	230	N/REP, 6.9 [§]	AMPHIPHILIC
PALYTOXIN*	POLYOL, 2 AMIDE & A PRIMARY AMINE	C ₁₂₉ H ₂₂₃ N ₃ O ₅₄	2678	263, 233	N/REP	AMPHIPHILIC
MAITOTOXIN	POLYOL, FOUR FUSED RING SYSTEMS	C ₁₆₄ H ₂₅₆ O ₆₈ S ₂ NA ₂	3422	230	N/REP	AMPHIPHILIC

* Palytoxin from *Palythoa toxica*; N/a = not applicable; N/rep = not reported

§Fux and Hess (unpublished observations) determined chromatographically (for YTX the pKa₁ was too low to be determined chromatographically, pKa₂ is given)

4.1.1.2. Algal toxins and bivalve molluscs

Shellfish toxins are produced by algae which are consumed by bivalve molluscs as part of their natural diet. Thus, toxins are accumulated actively by shellfish, and concentrated in the hepatopancreas of bivalves, their digestive organ. The factors influencing this accumulation are studied intensively. Although some toxins are accumulated very regularly by specific shellfish species in some areas, prediction of contamination levels in general remains very challenging due to a number of factors:

- Factors related to the occurrence of algae:
 - *Physical parameters:* weather and climate-related parameters (temperature, wind, light conditions), hydrography

- *Chemical parameters*: nutrient nature and availability (e.g. eutrophication), oxygen availability, anthropogenic pollution, ocean acidity
- *Biological parameters*: evolution of algal community structure, occurrence of grazing and parasitic micro-organisms

➤ Factors related to shellfish

- *Culture conditions*: bottom- or rope-growth of mussels, sub-tidal or inter-tidal growth, water-depth (and mixing of water-column), maintenance of support structures (biofouling may nurture growth of toxic benthic algae)
- *Filtration*: feeding status, species-specific filtration rates and selectivity, micro-organisms affecting shellfish (pathogenic bacteria and viruses, algae and cyanobacteria affecting shellfish, nuisance organisms)
- *Metabolism*: species-specific differences, metabolic changes in bivalves due to seasonal variation, reproduction status and environmental stress.

Many of the above parameters are inter-related and result in very complex and changing scenarios. For instance, duration of contamination may be related to season, and the occurrence of the same alga in summer may lead to shorter contamination episodes than its occurrence in autumn or winter time. While many of the factors affecting shellfish can be actively managed, in particular those related to culture techniques and conditions, many factors affecting the occurrence of algae are impossible to control and difficult to predict. As temperature and light conditions affect the growth of algae directly, many models for prediction are based on those parameters. However, the forecasting capability of these models remains limited due to the poor ability to forecast weather for more than 1 week, which is generally not sufficient warning for the shellfish industry to change harvest patterns or to relocate large quantities of shellfish. Also, prediction models have difficulty in incorporating the biological parameters, in particular inter-annual variations of the phytoplankton community structure and occurrence of parasitic organisms of micro-algae or conditions leading to significant cyst formation and hatching.

Significant differences in accumulation level and detoxification rate may appear between shellfish species, probably related to differences in metabolism, filtration rates and selectivity in the filtration of the algal food. These species-specific differences can be established and appropriate selection of species can be made to avoid the impact of certain toxic algae in specific areas.

Although for many toxins the producing algae are now known (Table 2), the causative relationship is not always clear and often requires many years of intense study. Examples of such studies are the confirmation of *Protoceratium reticulatum* as a causative organism of YTX by Satake et al. (1999), or the discovery of *Azadinium spinosum* as a producer of Azaspiracid (Krock et al., 2008; Tillmann et al., 2009).

Table 1.1.2. Toxins and their biogenetic, micro-algal origins* Apart from domoic acid which is produced by the diatom *Pseudo-nitzschia spp.*, other phycotoxins are produced by dinoflagellates (STX is also produced by certain cyanobacteria*).

TOXIN-GROUP	ABBREVIATION	ALGAE ASSOCIATED
AZASPIRACID	AZA	<i>AZADINIUM SPINOSUM</i>
BREVETOXIN-B	BTX	<i>KARENIA BREVIS</i>
DOMOIC ACID	DA	<i>PSEUDO-NITZSCHIA SPP.</i> [§]
GYMNODIMINE	GYM	<i>KARENIA SELLIFORMIS</i>
OKADAIC ACID	OA	<i>DINOPHYSIS SPP.</i> [§] , <i>PROROCENTRUM SPP.</i> [§]
PALYTOXIN	PLTX	<i>OSTREOPSIS SPP.</i> [§]
PECTENOTOXIN-2	PTX	<i>DINOPHYSIS SPP.</i> [§]
PROROCENTROLIDE	PCL	<i>PROROCENTRUM SPP.</i> [§]
SAXITOXIN	STX	<i>ALEXANDRIUM SPP.</i> [§] , <i>G. CATENATUM</i> , <i>P. BAHAMENSE</i>
13DM-SPIROLIDE C	SPX	<i>ALEXANDRIUM OSTENFELDII</i>
YESSOTOXIN	YTX	<i>P. RETICULATUM</i> , <i>L. POLYEDRUM</i> , <i>G. SPINIFERA</i>

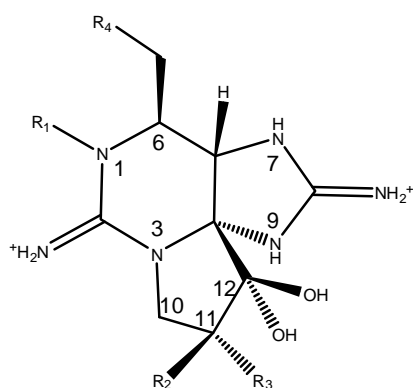
* For a complete list of harmful algae and associated toxins see website of Intergovernmental Oceanographic Commission (IOC): <http://www.marinespecies.org/hab/index.php>

[§] denotes the plural of species, i.e. several species of the indicated genus are reported to produce toxins from this group

Saxitoxins

Saxitoxin (STX) -group toxins are closely related compounds based on a tetra-hydro purin skeleton. The basic character of the hydro purin group renders the molecule highly water-soluble. More than 30 saxitoxins, mainly from marine dinoflagellates and shellfish that feed on toxic algae, have been identified (Dell'Aversano et al., 2004 and 2008; FAO, 2004), at least 18 have toxicological relevance (Fig. 1). They are mainly produced by dinoflagellates belonging to the genus *Alexandrium*: e.g. *A. tamarensis*, *A. minutum* (syn. *A. excavata*), *A. catenella*, *A. fraterculus*, *A. fundyense* and *A. cohorticula*. Also other dinoflagellates such as *Pyrodinium bahamense* and *Gymnodinium catenatum* have been identified as sources of STX-group toxins (FAO, 2004). In addition, some analogues have also been identified in some cyanobacteria which may occur in fresh and brackish waters.

STX analogues do not exhibit a strong ultraviolet (UV) absorbance or fluorescence. They are typically stable to heat treatment up to 100 °C. Different acid and base treatments will lead to various transformations. In particular, all C11-epimeric pairs (e.g. GTX2 and 3 or GTX1 and 4) will interconvert and equilibrate to a constant ratio at high pH. Similarly, carbamoyl- and sulfocarbamoyl-derivatives will convert to decarbamoyl-analogues through cleavage of the carbamoyl-ester group at high pH (e.g. C1 to dc-GTX2 and C2 to dc-GTX3). Under acidic conditions, the carbamoyl-ester is relatively stable but the sulphate ester will be cleaved to convert sulfocarbamoyl-groups into carbamoyl groups (e.g. C1 to GTX2 and C2 to GTX3). These transformations may only occur partially when shellfish tissues or human tissues or fluids contaminated with STXs are exposed to these conditions, as biological tissues typically buffer the pH. Since conversion reactions can result in a several fold increase in toxicity, a potential danger of these toxins was suggested (Hall and Reichardt, 1984). To examine this phenomenon experimentally, B1 (GTX5) was incubated at conditions simulating the human stomach and analysed by the mouse bioassay. After 5 h incubation at 37 °C, a two-fold increase of toxicity corresponding to 9% conversion of toxin was observed in the artificial gastric juice at pH 1.1 and no apparent increase of toxicity in rat gastric juice at pH 2.2 (Harada et al., 1984).



<u>R₁</u>	<u>R₂</u>	<u>R₃</u>	<u>R₄</u>	<u>Toxin</u>
H	H	H		STX
H	H	OSO ₃ ⁻		GTX2
H	OSO ₃ ⁻	H		GTX3
OH	H	H		NEO
OH	H	OSO ₃ ⁻		GTX1
OH	OSO ₃ ⁻	H		GTX4
H	H	H		GTX5 (B1)
H	H	OSO ₃ ⁻		C1
H	OSO ₃ ⁻	H		C2
OH	H	H		GTX6 (B2)
OH	H	OSO ₃ ⁻		C3
OH	OSO ₃ ⁻	H		C4
H	H	H		dcSTX
H	H	OSO ₃ ⁻		dcGTX2
H	OSO ₃ ⁻	H		dcGTX3
OH	H	H		dcNEO
OH	H	OSO ₃ ⁻		dcGTX1
OH	OSO ₃ ⁻	H		dcGTX4

Figure 1.1.1. Saxitoxin and analogues

The marine organisms most often affected are mussels and oysters, but also puffer fish and marine snails (e.g. abalone) have been reported to accumulate dangerous concentrations. The hydrophilic character of the compounds may partially explain the relatively rapid depuration of these toxins from mussels. This rapid depuration complicates the regulatory surveillance for these toxins which is therefore most often complemented by observations of the algae responsible for in situ production.

Complex toxin profiles, possible conversions and lack of reference materials have led most countries to maintain the mouse bioassay introduced by Sommer and Meyer (1937) and validated as AOAC method (959.08) (AOAC, 2005a). This assay allows for the quantitation of levels above 350 to 400 µg/kg. Alternative methods have been proposed based on chromatography and fluorescence detection by Oshima (1995), and Lawrence et al. (2004), the latter also being officially validated as AOAC method (2005.06) (AOAC, 2005b). These HPLC methods are technically challenging, time-consuming and depend on continuous supply of a large number of toxin standard as reference compounds. Due to the hydrophilic character of STXs, their complete

chromatographic separation has proven difficult until the introduction of hydrophilic interaction chromatography by Dell'Aversano et al. (2005). Also, the physicochemical determination of STXs has relatively high quantification limits which are slightly lower than or similar to the detection limits of the mouse bioassay for complex toxin profiles.

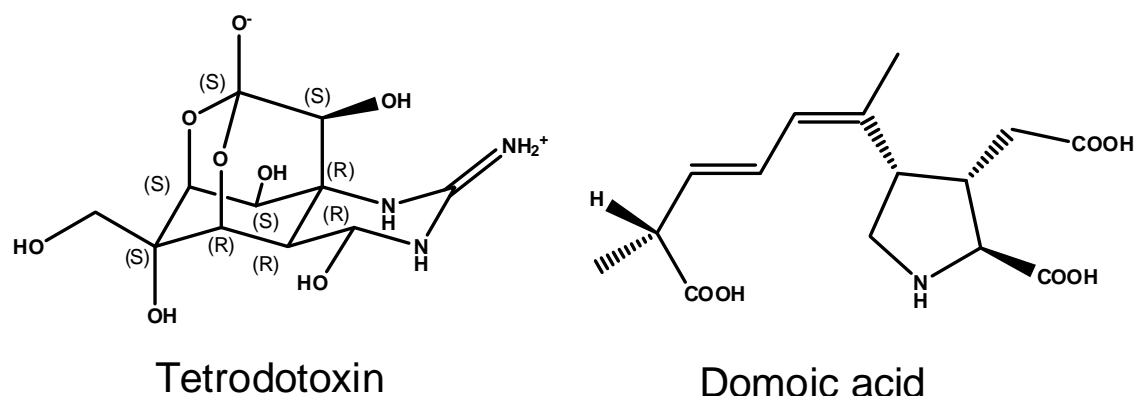


Figure 1.1.2. Small water-soluble toxins : (a) tetrodotoxin, (b) domoic acid

Domoic acid group

Domoic acid (DA) is a small cyclic amino acid (311 da), with three carboxylic acid groups (Fig. 2b). These groups are responsible for its solubility in water and its relatively great polarity, resulting in early elution in reverse-phase chromatography Quilliam et al., 2001. The acid constants ($pK_{a,s}$) of the three carboxylic acids and the cyclic amino group have been determined using NMR techniques by Walter et al. (1992) (Tab. 1). Although numerous isomers and several analogues have been reported (Holland et al., 2005; Maeda et al., 1986 and 1987; Walter et al., 1994; Wright et al., 1990), so far only DA and its C5-diastereomer have been shown to be of toxicological relevance Ramsdell, 2007. DA transforms into its diastereomer through heat or long-term storage (Quilliam et al., 1995) and analysis has focused on determination of the sum of these two isomers as best estimate of the total toxicity. A conjugated double bond in the aliphatic side chain allows detection of DA by UV absorbance and both UV and MS detection are commonly used for the physico-chemical determination of DA (Hess et al., 2005). The conjugated double bond also leads to light-sensitivity and is the cause of radical-mediated oxidative metabolism. As a contaminant in shellfish tissues, DA is heat stable and cooking does not typically destroy the toxin. However, protein coagulation leads to retraction of the tissues and DA as a water-soluble compound may be transferred significantly to cooking fluids (McCarron and Hess, 2006). Its stability under various conditions has been studied, and storage of raw or autoclaved tissues only resulted in ca. 50% degradation of the toxin after 5 months (McCarron et al., 2007).

Domoic acid has been reported in a wide variety of seafoods, including mussels, scallops and anchovies. Due to the common occurrence of its source organisms (diatoms of the genus *Pseudo-nitzschia* spp.), DA is spread world-wide. Thanks to lightly diarrhoeic properties caused by the macro-alga *Chondria armata* (of which DA is the active ingredient), it has been used in Japan as anti-worming agent (reviewed in Ramsdell, 2007). However, the severe poisoning in 1987 in Canada of over 100 people following consumption of mussels, including 3 fatalities, stopped this practise. The water soluble character also results in relatively rapid depuration from shellfish (similar to STX), and regulatory surveillance is complemented by screening of shellfish production waters for *Pseudo-nitzschia* to allow early warning in an attempt to prevent human poisoning.

Azaspiracid group

Azaspiracid (AZA1) is an intermediately sized polyether toxin (MW 841 dalton). The chemistry, ecology and toxicology of azaspiracids have been extensively reviewed by Twiner et al. (2008). Although its geographical distribution was initially believed to be restricted to Europe, recent work has also shown that the compound has been found in shellfish from North Africa, and in Canadian waters (Taleb et al., 2006; Twiner et al., 2008). Most recently, AZA2 has also been identified in the sponge *Echinoclathria sp.*, collected from Japanese waters, indicating that its producers occur world-wide (Ueoka et al., 2009). However, so far poisoning directly attributed to AZA has only been reported from Europe, either due to environmental conditions not being appropriate for the producer to reach seafood in other locations, or due to the predominant screening for marine biotoxins with the mouse bioassay, an intrinsically unspecific method of detection of toxins. Also, the symptoms of AZA in human poisoning events is similar to DSP from okadaic acid group compounds and may therefore not always be followed up with further investigation. The mouse bioassay, initially introduced by Yasumoto et al. (1978), for the detection of diarrhoeic shellfish poisoning toxins, also detects AZAs at similar levels (Hess et al., 2009).

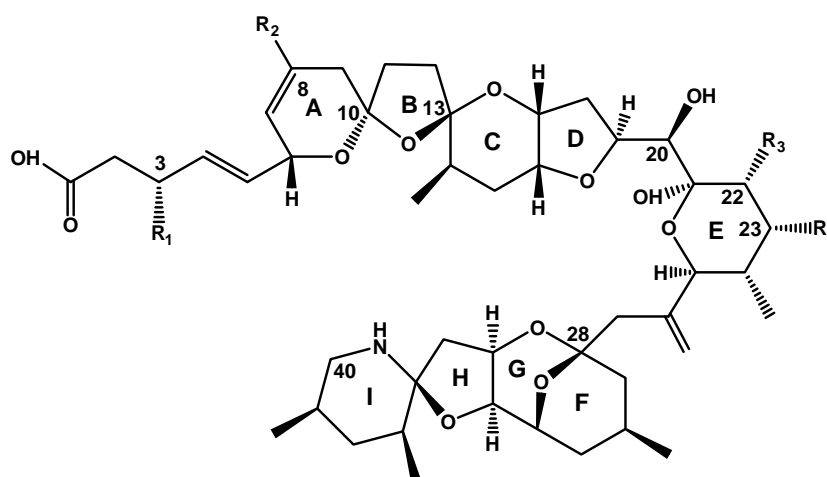


Figure 1.1.3. Azaspiracids: AZA1 ($R_{1,2,4} = H$; $R_3 = CH_3$), AZA2 ($R_{1,4} = H$; $R_{2,3} = CH_3$). The initial structure proposed by Satake et al. [20] was corrected by Nicolaou et al. (2003a and b; 2004a and b). The corrected structure is shown.

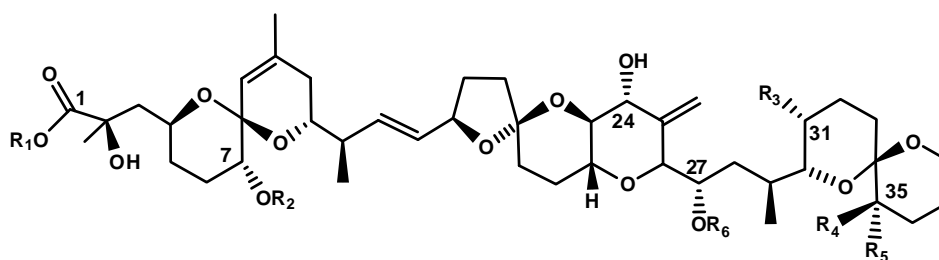
Chemically, azaspiracid is characterised by a cyclic amine group, a carboxylic acid and a unique tri-spiro ring assembly. Similar to okadaic acid, it is likely that the acid-labile character of the compound is related to the spiro-keto assembly (rings A, B and C in Figure 3) (Nicolaou et al., 2003a). Contrarily to okadaic acid, azaspiracids are also labile to strong bases, i.e. their destruction can be completed in methanolic solution through treatment with NaOH for 10 min at 76 °C. The mechanism for this reaction remains to be clarified. Due to the absence of conjugated double bonds or aromatic rings, the molecule has no chromophore or specific UV absorbance above 200 nm, therefore, physico-chemical determination is mostly based on separation by liquid chromatography followed by detection using mass spectrometry. An initial proposal of the chemical structure was made by Satake et al. (1998), but a correction was made to this initial proposal after chemical synthesis by Nicolaou et al. (2003a and b; 2004a and b). Approximately 20 analogues have been reported to occur naturally in shellfish (Rehamn et al., 2008). However, only two of these, AZA1 and AZA2, have been reported to be produced by the previously unknown dinoflagellate *Azadinium spinosum* (Krock et al., 2009; Tillmann et al., 2009). Due to the minuscule nature of the causative organism (< 20 µm), it cannot be easily identified using light microscopy and had only been discovered 12 years after the first poisoning event that was

attributed to this toxin group (McMahon and Silke, 1996). The metabolism of AZA1 and -2 in mussels is presumed to follow an oxidative path at C3 and C23 and the methyl group at C22. Following the initial observation by Hess et al. (2005) of increased AZA-concentrations after heat-treatment of AZA-contaminated mussel tissues, McCarron et al. (2009) postulated that a carboxylic acid located at C22 is a product of such metabolism and that heat-treatment leads to decarboxylation and further analogues of AZAs. In shellfish it is anticipated that the decarboxylation happens spontaneously over time. There is no reports on mammalian metabolism of AZAs. From the lipophilic nature of AZAs (Fux and Hess, unpublished observations), it is presumed that AZAs can pass the intestinal barrier, if they are sufficiently bioavailable. Stomach simulation experiments by Rehmann, and Alfonso et al. (Alfonso et al., 2008; Rehmann et al., 2008), suggest that there may be limited bioavailability due to the lipophilic character of AZA1, however, further in vivo studies will be required to clarify such behaviour. Initial evaluation of the compounds using intraperitoneal (i.p.) injection in mouse bioassays suggests that the hydroxyl-analogues are less toxic than the parent compounds Ofuji et al., 2001. Further structure-activity studies by Ito et al. (2006), showed that a synthetic stereo-isomer of AZA1 (C1-20 epi-AZA1) was 3 to 4 time less toxic than AZA1, and that a variety of smaller epitopes did not induce any effect similar to AZA1, thus suggesting that the entire skeleton is required to effectively interact with the biological target.

Okadaic acid group

Okadaic acid (OA) had originally been found in the sponge *Halichondria okadaii* (Tachibana et al., 1981) but was identified as a shellfish contaminant following a series of poisoning events in 1976 by Yasumoto et al. (1978) (Fig. 4). In 1980, Yasumoto et al. (1980) clearly demonstrated that diarrhoeic shellfish poisoning was associated with blooms of *Dinophysis fortii*, a dinoflagellate in which the authors also isolated an analogue of OA, dinophysistoxin-1 (DTX1). The same compound class was rapidly found as the causative agents of diarrhetic shellfish poisoning in Europe (Kumagai et al., 1986). Dinophysistoxin-2 (DTX2) has been discovered as a third main analogue by Hu et al. (1992), explaining shellfish toxicity found in Irish mussels. OA and DTXs are produced by a variety of different dinoflagellates from the *Dinophysis* and *Prorocentrum* genera, including *D. acuta* and *D. acuminata*, as well as *P. lima* and *P. belizeanum*. Although the toxins of the OA group have been mainly reported from Japan and Europe, recent evidence in the gulf of Mexico demonstrates that *Dinophysis* in these regions may also produce the same compounds under appropriate environmental conditions (Swanson et al., 2008). Therefore, a global distribution of these toxins is now widely accepted and monitoring should occur during shellfish production in potentially affected areas.

Chemically, OA is one of the many polyether toxins among the phycotoxins. Its structure is characterised by a carboxylic acid group and three spiro-keto ring assemblies, one which connects a five with a six-membered ring. OA, DTX1 and -2 withstand a wide pH range from mildly acidic to strongly basic, e.g. no degradation is found for up to 40 min at 76 °C in 0.3 molar methanolic NaOH solution. Treatment with strong mineral acids, e.g. HCl, leads to rapid degradation: OA and DTX1 are completely destroyed within 20 min at 76 °C of 0.3 molar methanolic HCl, even in the presence of shellfish matrix in the extract. However, without the addition of acid, the compounds are rather stable to heat. Also, recent work on stomach simulation experiments in the author's laboratory suggests that the food itself has a buffering capacity on the acid and the toxins may not be destroyed significantly in the gastric juice. In normal cooking procedures the toxins are not destroyed, although the coagulation of proteins in shellfish tissues may lead to redistribution within the organs of shellfish and some toxins may be released into the cooking fluids (McCarron et al., 2008).



	<u>R1</u>	<u>R2</u>	<u>R3</u>	<u>R4</u>	<u>R5</u>	<u>R6</u>
OA	H	H	CH3	H	H	H
DTX1	H	H	CH3	CH3	H	H
DTX2	H	H	H	H	CH3	H
DTX3	H	acyl	as parent	as parent	as parent	H
Diol-ester	diol	H/acyl	as parent	as parent	as parent	H
DTX4	Diol-ester	H	as parent	as parent	as parent	H
DTX5	Diol-ester	H	as parent	as parent	as parent	H
27-O-acyl	H	H	CH3	H	H	acyl

Figure 1.1.4. OA-group toxins derive from three main structures: OA, DTX1 and DTX2, all three independently produced by micro-algae. All other compounds are derivatives of these three, either identified in algae or shellfish. DTX3-type compounds are ester-derivatives of OA, DTX1 and -2 and have only been found in shellfish so far. The 27-O-acyl derivative has only been found in a sponge. DTX4 and -5 derivatives of OA, DTX1 and -2 have been found mainly in algae but recently also in shellfish. Stereochemistry at C31 and C35 was clarified by Larsen et al. (2007).

Different types of esters of OA and DTXs have been reported. In algae (so far mainly *P. lima* and *P. belizeanum*), esters of allylic diols with the carboxylic acid at C1 of OA and DTXs have been reported (Hu et al., 1992 and 1995; Yasumoto et al., 1989); these esters were named DTX4, -5 etc. When the algae enter shellfish through natural filter-feeding, it is believed that these esters are rapidly degraded (Vale, 2007). The shellfish then further metabolise OA and its analogues to form esters of OA and DTXs with fatty acids (at the C7-OH group); these esters were initially identified for DTX1 as shellfish derivatives (Yasumoto et al., 1985) and their toxicity has been described to be similar to the parent compounds, although the onset appears later in the intraperitoneal (ip) mouse model (Yanagi et al., 1989). A further fatty acid ester of DTX1 at the C27-OH group has been reported in a sponge (Britton et al., 2003), and most recently, Torgersen et al. (Torgersen et al., 2008) also reported mixed esters of diols (at the C1 carboxy-end) and fatty acids (at the C7-OH position) in shellfish, suggesting that partial degradation and simultaneous metabolism may co-occur during digestion of algae by shellfish. The multitude of compounds potentially present in shellfish (free toxins, diol esters and their derivatives, fatty acids and mixtures of diol- and fatty acid esters) leads to a difficulty in determining the complete toxin content in shellfish samples. This complexity has added to the difficulties in estimating the potency of these toxins and evaluation of their risk. The ester-bond has not shown any degradation in long-term stability studies in the authors' laboratory, however, fatty acids have been reported to oxidise easily if they contain double bonds. Any of the esters discussed above (either at the C1-carboxy or at the C7-

OH) group may be quantitatively cleaved through treatment with strong base, e.g. 0.3 molar methanolic NaOH at 76 °C for 10 to 40 min; this characteristic, in combination with the stability of the parent compounds (OA, DTX1 and -2) to base treatment, has been extensively used to quantitatively determine the equivalent of parent compound present in any given shellfish sample (Lee et al., 1989).

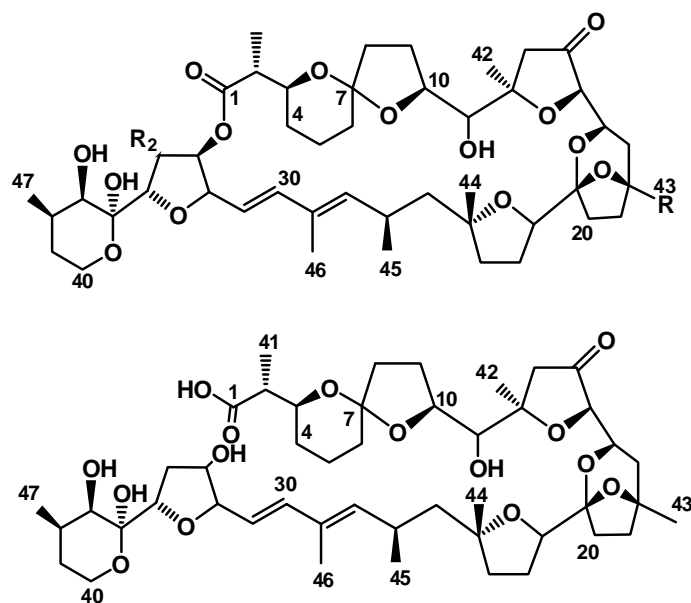
A recent review of recorded poisoning events suggests that esters of OA and DTXs have very similar toxicity to the parent compounds in human poisoning (EFSA, 2008). OA and DTX1 are considered to be of approximately equal toxicity when injected intraperitoneally into mice, while DTX2 has been reported to have only ca. 50 to 60 % of the toxicity of OA (Aune et al., 2007), both by ip injection into mice and by assessment of their inhibitory character towards phosphoprotein phosphatases.

Pectenotoxin group

Pectenotoxins (PTXs) are produced by *Dinophysis*, one of the main producers of OA and analogues. Pectenotoxin-2 (PTX2) is the main compound produced by *Dinophysis*. For this reason, PTXs have been associated initially with diarrhoeic poisoning, however, later studies clearly demonstrated that PTXs have a distinct mechanism of action which is different from that of OA and analogues.

PTXs are a group of polyethers with molecular weights similar to the OA and AZA-group, and PTXs also have two spiro-ketal ring assemblies. Contrarily to OA and AZA, active forms of PTXs represent a macrocyclic intra-molecular ester and do not possess a free carboxylic acid group (Fig. 5). Thus, most PTX2 behaves chromatographically like a neutral compound of high lipophilicity (Fux and Hess, unpublished observations). A comprehensive review of the occurrence, chemistry and shellfish metabolism of PTX analogues is given by Miles (2007). We describe here several analogues which exemplify the three main routes of metabolism in shellfish. In the Japanese scallop (*Patinopecten yessoensis*), PTX2 is successively metabolised to PTX1, -3 and finally PTX6 (Suzuki et al., 2005). In all these analogues the macrocycle is maintained, which means their lipophilicity is only slightly altered. On the contrary, in mussels (*Mytilus edulis*), PTX2 is metabolised to a seco-acid (PTX2sa), in which the macrocycle is opened (Miles et al., 2004). This ring opening is clearly related to a loss in the bioactivity, as PTX2sa shows no activity when injected i.p. in mice. A further route of metabolism in mussels is the esterification of PTX2sa with fatty acids to yield PTX2sa-fatty-acid-esters (Wilkins et al., 2006). Although the toxicity of these compounds has not yet been evaluated, it is anticipated that it is relatively reduced as the parent PTX2sa already does not show any toxicity.

Ito et al. (2008), also showed evidence for a reduced oral toxicity of PTX6, compared to PTX2, thereby suggesting that the main issue with pectenotoxins would be the presence of still un-metabolised PTX2. It is neither clear whether such remaining PTX2 is bioavailable (due to its high lipophilicity it may not be effectively liberated during human digestion of shellfish tissues) nor whether it withstands human digestive conditions. PTX2 has been shown to be rather labile, even under lightly acidic or lightly basic conditions and very rapid metabolism to non-toxic seco acids is likely if the compound is effectively liberated during digestion.



	R (C43)	C7
PTX-1:	CH ₂ OH	R
PTX-2:	CH ₃	R
PTX-3:	CHO	R
PTX-4:	CH ₂ OH	S
PTX-6:	COOH	R
PTX-7:	COOH	S
PTX-2 SA:		R
7-epi-PTX-2 SA:		S

Figure 1.1.5. Pectenotoxins: Upper structure shows the intact macrocycle (PTX1 – 7), lower structure shows the hydrolysed macrocycle (PTX2sa and 7-epi-PTX2sa).

Yessotoxin group

Yessotoxin (YTX) and analogues are also polycyclic ether compounds; special characteristics consist of the 11 contiguously transfused ether rings, an unsaturated side chain and 2 sulphate ester groups (Fig. 6). The contiguously transfused rings make YTX and analogues chemical relatives of brevetoxins and ciguatoxins; this structural characteristic has also led to the classification of ladder-shaped polyethers. Although this rigid structure constitutes a rather unpolar part of the molecule, YTX is considered of intermediate lipophilicity, as it also features 2 sulphate ester groups. The biogenetic origin, its chemistry, synthesis and structure-activity relationships of analogues have been recently reviewed by Hess and Aasen (2007).

YTX was first isolated from the digestive glands of scallops *Patinopecten yessoensis* in Japan (Murata et al., 1987). Because of its discovery through the mouse bioassay (MBA) originally developed by Yasumoto et al. (1978), for the detection of diarrhetic shellfish toxins, and due to its frequent co-occurrence with truly diarrhetic toxins, YTX was initially misclassified as one of the Diarrhetic Shellfish Poisoning (DSP) toxins. Later it was shown that YTX causes no diarrhetic effects when administered orally to mice (Aune et al., 2002; Tubaro et al., 2003 and 2004). Yessotoxin and its analogues are produced by the dinoflagellate algae *Protoceratium reticulatum* (Ciminiello et al., 2003; Samdal et al., 2004; Satake et al., 1997 and 1999), *Lingulodinium polyedrum* (Draisci et al., 1999) and recently reported to also be produced in *Gonyaulax spinifera* (Rhodes et al., 2006). Since the initial discovery of YTX several more analogues of YTXs have been discovered in many parts of the world including Japan, Norway, Italy, Scotland and Chile

(Draisci et al., 2000). Over the last years, this toxin group has been shown to contain a large number of analogues including 45-hydroxyYTX, carboxyYTX, 1- desulfoYTX, homoYTX, 45-hydroxyhomoYTX, carboxyhomo-YTX, heptanor-41-oxoYTX, heptanor-41-oxohomoYTX, trinorYTX, adriatoxin, (44-R,S)-44,55-dihydroxyYTX. 9-methylYTXs (Finch et al., 2005). In a paper by Miles et al. (2005a), numerous analogues of YTX in *P. reticulatum* are described.

Although different toxicities have been reported for YTX itself when using different mouse strains (Aune et al., 2002; Murata et al., 1987), crude estimates of relative toxicities can be obtained when using the same strain of mice for comparison of analogues, preferably in parallel. In this way, it is clear that YTX and homo-YTX have approximately the same toxicity (Satake et al., 1997), and that all other analogues have lesser toxicity than YTX, with hydroxyl and carboxy derivatives being approximately 5 times less toxic than the parent compounds. Some derivatives such as the trihydroxylated amides of 41-a-homo YTX and 1,3-enone isomer of heptanor-41-oxo YTX have not shown any toxicity by intraperitoneal injection into mice at levels > 5000 µg/kg bodyweight (Miles et al., 2004 and 2005b).

Another major pharmacological phenomenon directly related to the chemical structure is the large difference observed between toxicity of YTX in mice injected intraperitoneally and those orally exposed to YTX (Aune et al., 2002). This study showed that 2 out of 3 mice died when injected with a dose of 0.75 mg YTX / kg bodyweight and 3 of 3 mice injected with a dose of 1 mg YTX / kg bodyweight, while all mice survived when exposed orally to a dose of 10 mg/kg. Similar observations were made by Munday and coworkers (quoted in FAO, 2004). The difference in intraperitoneal and oral toxicity of YTX is probably related to low YTX absorption in the gastrointestinal (GI) tract. While the solubility of YTX in water facilitates bioavailability of YTX, it is probably also the reason for very short residence time in the gastro-intestinal tract, thus diminishing overall absorption. The large differences in toxicity between YTX and its oxidized analogues 45-hydroxy-YTX and carboxy-YTX are likely to be related to the further increase in water solubility. A different approach on determining the relationship between structure and activity was taken by Ferrari et al. (2004), where different YTX analogues were dosed onto cultured cells. The authors obtained different toxic equivalence factors with the 45-hydroxy and the 55-carboxy analogues being ca. 20-50 times less toxic. These differences could be related to the more complex toxicology in live animals or to differences in the standards used.

	R	n	m/z
YTX	H	1	1141
45-hydroxyYTX	H	1	1157
carboxyYTX	H	1	1173
1a-homoYTX	H	2	1155
45,46,47-trinorYTX	H	1	1101
ketoYTX	H	1	1047
40-epi-ketoYTX	H	1	1047
41a-homoYTX	H	1	1155
9-Me-41a-homo YTX	CH ₃	1	1169
44,55-dihydroxyYTX	H	1	1175
45-hydroxy-1a-homoYTX	H	2	1171
carboxy-1a-homoYTX	H	2	1187

Figure 1.1.6. Yessotoxins. Yessotoxins show an amazing diversity of structural variants. Hydroxy-, carboxy and hydroxy-carboxy-analogues have been identified as shellfish metabolites of the compounds produced by algae (figure adapted from Hess and Aasen, 2007).

Palytoxin group

With a continuous chain of 115 carbons, palytoxin (PITX) is one of the largest polyether-type phycotoxins (Fig. 7). The many hydroxyl groups in the molecule characterise it as a polyol and together with the amine and amide groups are responsible for its hydrophilicity. The long carbon chain constitutes a lipophilic part. Thus, PITX has a mixed hydrophilic and lipophilic character which also results in soap-like behaviour at larger concentrations in aqueous solutions. The structure of palytoxin was clarified in 1981 (Moore and Bartolini, 1981; Uemura et al., 198a and b). Recent reviews demonstrate that there is a lack of understanding on possible origins of PITX and related compounds (Katikou, 2008; Kita and Uemura, 2008). While the compound was originally reported from the coelenterate zoanthids *Palythoa toxica* (Moore and Scheuer, 1971) and *Palythoa tuberculosa* (Kimura et al., 1972), it is now clear that some micro-algae also produce PITX and a number of related compounds: *Ostreopsis siamensis* (Onuma et al., 1999), *Ostreopsis ovata* (Penna et al., 2005) and *Ostreopsis mascarenensis* (Lenoir et al., 2004).

Symbiotic micro-organisms have been postulated as the true source of PITX (Moore et al., 1982) and bacterial involvement is still not excluded (Frolova et al., 2000). This toxin group was traditionally associated with fish poisoning and aerosol problems in the tropics. More recently, *Ostreopsis spp.* as well as palytoxin and related compounds were also found in Southern Europe (Spain, Italy and Greece), mostly causing problems to bathing people at beaches on the Italian coast of Genoa (Ciminiello et al., 2008) and in mouse assays in shellfish from Greece (Aligizaki et al., 2008).

Cyclic Imines

Several compound groups have been found in this category: gymnodimines, spiroolides, pinnatoxins and pteriatoxins, pinnaic acids and halichlorines, prorocentrolides and symbio-imines (Hu et al., 1995; Lu et al., 2001; Seki et al., 1995; Takada et al., 2001; Uemura et al., 1995). A selection of chemical structures for some of these groups are shown in Figure 8. A common structural feature of all these compound groups is the hexa- or hepta-cyclic imine ring which is believed to contribute substantially to the bioactivity of these compounds. The opening of this ring has been related to loss of bioactivity at the example of spiroolides (Hu et al., 1996). However, this is not the only contributing factor as intricate stereochemical features may also play important roles, as demonstrated by McCauley et al. (1998), who were able to show that natural (+)-Pinnatoxin A was very toxic while synthetic (-)-Pinnatoxin A was non-toxic.

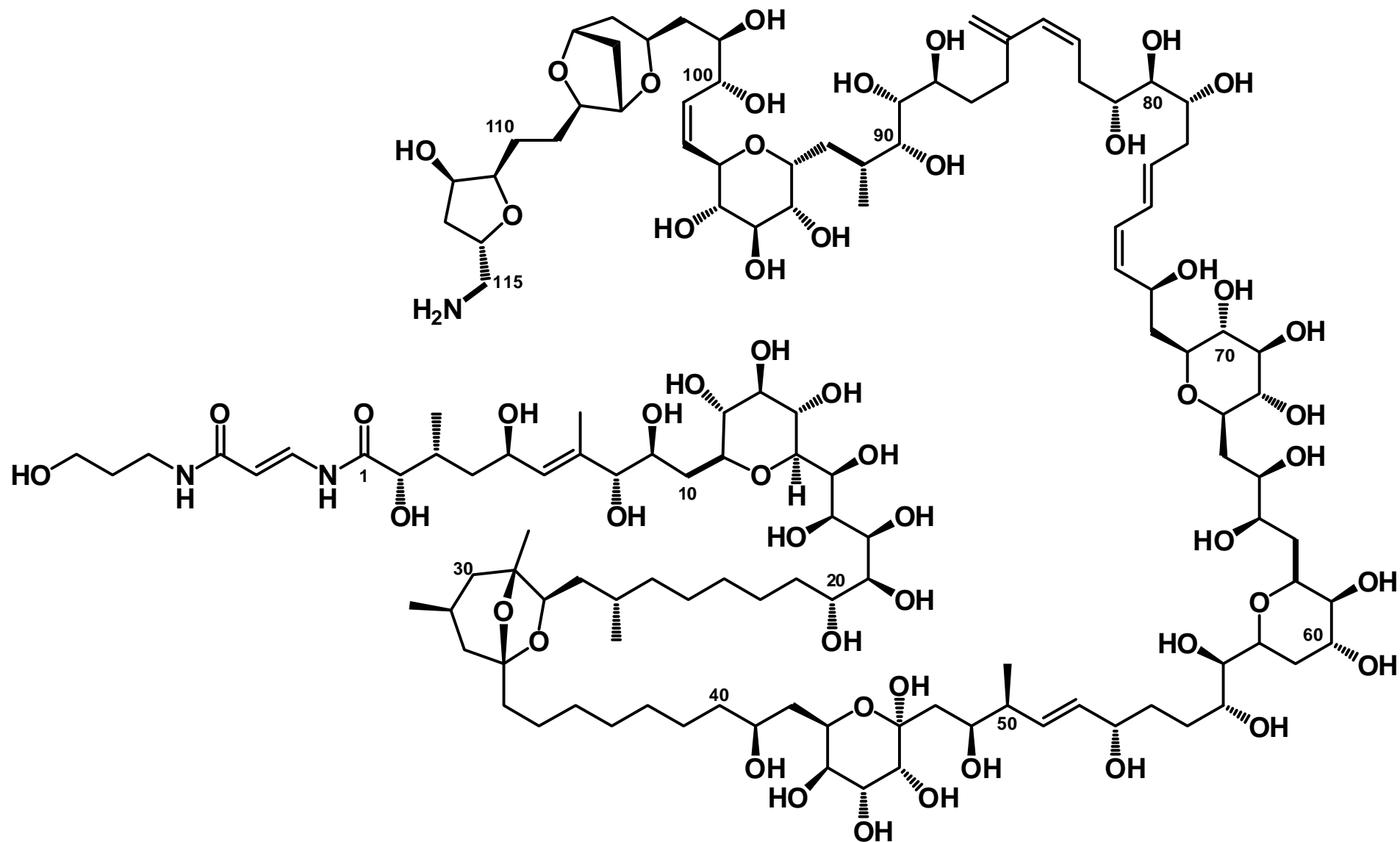


Figure 1.1.7. Palytoxin, originally isolated from the soft coral *Palythoa toxica*, later also found as a metabolite of benthic dinoflagellates of the genus *Ostreopsis* spp. One of the largest and most toxic, non-proteinaceous phycotoxins.

The unique neurotoxicity of cyclic imines is visible in mice following intraperitoneal injection of the toxin, which leads to rapid death with minutes; this feature has led to grouping cyclic imines together as “fast-acting toxins”. Due to the structural variety of the group, it is difficult to give physicochemical details in the frame of this chapter. Recent reviews give an overview of the chemistry and toxicology of these groups (Molgo et al., 2007; Cembella and Krock, 2008; Munday, 2008).

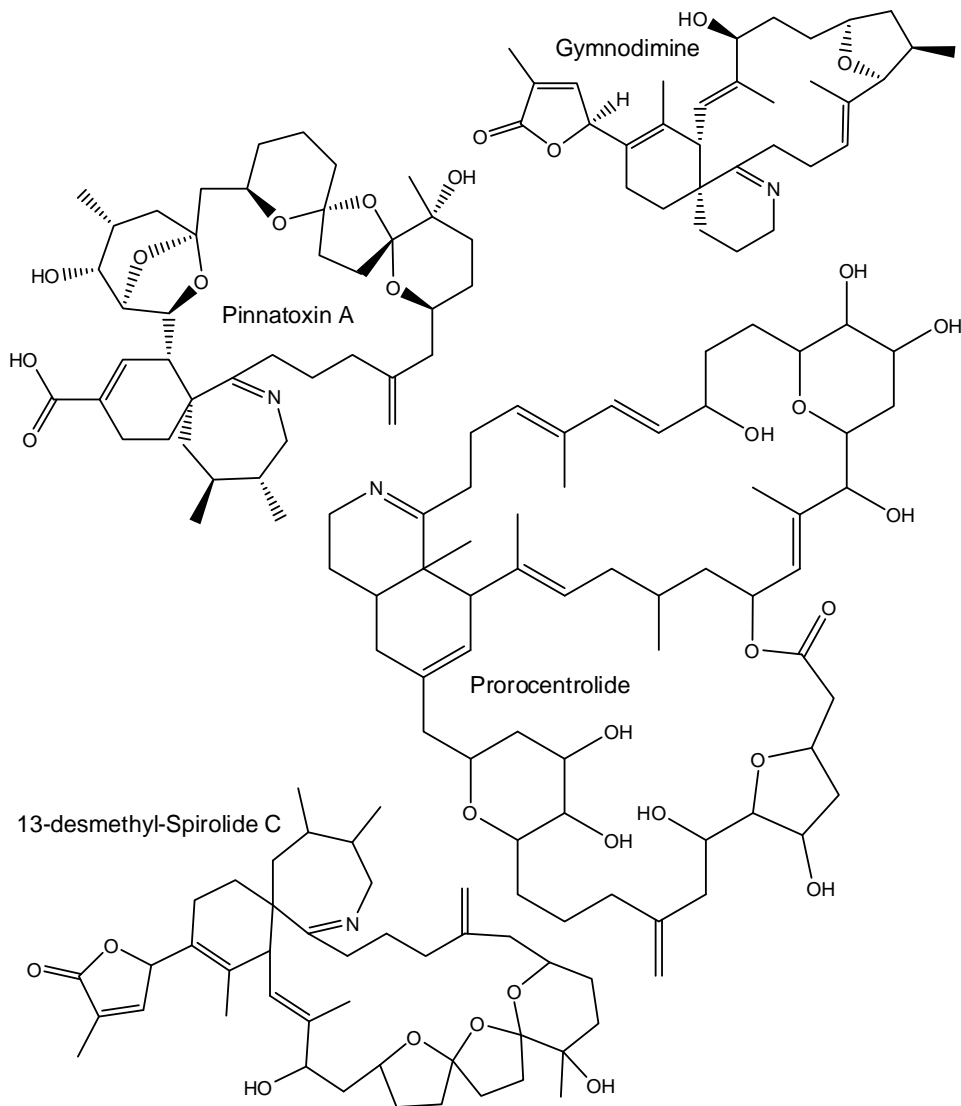


Figure 1.1.8. Cyclic imines. A variety of structures from different source organisms. All contain the cyclic imine function within their molecule, related to their fast acting toxicity in mice.

Brevetoxin group

Brevetoxins are a group of polyether toxins produced by the dinoflagellate *Karenia brevis*, and they also belong to the class of ladder-shaped polyethers, such as yessotoxin and ciguatoxin. Two types of abbreviations have been used (BTX and PbTX), sometimes leading to confusion. There is two basic skeletons (Fig. 9), a type-A skeleton, with 10 fused polyether rings, and a type-B skeleton with 11 fused polyether rings. BTX-A group compounds include the analogues PbTX-1, -7 and -10, while BTX-B compounds include PbTX-2, -3, -5, -6 and -9. The structure of PbTX-4 has never been confirmed, and PbTX-8 is an artefact from extraction procedures during the preparative isolation of brevetoxins (Baden et al., 2005). *K. brevis* had undergone a number of name changes and was previously referred to as *Gymnodinium brevis*, *Gymnodinium breve* and *Ptychodiscus brevis*, the latter name leading to the abbreviation PbTX. Interestingly, another *Karenia* species, *K. mikimotoi*, produces a related ladder-shaped polyether toxin, Gymnocin, structure not shown (Satake et al., 2002). PbTX-2 (= BTX-B) is the main analogue produced by *K. brevis* and tends to be the main analogue found in seawater during *K. brevis* blooms, however, it is rapidly transformed into the 10-times more toxic PbTX-3 (dihydro-PbTX-2), which is the main constituent in marine aerosols (Pierce et al., 2005). Brevetoxins had initially been only reported from US and the Mexican gulf, but have subsequently also been found in New Zealand waters.

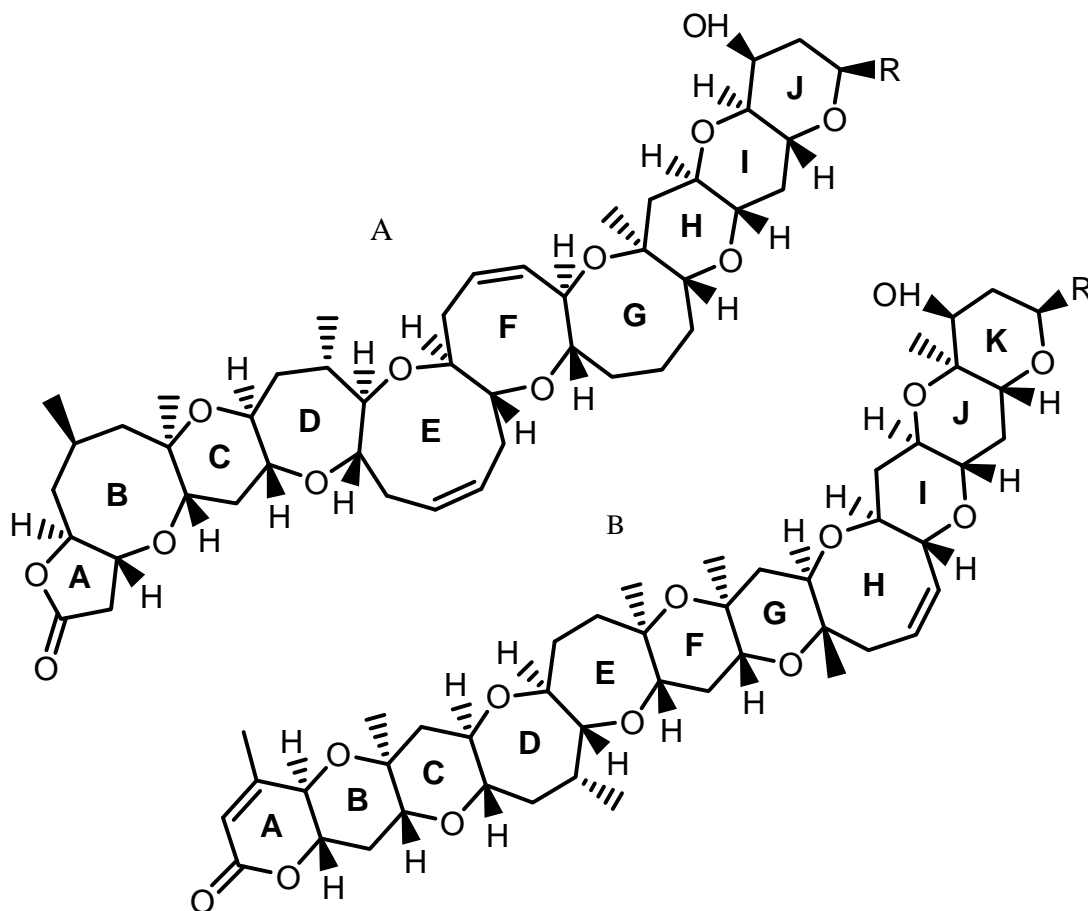


Figure 1.1.9. Brevetoxins are secondary metabolites of *Karenia brevis*, a dinoflagellate mainly reported from the Gulf of Mexico. For PbTX-1 and -2, the substituent R equals $\text{CH}_2\text{C}(\text{CH}_2)\text{CHO}$ in the A and B skeleton, respectively.

Although the illness is known since the mid 19th century, full structure elucidation was only possible during the 1980s (Alam et al., 1982; Lin et al., 1981; Shimizu et al., 1986). In Florida, *K. brevis* is known as a red tide organism, and the effects of the algae are three-fold: aerosol exposure leading to skin-damage and respiratory problems as well as accumulation in seafood leading to neurotoxic shellfish poisoning (see sections 3 and 4). While the brevetoxins produced by algae are very lipophilic compounds, some metabolites in shellfish have a slightly more hydrophilic character, due to the biotransformation to cystein-conjugates (Plakas et al., 2004; Wang et al., 2004). More recently, Abraham et al. (2006) also reported more polar metabolites from marine aerosols, in which the A-ring is opened, leading most likely to a reduced toxicity if the same structure activity relationship applies as found by Rein et al. (1994). Bourdelais et al. (2004) have isolated an interesting compound from *K. brevis*, namely brevenal. This compound is potentially a biosynthetic precursor to brevetoxins but has been shown to completely inhibit PbTX-action on Na-channels by competitive binding and is not toxic to fish. Dechraoui et al. (2006) have studied the binding of analogues of this group to voltage-gated Na-channels, further contributing to potential knowledge on the relative toxicity of analogues.

Ciguatera-related toxins

The toxins related to ciguatera fish poisoning comprise multiple groups. Although okadaic acid, palytoxin and other compounds have been implicated in some cases of ciguatera, we will focus here on those toxins that are part of the ciguatera complex that have not been described previously. Ciguatoxins, gambierol and maitotoxin are three groups of compounds among the toxic metabolites produced by *Gambierdiscus toxicus*, the main dinoflagellate responsible for contamination of fish by ciguatera toxins (Fig. 10). Maitotoxin is amphiphilic and is thus soluble in water, methanol and dimethylsulfoxide. It is relatively stable in alkaline but not in acidic conditions (Murata and Yasumoto, 2000). Maitotoxins as polyhydroxy-polyethers with two sulphate ester groups are amongst the more hydrophilic polyethers, do not migrate up the food web and are restricted to herbivorous fish (and potentially other grazing organisms). Gambierols and ciguatoxins are much more lipophilic polyethers and thus will persist and move up the food chain easier to predatory (piscivorous) fish. The CTX- analogue shown in Figure 9 is P-CTX-4B, one of the primary compounds produced by *Gambierdiscus toxicus* in the pacific. It appears to be a precursor to P-CTX-1 from Moray eel (Murata et al., 1989 and 1990), which is the major constituent in most piscivorous fish in the Pacific also frequently contributing > 90% to the overall toxic equivalents (Legrand et al., 1992; Lewis et al., 1991). A number of additional, often minor, analogues were isolated from the Indian, Pacific and Caribbean oceans (Murata et al., 1989 and 1990; Lewis et al., 1991; Poli et al., 1997; Satake et al., 1993, 1997 and 1998). The very low doses, which may already cause problems to consumers, result in challenges of ultra-trace detection in the range from 0.1 µg/kg to several µg/kg. For this reason, there are few methods available for the detection of these toxins, and worldwide, there are only few groups capable of analysis of ciguatoxins, mainly located in Canada, US, Japan and Australia (Dickey, 2008). Preference of *G. toxicus* to warm water temperatures was demonstrated through correlation of sea surface temperatures with the occurrence of the organism (Chateau-Degat et al., 2005). Thus ciguatoxins are currently restricted to tropical and subtropical latitudes, however, distribution may well increase through raising sea surface temperatures in many areas. They are globally distributed across the Indian, Pacific and Caribbean oceans. A recent review by Dickey (2008) describes difficulties in the analysis and diagnosis of this complex illness. The organism *G. toxicus* was discovered by Yasumoto et al. (1977) and first described by Adachi and Fukuyo (1979).

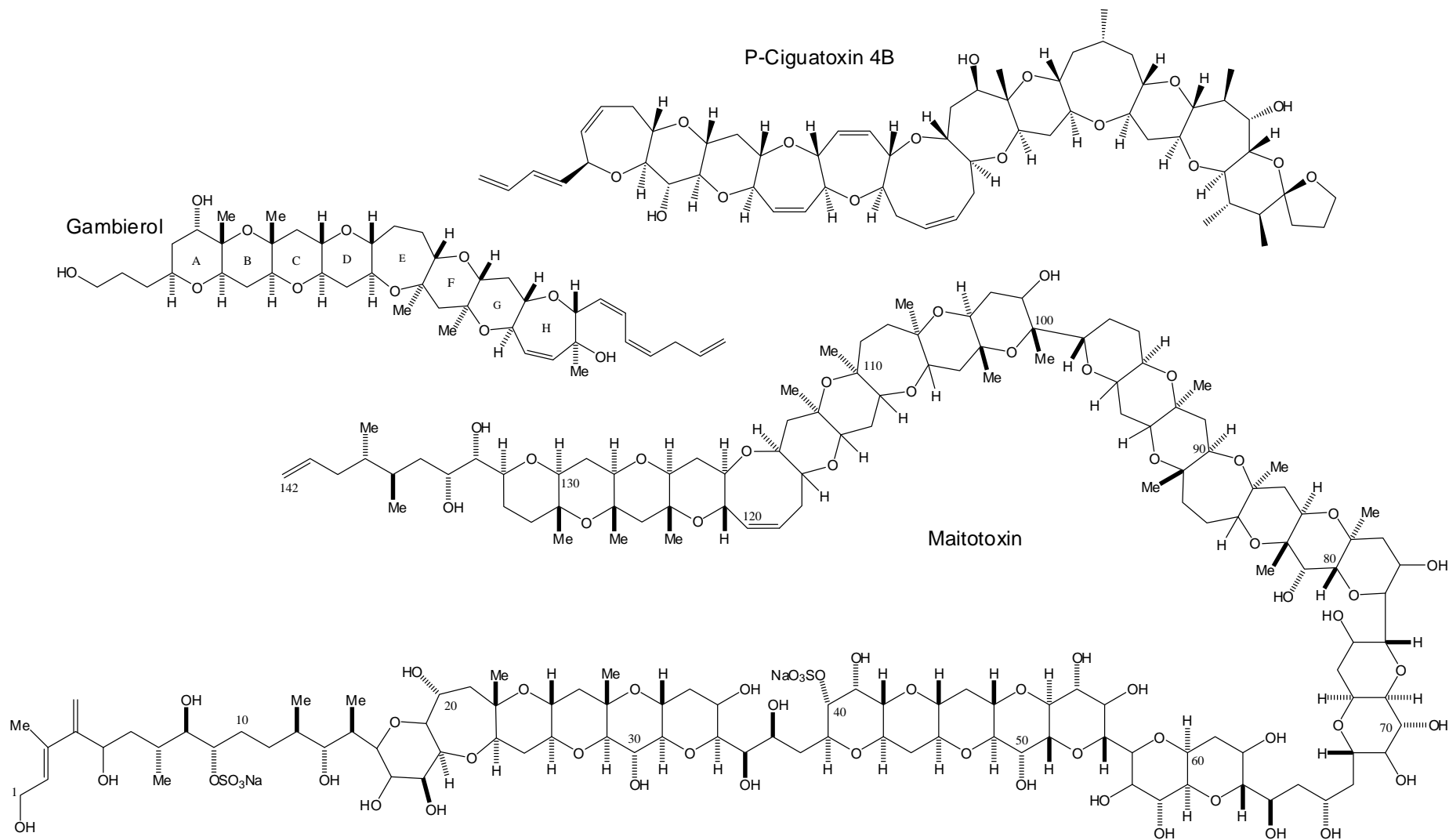


Figure 1.1.10. Toxins produced by the benthic dinoflagellate *Gambierdiscus toxicus*. While ciguatoxin and gambierol are very lipophilic compounds, maitotoxin is water-soluble and does not biomagnify in the marine food web. Maitotoxin is the largest non-proteinaceous marine toxin.

4.1.1.3. Shellfish poisoning

The route of human exposure to phycotoxins most often is oral, through ingestion of food contaminated with toxins. In some instances, however, living beings may become exposed to phycotoxins through other routes, as in the case of breathing aerosols containing toxins and/or direct skin contact with toxins. In this section, major symptoms are summarised and, whenever available, possible remedies to the most relevant poisonings that may occur in humans exposed to phycotoxins, independently of the routes of toxin entry into the body. The poisonings have been classified mostly on the basis of recorded symptoms, and our description will follow the existing classification.

The methods used for the detection of phycotoxins have historically been mainly influenced by the lack of knowledge of the exact causative agents. Without exact knowledge of all toxicologically relevant chemical entities, it is difficult to develop and validate specific, quantitative methods of analysis. In the early stages of test development, it was not even clear whether illness was caused by chemical or microbiological agents (Virchow, 1885; Wolff, 1887). Due to this lack of knowledge on the causative agents, early classifications of shellfish poisons were based on the symptoms experienced by humans following consumption of contaminated shellfish. Four categories are distinguished:

- Paralytic shellfish poisoning (caused by saxitoxins and tetrodotoxin)
- Neurotoxic shellfish poisoning (caused by brevetoxins)
- Diarrheic shellfish poisoning (caused by okadaic acid)
- Amnesic shellfish poisoning (caused by domoic acid)

Recently, azaspiracid shellfish poisoning was discovered as a fifth category of shellfish poisoning (McMahon and Silke, 1996; Satake et al., 1998), the symptoms resemble those of diarrheic shellfish poisoning. Ciguatera and tetrodotoxin poisoning are other types of diseases associated with seafood but these illnesses mostly arise from the consumption of fish, and are not further discussed in this context.

The exposure route for shellfish poisoning is through the consumption of shellfish. However, other routes of exposure such as through skin contact and inhalation have been observed for specific algal toxins, these include mainly brevetoxins and palytoxins. The main interest in this introduction is on the exposure through consumption of molluscan bivalve shellfish. From a medical point of view, it is now clear that the symptoms of these poisoning syndromes can be easily distinguished from microbiological poisoning by bacteria or viruses through the earlier onset: most bacterial or viral infections require incubation periods of 12 to 24 h before sickness is experienced by shellfish consumers, while illness from shellfish toxins typically occurs as early as 30 min after consumption (in case of STX or TTX) or 2 to 4 h (for most of the other compound groups).

Paralytic shellfish poisoning (PSP) has been reported worldwide (FAO, 2004a). Mild symptoms include altered perception (burning or tingling sensation and numbness of the lips, that can spread to the face and neck), headache, dizziness and nausea. More severe symptoms include incoherent speech, a progression of altered perception to arms and legs, a progressive loss in the coordination of limbs, and general weakness. Respiratory difficulty is a late symptom, as a consequence of muscular paralysis progressing in the whole body, and death may be the outcome of PSP by respiratory paralysis (Gessner and McLaughlin, 2008).

Brevetoxins are the causative agents of neurotoxic shellfish poisoning (NSP), that may ensue after both inhaling aerosol containing the toxins and as a consequence of ingestion of contaminated seafood. When poisoning is through the respiratory tract, the exposure usually occurs on or near the waters where a bloom of PbTX producers has developed. NSP has been recorded primarily in the southeastern coast of the United States, the Gulf of Mexico, and New Zealand (FAO, 2004a; Gessner and McLaughlin, 2008; Ishida et al., 1996). The symptoms due to contaminated shellfish appear after minutes/hours from its ingestion, and are more severe than those found when contaminated aerosol is involved. In the former case, symptoms are both gastro-intestinal (nausea, diarrhea, and abdominal pain) and neurological (circumoral paresthesia and hot/cold temperature reversal). In more severe cases, the muscular system (altered heart contractions, convulsions, and respiratory difficulties) may be affected. Death from NSP has never been reported in humans and, symptoms resolve within a few days after exposure to the toxins (FAO, 2004a; Gessner and McLaughlin, 2008).

The contamination of seafood by OA and related compounds is very common in European and Asia-Pacific Countries (FAO, 2004a). The symptoms of DSP appear within 1 hr from ingestion of contaminated seafood, and affect the gastro-intestinal tract with nausea, vomiting, abdominal cramps and diarrhea (FAO, 2004a). The symptoms do not last long and usually disappear within a few days. No death has been recorded due to DSP.

The symptoms due to ingestion of domoic acid contaminating shellfish appear within the first few hours from its ingestion, and in most severe cases, may persist for months (Perl et al., 1990; Quilliam and Wright, 1989; Teitelbaum et al., 1990). Initial symptoms affect the gastro-intestinal tract with nausea, vomiting, abdominal cramps and diarrhea. These are followed by headache and other neurological symptoms, that often result in disturbances to memory, an effect that has led to the naming to this shellfish poisoning. In most severe cases, death may ensue. The neurological symptoms of ASP have been shown to evolve in the weeks (months) following poisoning, and anterograde memory disturbances can be accompanied by confusion, disorientation, peripheral nerve damage and changes in memory threshold.

The symptoms of azaspiracid poisoning in humans are very similar to those described for DSP, including nausea, vomiting, abdominal cramps and diarrhea, that disappear within a few days from the ingestion of contaminated shellfish (McMahon and Silke, 1996).

Overall, it is difficult to assess the true occurrence of shellfish poisoning in the human population, as for most diseases. Gastrointestinal disturbance as such is not a notifiable disease in many countries, and due to the rapid disappearance of the gastro-intestinal symptoms many shellfish consumers do not even declare the illness to a medical doctor. However, in some cases, in particular when many people get sick from the consumption of traceable lots of shellfish, the illnesses can be properly diagnosed as shellfish poisoning. It is mostly these cases that are used in the assessment of how much toxin will start to cause symptoms in shellfish consumers. In US, during the period from 1990 to 1998, PSP outbreaks were responsible for about 20% of seafood borne diseases traced to molluscan shellfish (FAO, 2004b). Frequent low incidences of shellfish toxins, many of which are not reported or under-reported, are sometimes overshadowed by large-scale incidences where several tens or hundreds of people become ill (see also Table 3).

Table 1.1.3. Examples of shellfish poisoning incidents. Note that no new toxin groups have been reported since the discovery of azaspiracids in 1995 (Hess P., 2008). Large-scale poisoning events for okadaic acid group toxins have still occurred during the last decade despite the toxic algae and toxins involved being known for over 20 years.

POISONING	NO. OF CASES	SHELLFISH SPECIES	LOCATION OF ILLNESS	REFERENCE
DSP	164	MUSSELS AND SCALLOPS	JAPAN	YASUMOTO ET AL., 1978
DSP	> 300	BLUE MUSSELS (<i>M. EDULIS</i>)	NORWAY, SWEDEN	UNDERDAHL ET AL., 1985
ASP	107	BLUE MUSSELS (<i>M. EDULIS</i>)	CANADA	PERL ET AL., 1990
PSP	187	CLAMS (<i>A. KINDERMANII</i>)	GUATEMALA	RODRIQUE ET AL., 1990
NSP	48	EASTERN OYSTER (<i>C. VIRGINICA</i>)	UNITED STATES	MORRIS ET AL., 1991
AZP	24	BLUE MUSSELS (<i>M. EDULIS</i>)	IRELAND	MCMAHON AND SILKE, 1998
DSP	> 300	BLUE MUSSELS (<i>M. EDULIS</i>)	BELGIUM	DE SCHRIJVER ET AL., 2002
DSP	200	BROWN CRAB (<i>C. PAGURUS</i>)	NORWAY	AUNE ET AL., 2006
DSP	159	BLUE MUSSELS (<i>M. EDULIS</i>)	UNITED KINGDOM	UK COT, 2006

Uncertainties existing on the risks posed by some phycotoxins

We are not aware of cases of human intoxication that can be attributed with certainty to ingestion of food contaminated with either YTX, or PTX, or cyclic imines.

Animal studies aimed at characterizing the toxicity of these three groups of compounds have shown that symptoms of acute toxicity can be detected only after oral administration of high doses (in the mg/kg range) of those compounds (Munday, 2008; EFSA, 2009). Thus, the inclusion of YTX, PTX, and cyclic imines among toxins that can pose risks to the consumer through ingestion of contaminated seafood is debated.

4.1.1.4. Biological targets and the mechanism of action of phycotoxins

In biology, if a compound exerts a desired effect, it is called a medicine or drug (pharmacology), if the compound causes undesirable effects it is referred to as a toxic compound or toxin (toxicology). All medicines also have undesired effects (at a certain dose) and thus the consideration of whether a compound is a toxin or a drug depends very much on the dose level considered (Paracelsus). Alfred J. Clarke (1885 – 1941) has been recognised for the introduction of the receptor concept in pharmacology, i.e. the idea that the effect of a compound in biological systems is related to its interaction with a specific biomolecule. This interaction is also referred to as mode or mechanism of action, and includes mention of the specific molecular targets to which the compound binds, such as an enzyme or receptor. For example, the mechanism of action of aspirin involves irreversible inhibition of the enzyme cyclooxygenase, which suppresses the production of prostaglandins and thromboxanes, thereby reducing pain and inflammation and making it an effective drug. However, at larger concentrations or repeat dosing, aspirin may also cause stomach bleeding, which means it could be considered a toxic substance.

Biological targets are complex biopolymers such as proteins or nucleic acids and can be classified into broad categories such as compounds bound to membranes or those in the cytosol. Membrane-bound targets include receptors and ion channels as both are integrated into the cellular membrane. Cytosolic targets mainly include enzymes as well as type I nuclear receptors. Finally, DNA itself can also be a target, in which case effects would include mutagenic activity.

Among membrane-bound receptors, transmembrane receptors are most common. These receptors have intra- and extracellular components and can be classified into the number of times they cross the membrane. The group of seven transmembrane (7TM) or G-protein receptors (GPCRs) are metabotropic receptors, e.g. the histamine receptor or the receptor of γ -Aminobutyric Acid (GABA).

According to Landry and Gies (2008), biological targets can be classified according to their cellular function location, i.e. enzymes, membrane-transporters, voltage-gated and ligand-gated ion channels, receptors with intrinsic enzyme activity, receptors coupled to cytosolic proteins, G-protein-couple receptors, which are generally considered to be the largest class of receptors, and finally nuclear receptors.

Depending on their functions and ligands, several types of receptors may be classified as follows:

- Some receptor proteins are peripheral membrane proteins.
- Many hormone and neurotransmitter receptors are transmembrane proteins: transmembrane receptors are embedded in the phospholipid bilayer of cell membranes, that allow the activation of signal transduction pathways in response to the activation by the binding molecule, or ligand.
 - Metabotropic receptors are coupled to G proteins and affect the cell indirectly through enzymes which control ion channels.
 - Ionotropic receptors (also known as ligand-gated ion channels) contain a central pore which opens in response to the binding of ligand.
- Another major class of receptors are intracellular proteins such as those for steroid and intracrine peptide hormone receptors. These receptors often can enter the cell nucleus and modulate gene expression in response to the activation by the ligand.

The external stimulus or ligand physically binds to the biological target. The interaction between the substance and the target may be:

- noncovalent
- reversible covalent - A chemical reaction occurs between the stimulus and target in which the stimulus becomes chemically bonded to the target, but the reverse reaction also readily occurs in which the bond can be broken.
- irreversible covalent - The stimulus is permanently bound to the target through irreversible chemical bond formation.

Depending on the nature of the stimulus, the following can occur:

- There is no direct change in the biological target, except that the binding of the substance prevents other endogenous substances such as activating hormone to bind to the target. Depending on the nature of the target, this effect is referred as receptor antagonism, enzyme inhibition, or ion channel blockade.
- A conformational change in the target is induced by the stimulus which results in a change in target function. This change in function can mimic the effect of the endogenous substance in which case the effect is referred to as receptor agonism (or channel or enzyme

activation) or be the opposite of the endogenous substance which in the case of receptors is referred to as inverse agonism.

Currently, over 600 biological targets are known in pharmacology, and this number has been exponentially rising over the last couple of decades. While interactions of small molecules with biological targets had been studied intensively since the beginnings of pharmacology, more recently, the interactions of protein ligands with biomolecules have increasingly been revealed.

As could be expected from chemically diverse groups of compounds, different groups of marine biotoxins interact with many distinct biological targets. For several groups of toxins the targets are known (e.g. BTX, DA, OA, STX) but for many others full elucidation of the biological activity at molecular level still remains to be completed (AZA, YTX etc.).

Many marine biotoxins have been shown to interact primarily (but not exclusively) with ion channels (BTX, CTX, cyclic imines, DA, STX, TTX), as reviewed by Rossini and Hess, 2009. As many basic biological processes depend on the maintenance of specific ion gradients across the plasma membrane an alteration of the intracellular concentration of one ion can trigger a secondary change in the intracellular concentrations of other ions in the course of the molecular events triggered by the interaction of a toxin with its receptor. Through their action on ion channels, these toxins disturb the normal functioning of excitable cells and tissues such as nervous tissue, and are thus often referred to as neurotoxins.

Several of the abovementioned toxins interact with the voltage-gated sodium channel (VGNC). Interestingly, some of the toxins (STX and TTX) interact with the same part of the ion channel, site 1 of the α -subunit (Hartshorne and Catterall, 1984). Even though their chemical structure is very different, their effect is the same: blocking of the ion transport (Hill, 1968 and 1975). BTXs and CTXs are larger molecules and interact with site 5 of the VGNC, and their effects lead to enhanced ion transport across the cell membrane (Castèle and Catterall, 2000; Lombert et al., 1987). Still, both interactions result in neuronal malfunctioning and paralytic effects. Different toxins produced by the same algae may also have different mechanisms of action. Thus, maitotoxin (MTX) and gambierol (Gb) are also bioactives produced by *Gambierdiscus* toxins (the dinoflagellate known to produce CTX), however, unlike CTX, MTX interacts with non-selective cation channel (NSCC), while Gb interacts with the voltage-gated potassium channel (VGKC). Palytoxin has been shown to interact with the Na^+/K^+ -ATPase (Habermann et al., 1981; Redondo et al., 1996).

The mechanism of action of domoic acid (DA) involves toxin binding to non-N-methyl-D-aspartate (non-NMDA) glutamate receptors. Glutamate is one of the major neurotransmitters in the brain (Meldrum, 2000), and its importance in normal functioning of the central nervous system has been a primary drive for the extensive investigations on the mechanism of action of DA. DA binds to non-NMDA receptors in several regions of the central nervous system, and the effects ensue from a coordinated and synergistic action of receptors functioning at the two sides of the synapsis, resulting in altered neurotransmission (reviewed by Quilliam et al., 1995; Doucette and Tasker, 2008; Pulido, 2008). Non-NMDA receptors include α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors, that represent ligand-dependent ion channels, and the binding of DA determines the opening of the channels (Ozawa et al., 1998; Lerma et al., 2001). When DA binds to AMPA channels, influx of Na^+ into the cell occurs, whereas influx of extracellular Ca^{2+} ions into the cell is induced by DA binding to kainate receptors (Ozawa et al., 1998; Lerma et al., 2001). The molecular mechanism of action of DA, however, may not be reduced to these two receptor systems, because other ion channels and receptors are recognized to participate to the response induced by the toxin (Rossini and Hess, 2009).

OA and its analogues have been demonstrated to interact with serine/threonine phosphoprotein phosphatases (PPase), and the 2A-isoform has been shown to be specifically sensitive to this interaction (Bialojan et al., 1988; Takai et al., 1991). A recent study (Larsen et al., 2007) on the isolation of DTX1 and -2 and their stereochemical characterisation using NMR has enabled molecular modelling of their interaction with PP2A and our understanding of their differential activity compared to OA, as observed *in vitro* and *in vivo* by Aune et al., 2007.

Several toxins have also been shown to interact with cytoskeletal constituents (PTX) and cell-adhesion proteins (AZA, YTX). Thus, PTX has been demonstrated to form a 1:1 complex with F-actin, the alteration of which could explain the cell death observed in many cell lines affected by PTX. Both AZA and YTX have been shown to affect E-cadherin, a cell-adhesion protein, which explains the disruption of epithelial cell layers observed in experiments measuring the trans-epithelial electrical resistance. However, it remains questionable whether these effects are the only ones (or even the major ones) caused by those toxins and *in-depth* review suggests the need for further studies to clarify the mechanism(s) of action (Rossini and Hess, 2009).

4.1.2. Risk evaluation and management of shellfish toxins

4.1.2.1. Difficulties in protecting the consumer

As mentioned above, shellfish toxins pose particular problems to public health protection due to a number of differences compared to other contaminants. In particular, the lack of prediction capability of the occurrence of shellfish toxins is a major complicating factor. In combination with the requirement to produce live bivalve molluscs this results in the need for continued monitoring of shellfish harvesting areas. To illustrate the difficulties encountered in protecting the consumer from the risk of shellfish toxins, several scenarios can be examined. The accumulation of toxins in blue mussels has been followed using the MBA and an LC-MS based method in parallel (Fig.1). This event could be described as a classical event, as it involves toxins that have been reported to make shellfish consumers sick at levels incurred during the event (in this case, no sickness occurred as monitoring results were known to regulatory authorities immediately, and closure of the harvest areas prevented any risk to the public). Part c of Fig.1 outlines the results of the MBA, which is the regulatory test in many countries, including Ireland. It is apparent that using the MBA, the toxicity appears without warning, i.e. from the week of 9th to 16th of July 2001. If the chemical monitoring which was ongoing in parallel had not already indicated low levels of toxins of the AZA-group (see part a) of Fig. 1), the area may only have been closed on the 18th of July 2001, i.e. 9 days from the last “non-toxic” sample date (weekly sampling plus 48 h from the sample taken to the result obtained). This would have resulted in harvesting of the area for probably 3-5 days with high toxicity present in shellfish which may have led to illness if these shellfish had been marketed. The rapid accumulation of shellfish toxins is a phenomenon which is often underestimated and may lead to severe public health problems as well as to significant economic losses if end-product testing is carried out efficiently and timely.

In the same graph, it is also apparent that two shellfish toxin groups may co-occur independently, in this case OA-group toxins and AZA-group toxins. This co-occurrence may be governed by hydrographic and environmental conditions but it is not necessarily reproducible, as shown by comparison of Fig.1 with Fig. 2 (2001/2 versus 2008/9). Due to the possible co-occurrence of

several toxin groups, the methods used in official control of harvesting areas must be comprehensive. The mouse bioassay is capable of detecting both OA and AZA-group toxins, as is the LC-MS based method. If an AZA-specific ELISA had been the only technique used to detect toxins, the toxicity of the OA-group could have been neglected, and the shellfish growing area could have been reopened prematurely in January 2002 for the 2001 event (Fig.1). Similarly, if only a protein phosphatase assay (specific for OA-group toxins) had been used in official control of the harvesting area in Ireland in 2008, the area would have remained open in the month of June; yet, serious illness would have befallen the consumers of the shellfish due to the presence of AZA-group toxins (Fig. 2). Therefore, shellfish producers and official control authorities need to know all the agents potentially causing hazards in specific areas such that methods appropriate for public health protection can be implemented.

Another aspect of shellfish area management is also illustrated in Figures 1 to 2: the natural detoxification of shellfish in the growing area is significantly slower than the accumulation period. Thus, although the presence of (potentially) toxic algae may only last several days or weeks, the toxicity may persist in shellfish for many months after the algal bloom has disappeared. In this case, toxicity was still above threshold at 6 months after the algal appearance. These prolonged closure periods are potentially a problem for public health protection authorities, as they involve much effort in risk communication; many consumers in shellfish producing countries would be aware of the occurrence of shellfish toxicity during summer months, however, prolonged toxicity into winter months is a more recent phenomenon and requires additional efforts in managing the risks. In addition, it has been noted that detoxification rates are higher in the beginning of the detoxification, and the very slow detoxification over prolonged periods causes many problems to shellfish producers. Therefore, competent authorities also frequently face further difficulties in effectively implementing closure of production areas over these long periods.

4.1.2.2. Challenges in the production of safe shellfish

The challenges in the production of shellfish are multiple. Production efficiency is related to environmental parameters and local conditions as well as production mode and implementation. In addition, natural factors such as summer mortality, shellfish pathogens and storm conditions may significantly reduce annual production by up to 80 % in some years. Further food safety risks also arise at a high level from microbiological human pathogens, such as viruses (in particular norovirus and hepatitis A virus) and bacteria (in particular vibrios). While official microbiological classification of harvest areas results in a continuous cost of producing safe live bivalve molluscs (in class B or moderately polluted areas), peak occurrences of pathogens may also lead to unpredictable closures reducing the productivity of a given harvest area.

With the exception of oysters, bivalve molluscs as a raw product are considered low-cost food in most countries; typical prices being less than 1000 €/metric ton at production level. This is very much in contrast with many other foods, e.g. crustaceans such as lobster or crab, which may easily yield 5 to 10 times higher income to producers, even though meat yield may be very similar. In view of this low value, end-product testing of shellfish safety becomes a major challenge. In the production of shellfish, the price for a single end-product test may be around 100 to 200 €. As algae occur often in thin layers, and with patchy structures, production lots may often be contaminated very inhomogeneously. Depending on what would be considered a representative number of tests to conduct per production batch, proper end-product testing could cost as much as 10 % of the total product value.

A recent in-depth review of the Irish rope mussel sector (BIM, March 2006) can serve as an example of the economic status of the bivalve mollusc sector. This report indicates that profit margins varied between 1 and 8 % on average (for the years 2003 to 2005), depending on the production year. The number one factor influencing productivity and profitability of the rope mussels sector are marine biotoxins. This also means that in years of high biotoxin occurrence, some producers will invariably be in red figures. In addition, the structure of the shellfish industry is still dominated by a large percentage of small and medium sized enterprises. Therefore, if biotoxins occur at high levels during consecutive years, some small producers will risk bankruptcy due to lack of income.

Apart from these economic boundaries in which the shellfish producers operate, there is also challenges associated with emerging toxins and the type of testing used in official control. As mentioned above, official control has historically relied upon animal testing to assess the toxicity present in shellfish samples. Over the years, the compound groups responsible for causing shellfish poisoning have been identified, yet due to lack of pure compounds toxin-group specific methods have not been implemented as official methods for most toxin groups (apart from DA and STX). The scenario shown in Fig. 3 exemplifies differences in the interpretation of toxin events, depending on the method used in the official control of harvesting areas. In this area, okadaic acid-group toxins known to cause human poisoning exceed the regulatory limit only during one week during the summer of 2005. Other toxin groups have also been monitored, including pectenotoxins, which occurred at very low levels, and always below regulatory levels. Additionally, all other regulated lipophilic toxins (YTXs and AZAs) and known non-regulated bioactive compounds (gymnodimines and spirolides) were either totally absent or present at levels more than 10-fold lower than the regulatory limits. Yet, the MBA as reference test gave many positive results for the area over the whole summer period. In fact, the area could not be opened during summer 2005 due to the sporadic occurrence of positive results.

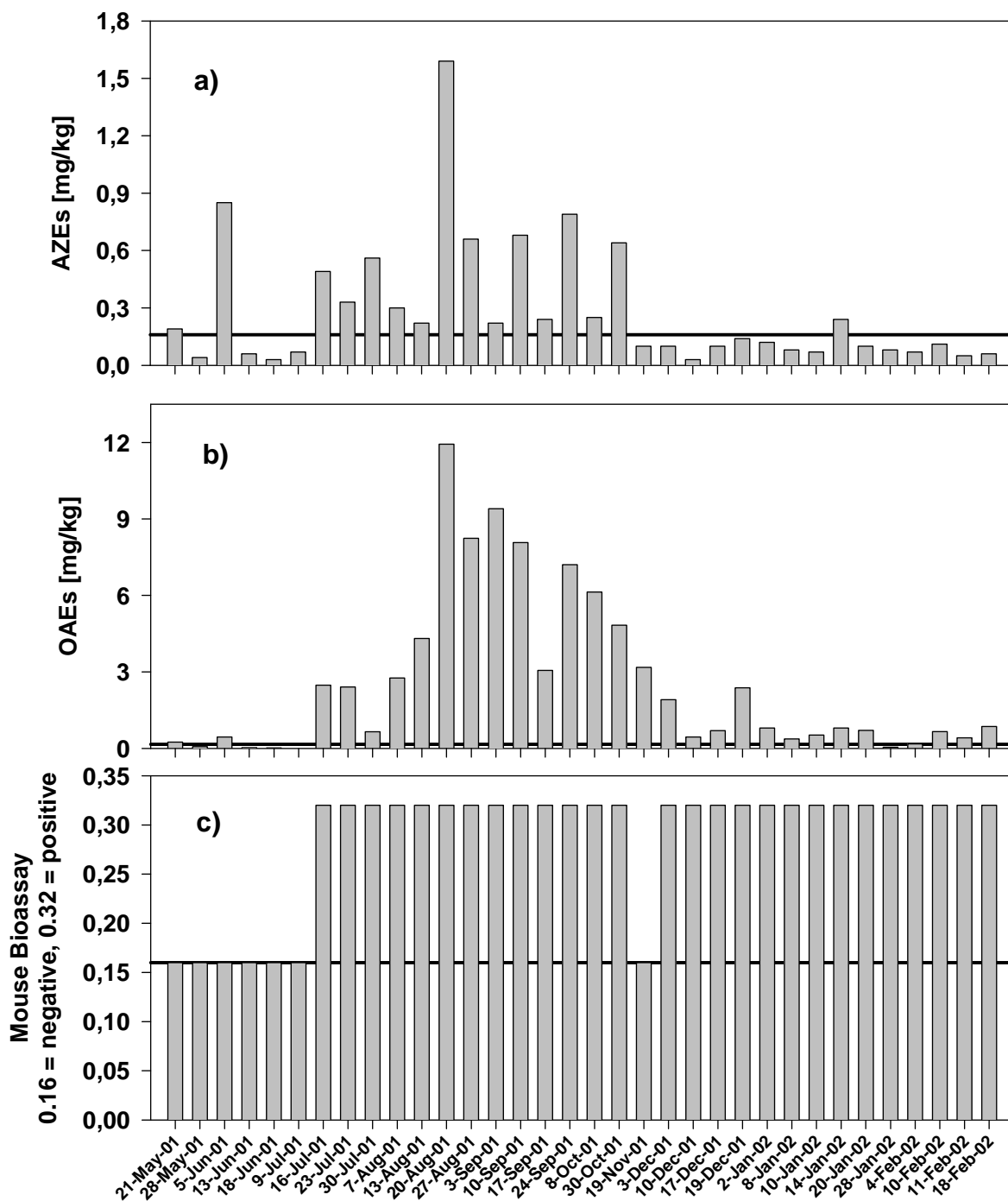


Figure 1.2.1. Temporal trends of azaspiracid group toxins, okadaic acid group toxins and mouse bioassay results in mussels (*M. edulis*) from Castletownbere, southwest Ireland from May 2001 to February 2002; a) azaspiracid-1 equivalents (AZEs) and b) okadaic acid equivalents (OAEs), both determined by LC-MS and measured and expressed in whole shellfish flesh, c) mouse bioassay (MBA) results for the same samples measured in hepatopancreas. The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and AZA-group toxins. Arbitrarily, and for visualisation purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives are represented as 0.32 mg/kg. (Figure adapted from Hess et al., 2003)

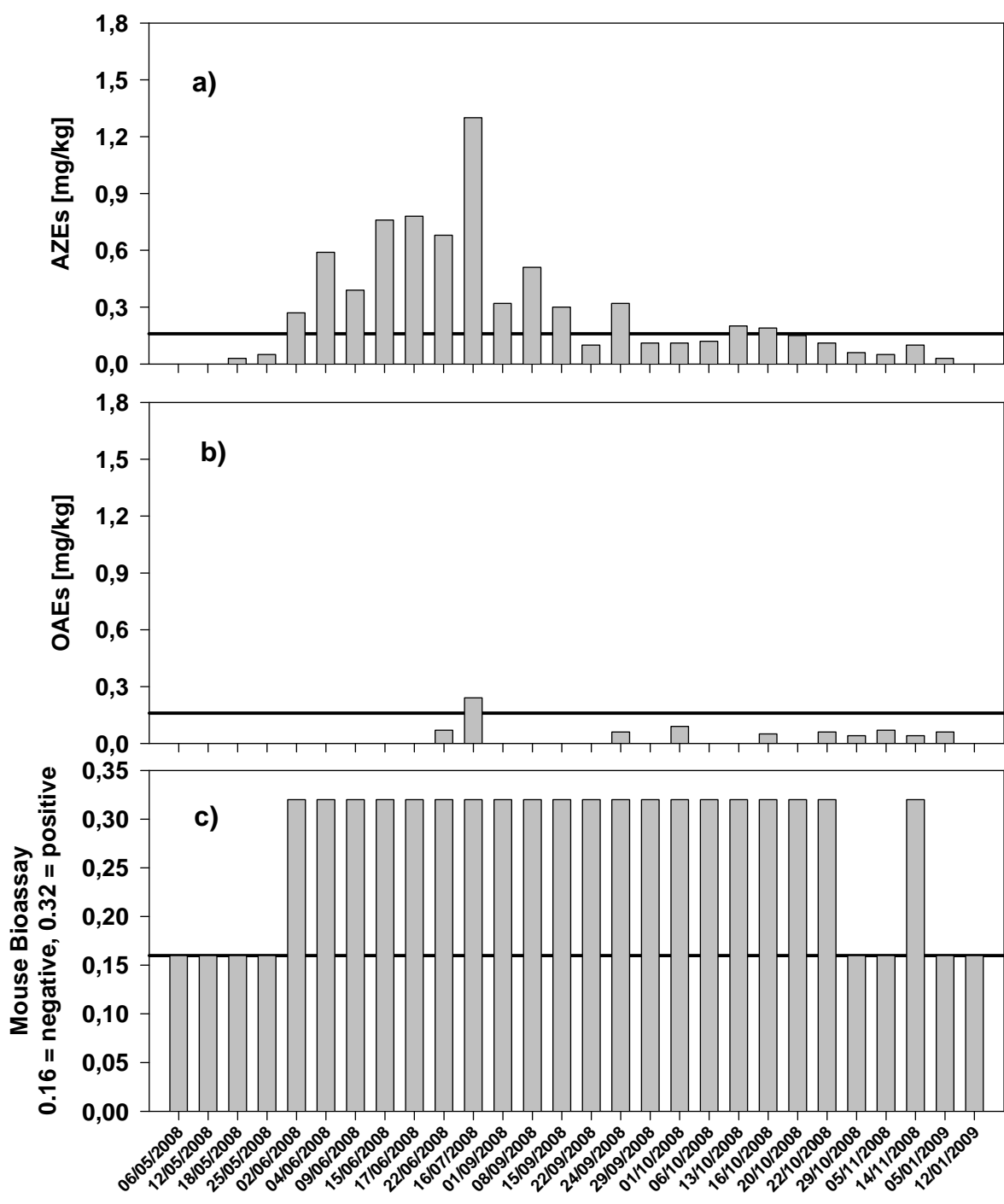


Figure 1.2.2. Temporal trends of azaspiracid group toxins, okadaic acid group toxins and mouse bioassay results in mussels (*M. edulis*) from Castletownbere, southwest Ireland from May 2008 to January 2009; a) azaspiracid-1 equivalents (AZEs) and b) okadaic acid equivalents (OAEs), both determined by LC-MS and measured and expressed in whole shellfish flesh, c) mouse bioassay (MBA) results for the same samples measured in hepatopancreas. The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and AZA-group toxins. Arbitrarily, and for visualisation purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives are represented as 0.32 mg/kg (Figure created from data available online from the Marine Institute, at <http://www.marine.ie>, accessed on 29/07/2009).

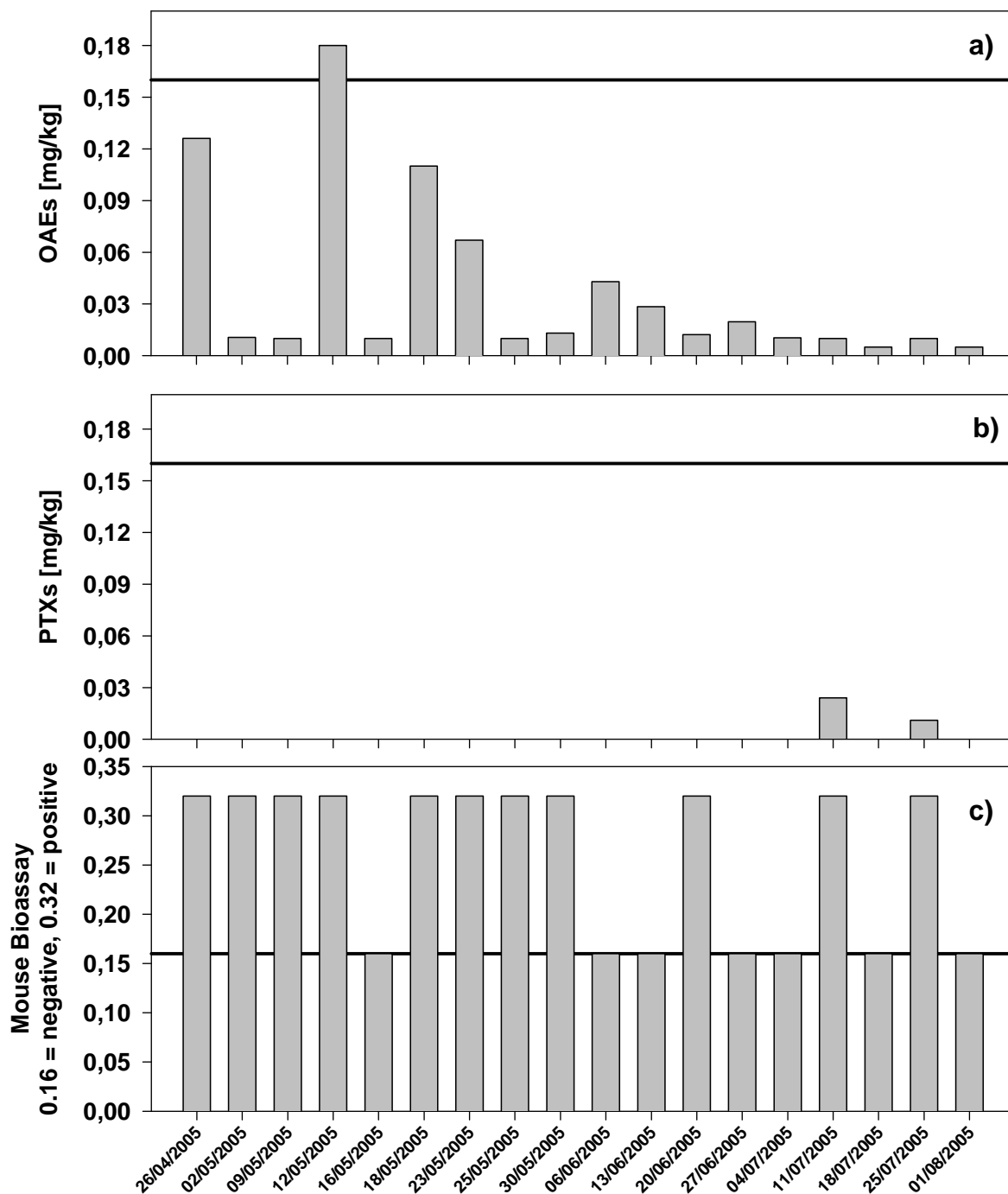


Figure 1.2.3. Temporal trends of okadaic acid group toxins, pectenotoxin group toxins and mouse bioassay results in oysters (*C. gigas*) from Arcachon, Bay of Biscay, French Atlantic coast from April 2005 to August 2005; a) okadaic acid equivalents (OAEs) and b) sum of pectenotoxins (PTX2 and PTX2sa), both determined by LC-MS (measured in hepatopancreas (HP) and expressed in whole shellfish flesh) and c) mouse bioassay (MBA) results for the same samples (measured in HP). The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and PTX-group toxins. Arbitrarily, and for visualisation purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives are represented as 0.32 mg/kg (Figure created from data available from REPHY (French national monitoring programme; extracted 27/07/2009).

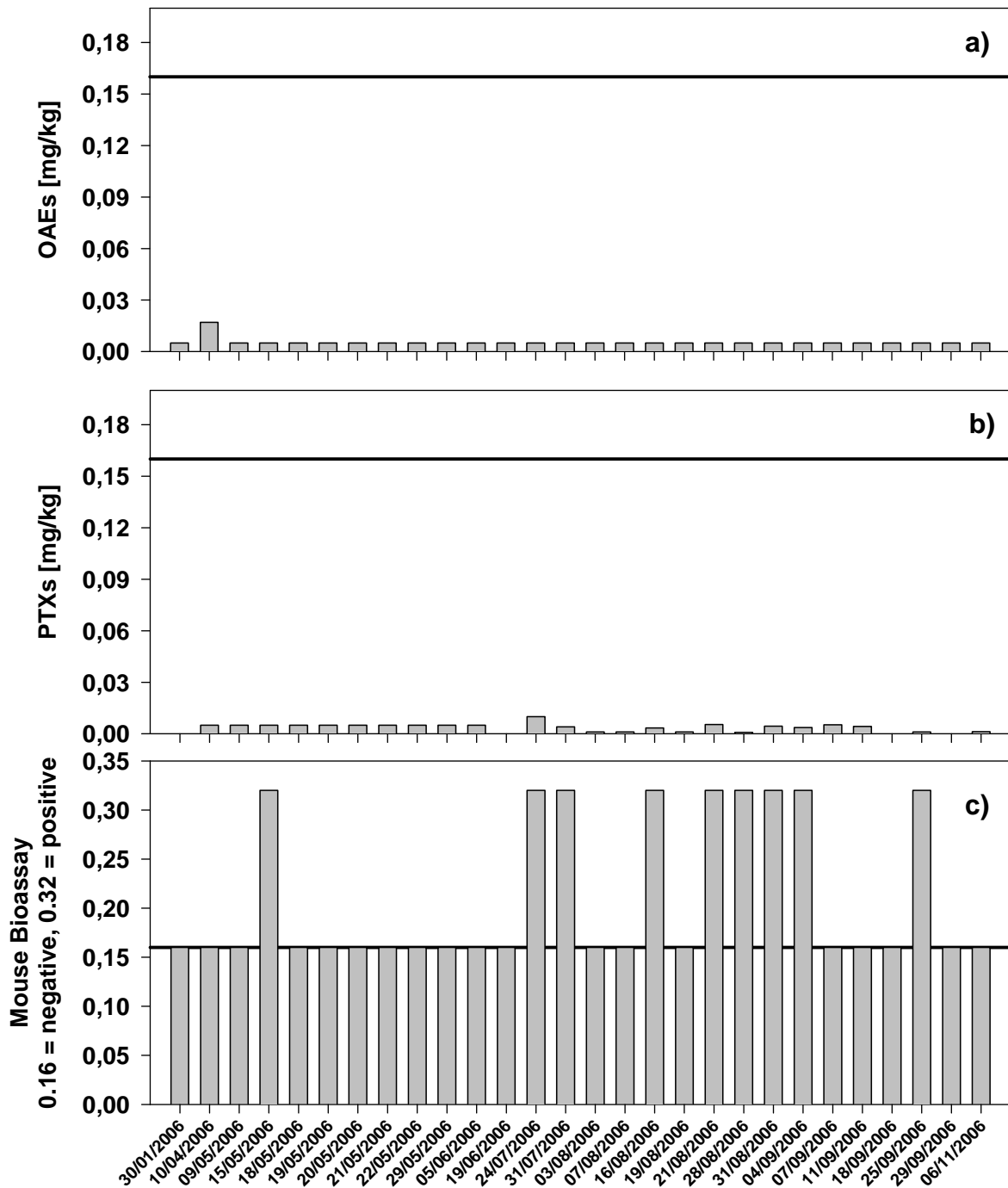


Figure 1.2.4. Temporal trends of okadaic acid group toxins, pectenotoxin group toxins and mouse bioassay results in oysters (*C. gigas*) from Arcachon, Bay of Biscay, French Atlantic coast from January 2006 to November 2006; a) okadaic acid equivalents (OAEs) and b) sum of pectenotoxins (PTX2 and PTX2sa), both determined by LC-MS (measured in hepatopancreas (HP) and expressed in whole shellfish flesh) c) mouse bioassay (MBA) results for the same samples (measured in HP). The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and PTX-group toxins. Arbitrarily, and for visualisation purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives are represented as 0.32 mg/kg (Figure created from data available from REPHY (French national monitoring programme; extracted 27/07/2009).

These positive results of the MBA may be related to yet unknown toxins of public health relevance, or they may be due to interference from bioactive compounds which are not relevant to public health. Thus, in a regime which had been based on chemical analysis (by LC-MS), production would have continued after a 3-week closure period (one week toxin levels exceeded regulatory limits and 2 consecutive clear tests are required to re-open an area). The following year showed an even more dramatic picture where MBA results were again sporadically positive between May and September, while all lipophilic toxins known to occur in this area (OA, AZA, PTX, GYM, SPX, YTX) were well below the threshold expected to result in positive results in mouse bioassays (Fig. 4). The situation in Arcachon in 2006 had been further complicated by the fact that anecdotal evidence provided by oyster producers from the area suggested that consumption of these oysters did not result in acute human illness. Therefore, the question remains on how to manage areas where parallel tests yield contradictory results. To look at this question, the next section describes the process of risk analysis.

4.1.2.3. Risk analysis principles in Codex Alimentarius

Codex has laid down working principles for the risk analysis of food stuffs in a guideline (CAC/GL 62-2007) to clarify the approach proposed to governments. In these guidelines, a clear role is attributed to each of the following three integral components of the process:

1. risk assessment
2. risk management
3. risk communication

This three-pronged approach to risk analysis should be applied consistently, in an open, transparent and documented manner. In addition, risk analysis should be evaluated and reviewed in light of newly generated, scientific data. There should be a functional separation of risk assessment and risk management to the degree practicable, in order to ensure the scientific integrity of the risk assessment, to avoid confusion over the functions to be performed by risk assessors and risk managers and to reduce any conflict of interest. Risk communication is required for consumers and food producers sake but also to improve understanding between risk assessors and risk managers, for instance to clarify elements of uncertainty in a risk assessment to risk managers. Risk assessment should be structured as a process including the elements of hazard identification and characterization, exposure assessment and risk characterization. Risk management also follows a structured approach including specific steps such as preliminary activities, evaluation of risk management options, implementation, monitoring and review of the decisions taken.

While these general principles make the international approach to risk analysis very clear, it must be noted that specific risk analyses are far from trivial, in particular because of the frequent lack of data on toxin analogues, relative toxicities, exposure and epidemiology. This lack in data often makes risk assessments provisional and requires frequent review of the assessment and the management options derived. This fact has also been recognised by Codex and therefore, the iterative character of the risk analysis process has been stressed in the guidelines.

In the following sections, a few important steps are addressed to exemplify why a risk analysis process may often take a number of years before a satisfactory process for managing the risk is achieved.



Figure 1.2.5. Steps involved in risk analysis: risk assessment includes steps 1 to 4, risk management includes steps 5 to 7. Risk communication ensures understanding of all steps by all stakeholders. The process is considered iterative as new information becomes available.

4.1.2.3.1. Hazard identification and characterization

When a new shellfish toxin emerges in an area of shellfish production, it is not easy to identify this hazard. Initially, there will be customer complaints about food in general, it needs to be established that shellfish were the likely source of the customer complaint. Once it is established that bivalve molluscs were actually at the origin of the illnesses reported, medical doctors need to establish whether the nature of the illness is of microbiological or chemical origin. As microbiological contamination of shellfish often generates similar symptoms of diarrhea, vomiting and sickness, it is not necessarily clear that a novel toxic agent is involved. As abovementioned, the most important information comes from the epidemiological reports on the onset of the sickness, since microbiological contaminations typically require 24 h incubation periods before symptoms develop (some, such as hepatitis virus may cause illness as late as 4 weeks after consumption of the shellfish. Subsequently, symptoms will need to be examined for their specificities, including neurological poisoning symptoms, memory loss, inversion of hot and cold sensation etc.; all these symptoms could arise from known shellfish toxins. However, often the unspecific symptoms of diarrhea, vomiting and sickness outweigh specific symptoms in most customers, in particular if the toxin has not occurred at very high levels.

Obviously, as soon as complaints are traced back to shellfish, both shellfish producers and government officials will trace back the products to their production site and will gather additional data available from routine monitoring of these sites for microbiological agents and (potentially) toxic phytoplankton. Only if it can be established that no known agents from this area could have caused the disease, work can begin on the identification of a new hazard. Most often, at the first occurrence of a new agent, the time between illnesses reported and the time when it was

established that a new toxin is probably the cause of the illness is so long that there is some likelihood that the agent may again have naturally depurated from the shellfish and the shellfish growing in the area at that time may not be contaminated anymore. However, sometimes this is not the case, and contaminated shellfish may be obtained from large production lots if these are not yet all consumed or from the area in which the shellfish are grown. For instance, when a new toxin was suspected from mussels in Killary, Ireland, 1995, the contamination has happened in autumn (September/October), and when government officials collected shellfish from the area in November, contamination was still sufficiently high (McMahon and Silke, 1996) to identify the toxin involved, probably due to slow detoxification in late autumn (colder water temperatures, fewer non-toxic algae present as food for mussels). However, due to a lack of specialised scientists in Ireland, it took the effort of international collaboration with experienced teams in Japan to identify the toxin within 2 years of the initial discovery of the illness (Satake et al., 1998). In times when virtually all scientific activities are accounted for through publicly funded projects, it is not always easy to mobilise scientific capacity, even if it is available in the country where the illness occurs. These logistic issues around finances and scientific capacity often result in an emerging hazard not being identified for years.

As abovementioned, natural toxins typically occur as mixtures of analogues. Often, the toxin-producing phytoplankton species produces 2 or more main analogues of a toxin, and these are further metabolised by shellfish into a multitude of analogues. As it is not guaranteed that the most abundant metabolite is also the most toxic one, hazard identification is also about the identification of all relevant analogues and about the assessment of their relative toxicity. In the case of azaspiracids, it should be noted that two of the three currently regulated analogues were only discovered and crudely characterised for their toxicity a further 2 years later (Ofuji et al., 1999). By 2008, twenty analogues of this toxin group are known (Rehmann et al., 2008) and metabolism in shellfish starts to be understood (McCarron et al., 2009).

The identification of the toxin alone is only the first step of a full hazard identification. In the case of shellfish toxins it is important to identify the biological source organism of the toxin, mostly unicellular algae (diatoms and dinoflagellates). Once these producing organisms are known, the surveillance system can observe the frequency of their occurrence and the conditions leading to toxin production, such that the full extent of the occurrence of the toxin can be understood. In the case of azaspiracids, for instance it took 12 years from the discovery of the toxin until one azaspiracid-producing organism was discovered (Krock et al., 2008; Tillmann et al., 2009). Again, this work was far from trivial as it involved the discovery of a new species and a new genus, in an area completely different (North Sea) from the area of the initial discovery of the toxin (Irish Atlantic coast).

In order to fully characterize the hazard deriving from a new toxin (group), it is also necessary to understand the toxicity of the chemical substances involved. Many questions need to be answered for the characterization of the hazard:

- Is it the substance itself (produced by the alga) which is toxic or is it the metabolites in shellfish or the metabolites in humans which cause the actual toxic effect ?
- What is the toxic effect ? Is it only digestive trouble, or are there other more grave or more subtle effects ?
- Is there only acute toxicity or are there also long-lasting, chronic effects ?
- What is the molecular mechanism of action of the toxin ?

Depending on the availability of the compounds involved and the complexity of the toxicity, it may take several years, sometimes decades to answer the above questions. In fact, while for some toxin groups one mechanism of action is known (BTX, DA, OA, STX), it remains questionable whether other mechanisms of action do not also contribute to the overall toxicity and need to be considered in the risk assessment. For other toxin groups, such as YTX, cyclic imines and AZA, the mechanism of action is not yet clarified, although these toxins were discovered in 1986 and 1995, respectively.

The problems of identification of the compounds and the characterization of their toxic effects are closely intertwined through the need for isolation of large quantities of highly purified compounds. In the identification of the molecule, high purity is required to unequivocally identify the structure of the molecule, mostly through mass spectrometric and nuclear magnetic resonance techniques. In toxicological evaluation, the purity of the compound is important to associate a certain level of toxicity with one analogue only to establish its relative toxicity; if other analogues remain as impurity, the assessment of this particular analogue may be strongly falsified, as even structurally closely related analogues may differ 100- or 1000-fold in their toxic activity. The efforts required to identify a completely novel bioactive compound are often heavily underestimated. As a rule of thumb, it should be noted that preparative isolation procedures typically only recover about 5 to 10% of the compound originally present in shellfish. Even though shellfish toxins typically cause illness at mg/kg level, 10-fold larger concentrations are typically required to efficiently purify novel compounds. Also, as the mode of action is typically not known at the beginning, the isolation procedure often relies on insensitive animal testing for the activity-guided fractionation and isolation of the toxin, thus consuming the toxin during its isolation. In many cases, the collection and dissection of hundreds of kg of shellfish is required to recover sufficient toxic material for successful identification of the compound. Toxicological evaluation requires significantly higher quantities of the compound as many experiments need to be repeated in animals and thus for most shellfish toxins, information on chronic effects is still not available.

4.1.2.3.2. Exposure assessment

The assessment of the extent to which a single consumer or a population is exposed to a specific shellfish toxin is derived from two main variables: consumption of shellfish and occurrence of the shellfish toxin.

Consumption of shellfish, as of any other commodity, is mostly determined through consumption surveys. A difficulty with bivalve molluscs is the multitude of portion sizes that may be consumed which may change from region to region, consumer to consumer and by shellfish species. A difficulty in interpreting many of the surveys consists in the fact that surveys were poorly designed in the definition of the portions consumed: it is not always clear whether the weight reported refers to the shellfish with or without their shell and intervalvular fluid, or whether it refers to the shellfish flesh consumed. In addition, it is not clear whether the weight refers to the raw weight or to the cooked weight. These difficulties result in significant uncertainty in the exposure assessment and in the evaluation of the dose that made people sick (see next section).

The occurrence of the new shellfish contaminant is not well known at the stage of its appearance. This lack of knowledge is one of the major reasons for the iterative character of risk analysis. As described above under hazard identification and characterisation, it may take several years before the chemical structure and behaviour of a new toxin is actually known. Toxin-specific methods

can obviously only be developed at that stage, and the concentration levels of the toxin may only be known after some time. If shellfish tissues from the poisoning event are retained and well preserved through freezing at low temperatures, it may later be possible to retrospectively establish the concentration of the toxin in the shellfish the consumption of which had made people ill. However, more often than not, this is not the case. Therefore, it is most important that one of the risk management options for the establishment of the extent of the problem includes regular monitoring of the toxin responsible for causing the sickness encountered. The methods used for such monitoring are typically not yet fully validated through interlaboratory trials as there is no large-scale interest in working on a toxin as long as the extent of the problem has not been established.

Once monitoring reveals that the compound does occur with some regularity, and once toxicological evaluation has clearly established that the compound poses a significant risk, work on methods needs to be intensified. Such studies include the establishment of quality control tools referred to as reference materials. Steps involved in the making of a reference material are outlined in Figure 6.

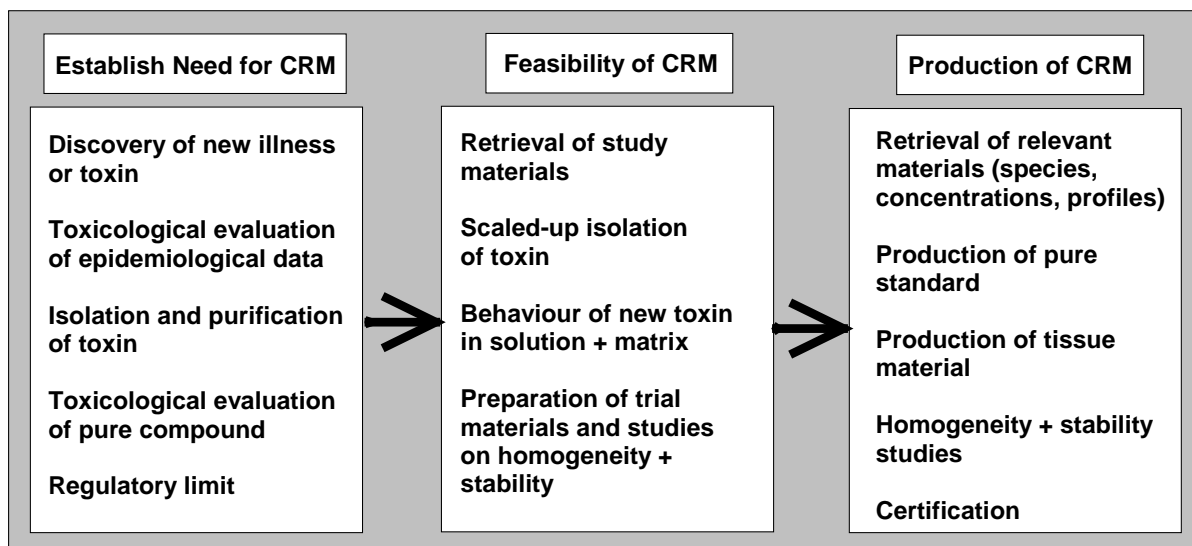


Figure 1.2.6. Stages in the development of certified reference materials (CRMs). CRMs are tools that are considered major milestones of quality control of methods for the detection of trace contaminants such as phycotoxins (adapted from Hess et al., 2007). Once the need has been established to produce a CRM, the feasibility must be explored; only after feasibility has been established, work can begin on the production of the material.

Once reference materials are available for a toxin group (including certified standards and shellfish tissue reference materials), a method typically progresses to the stage that method validation may proceed. Ideally, a method is characterized through an interlaboratory study, an exercise for which further reference materials are required.

Best data on occurrence of shellfish toxins are obtained after methods have been validated and regular monitoring is ongoing with quantitative, toxin-specific methods, such as LC-UV for domoic acid or LC-MS for azaspiracids. Unfortunately, for many lipophilic toxins, the reference method has remained the mouse bioassay for lipophilic toxins since the introduction of EU legislation on marine biotoxins in 1991. The existence of this reference method means that most regular monitoring is carried out with this method and unfortunately, neither quantitative nor toxin-specific data are available from this surveillance. Therefore, the situation for many lipophilic

toxin groups is very complex to assess, and exposure assessment relies on parallel testing which is sometimes carried out in countries that have recognised the value of quantitative toxin-specific methods beyond regulatory reference tests.

The difficulty in predicting the occurrence of toxic algae and thus the sporadic occurrence of shellfish toxins also affects the monitoring necessary for exposure assessment. It is typically not sufficient to monitor shellfish toxins for a one-year period to assess their overall frequency of occurrence (see also Figures 1 and 2). In many cases, toxins may not occur for several years and then re-appear for one or several years. Therefore, regular surveillance over many years is required to assess the exposure of a population to a toxin group.

4.1.2.3.3. Risk characterisation

Risk characterisation investigates in depth the nature and extent of the adverse health effects caused by a toxin group. In particular, risk characterisation should examine whether only acute effects are encountered or if also long-term effects exist. These considerations will result in the recommendation of an acute reference dose (ARfD) for protection against immediate effects, or of a total allowable daily intake (TDI) for protection against chronic effects.

Wherever possible risk characterisation should be quantitative, and should also outline options for risk management. For instance, such options will be given in the toxin-specific sections for covering different proportions of the population of shellfish consumers.

One of the most central questions of risk characterisation is to establish the smallest concentration of toxin consumed that will likely cause health problems; this level will be called the lowest observable adverse effect level (LOAEL), expressed in mg/kg body weight (b.w.). As abovementioned, data acquired with validated methods are the best available for the investigation of which dose of toxin will make people sick. Unfortunately such data are not typically available at the early stages of risk analysis. This constellation is another example of the iterative nature of risk analysis. Indeed, the best data on poisoning events are acquired at an advanced stage when the toxin is already known to cause a problem and when quantitative methods are well advanced in their performance credibility. Obviously, at this stage, the government officials should already have organised monitoring of the toxin to prevent poisoning events, and thus, paradoxically, the best data are obtained from failures of monitoring systems. Such failures sometimes occur when shellfish farms are newly established and the first crop consumed at inaugural events has not been analysed; this has been recorded both in Norway for okadaic acid group toxins (Aune, 2001), and in Ireland for azaspiracids (McMahon and Silke, 1998). Other occasions for acquiring solid data for the estimation of the dose of toxin making people sick arise when a toxin group is known to occur in one country (A) but not in another (B). When the toxin appears in country B, poisoning may occur in the population of shellfish consumers and characterisation of the concentrations causing the sickness may happen with methods applied or developed in country A. Unfortunately, failures of monitoring systems also occur in situations where toxins are recognised as a problem but regulations are not followed closely, this is the case for most of the data obtained for the risk assessments described in the toxin specific sections.

Once the LOAEL has been established, consideration will be given to the nature of the risk, i.e. the effect, and its gravity. From these considerations and from uncertainties associated with establishing the LOAEL, a safety factor will be applied to estimate a no-observable adverse effect level (NOAEL), again expressed in mg/kg b.w. This NOAEL can also be expressed as a dose,

called the acute reference dose = ArfD, expressed in µg or mg (typically NOAEL * 60 for a 60 kg person).

The size of the safety factor depends very much on the data available to estimate the LOAEL. The first preference is always to derive the LOAEL from observation in humans. If such data are available then most typically, a safety factor 10 is applied to account for variability between humans (different susceptibilities due to age, sex, genetic predispositions). In some cases, where large data sets are available on poisoning of many different people, including male and female, as well as children and aged consumers, it may be possible to reduce the safety factor to less than 10. However, this will also depend on the gravity of the effect at the LOAEL. If insufficient data are available from observations in humans, observations from animal experimentation will be considered to derive a NOAEL, in this case the typical safety factor will be 100 or 1000, depending on the nature and gravity of the effect and other uncertainties.

Risk characterisation should also quantitatively evaluate the risk as a function of the exposure data. This is particularly important for risk managers as it puts the gravity of risk in relation to the likelihood of occurrence. For instance, if long-term monitoring of a toxin indicates that concentrations in shellfish very rarely or never exceed a certain level, and that no effects have been observed below this level, it would not be necessary to have a regulation in place for such a compound.

4.1.2.4. Risk Management

As abovementioned, risk management is an integral part of the risk analysis approach. Once a risk has emerged, one of the initial activities of risk management is the organisation of a risk evaluation or assessment. The outcome of such an assessment usually contain several options for risk managers. Thus, the next step for risk managers is the evaluation of the management options in light of their impact on shellfish consumers and producers. Two very extreme options for risk managers are to either not take any further action (if the risk is considered negligible) or to completely prohibit shellfish production in an area (if the risk is considered too big for any consumption). Most often though, risk managers will make use of some or all of the many options in between these two extremes:

- Initiate studies to preparatively isolate the contaminant, produce calibration standards and reference materials, encourage method development for the specific and quantitative monitoring of the contaminant
- Monitoring of the emerging contaminant and closure of production area when level of contaminant rises above background
- Initiate studies on deeper understanding of the toxicity caused by emerging contaminant
- Review effectiveness of management procedure
- Enter second phase of risk assessment based on novel data from monitoring and toxicity studies

Especially the last point of re-evaluation of the risk after having obtained new data may often be undertaken in conjunction of moving up a hierarchical level of management. For instance, in the case of azaspiracids, an initial risk assessment by the Food Safety Authority of Ireland (Anderson et al., 2000) has led the Irish government to take early steps in managing the risks from azaspiracids occurring in shellfish produced in Ireland. After monitoring the risk for some time, re-evaluation of the risk was carried out at European level (EU, 2001), and risk management steps

following this re-evaluation included the regulation of maximum levels of AZAs in bivalve mollusks (Anonymous, 2002).

Such iterations of risk assessment and management from regional to national level, or from national to international level are often conducted when it emerges that toxins may actually also occur not just in a specific area but also at a much wider spread level. The move up to international and even global level is also most often necessary to ensure trade is not compromised between different countries due to import restrictions or monitoring requirements.

4.2. Method development for the analysis for phycotoxins

In terms of methodology, an early breakthrough was made by Sommer and Meyer (1937) with the development of a mouse bioassay for the detection of the agents involved in “paralytic shellfish poisoning”. This procedure is based on extraction of water-soluble (hydrophilic) compounds using hydrochloric acid as extraction solvent and detection by injection of filtered crude extracts into mice. Thus, the procedure is based on the toxic response of any water-soluble acid-stable compound in mice. As such it is also capable of detecting domoic acid in shellfish, albeit at levels higher than the currently regulated levels. The quantification of paralytic toxins with this method was further improved through the work of Schantz et al., 1957 and 1958, and the method was finally validated as official method in 1990 (AOAC, 2005). Similarly, although neurotoxic shellfish poisoning had been reported in US since the mid-19th century, formal control methods were only developed following work by McFarren (1959), and standardised as official control procedures by the American Public Health Association in 1970 (Anonymous, 1970). Again, the APHA-protocol is based on the detection of toxic principles in shellfish by injection of extracts into mice, however, in this case a lipophilic extraction solvent is used, diethyl-ether. A similar method was developed by Yasumoto et al. (1978) for the detection of another lipophilic toxin group: okadaic acid and analogues; the protocol in this case is based on extraction of toxins with acetone. The water-miscible nature of this extraction solvent has led to many interferences, such as low levels of saxitoxins and domoic acid; therefore, the protocol has later been amended to include a solvent partition step between water and diethyl-ether to eliminate these water-soluble compounds from the acetone crude extract (Yasumoto et al., 1985). This procedure is also capable of detecting azaspiracids up to certain levels (Hess et al., 2009). Furthermore, a rat bioassay has been developed by Kat et al., 1978. This assay detects shellfish toxins through their diarrheic effects in rats following oral feeding of shellfish tissues. The rat bioassay is only capable of detecting okadaic acid and azaspiracids, as these are the main diarrheic toxins. Thus, the main five toxin groups responsible for shellfish poisoning can be detected by procedures involving toxicity testing with mice or rats. However, these bioassays may not detect all toxins at the levels required for protection of public health, and generally, mouse bioassays (MBAs) suffer from a number of disadvantages, including a lack of specificity, non-toxic interferences, and ethical issues around animal welfare. In addition, the bioassays for lipophilic toxins are not quantitative and thus do not lend themselves to effective monitoring practises. Also, the MBAs for lipophilic toxins have not been validated through inter-laboratory trials and their performance characteristics are not well established.

Due to these shortcomings of bioassays, a strong need for alternative methods has emerged (Hess et al., 2006). In particular, there is a requirement to detect specific compound groups to be sure of the nature of a toxic event, and also to be able to detect the quantity present to be able to implement limits for these toxic compound groups.

For the development of quantitative and specific methods, the availability of pure reference compounds (the toxins themselves) is a major prerequisite. Onoue et al. (1931) started work on the isolation of saxitoxin analogues as the toxic principles of paralytic shellfish poisoning. The efforts were significantly advanced by Schantz et al. (1957, 1958). However, it was not until 40 years after initial isolation efforts that the structure of saxitoxin was finally confirmed by Wong et al. (1971). The characterisation process has been hampered for many toxins in a similar fashion due to the lack of compound mass for the studies. This lack can be understood from the fact that the organisms producing the toxin cannot always be cultured, and scientists thus rely on the natural occurrence of the compounds. In addition, the structure elucidation in early days was mostly based on chemical reaction of the compounds. The onset of more powerful non-destructive

techniques such as nuclear magnetic resonance (NMR) has allowed for the characterisation of smaller quantities: while several hundreds of mg were required to characterise a toxin in the 1950/60s, nowadays 10 to 100 µg of compound may be sufficient to complete the structure elucidation of a novel compound. Thus, the discovery of domoic acid as a shellfish toxin could be completed within weeks from the poisoning event (Quilliam and Wright, 1989). More typically, it takes one to several years from the initial poisoning event to the identification of the chemical responsible for the toxic effect, e.g. for the identification of okadaic acid and azaspiracids (Yasumoto et al., 1978; Satake et al., 1998). Currently, at least one reference compound is available for every compound group, however, for several toxicologically relevant analogues, there is still no certified reference compound.

With reference compounds becoming more and more available, methods alternative to the bioassays have been developed. In the early stages, many of these methods were based on liquid chromatographic (LC) separation of the toxins (gas chromatography is excluded due to the non-volatile nature of the compounds), followed by detection of UV-absorbance or fluorescence of the toxins in solution (for specific methods see compound-specific sections). However, most compounds have no specific UV absorption or fluorescence in their natural form, and thus these methods were often cumbersome due to complicated derivatisation and purification steps. Since the 1990s, methods based on LC followed by mass spectrometric (MS) detection (LC-MS) have been developed and their ease of use has led to relatively widespread application of such methods in the new millennium. The LC-MS technique was very expensive in the early stages, therefore, these methods had initially been mainly restricted to developed countries (North America, Europe, Australasia). Nowadays, the technique is less expensive but still costly and sophisticated, requiring specialised staff. However, thanks to robotics, the price per analysis has decreased thus allowing for a wider range of users to access the technique. The main advantages of this technique consist in the fact that individual analogues can be distinguished and quantified. These features result in a maximum of information on possible causative organisms and risks encountered. However, the toxic potency of each analogue must be known to calculate the total toxicity associated with a sample of shellfish. Other non-animal alternatives are available for some toxin groups, including methods based on antibody technology, such as dip stick tests or enzyme-linked immunosorbent assays (ELISAs). The advantage of these tests compared to LC-MS based methods lies in their ease of use and low cost. However, these tests can only give a single response per toxin group, which lacks information on individual analogues and also on total toxicity present. For some groups of toxins, e.g. okadaic acid and saxitoxin, functional assays have been developed. Functional assays are based on the mechanism of action and thus give information on the total toxicity present, even if there is no information on individual analogues.

4.2.1. Mass spectrometry and paralytic shellfish toxins

As abovementioned, the traditional method for the detection of saxitoxin (STX) and its analogues and to a lesser extend for tetrodotoxin (TTX) has been a mouse bioassay (MBA) for hydrophilic toxins. This assay is based on the intraperitoneal injection of 1 mL of a HCl-extract into a 20 g mouse, and has been validated as an AOAC-procedure (AOAC, 2005). Even though the assay has been widely used and shows good precision, it has been criticised for matrix suppression effects at the legal limit, as well as for reasons of animal welfare. In an attempt to develop alternatives to the mouse bioassay, a number of techniques have been used, including antibody-based assays (ELISA and lateral flow immuno-chromatography), cell culture – based functional assays (neuroblastoma assay) and physico-chemical determination using liquid chromatography. As STX and its analogues do not have a chromophore, oxidative derivatisation has been used to create derivatives

which are fluorescent. The derivatisation may be carried out prior to chromatographic separation or post-chromatography. The pre-chromatographic oxidation has the advantage of easy sample handling but has the inconvenience of poor separation between analogues, as some analogues have the same derivatisation products. Also, the method must be well-controlled as the derivatisation products are not stable at room temperature and rapid analysis, preferably using cooled auto-samplers, is of adamant importance. The post-chromatographic derivatisation has the advantage of adequate separation to distinguish analogues of different toxicities, and less problems with the stability of derivatives; however, it is difficult to implement and needs careful maintenance to provide reproducible results. As saxitoxins are very hydrophilic (water-soluble) compounds, their chromatography on reverse-phase columns is not easy, and ion-pair reagents have to be used to achieve adequate separation for some methods (in particular post-column derivatisation methods). The use of mass spectrometry as a detector for STX and analogues virtually imposes itself as it overcomes the need for derivatisation of the analogues prior to detection. However, mass spectrometric detection does not answer to the difficulties in separating STX-analogues chromatographically. Therefore, we have studied novel combinations of hyphenated techniques, including different types of chromatography.

4.2.1.1. Ion exchange chromatography coupled to mass spectrometry for the detection of saxitoxin group toxins

This work was carried out as a collaboration between the University of Jena and the Marine Laboratory Aberdeen (Jaime et al., 2001). The set-up used in this study allows for MS detection or electrochemical derivatisation and fluorescence detection; a fraction collector could be included for the collection of previously unidentified STX-analogues (Figure 2.1.1).

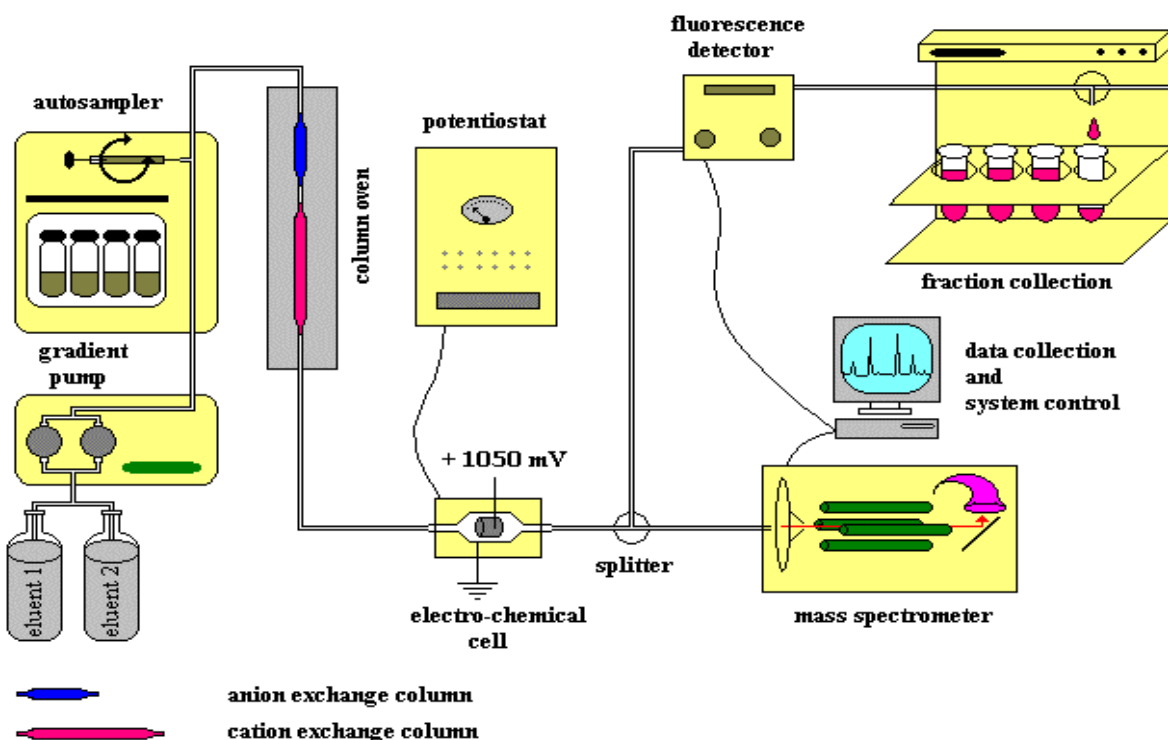


Figure 2.1.1. Automated HPLC system with ion-exchange columns, electrochemical cell with fluorescence detector and mass spectrometer (fraction collection as additional option)

The chromatographic separation obtained is acceptable as it achieves both separation of epimers (GTX2 and -3, GTX1 and -4) and other analogues such as dc-STX and STX that may be difficult to separate (Fig. 2.1.2).

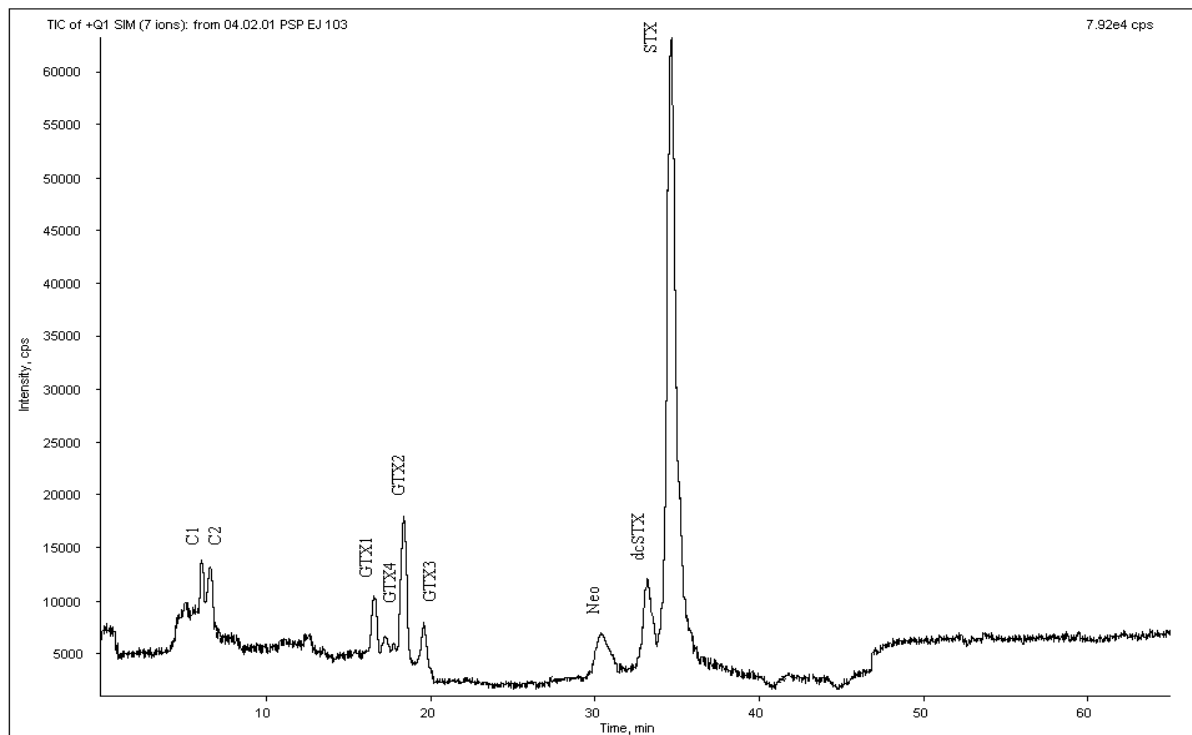


Figure 2.1.2. LC-MS determination of PSP toxins: C1/ C2, GTX1 (17.2 ng), GTX4 (8.9 ng), dcGTX3, dcGTX2, GTX2 (28.7 ng), GTX3 (10.4 ng), Neo (34.8 ng), dcSTX (25.0 ng), STX (39.9 ng).

The retention mechanism of ion exchange chromatography allowed for the use of a mobile phase which did not contain ion-pair reagents. This was a major advantage compared to previous methods, as ion-pair reagents had been reported to contribute to ion suppression and methods based on ion-pair chromatography thus resulted in poor sensitivity. However, the method still presented several disadvantages: the length of separation and the high detection limits. Despite the absence of ion pair reagents, the detection limits by MS detection were still between 1.25 and 50-fold higher than by fluorescence detection with the same chromatography (Table 2.1.1). Unfortunately, these high detection limits prevented the application of the method in a regulatory setting.

Table 2.1.1. LODs (signal/noise 3:1) of PSP determination by application of ion-exchange chromatography coupled to electrochemical oxidation with fluorescence detection and MS detection

Toxin	LOD (ng) Fluorescence	LODs (ng) MS
GTX1	0.8	1
GTX2	0.01	1.5
GTX3	0.01	0.5
GTX4	0.5	1.5
Neo	0.6	2
dcSTX	0.02	1
STX	0.03	0.5

While the long separation resulting in large peaks contributes to the high detection limits, the critical factor affecting sensitivity was the use of single quadrupole MS, a flaw that was addressed in the study presented in the following section.

4.2.1.2. Hydrophilic interaction chromatography coupled to mass spectrometry for the detection of saxitoxin group toxins

The study described in this section (Dell'Aversano et al., 2005) was a combination of two efforts to improve the deficit of the methods previously used for the physico-chemical analysis of saxitoxin-group compounds. Firstly, we examined the potential of a novel chromatographic technique: hydrophilic liquid interaction chromatography (HILIC). Secondly, the introduction of tandem mass spectrometry as detection technique was evaluated for the determination of STX and its analogues.

The systematic investigation of the HILIC chromatography showed that this type of chromatography had both characteristics of normal phase and of ion exchange chromatography. The ion exchange character of HILIC chromatography was seen in the influence of pH, buffer concentration and temperature on the separation (Figs. 2.1.3, 2.1.4 and 2.1.5), while the normal phase character of the separation became clear in the behaviour of compounds towards change in the percent organic of the mobile phase.

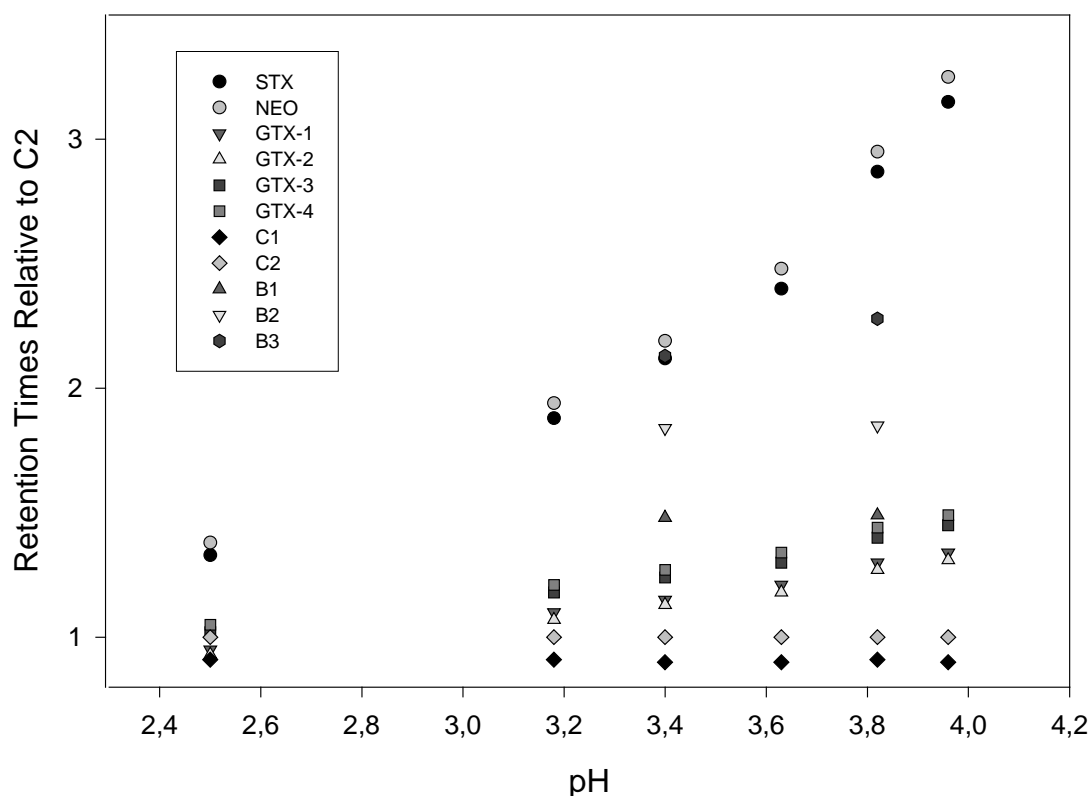


Figure 2.1.3. Influence of pH on retention time of saxitoxin group toxins relative to C2 in HILIC chromatography. Retention times of STX and NEO are most affected by increasing pH as they are doubly charged ions in acidic medium while those of sulfocarbamoyl-toxins B1/B2 (singly charged ions) and C1/C2 (neutral molecules) are less affected. Percent organic was kept at 65% B (A = 100% aqueous, B=95% acetonitrile + 5% aqueous)

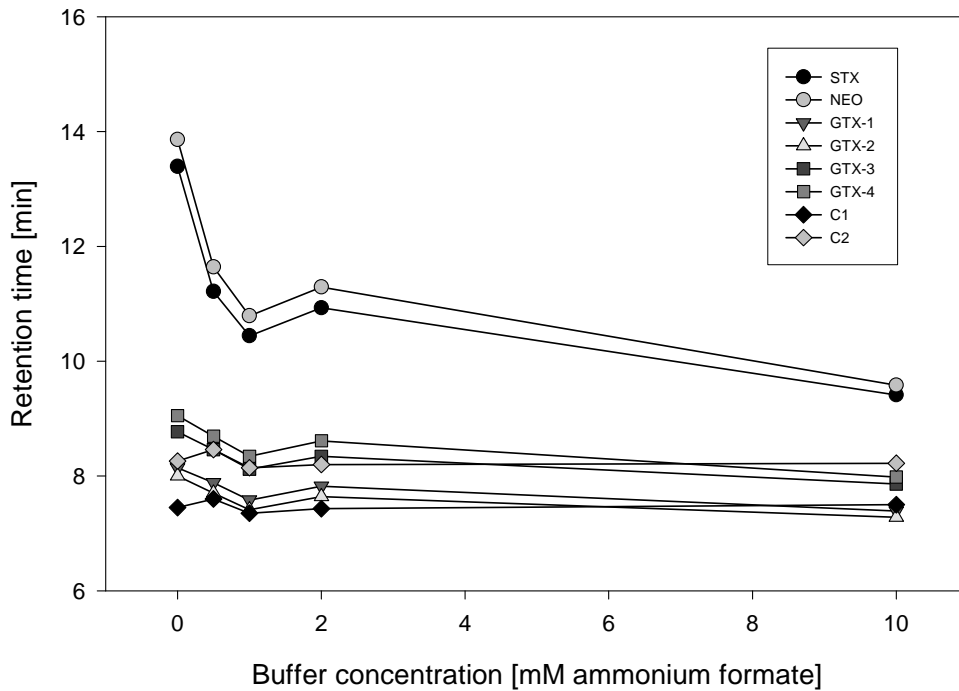


Figure 2.1.4. Retention time of saxitoxin and analogues in HILIC chromatography as a function of buffer concentration in the mobile phase. Similar to pH, the effects of the buffer concentration on retention time can be understood from the charge state of analogues.

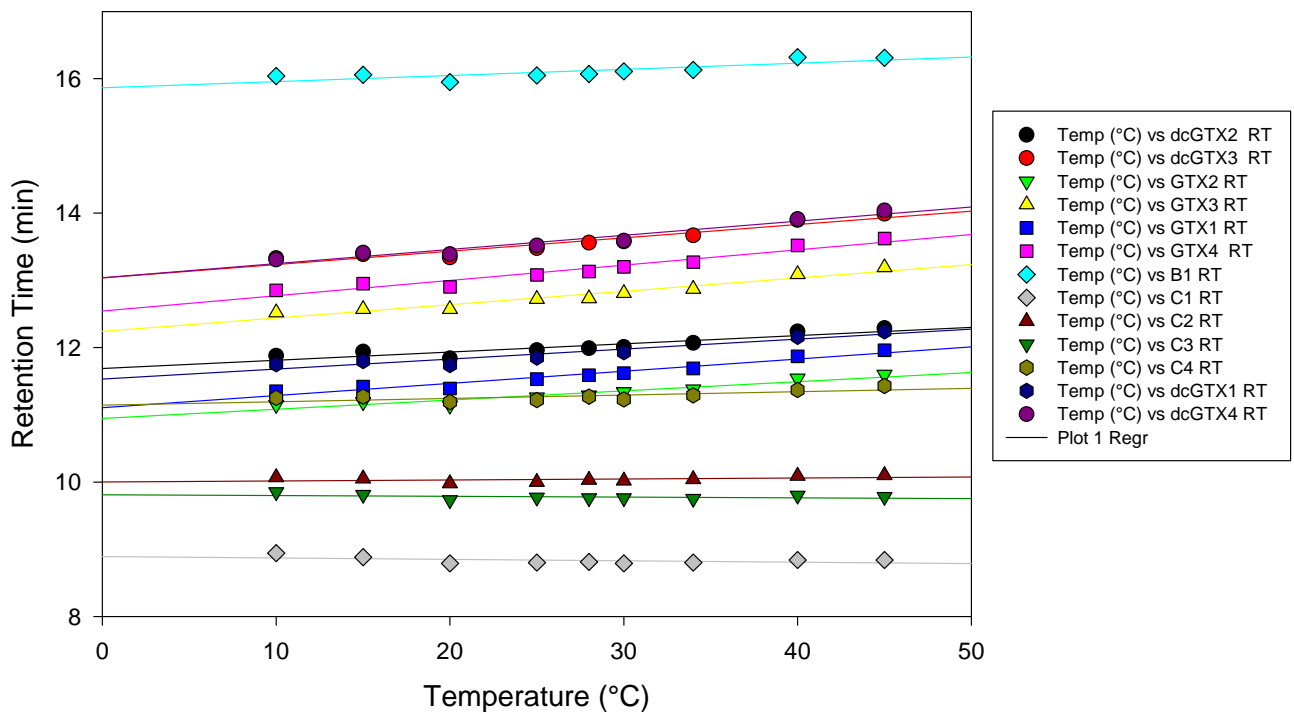


Figure 2.1.5. Influence of temperature on retention time of saxitoxin group toxins in HILIC chromatography. Temperature plays a minor role in the optimisation of parameters, however, as for pH and buffer concentration, compounds with higher charge state are more affected than others.

While pH, buffer strength, and to a lesser extent temperature, are parameters that influence both retention time and selectivity of the separation, the percentage of organic solvent in the mobile phase affects merely retention time (Fig. 2.1.6).

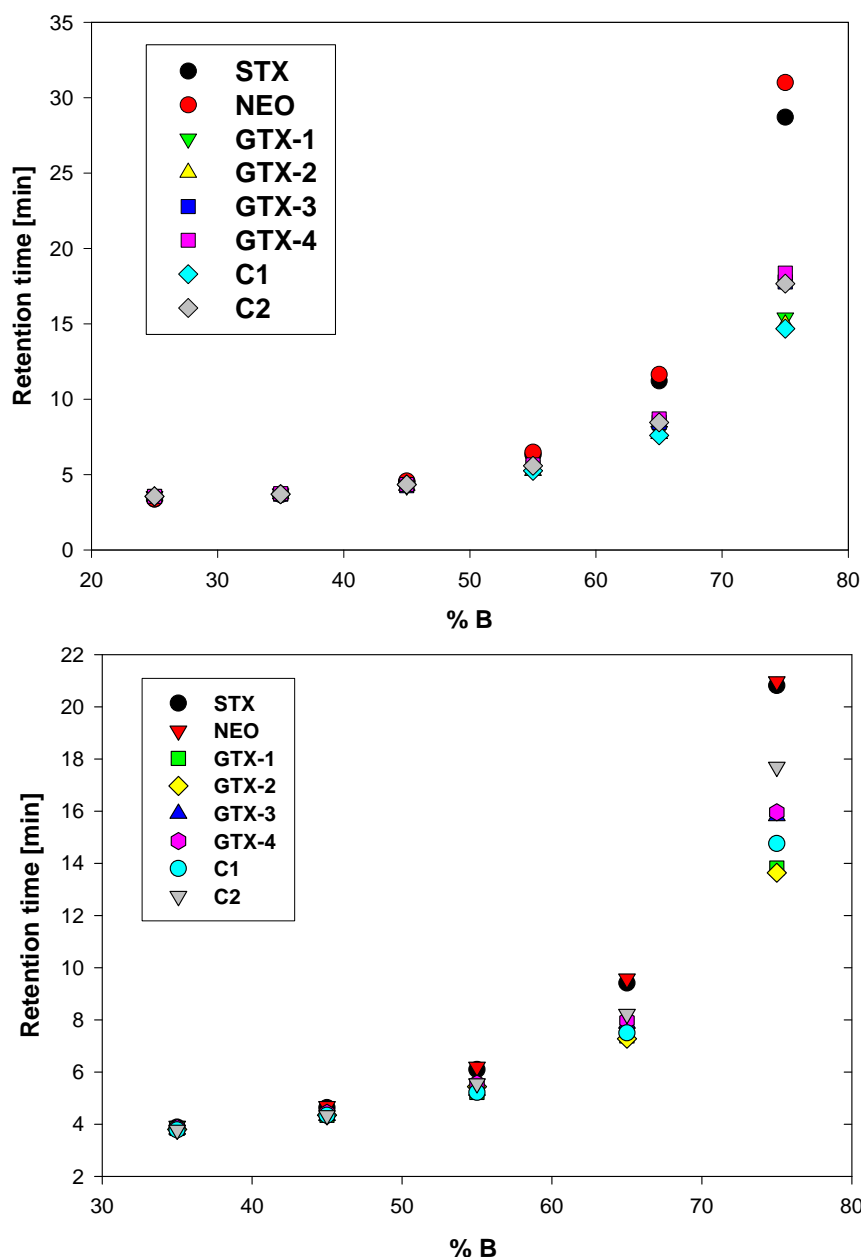


Figure 2.1.6. Retention time as a function of percentage organic in mobile phase B (A = 100% aqueous, B=95% acetonitrile + 5% aqueous). As in normal phase chromatography, water-soluble compounds are easily retained by their interaction with the polar stationary phase, and retention times are the longer the less the mobile phase is polar. Top graph at buffer strength of 0.5 mM ammonium formate, bottom graph at 10 mM ammonium formate.

As the pH influences the ionisation process, the sensitivity was also slightly affected by pH and buffer concentration, with best sensitivity being obtained at lower pH (2.5) and lower buffer strength. Therefore, the optimised conditions would be a function of the instrument sensitivity, and the capability of distinguishing overlapping compounds in the detector.

As previously mentioned in the section on ion exchange chromatography, the use of tandem MS was thus not only used to overcome sensitivity issues but also to increase the selectivity to allow for best use of the chromatographic conditions imposed by HILIC chromatography.

Thus we carried out a series of full scan acquisitions with a single quadrupole instrument (API 165) to investigate the fragmentation pattern of all saxitoxin analogues, an example is given for GTX1/4 (Fig. 2.1.7).

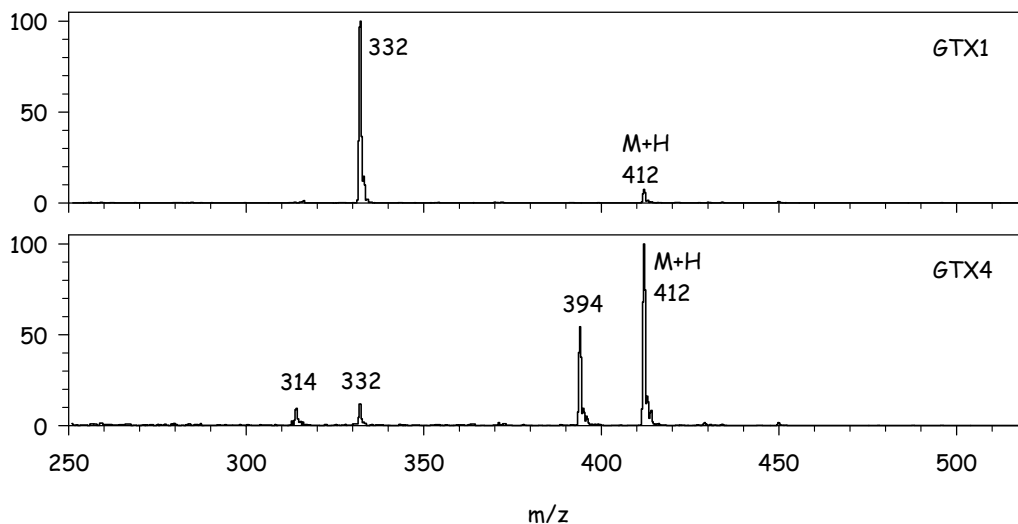


Figure 2.1.7. Full scan MS of GTX1/4: epimers of the same mass show distinct differences in their fragmentation behaviour. For GTX4, the molecular ion ($m/z = 412$) dominates the spectrum while the loss of a sulphate group is dominant in the spectrum of GTX1 ($412 \rightarrow 332$, loss of 80).

Subsequently, the relevant ions were monitored in multiple reaction monitoring using a triple quadrupole instrument (Fig. 2.1.8).

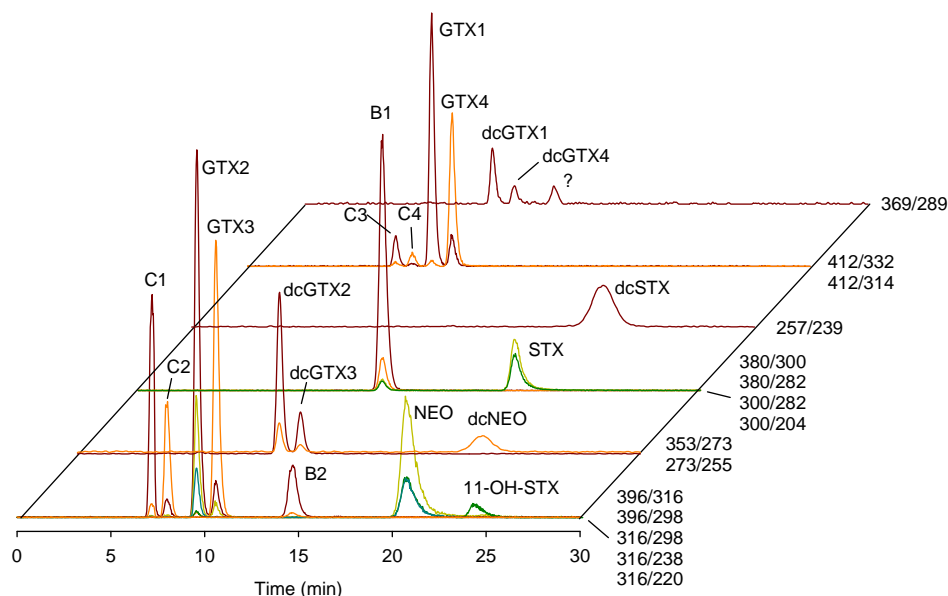


Figure 2.1.8. Multiple reaction monitoring of saxitoxin analogues using triple quadrupole mass spectrometry (API 4000) and HILIC chromatography (Amide 80). Separation of all relevant epimers can be achieved in less than 30 min, while tandem MS allows for sufficient selectivity and sensitivity to apply this technique in a regulatory setting.

Table 2.1.2. Estimated detection limits (LOD, S/N = 3) for saxitoxin group toxins (nanomolar concentration, 5 μ L injected on column) for tandem MS (Dell'Aversano et al., 2005) and LC with post-column oxidation and fluorescence detection (Oshima, 1995).

Toxin	MS-SRM	LC-FLD
	API4000	Post-column
STX	20	60
NEO	30	60
GTX2	20	20
GTX3	10	5
GTX1	10	20
GTX4	5	30
B1	10	100
C1	20	30
C2	10	20

For STX, which has a molar weight of 300g/mole, the LOD of 20 nM equates to 0.006 μ g/mL in solution. The current regulatory limit of 800 μ g/kg translates to a maximum concentration in a crude extract of ca. 0.4 μ g/mL, i.e. ca. 65 times higher (or ca. 20 times higher than the limit of quantification). Thus, even with several analogues present, the tandem MS method can quantify levels below the current regulatory limit. The detection limits reported in Table 2.1.2 demonstrate also that the tandem MS method developed was similarly sensitive to the HPLC-FLD method by Oshima (1995), which is slightly more sensitive than the Lawrence method (already accepted in the EU regulatory framework for official control of saxitoxin group toxins). Hence, we anticipate that this method will find further application in the official control of STX and its analogues.

4.2.2. Mass spectrometry and lipophilic toxins

Contrarily to saxitoxin and its analogues, the sensitivity of mass spectrometric (MS) detectors for lipophilic toxins is generally considered sufficient to determine values below the current regulatory limits. Additionally, chromatograms obtained using tandem mass spectrometric detection (LC-MS-MS) tend to show little interference, and thus interpretation of the chromatograms is much easier than that of chromatograms obtained in HPLC-FLD. Thanks to the ease of interpretation of mass spectral measurements and the sensitivity of the most recent instrument generations, LC-MS has been “hailed” as the obvious technique to replace animal testing for lipophilic toxins.

I have initially worked on the development of LC-MS methods for the detection of lipophilic toxins in UK and Canada, in particular under the guidance of Dr. Michael Quilliam on the development of LC-methods for the detection of multiple groups of toxins (Quilliam et al., 2001). Subsequently, I moved to Ireland (2001) to implement LC-MS methodology for the support of official control, where accreditation of the method became rapidly a prime concern. As no multi-toxin LC-MS method was validated through interlaboratory trials at the time, single-laboratory validation was the only route to achieve accreditation, and thus the method underwent scrutiny for a number of performance characteristics that need to be described for acceptance by the accreditation body; these parameters included scope, specificity, limits of detection and quantification, linear range, repeatability (within batches) and reproducibility (between batches), accuracy, robustness and comparability between laboratories. While most parameters (scope, specificity, LOD, LOQ, linear range, repeatability and reproducibility) could be rapidly established, some parameters (accuracy, robustness and comparability between laboratories) were more difficult to establish. Initial trials investigating robustness were carried out on early stages of the method (extraction solvent and amount, variation in sample mass, extraction duration and rotor speed, speed and duration of centrifugation etc.). These parameters showed to have little influence on the result as long as they were kept within reasonable ranges. Still, robustness was difficult to assess as there are also many technical factors influencing ionisation in LC-MS detection (temperature, gas flows, column age and the sample itself). Eventually, response stability throughout the chromatographic batch was chosen as the best indicator of robustness, and the difference of the slope of bracketing calibration curves was selected as the parameter. Slope difference of up to 20% have been found acceptable to keep the method within acceptable limits long-term. An in-house prepared LRM had also been used in every batch to verify the performance of the method. Accuracy was assessed using a certified reference material for OA, available at the time from the NRCC; unfortunately, no such material has been available for AZA. Therefore, repeated extractions were carried out to determine the number of extraction steps necessary to extract > 95% of toxin from the matrix. Further efforts were also undertaken to produce certified reference materials, these efforts are described in section 4.5.1. The comparability between laboratories was also a very difficult point as the Community Reference Laboratory focussed solely on the reference method at the time: the mouse bioassay. Also, there was no other, independent proficiency testing scheme in operation for shellfish toxins, which is the reason why we encouraged a provider of such schemes to take on these compound groups into their scope; efforts in this area are described in section 4.5.2. In addition, bilateral comparison was carried out with a Norwegian laboratory for AZA (Hess et al., 2005). Also, an intercalibration exercise had been organised by the Cawthron Laboratory (NZ) in 2003, and results of this exercise had been positive, suggesting that matrix effects were small or negligible in this type of analysis. In light of the difficulties encountered due to the lack of certified standards, certified reference materials and the lack of interlaboratory studies in this field, I participated in the proposal of an EU project, BIOTOX. Over the course of this project, we organised and participated in several

interlaboratory trials and we were able to investigate in more depth the critical parameters in LC-MS analysis of lipophilic toxins in shellfish.

4.2.2.1. Critical parameters in the analysis of lipophilic toxins by LC-MS

In the BIOTOX project, parameters influencing LC-MS performance were systematically evaluated; the data presented here are from different parts of Work Package 2, deliverable 7 (BIOTOX, 2008a). In the area of LC-conditions the most important parameters evaluated were the difference between isocratic and gradient chromatography, the pH of the mobile phase and the difference between HPLC and ultrahigh performance liquid chromatography (UHPLC).

Similar to the hydrophilic toxins, the pH is of particular interest in the selectivity of the separation. At acidic pH, PTX2, which is ionised in positive or negative mode, typically co-elutes with OA-group toxins, while it elutes after AZAs at basic pH (compare Fig. 2.2.1 and 2.2.2). Such variations are useful when instrumental settings do not allow for rapid switching between ionisation polarity.

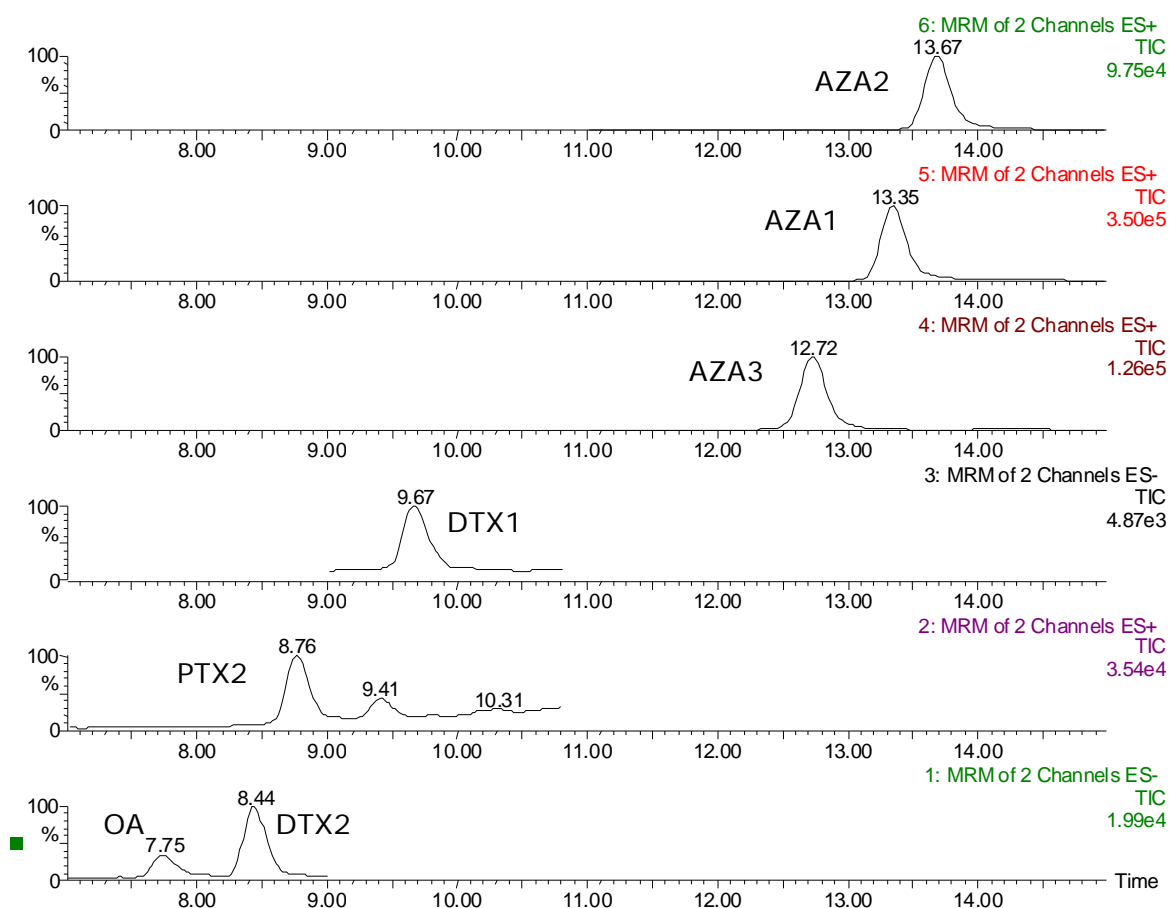


Figure 2.2.1. Separation of the lipophilic biotoxins on the BDS Hypersil C8 column (50*2mm, 3µm; guard column 10*2mm, 3µm) under acidic gradient conditions, at 0.2ml/min.

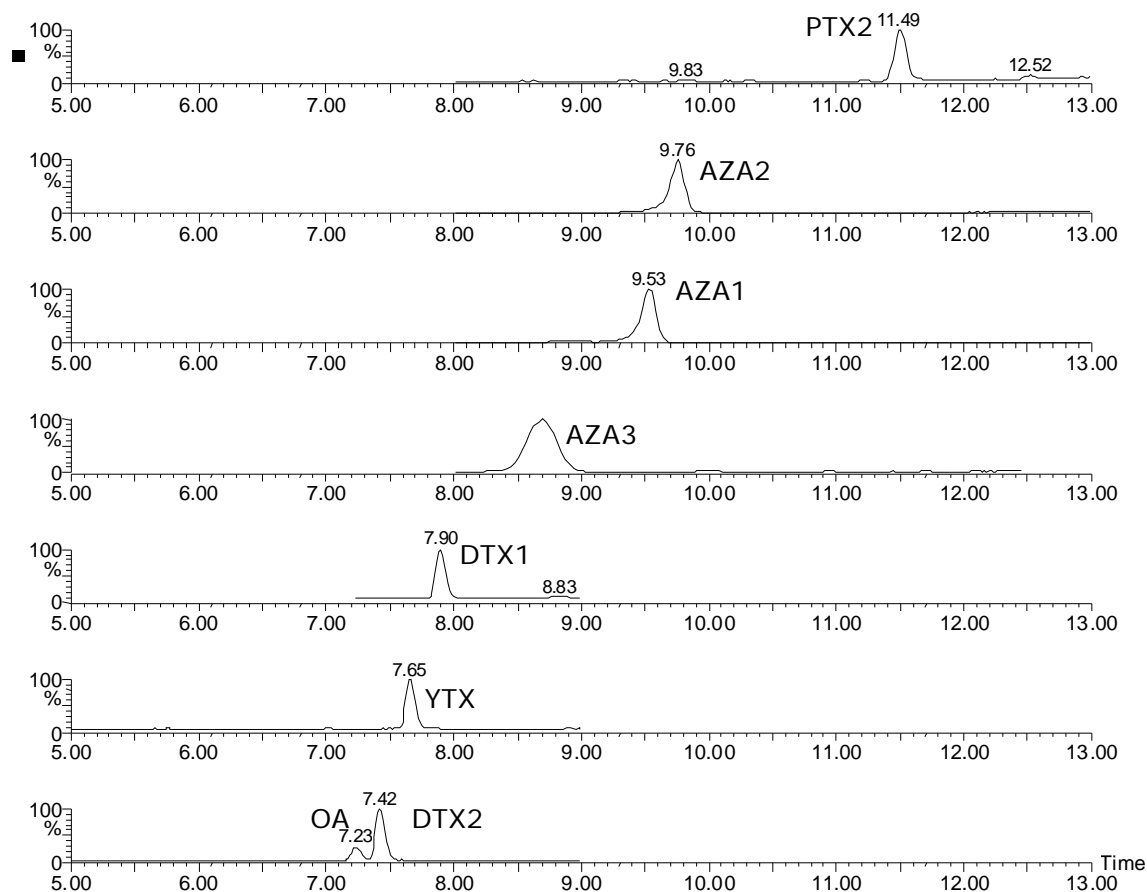


Figure 2.2.2. Separation of the lipophilic marine biotoxins on the Xbridge column (150*3mm, 5 μ m) using a binary gradient of mobile phases A (100% aqueous + 0.05% NH₄OH) and B (95% acetonitrile + 0.05% NH₄OH)

A further important parameter in mass spectrometric analysis is the ionisation. In the ion source, the mobile phase needs to be evaporated and the analyte needs to be ionised to enter the mass spectrometer. The evaporation and ionisation process depend heavily on the compounds present in the mobile phase as well as the analyte itself.

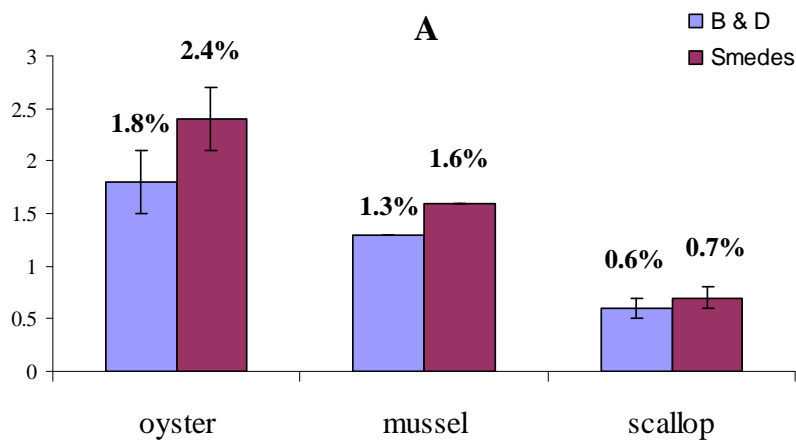


Figure 2.2.3. Total lipid content of oyster, mussel and scallop tissue extracted using adaptations of the methods of B&D and Smedes, SD \pm 1, (n=3)

The more compounds are present in solution, the more difficult it will be to evaporate the solvent, in particular if the solutes are non-volatile compounds such as lipids. As shellfish contain various amounts of lipids (Fig. 2.2.3), the shellfish type and concentration of shellfish matrix in the extract will affect the evaporation, and the ionisation.

To compare the effects of matrix content on the ionisation (mode and choice of transitions), an intercomparison study with six laboratories was carried out as part of the transition study (BIOTOX, 2007). In this study, we focussed on mussel matrix, as this shellfish species is one of the most contaminated and most produced species in Europe. Two different matrix strengths were chosen: solvent to sample ratio 5 and 10, the latter being 2 times less concentrated than the first. Three different ionisation and transition types were investigated when applicable:

- a) positive and negative ionisation mode
- b) parent ion monitoring versus tandem mass spectrometric transition monitoring
- c) different transitions monitored

For OA, all three parameters were evaluated. In parent ion monitoring, positive ionisation always led to larger between laboratory variability, irrespective of the matrix strength (Fig. 2.2.4). The mean value was also slightly higher in positive mode than in negative mode, suggesting larger matrix enhancement than with negative ionisation.

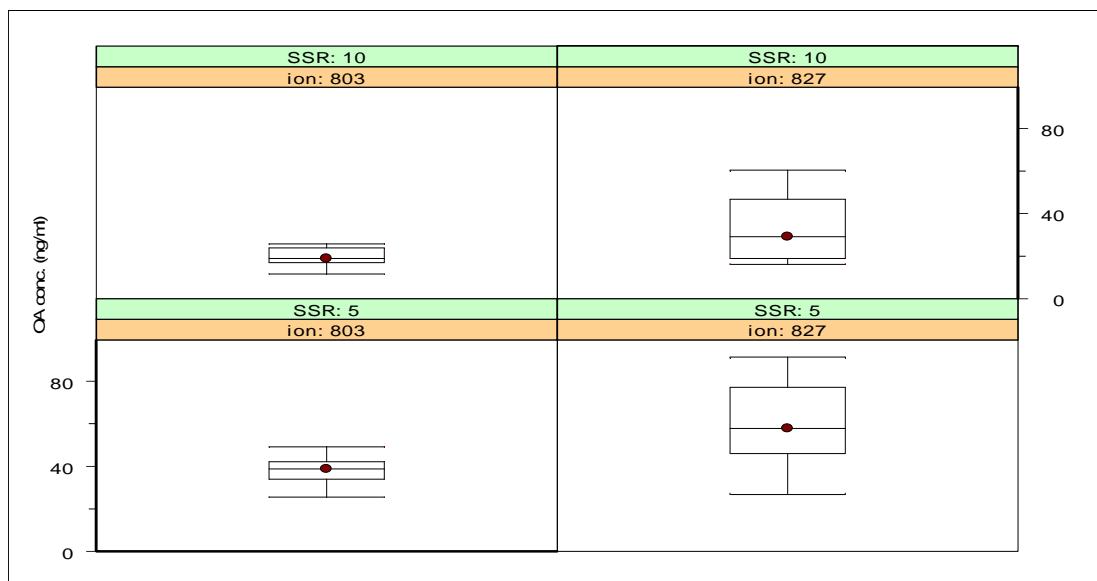


Figure 2.2.4. Distribution of the OA results acquired in parent monitoring mode in mussel (*M. edulis*) extract

The same trend of overestimation in positive mode accompanied by larger variability was observed in double transition mode (compare Figs. 2.2.5 and 2.2.6).

The difference between the two matrix strengths could also be clearly seen to influence the result (Fig. 2.2.6). When the transition 803 > 255 was monitored, there was a significantly higher variability in the more concentrated SSR5 solution.

Therefore, we recommended both negative monitoring and double transition monitoring of dilute solutions for OA-analysis.

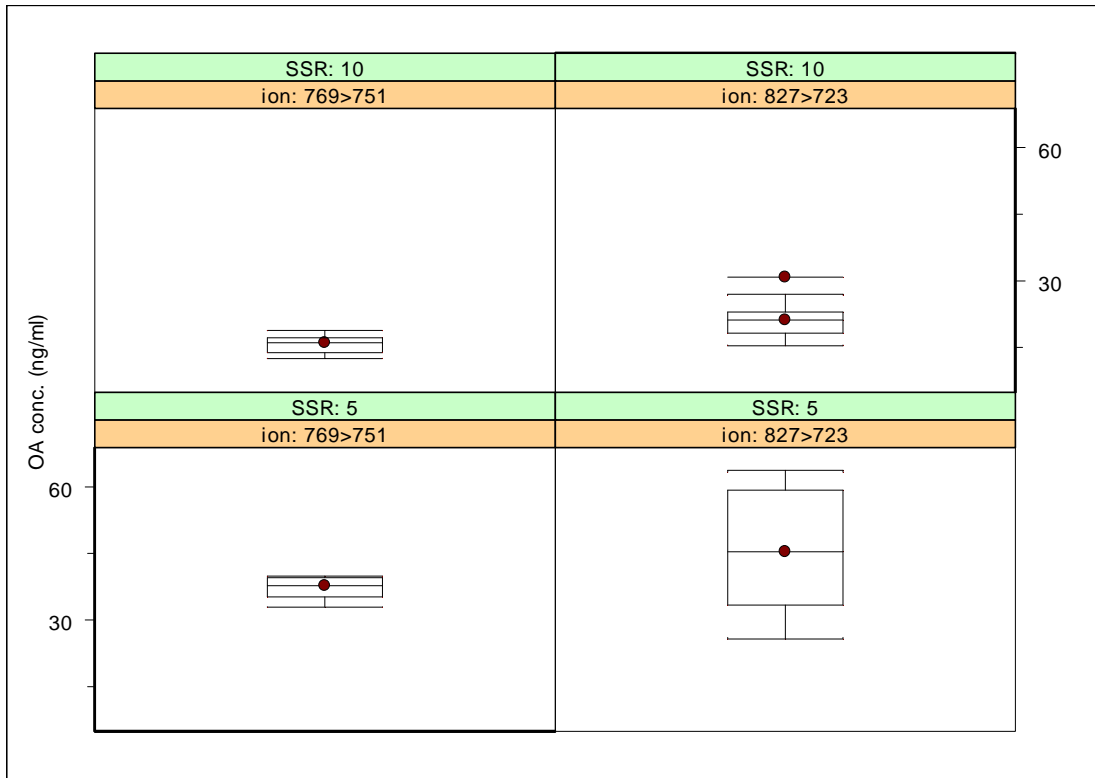


Figure 2.2.5. Distribution of the OA results acquired in positive double transition monitoring mode in mussel (*M. edulis*) extract

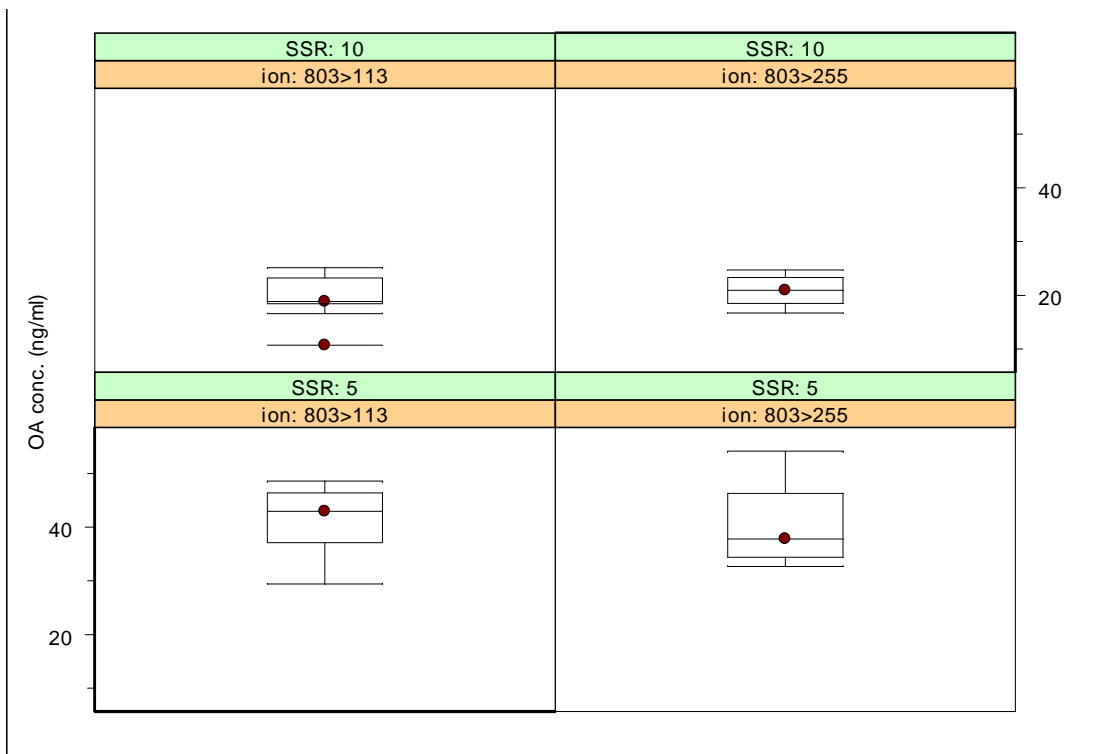


Figure 2.2.6. Distribution of the OA results acquired in negative double transition monitoring mode in mussel (*M. edulis*) extract

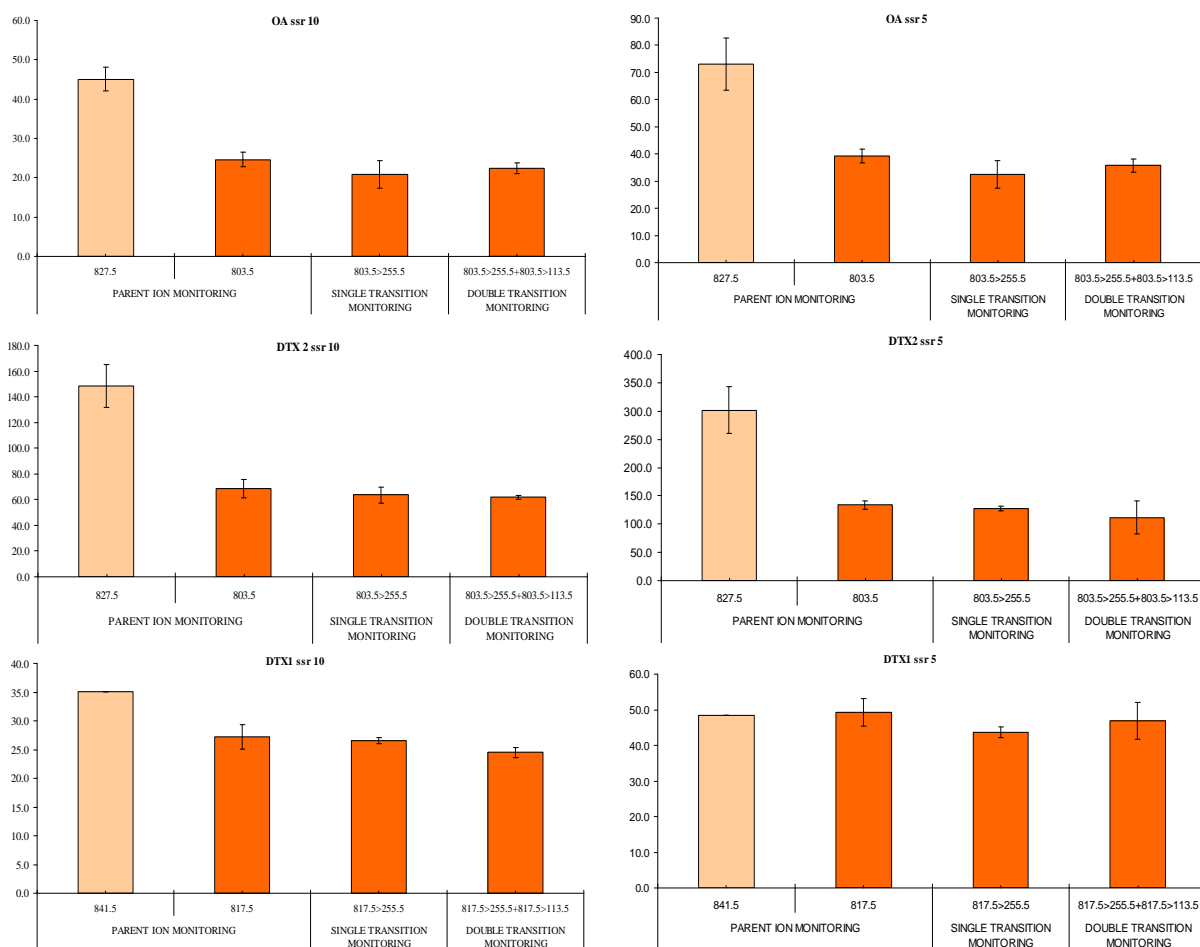


Figure 2.2.7. Concentrations of OA group toxins (OA, DTX1 and -2) in mussel (*M. edulis*) LRM material at two different matrix strengths (SSR 10 and 5) analysed using three different acquisition modes (parent ion monitoring (positive mode: light orange and negative mode: dark orange), single transition monitoring and double transition monitoring). Analysis conducted using LC-TSQ-MS. Error bars represent $SD \pm 1$, $n=3$

Also when comparing different analytes out of the same group (which often have to be determined from the same calibrant), it is clear that parent ion monitoring in positive mode is not an option for quantitative analysis of OA-group toxins (Fig. 2.2.7).

YTX was only analysed in negative mode, and AZA only in positive mode but for both compounds similar trends were observed for parent ion monitoring as shown for OA. PTX2 showed always large variability and enhancement, irrespective of the ionisation mode.

The transition study also allowed to some extent an evaluation of the influence of different types of mass spectrometers. In particular, it was noted that the use of classical ion trap mass spectrometry led to relatively high variability, and is thus not recommended for quantitative analysis.

4.2.2.2. Evaluation of matrix effects in mass spectrometry

The results presented in this section were obtained during the Master thesis of Mairead McElhinney, the degree thesis of Daniela Rode and the PhD thesis of Elie Fux and have been published in Fux et al., 2007 and 2008. Some data have not yet been published in the peer-reviewed press but have been reported (BIOTOX, 2008c).

The systematic investigation of matrix effects can be carried out using at least three different approaches:

- post-column infusion of analyte into the eluate of a blank matrix extract
- spike of the same analyte concentration into blank extracts of various matrix strength
- spike of increasing analyte concentrations into blank extracts of the same matrix strength (matrix matched standards)

The postcolumn infusion of AZA into mussel extract was attempted by Fux et al., 2007, and showed that no matrix effects are to be expected (Fig. 2.2.8). However, this was in contradiction to most previous observations using other techniques. Possibly, this disparity was related to the fact that the experiment had to be carried out using isocratic elution, which needed to be applied due to the rising baseline in gradient elution. Therefore, we decided not to use this approach further in our investigations of matrix effects.

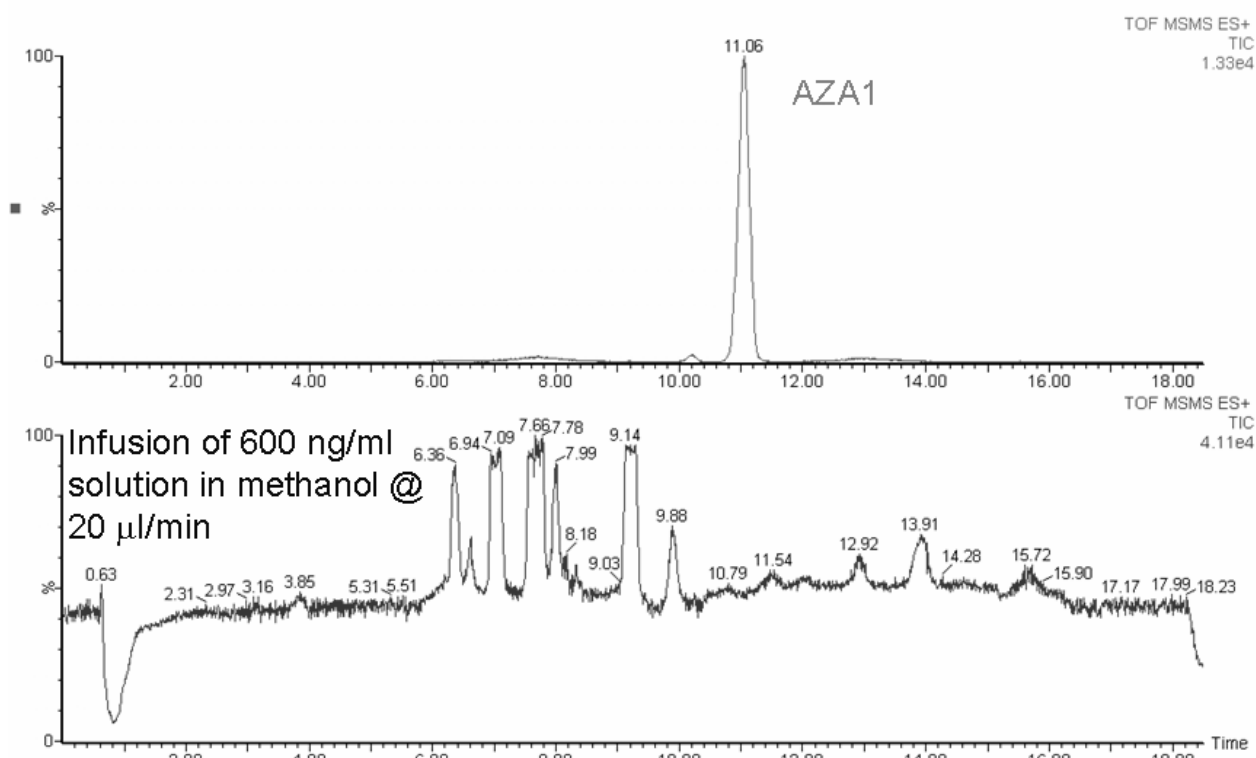


Figure 2.2.8. Examples of (A) AZA1 standard and (B) AZA1 post column infusion chromatograms obtained by step gradient HPLC with Q-ToF detection.

The other two techniques involving analyte spikes into matrix extracts (b and c above) gave much more consistent results. For example, the previously mentioned ion enhancement for OA-group toxins was observed both in the spikes of analytes into blank extracts of increasing matrix strength (Fig. 2.2.9) and in matrix matched standard experiments (Fig. 2.2.10 and Table 2.2.1).

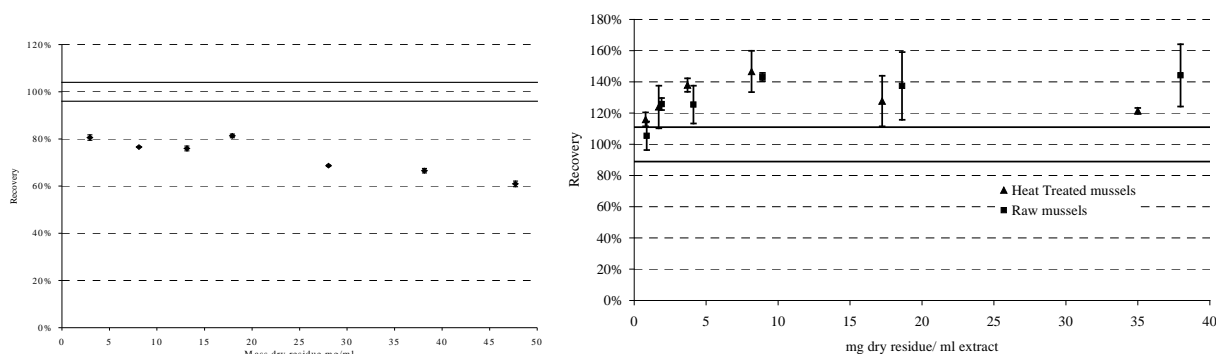


Figure 2.2.9. Post extraction addition of AZA (left graph) and OA (right graph) analysed using step gradient HPLC conditions and Q-ToF detection. Bold lines represent the precision obtained by duplicate injection of spiked matrix free solutions. The error bars were calculated from duplicate injection.

Also, even though the absolute effect may vary between shellfish species (Fig. 2.2.10) and even raw or cooked matrix (Table 2.2.1), the trends for a given analyte are almost always in the same direction: OA tends to show ion enhancement, AZA tends towards ion suppression, and PTX2 is always strongly enhanced, even at very strongly diluted extracts.

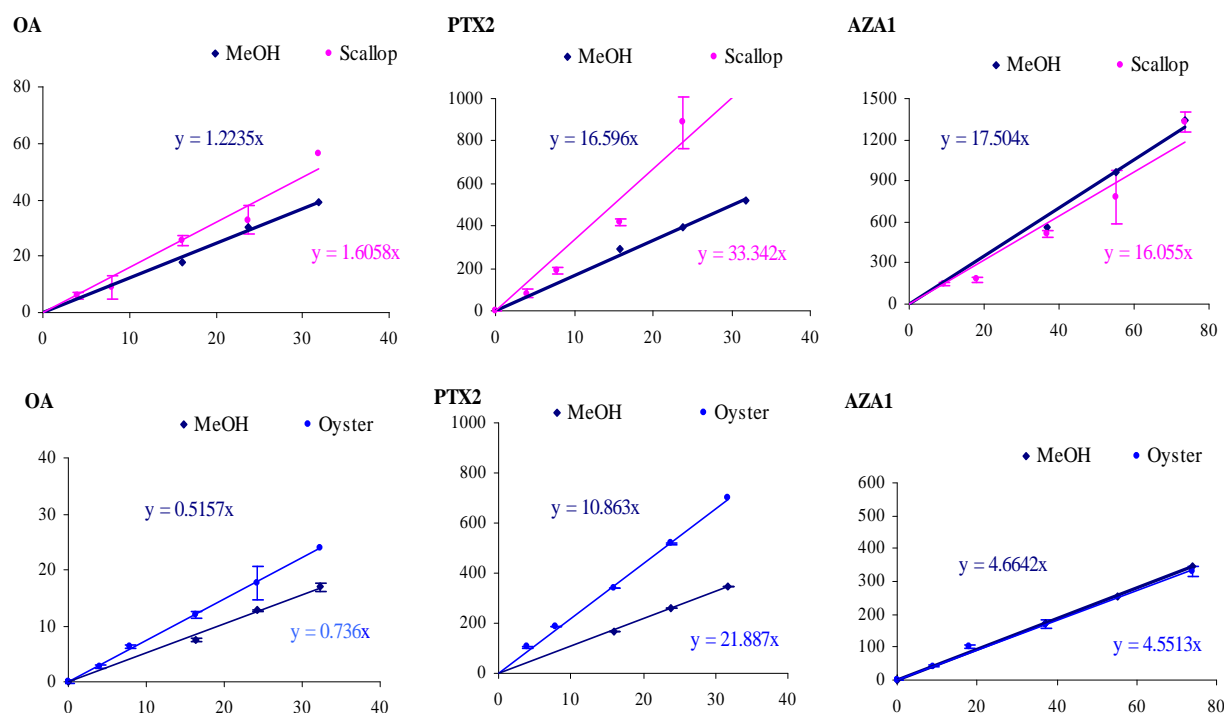


Figure 2.2.10. Matrix matched calibration as a measure for matrix effects. Top: OA, PTX2 and AZA1 in MeOH and scallop matrix extracts. Bottom: OA, PTX2 and AZA1 in MeOH and oyster matrix extracts.

Matrix effects for OA, AZA1 and PTX2 could be effectively reduced by switching from HPLC to UPLC (Table 2.2.1). However, this was not the case for all UPLC columns and the use of C8 reverse-phase chromatography is still recommended. Also, the reduction of matrix effects led to virtual elimination of effects for AZA1, while effects for OA and PTX2 still need to be accounted for. However, for OA, dilution of extracts is an effective tool to further reduce matrix effects (Table 2.2.1).

Table 2.2.1. Slopes, intercept (Int), correlation coefficients (R^2) and percentage of matrix effects (ME) observed for OA, AZA1 and PTX2 in extracts of heat-treated mussels (HM) and raw mussel (RM) flesh and standards using HPLC and UPLC equipped with BEH C₈ and C₁₈ column.

Matrix	HPLC C ₈ ¹				UPLC C ₈ ²				UPLC C ₁₈ ³			
	Slope	Int.	R ²	ME %	Slope	Int.	R ²	ME %	Slope	Int.	R ²	ME %
OA	RM SSR	116.0±	-135.0	0.9982	78.0	8.3	0.9969		92.1	10.9	0.9971	
	10	6.7	±3.7	±0.0019	±8.1	±13.6	±0.0023	21.2	±5.8	±18.1	±0.0032	19.3
	RM SSR	104.2±	33.3	0.9926	70.5	55.3	0.9975		81.6	99.1	0.9976	
	20	3.6	±11.4	±0.0066	±6.6	±86.7	±0.0020	9.5	±8.5	±59.6	±0.0028	5.7
	HM SSR	115.0±	-43.2	0.9961	85.3	-97.8	0.9956		98.3	-111.9	0.9942	
	10	17.9	±111.0	±0.0006	±5.5	±24.5	±0.0042	32.5	±5.8	±2.7	±0.0050	27.2
	HM SSR	99.0 ±	88.5	0.9973	75.7	70.1	0.9950		90.6	99.7	0.9932	
20	1.9	±33.7	±0.0022	±9.8	±65.1	±0.0041	17.6	±6.4	±56.7	±0.0040	17.4	
Std	77.2 ±	-13.7	0.9987	64.4	14.3	0.9994		77.2	57.6	0.9946		
	9.7	±152.1	±0.0016	±5.5	±121.4	±0.0002		±3.9	±140.4	±0.0071		
AZA1	RM SSR	1321.1	149.2	0.9997	694.8	29.5	0.9994		667.4	393.2	0.9997	-
	10	±126.9	±3.7	±0.0003	±49.7	±437.1	±0.0003	-2.4	±62.1	±818.2	±0.0004	34.4
	RM SSR	1189.5	2838.8	0.9987±0	692.5	850.2	0.9996		744.1	938.3	0.9990	-
	20	±13.1	±112.9	.0002	±45.4	±251.7	±0.0003	-2.7	±101.1	±31.6	±0.0010	26.8
	HM SSR	1196.2	-49.1	0.9995±0	712.1	115.2	0.9994		825.1	-3775	0.9779	-
	10	±17.9	±859.1	.0005	±62.0	±116.7	±0.0005	0.1	±26.8	±1017	±0.0094	18.9
	HM SSR	1232.0	1576	0.9989±	715.6	157.5	0.9997		885.6	-469.4	0.9981	-
20	±107	±172	0.0011	±74.3	±563.8	±0.0003	0.1	±28.8	±726.0	±0.0000	12.9	
Std	1456.8	-1947	0.9995±	711.7	-497.9	0.9995		1016.9	-1095	0.9967		
	±132	±530	0.0001	±46.4	±384.3	±0.0004		±76.6	±1109	±0.0052		
PTX2	RM SSR	1119.9	-580.8	0.9989±0	364.1	169.6	0.9994		432.6	-221.9	0.9962	
	10	±30.0	±165.4	.0012	±16.6	±52.5	±0.0005	-1.5	±40.3	±124.7	±0.0024	92.5
	RM SSR	1083.6	423.6	0.9981±0	433.3	-5.6	0.9995		380.6	113.1	0.9970	
	20	±22.2	±89.8	.0008	±31.4	±117.9	±0.0002	17.2	±33.4	±86.0	±0.0034	69.4
	HM SSR	1188.2	-576.3	0.9993±0	393.1	71.0	0.9978		397.5	-696.6	0.9942	
	10	±196	±440.0	.0001	±33.5	±58.6	±0.0028	6.4	±39.2	±86.3	±0.0033	76.8
	HM SSR	1042.2	347.8	0.9996±	439.4	21.5	0.9990±0		399.8	-62.9	0.9944	
20	±60.3	±33.7	0.0002	±32.7	±40.1	.0012	18.9	±102.4	±311.5	±0.0012	77.9	
Std	770.7 ±	119.4	0.9994±	369.7	126.1	0.9996±0		224.7	167.7	0.9923		
	80.8	±404.8	0.0007	±24.1	±130.7	.0002		±29.9	±291.7	±0.0106		

¹ Standard slope was obtained from triplicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from triplicate injections of a set of five solutions

² Standard slope was obtained from four replicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from triplicate injections of a set of five solutions

³ Standard slope was obtained from six replicate injections of a set of 7 solutions and slopes in mussel matrices were obtained from duplicate injections of a set of five solutions

We had also investigated the influence of day-to-day variation of the analysis on matrix effects, and noted that this may vary easily up to 15 % difference in absolute terms (Table 2.2.2). This variation should be further investigated as it probably related to the state of cleanliness of the instrument, and it may also depend on the source design of different manufacturers.

Table 2.2.2. Slopes, intercept, correlation coefficients and percentage of matrix effects observed on different days for OA, AZA1 and PTX2 in oyster and scallop extracts using conditions E1.

			Slope	Intercept	R2	%ME
OA	Day 1	Oysters	0.7 ± 0.1	0.2 ±	0.9960 ± 0.0026	37.4%
		MeOH	0.5 ± 0.0	-0.2 ±	0.9931 ± 0.0024	
	Day 2	Scallop	1.7 ± 0.2	-2.1 ±	0.9867 ± 0.0055	43.7%
		MeOH	1.2 ± 0.0	-0.1 ±	0.9984 ± 0.0007	
AZA1	Day 1	Oysters	4.3 ± 0.2	6.7 ±	0.9941 ± 0.0018	-7.1%
		MeOH	4.6 ± 0.1	-0.3 ±	0.9971 ± 0.0025	
	Day 2	Oysters	9.9 ± 0.1	-0.6 ±	0.9979 ± 0.0016	-1.9%
		MeOH	10.1 ± 0.5	-27.7 ±	0.9942 ± 0.0034	
	Day 3	Scallop	4.1 ± 0.1	1.9 ±	0.9961 ± 0.0028	3.4%
		MeOH	3.9 ± 0.3	-0.1 ±	0.9990 ± 0.0008	
PTX2	Day 1	Scallop	33.4 ± 1.0	-51.2 ±	0.9886 ± 0.0043	109.1%
		MeOH	16.0 ± 1.1	4.6 ±	0.9980 ± 0.0020	
	Day 2	Oysters	20.9 ± 1.0	7.1 ±	0.9975 ± 0.0013	93.3%
		MeOH	10.8 ± 0.1	-2.5 ± 3.1	0.9976 ± 0.0008	

In summary it needs to be reminded that matrix effects still remain complex phenomena, depending on a number of instrumental parameters (Table 2.2.3). Therefore, it is my opinion that method validation is difficult in this area, and should in any case be complemented with the requirement for regular participation in proficiency testing to ensure performance of laboratories.

Table 2.2.3. Summary of the effect of chromatography, stationary phase, instrument, shellfish species, shellfish heat treatment and extract dilution on the matrix effects affecting the analysis of OA, AZA1 and PTX2.

	LC	Stationary Phase	Instrument	Species	Heat Treatment	Dilution
OA	n/a	-	+	+/-	+	+
AZA1	-	+	-	+/-	+	-
PTX2	+	+	+/-	n/a	-	-

+ : observed effect; - : no observed effect; +/-: limited observed effect; n/a: not available

4.2.2.3. Clean-up techniques for the removal of matrix effects

Two common clean-up techniques have been evaluated in the frame of the BIOTOX project, liquid-liquid extraction (LLE) and solid phase extraction (SPE), results have been reported in various documents (Biotox, 2008b; Fux et al., 2008; Gerssen et al., 2009; McElhinney, 2007).

Partitioning with isopropyl-acetate (IPA) resulted in significantly higher deviations than with dichloromethane (DCM), suggesting that IPA is not very suited for analytical scale purposes. The effect of LLE on the toxin recovery was rapidly examined and showed a major loss of YTX, either when using DCM or IPA, Fig 2.2.11. The difficulty with the partitioning behaviour of YTX meant that it would be very difficult to gather all toxins classed “lipophilic” in the current EU legislation into a single fraction for subsequent chromatographic analysis. This would severely hamper the multitoxin approach in which many different analogues from a wide range of toxin groups are analysed in a single extract.

Further recovery losses were observed during evaporation steps which are typically necessary in combination with LLE due to the need for solvent exchange after the partitioning step. In particular, we observed problems when purified sample extracts were dried down in centrifugal evaporators (e.g. Speedvac®) or when reconstituting the dried extracts with less solvent than from which they had been evaporated.

In summary, the possible recovery losses during LLE and subsequent evaporation steps showed to be detrimental in such a way that SPE was investigated as a clean-up technique.

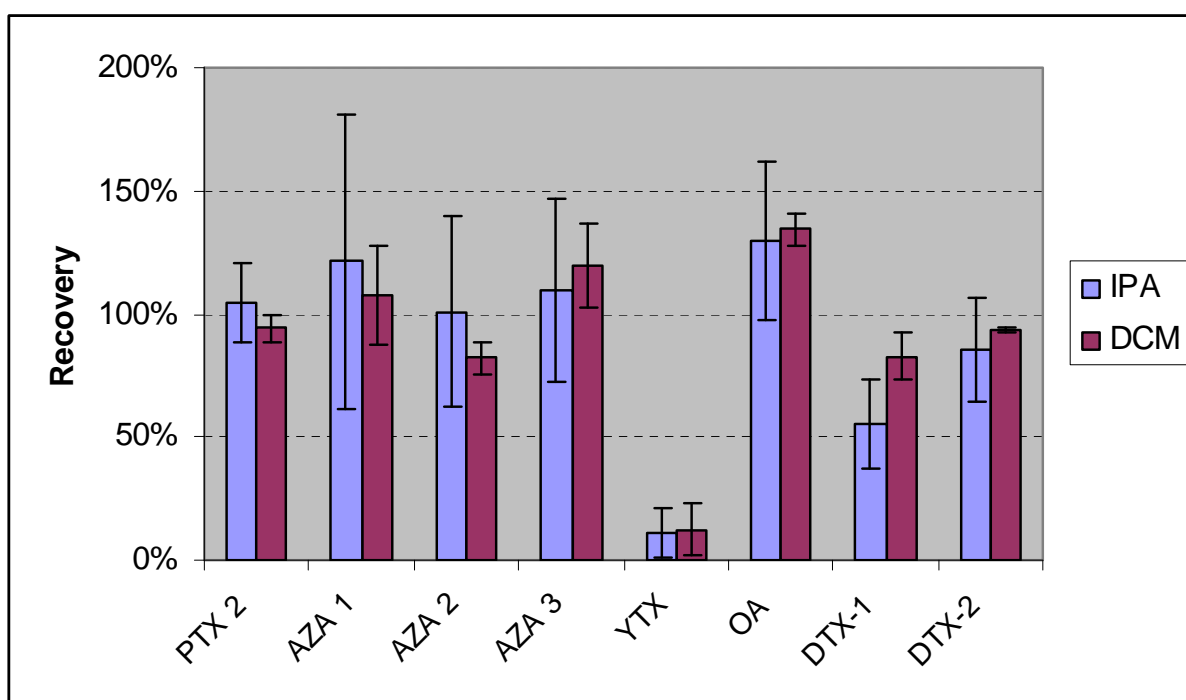


Figure 2.2.11. Recoveries of lipophilic toxins from liquid-liquid partitioning using either IPA or DCM. Error bars represent ± 1 SD ($n=3$). Note low recoveries for YTX and large standard deviations for IPA.

The efforts in development and optimisation of SPE as a clean-up have been described in the BIOTOX project report (WP2, deliverable 15; BIOTOX, 2008b), and have been published in the peer-reviewed press (Gerssen et al., 2009).

The first step of the SPE method development focused on finding an appropriate sorbent which retained the toxins sufficiently. With the Symbiosis system a rapid sorbent screening of 12 different sorbents ranging from very polar to strongly hydrophobic was performed. General settings of critical parameters were chosen such as wash solvent, wash volumes, percentages organic and flow rates. From the recoveries obtained it became clear that the WCX cartridge under neutral and acidic wash and elution conditions and the WAX and MAX cartridges under all conditions showed the highest recoveries for OA (2.2.12a). YTX was not retained on or eluted from the WAX and MAX cartridge, on the WCX and MCX cartridge the recovery was poor, except for the WCX cartridge where with a neutral wash and basic methanol elution a good recovery was obtained (2.2.12b). For AZA1 the WCX cartridge gave good recoveries under neutral and acidic wash and elution conditions. The MCX cartridge gave poor recoveries under all applied wash and elution conditions. The WAX cartridge gave good recoveries under neutral wash conditions and a neutral or basic elution, while the MAX cartridge gave good recoveries with all applied conditions except with an acidic wash and elution for AZA1 (2.2.12c). The WCX cartridge provided good recoveries under neutral and acidic conditions for PTX2, while the MCX cartridge provided only good recoveries under neutral conditions. With the WAX and MAX cartridge good recoveries under all applied conditions were obtained for PTX2 (2.2.12d).

From the other cartridges that were investigated (CN, C₂, C₈, C_{8ec} (endcapped), C₁₈, C₁₈, GP and SH) only the CN cartridge gave a poor recovery for OA. For YTX good recoveries were obtained at the C₁₈ HD and GP cartridge, while AZA1 showed good recoveries on all the cartridges except the SH cartridge. PTX2 showed a poor recovery on the CN cartridge but good recoveries on the other cartridges. From the data shown (Fig. 2.2.13) it is clear that the C₁₈ HD and GP cartridge in general showed the best recovery for all toxin groups.

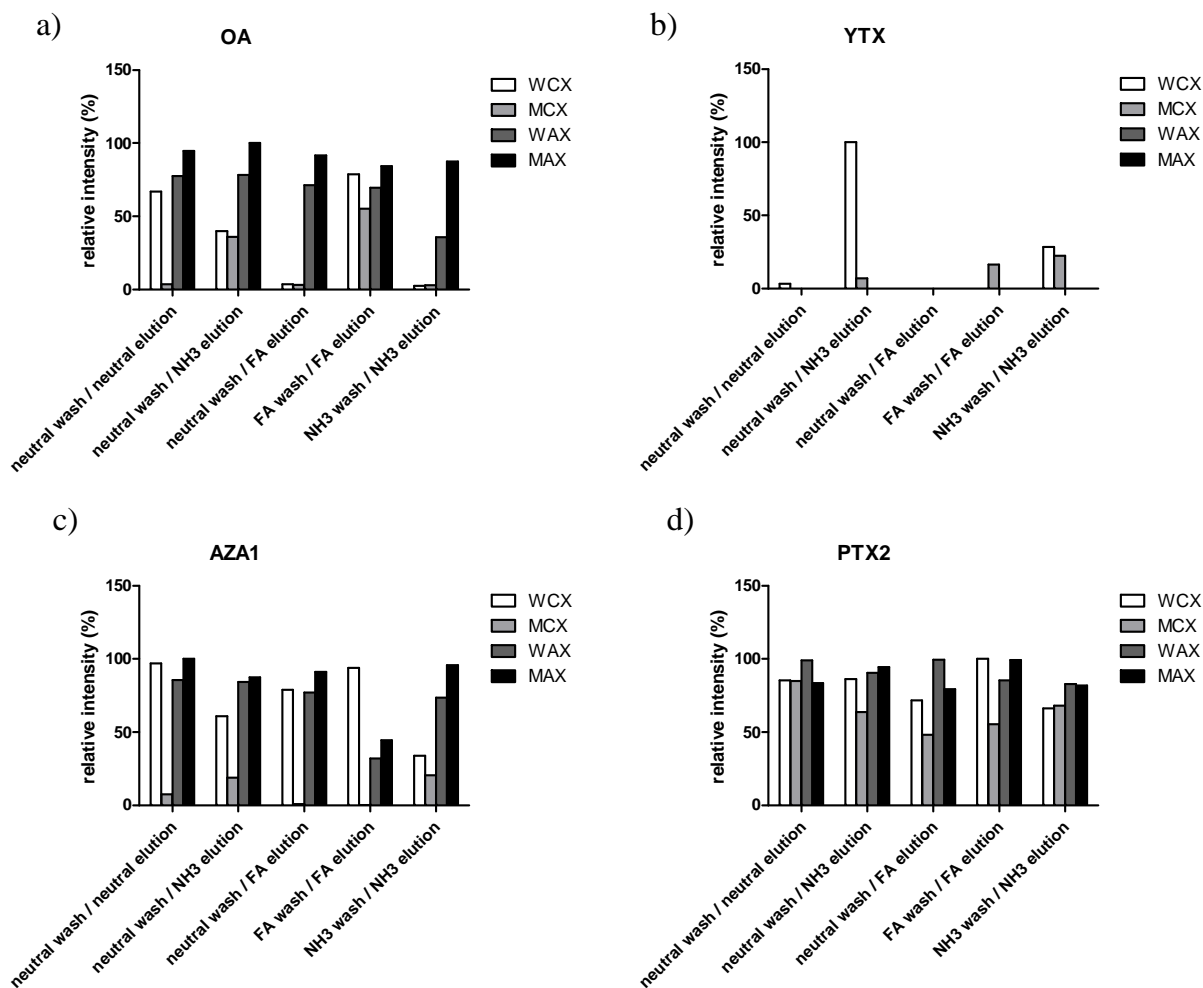


Figure 2.2.12. Mixed mode sorbent screening for a) OA, b) YTX, c) AZA1, d) PTX2 using the Symbiosis automated SPE system (highest intensity per toxin set to 100 %). Wash with either water (neutral), 1% formic acid (FA), 1% NH₄OH solution, elution with either methanol (neutral), 1% FA in methanol or 1% NH₄OH in methanol.

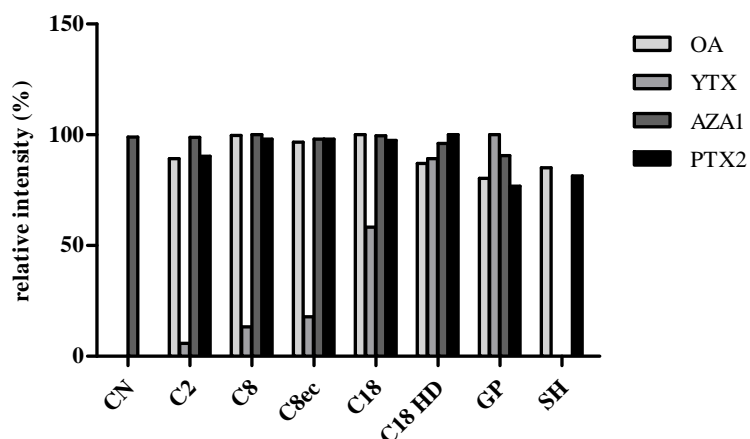


Figure 2.2.11: Sorbent screening using the Symbiosis automated SPE system (highest signal intensity per individual toxin set to 100 %). Wash with 20 v/v % methanol / water elution with 1 v/v % NH₄OH in methanol.

For each toxin different optimum conditions and different optimal sorbents were obtained. Especially for YTX it was difficult to find suitable conditions. This is not surprising, because the different classes of toxins also have different chemical properties. Therefore it proved difficult to select an SPE sorbent that would be useful for all toxins in the four different classes. Based on these results the focus changed towards polymeric columns that can be applied for a broad range of different toxins. Two different brands of polymeric sorbents were selected for further investigation, Oasis HLB and StrataTM-X. Because these cartridges were not available for the symbiosis system the further optimisation experiments were done off-line.

Optimisation of SPE clean-up protocol

For the optimisation 3 cc 60 mg cartridge were used containing either Oasis HLB and StrataTM-X sorbent. The retention capacity of both cartridge types was investigated by application of LRM extract diluted before application with 60 % water. The cartridges were washed with 50 v/v % MeOH and eluted with 100 v/v % MeOH. It became clear that OA, DTX1, DTX2 and YTX were better retained on StrataTM-X than on Oasis HLB (Figure 2.2.14) during application and the wash step. AZA1, AZA2, AZA3 and PTX2 were slightly better retained on the HLB. In general the toxins were somewhat better retained on the StrataTM-X under these conditions, therefore this cartridge was used for further optimisation experiments.

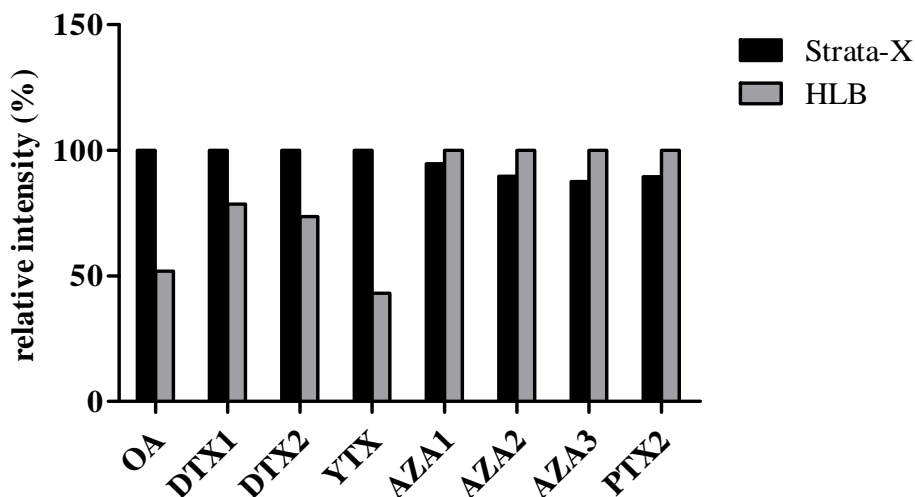


Figure 2.2.14. Relative intensity of the toxins on two different sorbents with the preliminary SPE protocol (highest intensity per individual toxin is set to 100 %).

Parameters of the solid phase extraction protocol that needed to be optimized are:

- Volume and organic solvent strength of the crude extract applied to prevent break-through.
- The wash step; pH, organic strength and volume to prevent losses due to wash-out.
- The elution step; pH, organic solvent and volume to prevent losses due to retention.

No break-through was observed when the crude methanol extracts (with a solvent-to-sample ratio of 10) were diluted to 30 v/v % methanol with water prior to application to the SPE cartridge. At higher percentage methanol some break-through of OA, DTX1, DTX2 and YTX was observed. Increasing amounts of sample; 2, 4 and 6 ml of the crude extract (diluted with 3, 8 and 14 ml water, respectively) were applied to the 3 cc 60 mg cartridge. For none of the extracts break-

through of any of the toxins was observed. Therefore, if necessary a concentration step can be incorporated into the extraction protocol.

The organic solvent strength of the wash step (3 ml) was optimised using 0-70% (v/v) for methanol / water mixtures. From the recoveries obtained it was clear that OA, DTX1, DTX2 and YTX were starting to elute at concentrations above 30 v/v % methanol. AZAs were retained on the cartridge up to 50 v/v % methanol and PTX2 even up to 70 v/v % methanol. A wash step of 20 v/v % methanol was incorporated to avoid losses during washing.

Next, the effect of using acidified, neutral and basic 20 v/v % methanol as wash solvent was investigated. The acidified and basic wash solvent contained 1 v/v % formic acid and 1 v/v % ammonium solution (25 %), respectively. No significant differences were obtained between the different washes, therefore a neutral wash step of 20 v/v % methanol was incorporated in the final method (Fig. 2.2.15).

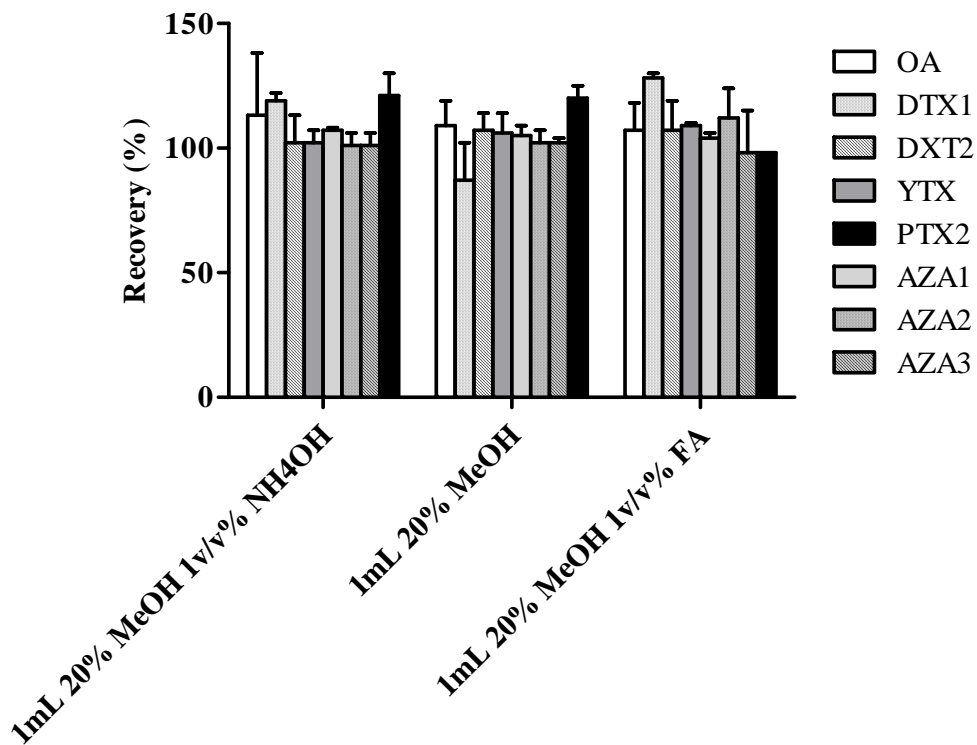


Figure 2.2.15. Optimisation of wash step: effects of different wash solvents (average \pm SD, n=2).

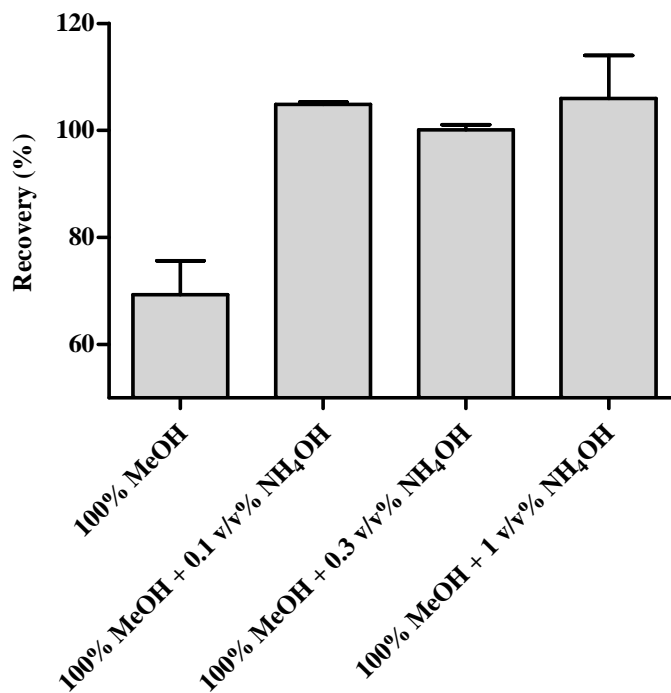


Figure 2.2.16 Optimisation of the elution step: YTX recovery using different elution solvents (average \pm SD, $n=2$).

Elution was performed with 2×2 ml methanol (absolute) and the toxins were collected in two different vials. By incorporating a second elution step the amount of remaining toxins on the cartridges could be estimated. The first elution resulted in an almost complete recovery (> 90 %) of OA, DTX1, DTX2, AZA1, AZA2, AZA3 and PTX2 but for YTX the recovery was somewhat lower (60-70 %). Around 10-20 % of YTX was eluted with the second extraction. The recovery of YTX was related to the pH of the wash step used before elution, with a basic wash step the recovery of YTX was higher (80-90 %), with a acidic wash step it was lower (50-60 %). By adding 0.3 v/v % ammonia solution (25 %) to the elution solvent the recovery could be increased to around 90-100 % for YTX without affecting the recoveries of the other toxins. Using higher concentrations of ammonia solution did not result in improved recoveries (Fig. 2.2.16).

The stability of the toxins under basic conditions in the presence of ammonia was tested. No degradation of any of the toxins was observed even at concentrations of 12.5 v/v % ammonia solution (25 %). The final optimised protocol was downscaled to the smaller 1 cc 30 mg cartridges in order to save valuable toxin standards (Fig. 2.2.17).

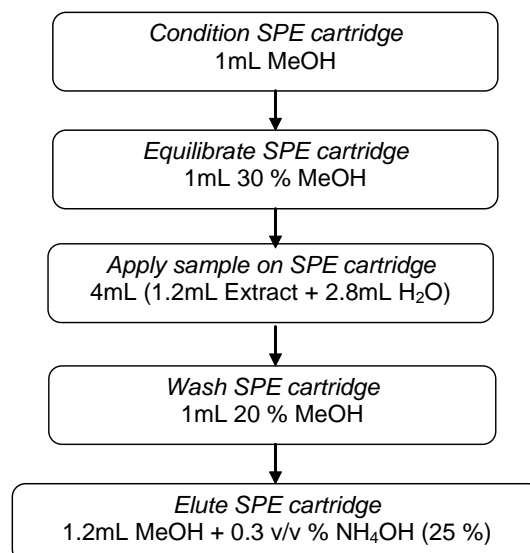


Figure 2.2.17. Final SPE protocol for 30 mg, 1ml Strata™-X cartridge.

Even though the SPE protocol developed showed excellent recoveries and could be optimized to recover all 4 toxin groups into a single SPE fraction, the clean-up effect was only demonstrated in a single laboratory. It remains to be demonstrated that the inclusion of an SPE step into a method validation protocol will still significantly reduce variability between laboratories.

However, if single group methods are investigated it is probable that even more effective clean-up techniques may be developed that demonstrate ruggedness and comparability between laboratories.

4.3. Distribution of algal toxins in the marine environment

The distribution of algal toxins in the marine environment can be described at several levels:

- temporal and geographical distribution
- ecosystem distribution

Data on temporal and geographical distribution are of importance for the optimization of official control programs. With in-depth knowledge on the periods and geographical occurrence of algal toxins, official monitoring can be focused on risk areas and periods. Thus, it has been established for most areas in Europe that summer and autumn are high risk periods for most toxin groups, with the exception of domoic acid which has been shown to reach maximum concentrations in mussels during spring time in Ireland (McCarron and Hess, 2006), or azaspiracids which have been observed to also occur late autumn and into winter (McMahon and Silke, 1996 and 1998).

The distribution of toxins can also be considered from the ecological perspective and this view will contribute to our understanding of the transport and the transformations of toxins as they migrate through the marine food web. In this area of research, it will be important to understand the interactions of different phytoplankton organisms and their relationship with bivalve mollusks:

- Are the primary producers directly taken up by bivalve species or is the accumulation mediated via different phytoplankton species?
- Are there differences in uptake between different bivalve species?
- Do biotransformations or chemical degradation play a role in the profiles encountered in different shellfish species?
- Are the uptake and detoxification curves of toxins in bivalves dependent on the bioavailability of toxins after algal blooms?
- Do toxins persist in the environment beyond the occurrence of toxic algae?

The answers to the above questions will heavily impact on the design of monitoring programs and aquaculture practices in the prevention and remediation of algal blooms.

The five sections below will outline contributions to some of the above themes, demonstrating that both continuous surveillance programs based on quantitative specific methods as well specific studies are required to progress knowledge in this area.

4.3.1. Okadaic acid in America

Okadaic acid (OA) and its analogues, diarrhetic shellfish toxins (DTXs), are a major public health and economic problem for many countries (Boni et al., 1993; Giacobbe et al. 2000) and are among the most important and widely distributed compounds contributing to HAB-associated poisoning syndromes (Van Dolah, 2000). On a global scale, Diarrhetic Shellfish Poisoning (DSP) is widespread, documented in Western Europe, East and Southeast Asia, South America, South Africa, New Zealand and Australia. The situation in North America is particularly interesting, since DSP toxins in shellfish have been reported only on rare occasions. Two events in Canada were attributed to the benthic dinoflagellate *P. lima* found in association with filamentous algae growing on raft cultures of mussels (Quilliam et al. 1993; Lawrence et al., 1998). The most significant event where DSP toxins were detected in North American shellfish occurred in 2008 in Southern Texas and was attributed to *D. acuminata* (Texas Department of State Health Services

news release, March 7th 2008). Until very recently, it has not been possible to culture *Dinophysis* species, and this handicap has slowed down much of the research needed to understand the geographical differences in the occurrence of okadaic acid. Park et al. (2006) successfully cultured *Dinophysis* using the ciliate *Myrionecta rubra* as an intermediate between the cryptophyte and *Dinophysis*. This groundbreaking work has laid the foundations for a recent study by Hackett et al., 2008.

The study by Hackett et al., 2008, demonstrated clearly that American *Dinophysis acuminata* can be cultured under similar conditions to those described by Park et al., 2006. The DTX1 and OA concentrations in the Woods Hole *D. acuminata* were approximately 203 and 18 fg.cell⁻¹ respectively (Fig. 3.1.1). These concentrations are at the low end of toxin content measurements for *D. acuminata*, which range from 100 fg . cell⁻¹ (Yasumoto 1990) to 158 pg . cell⁻¹ (Marcaillou et al., 2005) for DTX1 and 0.1 – 2.7 pg.cell⁻¹ for OA (MacKenzie et al, 2005).

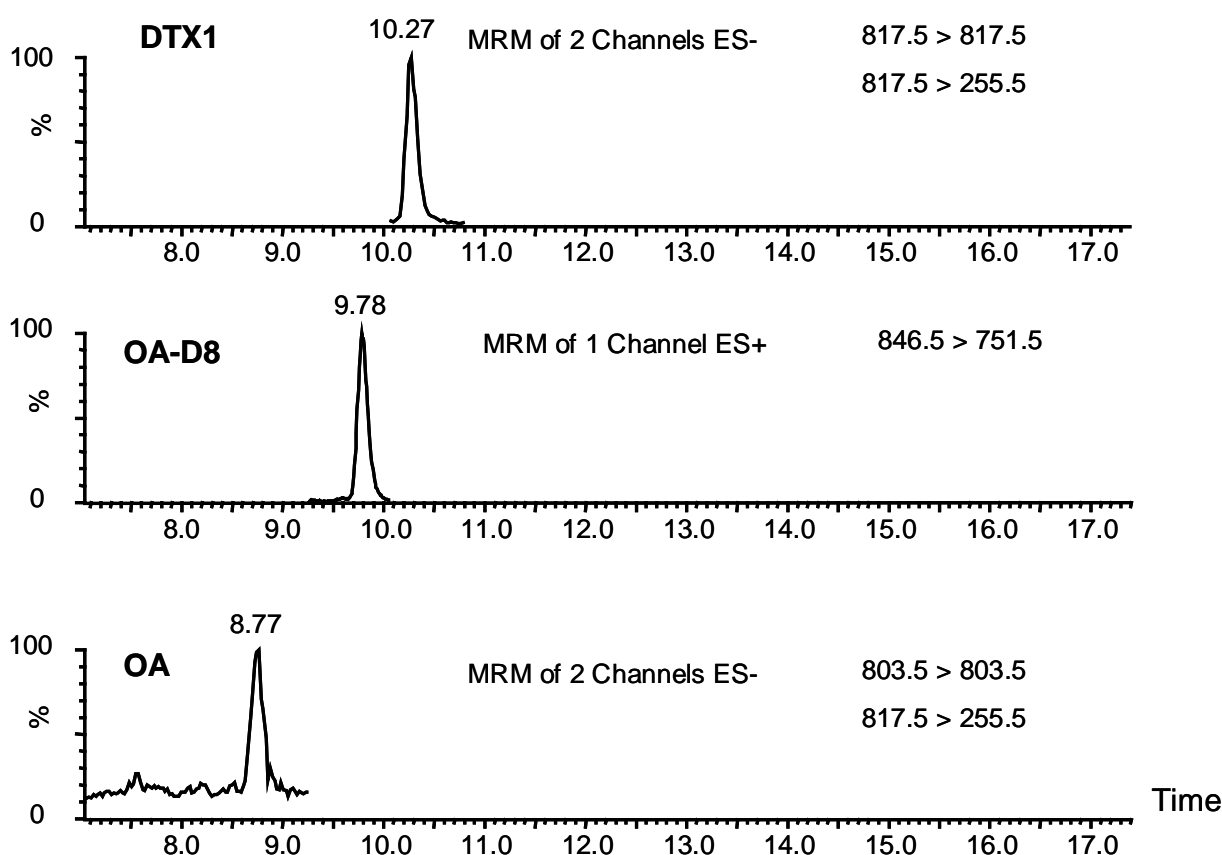


Figure 3.1.1. Example of chromatogram obtained from sample 100% 4°C 2/4/08 No2 obtained by LC-MS Triple-Quadrupole using gradient method on Hypersil C8 column. OA and DTX1 analyses were performed in negative ionisation mode and OA-D8 in positive mode (modified from Hackett et al., 2008)

This result was only possible through the collaborative efforts of the two groups in US and Ireland. The US group of Anderson optimised culture conditions of *D. acuminata* to produce several samples of 100,000 cells and more. At the same time, our group implemented specialised analytical techniques to allow for the detection of low quantities of toxins, as well as their identification. The identity of the compounds was confirmed both by chromatographic characteristics (retention time) and high resolution mass spectrometric characteristics (m/z values). While our study has shown that American *Dinophysis* is capable of producing OA and DTX1, it has also raised further questions as to the biosynthetic route of these toxins, since OA is produced mainly in the form of an OA-diol ester, while DTX1 is produced as the free acid.

4.3.2. Azaspiracids not just in Ireland

Azaspiracids (AZAs) were first detected in mussels (*Mytilus edulis*) from Ireland in 1995 (McMahon and Silke 1996). Since then, poisoning cases were reported in 1997, 1998 and 2000, the country of origin always being Ireland until 2005 (FSAI, 2006). Due to the official control in Europe being mostly conducted using mouse bioassays, it was not trivial to establish whether other countries also suffered from the occurrence of AZAs and whether their consumers would thus be exposed to the risk of AZA-poisoning. Regular surveillance in Norway was established as a collaborative project between our laboratory and the Norwegian National Reference Laboratory (NRL). After initial, unpublished reports of occurrence of AZAs in Norwegian mussels, a sample of mussels collected in 2004 was characterised by our laboratories to investigate whether concentrations of AZAs occurring in Norwegian shellfish were relevant to the EU regulatory limit at the time (160 µg/kg).

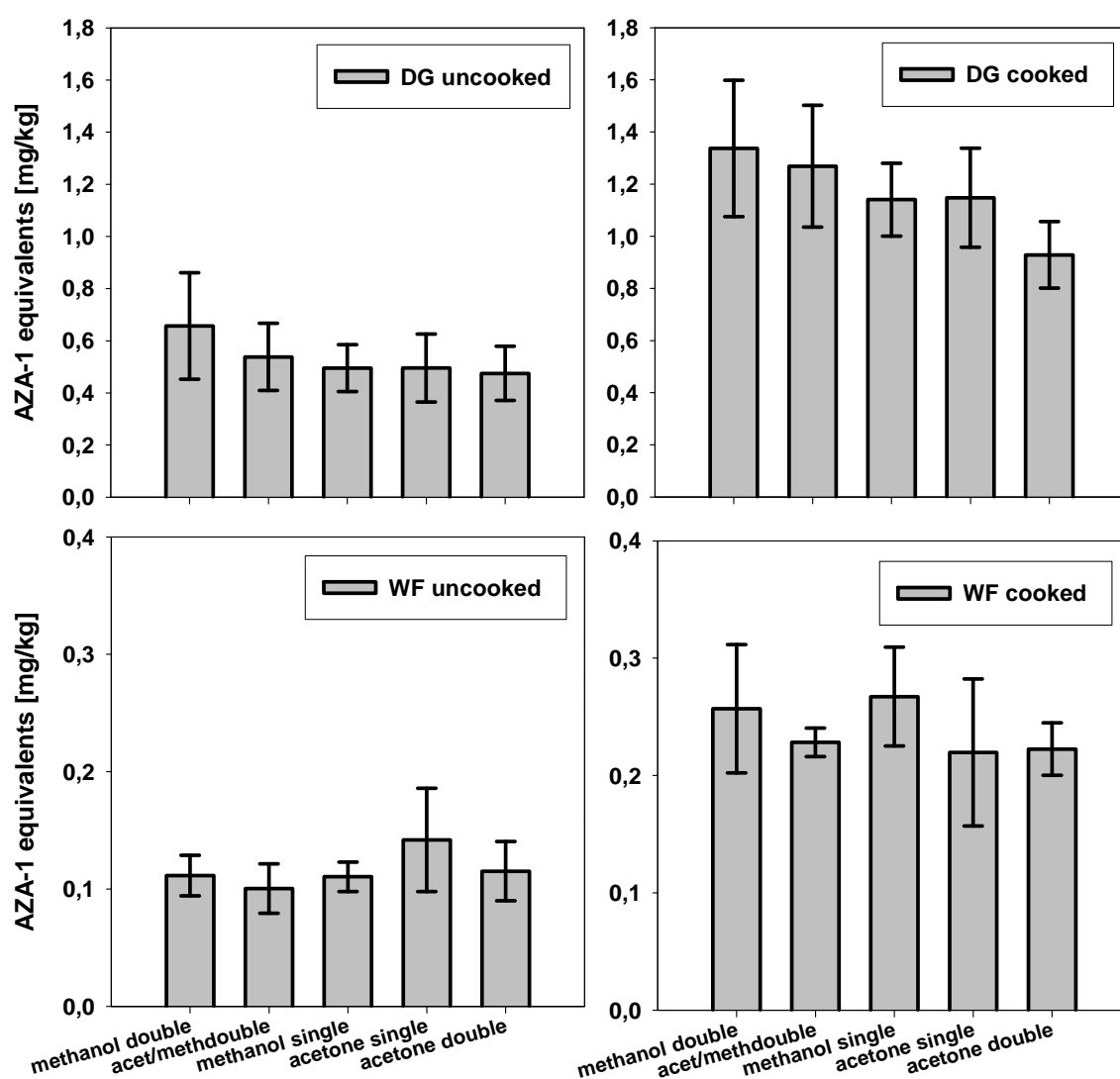


Figure 3.2.1. Comparison of AZA-1 equivalents (AZEs) obtained at the Marine Institute for the four tissue types (cooked vs. uncooked and whole flesh WF vs. digestive gland DG) using different extraction treatments (single and duplicate extraction) and different solvents (acetone, methanol and acetone/methanol). The four tissue types were all from the bulk sample obtained at Fisterfjorden near Stavanger on the West coast of Norway during 2004. Error bars represent 1 SD (n=5), adapted from Hess et al., 2005.

At the time, there was no collaboratively validated method available for the determination of AZAs. For this reason, the study comprised a comparison between two collaborating laboratories (Norwegian and Irish NRL), as well as an investigation of different method parameters that may influence the result (single versus duplicate extraction and different extraction solvents), carried out in one laboratory.

The extraction parameters showed that there was little difference between single and duplicate extraction or between methanol and acetone as extraction solvents (Fig. 3.2.1). A duplicate extraction with methanol was retained for further comparison, as there was a tendency for slightly higher recovery with this solvent and as the duplicate extraction would tend to be less matrix dependent.

The comparison between the two laboratories showed no statistical differences in the results, indicating that the methods were sufficiently optimised in the in-house validation procedure to yield comparable results, despite numerous differences in instrumental and chromatographic parameters (Fig. 3.2.2).

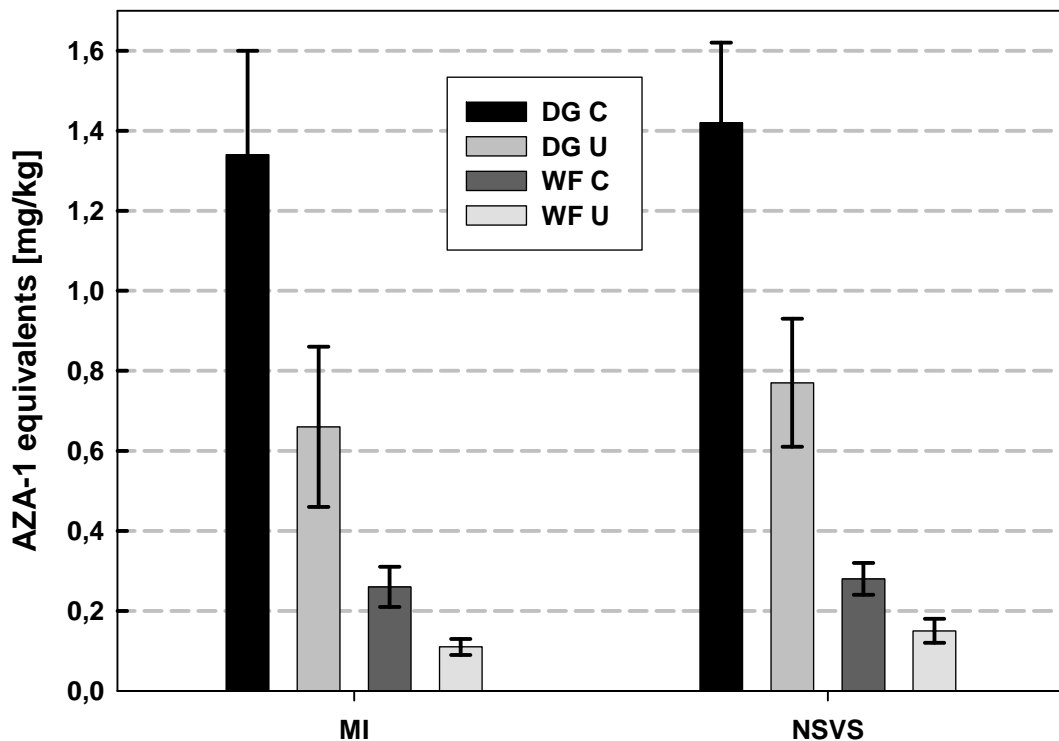


Figure 3.2.2. Comparison of azaspiracid-1 equivalents (AZE), bars are average results and error bars are standard deviations ($n=5$), obtained by the Marine Institute (MI) and the Norwegian School of Veterinary Science (NSVS) for the bulk sample obtained at Fisterfjorden near Stavanger on the west coast of Norway during 2004. Double extraction was carried out using 100% methanol. (DG = digestive gland, WF = whole flesh, C = cooked, U = uncooked).

Thus, the study convincingly demonstrated that the concentrations in shellfish from Norway may reach (or exceed) the EU regulatory limit. Interestingly, the study also revealed an influence of cooking on the concentration of AZAs in whole mussel flesh as well as hepatopancreas tissue. This phenomenon is more complex than initially anticipated and will be discussed in section 4.6.2.

Our knowledge regarding the extent of occurrence of AZAs has further increased, as other publications have revealed the existence of AZAs in all countries of the European and Moroccan Atlantic coast (Amzil et al., 2008; Taleb et al., 2006; Twiner et al., 2008; Vale et al., 2009).

Additionally, AZA2 has also been identified as a metabolite in a sponge sampled in Japan, a finding which stipulates the need for further research to clarify the full extent of distribution of AZAs in the marine environment, and the ecological role of their source organism(s).

4.3.3. Species differences in the uptake of OA and AZA

Blue mussels (*Mytilus edulis*) and pacific oysters (*Crassostrea gigas*) are the two main species of bivalve molluscs cultivated in Ireland. Flat oysters are a minor cultivation activity while scallops and razor fish are ranched and fished in some areas. Rapidly, after the introduction of chemical testing in Ireland in 2001, it was noted that mussels and oysters do not accumulate toxins in the same manner (Hess et al., 2003). In fact, both for okadaic acid group toxins and for azaspiracids, mussels accumulate higher concentrations on average than oysters, and consequently, the frequency of exceeding the regulatory limit is significantly higher for mussels compared to oysters (Tables 3.3.1 and 3.3.2).

Table 3.3.1. Occurrence of okadaic acid equivalents in shellfish from Irish waters, as analysed by LC-MS from July to October 2001

Species (n)	Average [$\mu\text{g/g}$]	Maximum [$\mu\text{g/g}$]	Percentage > 0.16 mg/kg
<i>Mytilus edulis</i> (452)	0.99	48.3	36 %
<i>Crassostrea gigas</i> (306)	0.06	7.76	2.0 %
<i>Ostrea edulis</i> (31)	< 0.01	0.05	0 %
<i>Ensis siliqua</i> (26)	< 0.01	0.02	0 %

Table 3.3.2. Occurrence of azaspiracid equivalents in shellfish from Irish waters, as analysed by LC-MS from July to October 2001

Species (n)	Average [$\mu\text{g/g}$]	Maximum [$\mu\text{g/g}$]	Percentage > 0.16 mg/kg
<i>Mytilus edulis</i> (452)	0.07	1.5	8.5 %
<i>Crassostrea gigas</i> (306)	0.03	0.42	1.6 %
<i>Ostrea edulis</i> (31)	< 0.01	0.04	0 %
<i>Ensis siliqua</i> (26)	< 0.01	0.08	0 %

These nationwide tendencies could theoretically be explained either through differences in habitat or through regional differences in the occurrence of toxic algae. However, the same trend could also be confirmed in bays where both species are cultivated in similar water depths. The difference can therefore be considered to be a species-specific characteristic of these two bivalves. The observation of this species specific difference has also been observed in other places, including France, and can be used to minimise the impact of harmful algae on the exploitation of shellfish harvesting areas.

However, the study did not allow the interpretation of the phenomenon at a physiological level: it is not clear whether the differences observed are due to differences in filtration rate or selectivity, i.e. intake of toxic algae, or whether the difference is due species-specific metabolism of the toxins. If indeed, oysters were able to metabolise toxins faster than mussels, these trait could possibly be enhanced if well understood. The differential metabolism could again be thanks to enzymatic differences in the two species or due to differences in bacterial co-inhabitants in the two species. Further research should focus on understanding enzymatic and filtration differences.

4.3.4. Domoic acid and scallops

Although no reports exist of amnesic shellfish poisoning from Europe, neither for humans nor for marine mammals, the obligation to test for DA since the late 1990s (Anonymous, 1997) had rapidly triggered studies investigating the contamination of shellfish with DA. For Scottish waters, we showed the extent of contamination in 1999 and 2000 (Gallacher et al., 2000; Hess et al., 2001; Campbell et al., 2001). In 1999, large scale blanket closures of scallop harvesting areas were put in place in Scotland, Ireland and Spain. In Scotland alone, 8,000 square miles were banned for harvest of scallops in 1999 (Gallacher et al., 2000). In these studies, we also demonstrated that the toxin was, like for other shellfish, concentrated mostly in the digestive organs of scallops. Thus, new legislation was subsequently put in place to allow for harvest of moderately contaminated scallops, and their placing on the market following dissection of the edible parts: adductor muscle and roe (Anonymous, 2002b). For the characterisation of the hazard in detail, see also section 4.6.2.1 (evisceration of scallops).

Despite regular surveillance, it appears that scallops are not only contaminated to the highest levels but also to the largest extent compared to other bivalves, such as mussels and oysters. Contamination of mussels above the regulatory level of 20 mg/kg is reported relatively rarely (e.g. McCarron and Hess, 2006). Thus, there is at least two scientific questions remaining, mainly concerned with how scallops become contaminated with DA and what the sources are causing the high variability observed for DA in scallops. The first question is an ecological question and the answer to it may also contribute to answer the second question. The question of how scallops become contaminated with DA is not trivial, as scallops live mostly on the seafloor, often at great water depths of more than 100 m, while DA is known to be produced by *Pseudo-nitzschia*, a pelagic diatom that requires light to flourish. Two hypotheses have been put forward to explain the contamination of scallops:

- d) *Pseudo-nitzschia* produces DA in the upper parts of the water column and when cells die, the remaining particles fall to the seafloor, where they are taken up by detritivorous organisms and subsequently enter the food chain of scallops.
- e) Separate benthic organisms produce DA, which enters the food chain independently of *Pseudo-nitzschia*

I had occasion to look at this topic through the co-supervision of a PhD (Yvonne Bogan). This work is presented here in 4 papers (Bogan et al., 2006, 2007a, b and c). Bogan et al., 2006, attempted to distinguish contamination at the surface and on the seafloor. In a one-year long study (February 2003 to February 2004), uncontaminated scallops were placed on the seafloor (12 to 15 m water depth) and in cages on longlines (2 m water depth). Concentration of DA peaked in April 2003, slowly declined until June 2003 and then remained virtually constant until February 2004. No difference could be detected between the scallops close to the surface and those on the seafloor at any time of the study. The fact that the peak of toxicity occurred close to the time when the spring bloom of phytoplankton occurs (dominated by diatoms) suggests that *Pseudo-nitzschia* does play a major role in the contamination of scallops.

If then toxin produced in the surface parts of the ocean is responsible even for contamination of scallops on the seafloor, one could expect the contamination on the seafloor to be fairly homogeneous. However, this is not the case. To establish the extent of variation of the contamination several studies were conducted.

An investigation of variability in domoic acid (DA) concentration in king scallop from 69 sampling sites within a limited area off the southeast coast of Ireland was performed (Bogan et al., 2007a). Variation in DA concentration was examined in the whole area, within smaller sub-areas, with size, age, and water depth. Mean DA concentrations in whole scallop ranged from 6.5 to 154.3 $\mu\text{g g}^{-1}$, with an overall mean of 40.6 \pm 30.8 $\mu\text{g g}^{-1}$. The concentration in gonad exceeded 20 $\mu\text{g g}^{-1}$ in 17 sites and in adductor muscle in 3 sites. Significant differences in DA concentration were detected between scallops from different sampling stations. Whole scallop tissue and individual scallop tissues with the exception of gonad, exhibited significant negative correlations with water depth. Highest DA concentrations were recorded in inshore, shallow sites and lowest DA concentrations in deeper offshore waters. Significant positive correlations between DA concentration in hepatopancreas and scallop size were exhibited at inshore sites but not at offshore sites. High inter-animal and spatial variability in toxin concentration demonstrated the importance of a reliable sampling protocol for the management of ASP outbreaks to ensure public safety and to avoid unnecessary fishery closures.

The negative correlation between DA concentration in whole scallop and individual tissues with water depth raised the question as to why such a relationship might exist (Fig. 3.4.2). Shallower sampling stations exhibiting higher DA concentrations in this study were closer inshore, however they were also directly opposite Waterford Harbour and the possibility cannot be ignored that high DA concentrations may in part have resulted from geographical location rather than from water depth alone. Differences in DA concentration between inshore sites and offshore sites may also be influenced by the prevailing oceanographic conditions.

In this study area off the southeast coast of Ireland the strongest currents occur in inshore waters particularly in the eastern part of the sampling area. These stronger currents may result in exposure to enhanced levels of phytoplankton, including toxic *Pseudo-nitzschia* cells, causing elevated DA concentrations, increased growth rates and larger gonads in scallops from inshore compared to offshore waters. The boxplots prepared for the spatial cluster groups at offshore east, offshore west and inshore east supported the relationship between DA concentration and water depth. However inshore west sampling stations exhibited a narrow range in DA concentration but wide range in water depth suggesting that factor(s) other than water depth influenced DA concentration.

Nutrient gradients observed in the study area also suggest that there may be a higher primary production closer to the coast (Fig. 3.4.3). Thus, algal growth of diatoms, including *Pseudo-nitzschia*, could be more prevalent in this area and be part of the cause of toxicity.

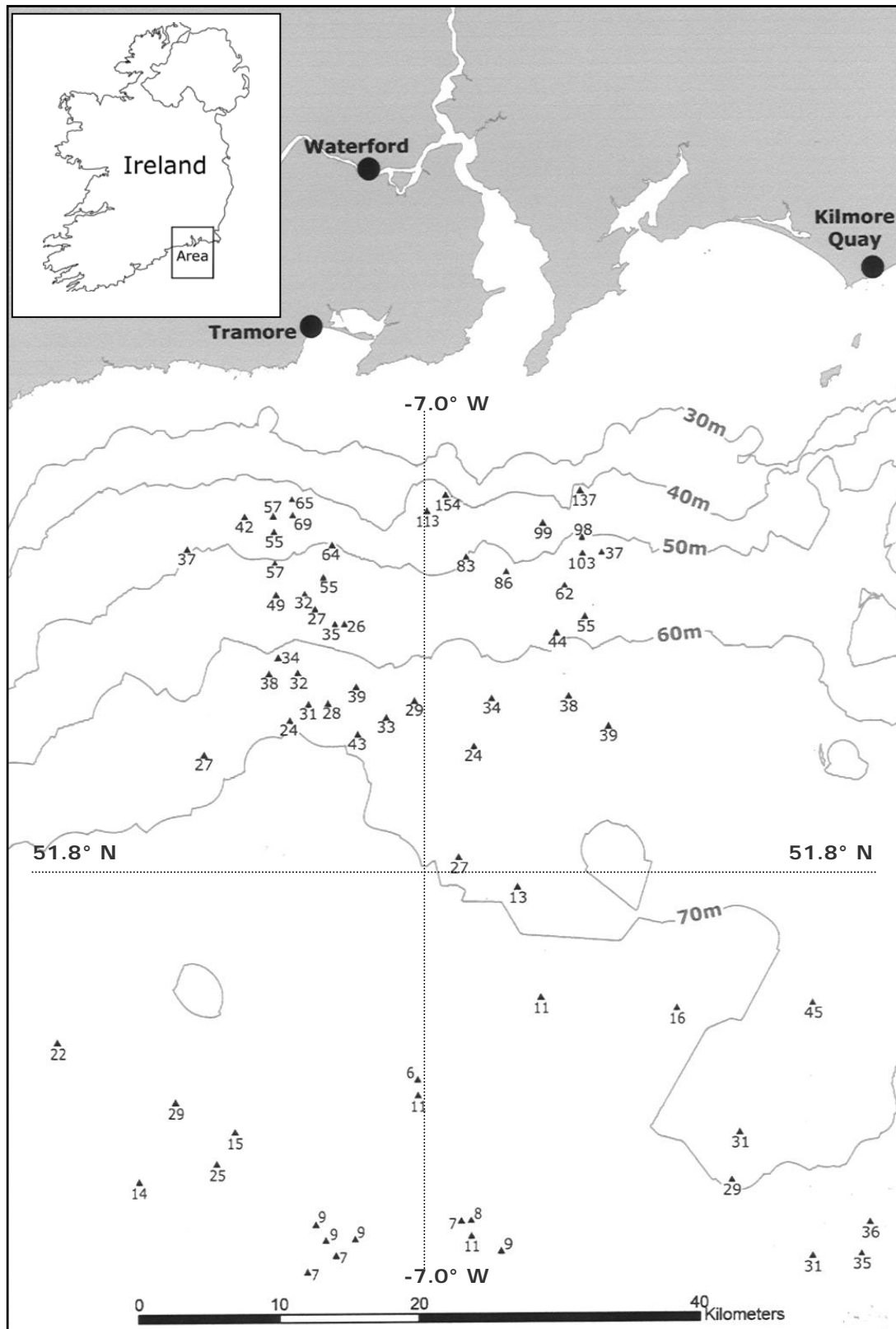


Figure 3.4.1. Domoic acid concentrations in scallops from the fishing grounds off Wexford (Southeast coast of Ireland, between Celtic and Irish Seas). Note that concentrations decrease from North to South (going away from the coast) and that they decrease with increasing water depth (from Bogan et al., 2007a).

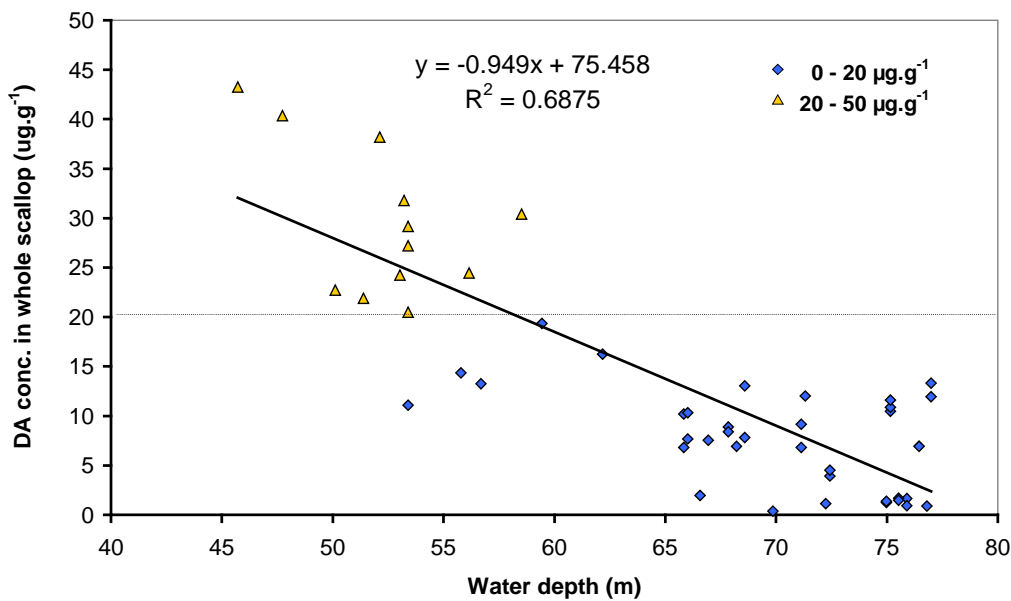
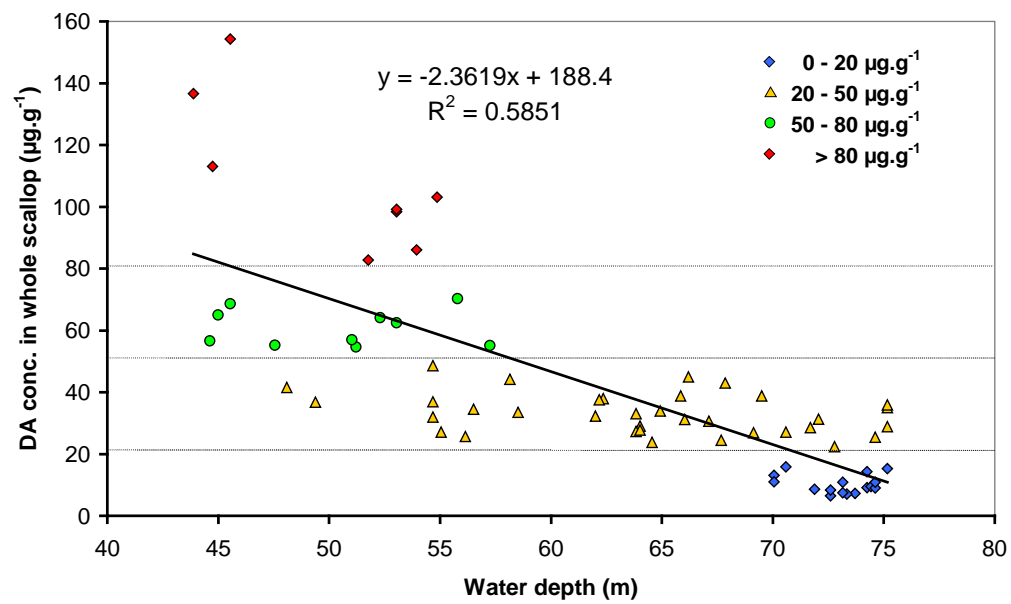


Figure 3.4.2. Relationship between DA concentration in tissue homogenates of whole scallop from the southeast coast of Ireland fishery and water depth in Sept/Oct 2003 (top) and Sept/Oct 2004 (bottom), from Bogan et al., 2007a.

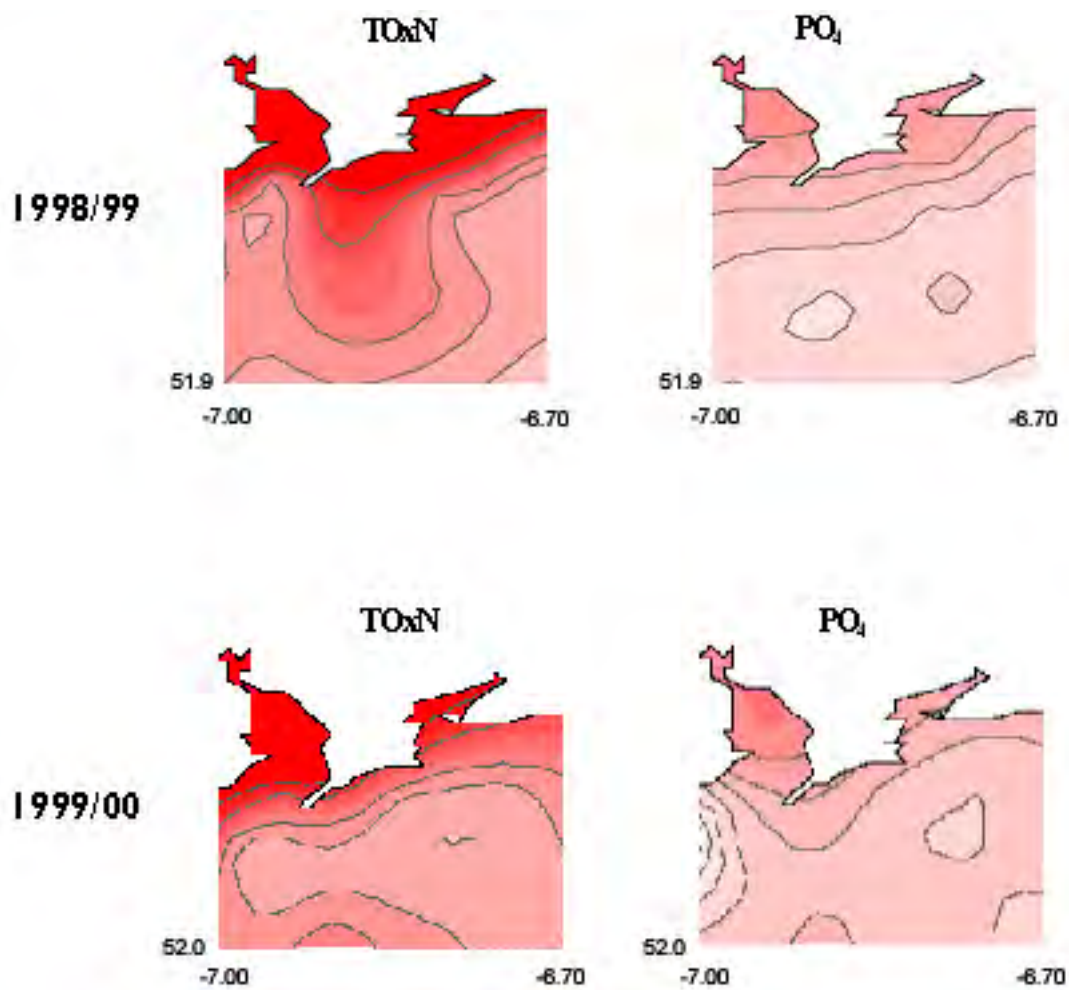


Figure 3.4.3. Nutrient concentrations off Wexford Harbour (Southeast coast of Ireland, between Celtic and Irish Seas). Note that concentrations decrease from North to South (going away from the coast) and that they decrease with increasing water depth (from Bogan et al., 2007a).

Domoic acid (DA), the toxin responsible for amnesic shellfish poisoning (ASP) can accumulate in king scallop (*Pecten maximus*) leading to extensive fishery closures. Approximately 59% of the total value of all fish and shellfish landed in the Isle of Man in 2004 comprised king scallop, hence the economy of the Manx marine sector is particularly susceptible to impacts from this biotoxin. Scallop from fishing grounds around the Isle of Man were sampled in October 2003, June 2004 and October 2004 to determine levels of inter-animal and spatial variability in DA concentration and factors that might influence toxin concentration such as scallop size and water depth. Mean DA concentrations in hepatopancreas ranged from $296.3 \mu\text{g g}^{-1}$ to below the detection limit, in gonad from $27.8 \mu\text{g g}^{-1}$ to below the limit of detection and in adductor muscle from $7.3 \mu\text{g g}^{-1}$ to below the limit of detection. High levels of inter-animal variability of DA concentration in hepatopancreas were recorded, with coefficients of variance (CVs) ranging from 16.1% to 70.0%. DA concentrations above $20 \mu\text{g g}^{-1}$ were recorded in gonads on all three sampling dates. Scallops from fishing grounds on the east of the Isle of Man were significantly less contaminated than those from the west and southwest (Fig. 3.4.4). A significant positive correlation between DA concentration and shell length was recorded in some sites, but there was no relationship with water depth.

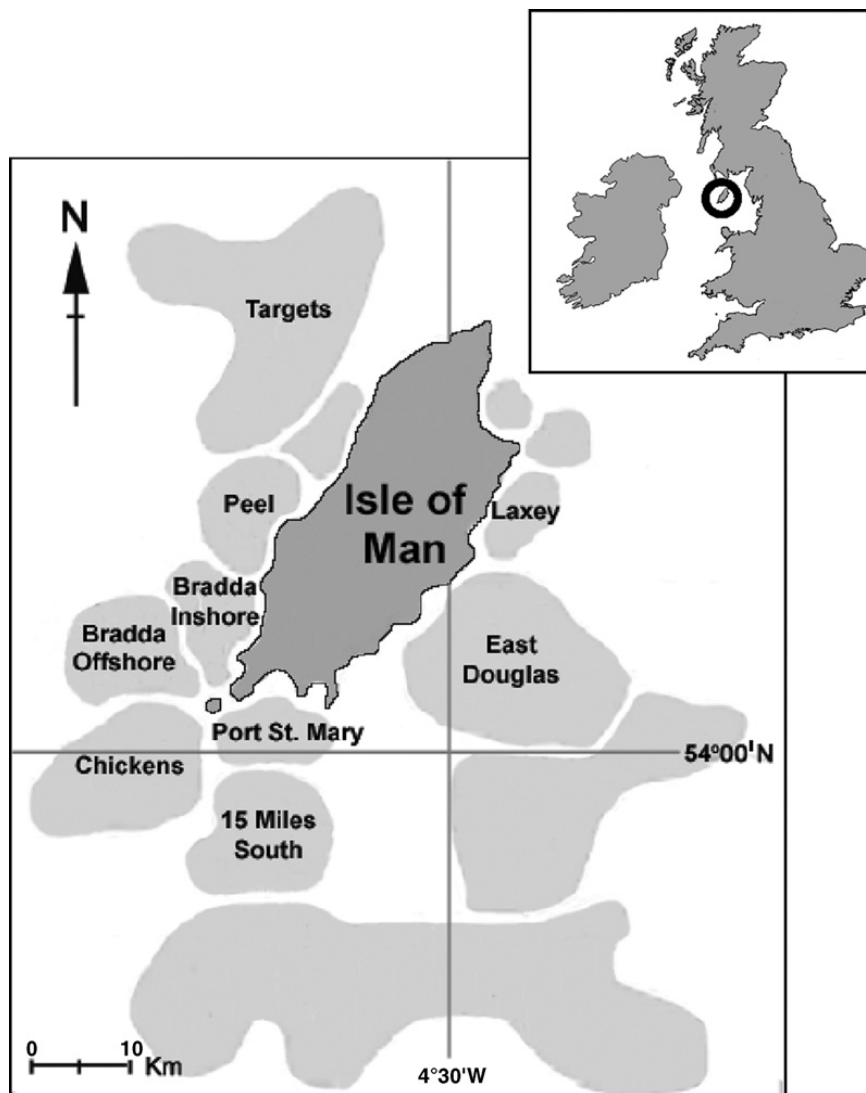


Figure 3.4.4. Scallop fishing grounds around the Isle of Man.

4.3.5. Environmental distribution of algal toxins

It has been demonstrated that polymeric resins can be used as receiving phase in passive samplers designed for the detection of lipophilic marine toxins at sea and was referred to as solid phase adsorption toxin tracking (SPATT, McKenzie et al., 2004). In a study I supervised in Ireland (Fux et al., 2008a), we described the uptake and desorption behaviour of the lipophilic marine toxins okadaic acid (OA) and dinophysistoxin-1 (DTX1) from *Prorocentrum lima* cultures by five styrene-divinylbenzene based polymeric resins Sepabeads1 SP850, Sepabeads1 SP825L, Amberlite1 XAD4, Dowex1 Optipore1 L-493 and Diaion1 HP-20. A scheme of possible adsorption mechanisms is shown in Fig. 3.5.1. All resins accumulated OA and DTX1 from the *P. lima* culture with differences in adsorption rate and equilibrium rate. Following statistical evaluation, HP-20, SP850 and SP825L demonstrated similar adsorption rates. However, possibly due to its larger pore size, the HP-20 did not seem to reach equilibrium within 72 h exposure as opposed to the SP850 and SP825L. This was confirmed when the resins were immersed at sea for 1 week on the West Coast of Ireland (Fig. 3.5.2). Furthermore, this work also presents a simple and efficient extraction method suitable to SPATT samplers exposed to artificial or natural culture media.

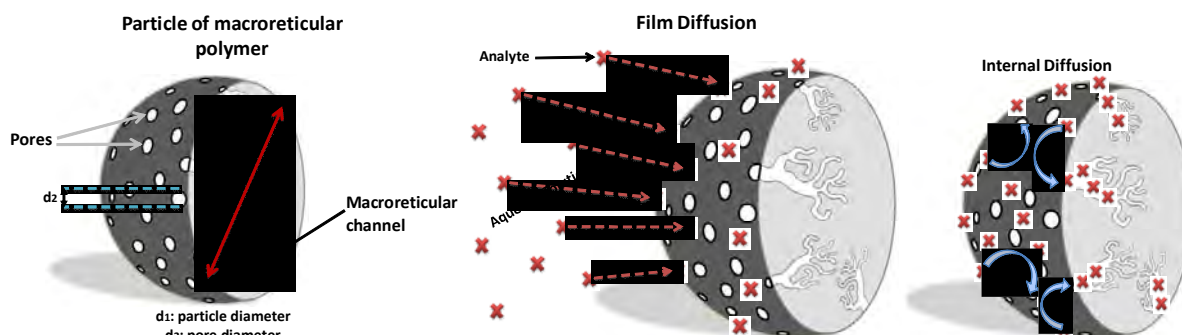


Figure 3.5.1. Simplified model proposed for diffusion processes in macroreticular polymer particles. Film diffusion is shown by the migration of analyte from the solution to the surface of the particles. Internal diffusion is shown by migration of analyte from the surface to the interior of the particle, adapted from Fux et al., 2008.

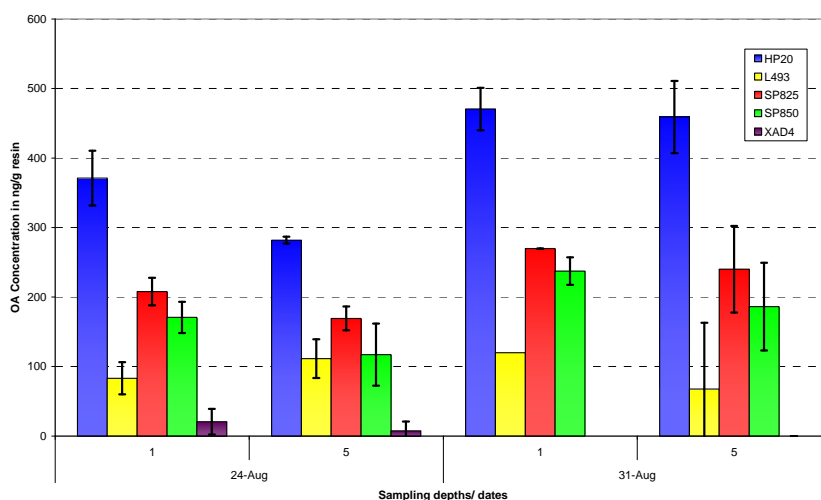


Figure 3.5.2: OA concentrations found in five polymeric resins immersed in Galway bay. Error bars represent the standard deviation obtained on three SPATT replicates. Quantitation was performed by triplicate injection on LC-MS QqQ, adapted from Fux, 2008 (PhD thesis).

Monitoring of lipophilic marine toxins was carried out in three shellfish production sites on the West Coast of Ireland. The toxins were monitored using passive samplers (solid phase adsorption toxin tracking; SPATT) and toxin-free mussels that were replaced weekly in the selected sampling stations. The toxin profiles and concentrations obtained in the SPATT and in the transplanted mussels were compared with those observed in indigenous (native) mussels from each production site as well as with the phytoplankton that was detected in the water. Numerous lipophilic toxins were detected in the SPATT discs by ultra-performance liquid-chromatography–mass spectrometry/mass spectrometry (UPLC–MS/MS) and included okadaic acid (OA), dinophysistoxin-2 (DTX2), pectenotoxin-2 (PTX2), AZA1, AZA2, yessotoxin (YTX) and SPX-13-DesMe-C. The accumulation rate of toxins in the indigenous mussels and in the SPATT discs correlated well. Toxins were detected in all SPATT discs from all locations, even in the absence of toxin-producing phytoplankton, as observed previously by other research groups. It is quite clear from our data that the presence of okadaic acid in the water (even at high concentrations) did not induce toxicity in the transplanted mussels in the absence of phytoplankton (Fig. 3.5.3). A severe toxic event of azaspiracids (AZAs) occurred in one of the sampling stations. The SPATT discs accumulated predominantly AZA1 and -2 suggesting that both toxins are biosynthesized by the AZA-producing organism. As opposed to the DSP event, the AZA event resulted in the contamination of the transplanted mussels for several consecutive weeks. This was also the first study that reports the presence of YTX and SPX-13-DesMe-C in Irish waters. In our study conditions, the SPATT did not enable the forecasting of shellfish contamination as the increase in toxin concentration occurred at the same time in the shellfish and in the SPATT.

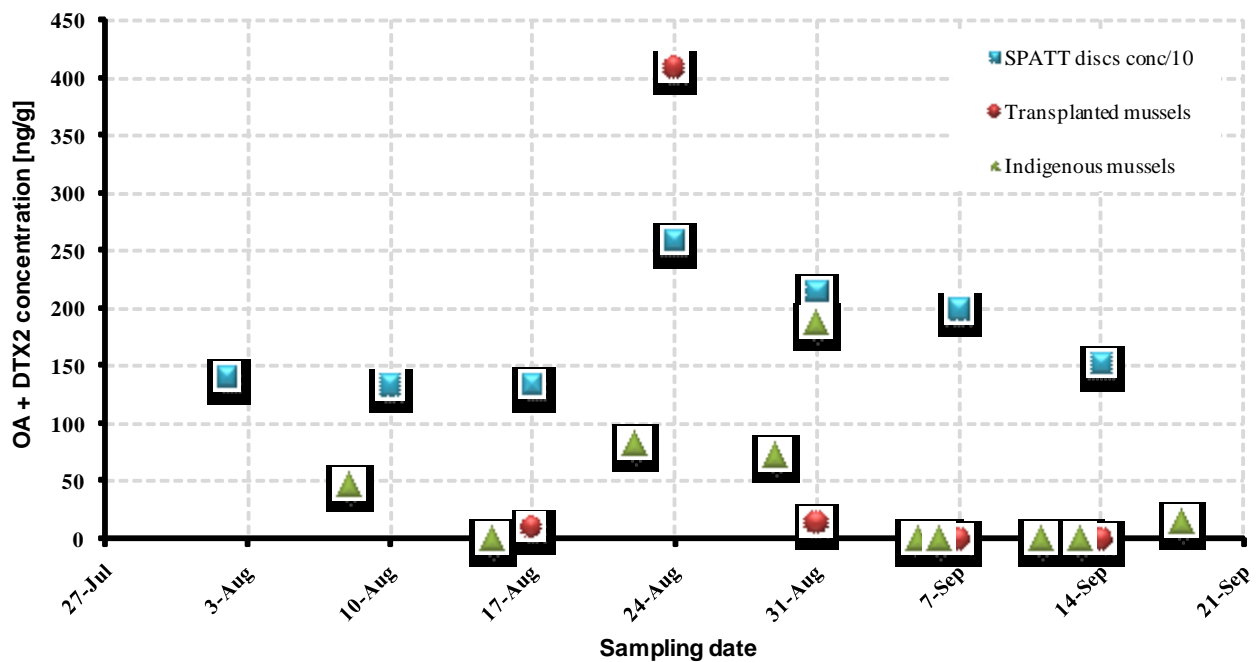


Figure 3.5.3: Comparison of the sum of the concentrations of OA and DTX2 accumulated in transplanted mussels (n=3), indigenous mussels (n=1) and in the SPATT discs (n=3) from 2/8/05 to 17/8/05; (n=9) from 17/8/05 to 14/9/05 immersed in Killary Harbour. For the SPATT discs and the transplanted mussels each data point represents the average of the three depths; adapted from Fux et al., 2009.

The use of SPATT did not provide an early warning of shellfish contamination as the concentration of toxins rose in SPATT discs and in mussels at the same time. Nevertheless, the passive samplers have shown the ability to respond rapidly to the presence of harmful phytoplankton (Fux et al., 2009). Previous experiments have shown that significant amounts of

OA and DTX1 accumulate in the SPATT after only 12 h exposure. Thanks to these rapid accumulation times, early warning may be possible if the duration of transportation of toxic algae along ocean currents and tides are significantly longer than the duration of toxin adsorption on the SPATT. In such a scenario, SPATT samples would need to be obtained at high frequency from sites that are upstream of the shellfish aquaculture sites. Thus, the early warning obtained would be similar to early warning obtained from phytoplankton with the added advantage of obtaining a spatially and time integrated response.

Overall, it appears that the SPATT-technique is particularly suited to indicate the presence of a toxin in a particular area. Therefore, it would also be appropriate to include sampling using these resins prior to allowing new shellfish farms to be created in areas where no shellfish occur.

4.4. Preparative isolation of natural products

Although the effects of marine toxins have been known for hundreds of years, the toxic principles involved were not discovered until the 20th century. The identification and characterization of toxins has been a lengthy process for some toxins. For instance, in the late 19th century, reports describe paralytic shellfish poisoning as a poisoning caused by the consumption of blue mussels (Virchow, 1885) the toxic principles of which also occur in starfish (Wolff, 1887), without the identity of toxic principles being revealed. Groundbreaking work was completed by Sommer and Meyer (1937) to link this toxicity to the occurrence of micro-algae and to conceive an assay that will remain the reference tool to our days, the mouse bioassay for paralytic toxins. Onoue et al. (1931) started work on the isolation of saxitoxin analogues as the toxic principles of paralytic shellfish poisoning. The efforts were significantly advanced by Schantz et al. (1957, 1958). However, it was not until 40 years after initial isolation efforts that the structure of saxitoxin was finally confirmed by Wong et al. (1971). The characterization process has been hampered for many toxins in a similar fashion due to the lack of compound mass for the studies. This lack can be understood from the fact that the organisms producing the toxin cannot always be cultured, and scientists thus rely on the natural occurrence of the compounds. In addition, the structure elucidation in early days was mostly based on chemical reaction of the compounds. The onset of more powerful non-destructive techniques such as nuclear magnetic resonance (NMR) has allowed for the characterisation of smaller quantities: while several hundreds of mg were required to characterise a toxin in the 1950/60s, nowadays 10 to 100 µg of compound may be sufficient to complete the structure elucidation of a novel compound. Thus, the discovery of domoic acid as a shellfish toxin could be completed within weeks from the poisoning event (Quilliam and Wright, 1989). More typically, it takes one to several years from the initial poisoning event to the identification of the chemical responsible for the toxic effect, e.g. for the identification of okadaic acid and azaspiracids (Satake et al., 1998; Yasumoto et al., 1978).

4.4.1. AZAs

Since 1999, there was a number of reasons to implement chemical analysis of azaspiracids in Ireland on a routine basis. Firstly, numerous mussel lots produced in Ireland and exported to France had been rejected on the basis of positive animal assays conducted in France, suggesting that the testing systems had not been sufficiently harmonized across Europe. Chemical analysis should allow for quantitation of levels close to the mouse detection limits and thus prevent that toxic shellfish could reach the market. Furthermore, quantitative data were needed to complete risk assessment for azaspiracids to consolidate the legislation, which, at the time, had been solely based on animal assays. However, one of the major requirements prior to the implementation of chemical analysis is the availability of calibration standards. Thus, availability of purified AZAs became a priority. Most often, chemical synthesis is too labor-some to be carried out on a routine basis, such synthesis had also not been achieved until 2004 (Nicolaou et al., 2004a and b). Thus, preparative isolation of marine biotoxins remains an important part of research even after the structure of the molecules have been identified. In the case of azaspiracids in particular, the causative organism had not been identified until 2007, and hence, isolation had to be based on the purification of AZAs from contaminated mussel (*M. edulis*) tissues.

The initial isolation of azaspiracid and some analogues (AZA-2 to -5) had been completed by Japanese workers (Satake et al, 1998; Ofuji et al., 1999 and 2001). In 2001, I had the opportunity to spend 3 weeks at Sendai University (Japan) with Drs. Satake and Ofuji on the isolation of AZA. During this time, it was possible to obtain ca. 2 mg of AZA1 in pure form (> 95%, by NMR, < 1%

impurities of analogues by LC-MS) from Irish blue mussels. This amount was sufficient for in-house method validation at the Marine Institute, Ireland, and for use as calibrant in routine (daily) LC-MS-MS analysis of AZAs from 2001 to 2007. The procedure developed by the Japanese was slightly modified by Rehmann et al., 2008, and adapted to suit available equipment in Ireland and Canada (Fig. 4.1.1). This modified procedure had also been used to produce a certified calibrant for AZA1 (NRCC, 2007).

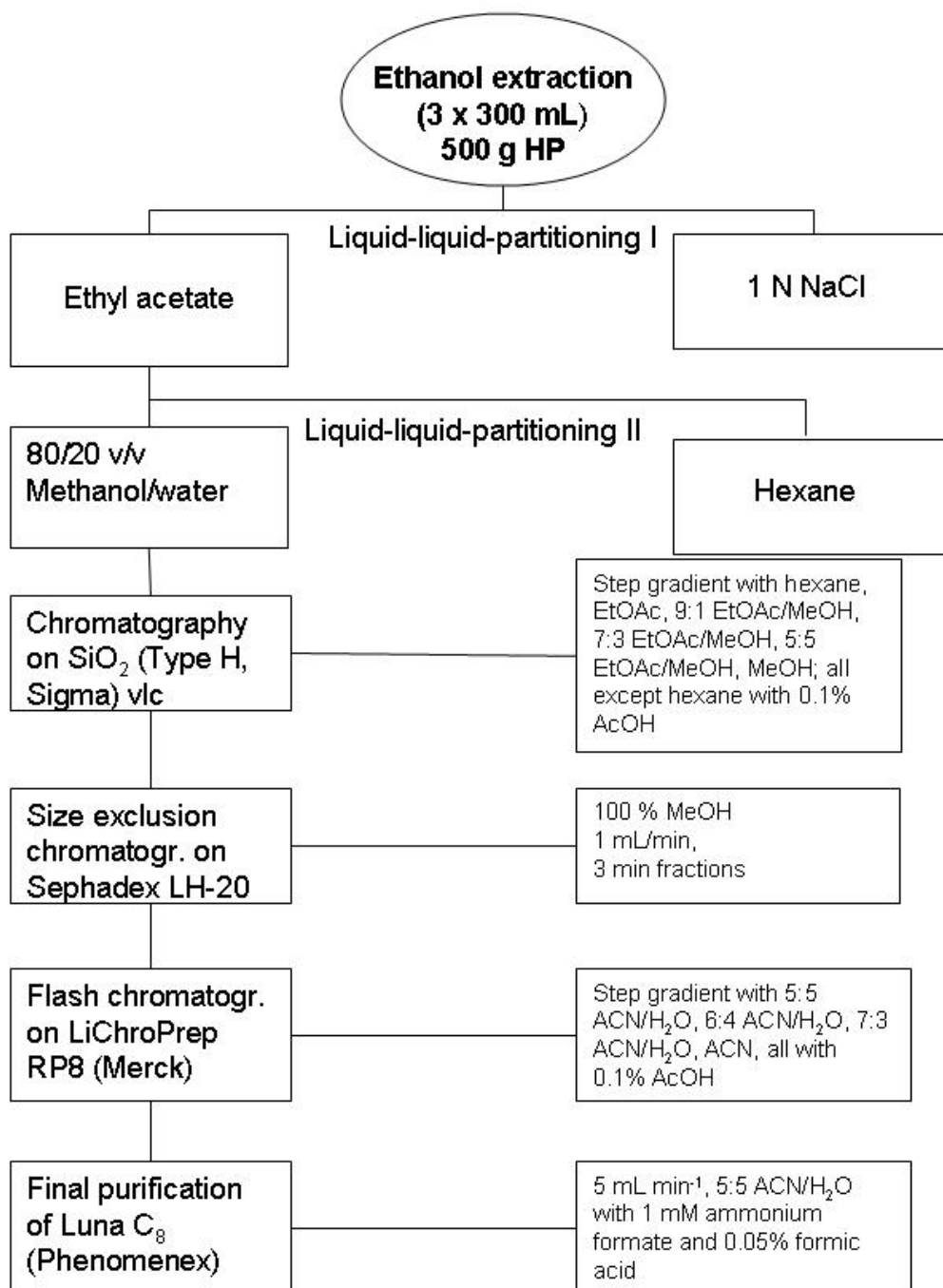


Figure 4.1.1. Preparative isolation scheme for AZAs as used by Rehmann et al., 2008, for discovery of numerous analogues and for the production of a certified AZA1 calibrant (NRCC, 2007).

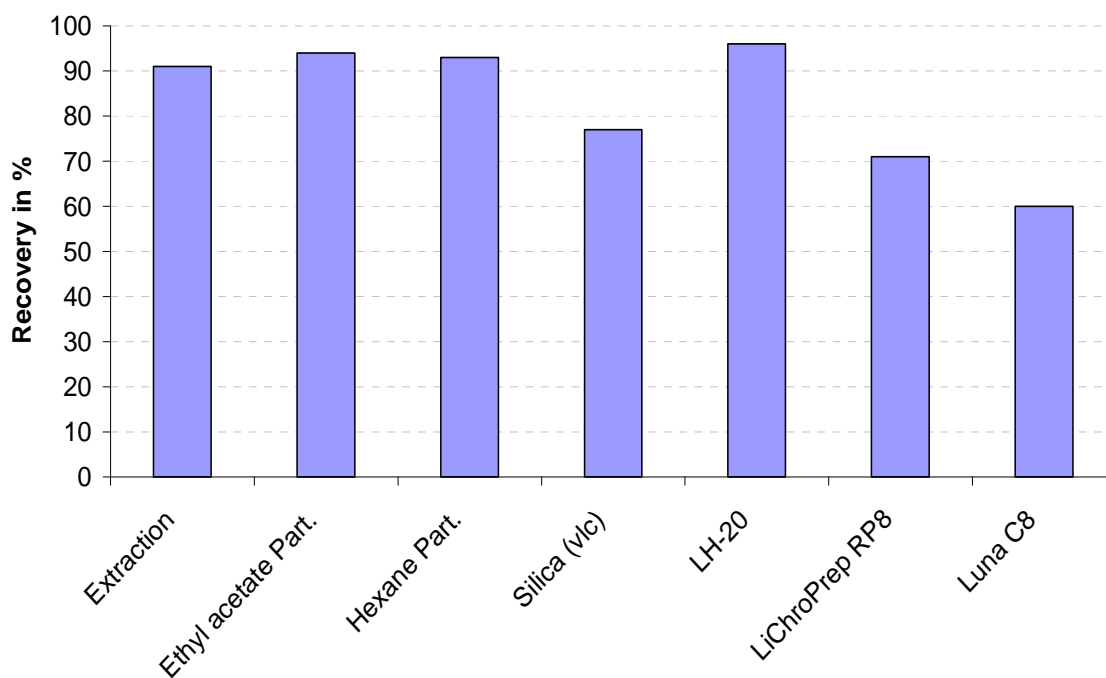


Figure 4.1.2. Recovery of AZA1 in purification steps of the preparative isolation procedure by Rehmann (2008). Overall recovery ca. 24%.

Significant improvements were made on the recovery of some chromatographic steps, in particular the low pressure normal phase chromatography on silica gel, which had shown low recoveries (ca. 35%) in initial trials after transfer of the method from Japan (Fig. 4.1.2). This improved procedure was also used in the certification of AZA1, -2 and -3 (Fig. 5.1.1 and 5.1.2).

4.4.2. DTX2

Procedures similar to those used for AZAs were also applied to the preparative isolation of DTX2 (Larsen et al., 2007; Rehmann, 2008; Wilkins et al., 2006).

The collaboration colleagues from New Zealand and Norway allowed for the clarification of the stereochemistry of DTX2 (Larsen et al., 2007), in particular the stereochemistry of C35 (Fig. 1.1.4).

The increased knowledge of the structural differences of OA, DTX1 and DTX2 also contributes to the understanding of the interaction of the DTXs with protein phosphatases (Fig. 4.2.1), which confirms the observation that DTX2 has less binding affinity to PP2a and less toxicity towards living systems (Aune et al., 2007).

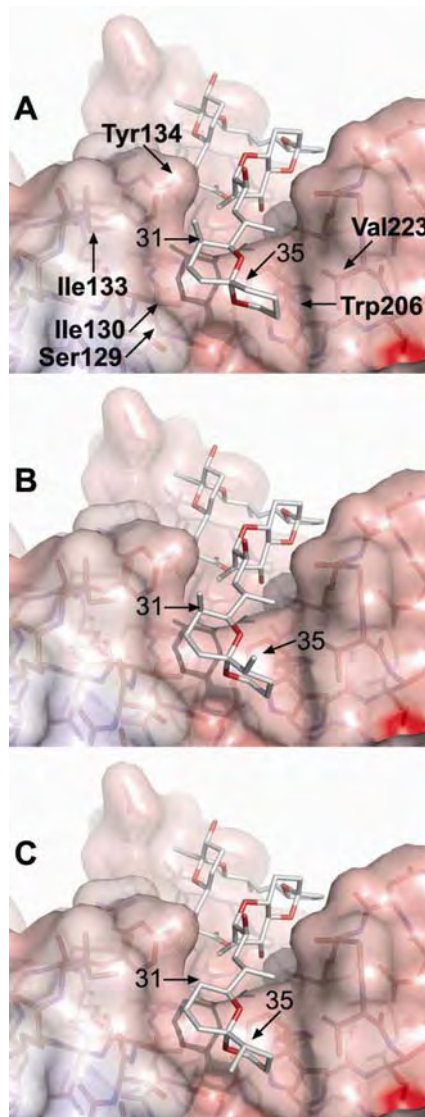


Figure 4.2.1. Adapted from Larsen *et al.*, 2007: Molecular models of okadaic acid analogues docked to protein phosphatase-1 (PP1), based on the crystal structure of okadaic acid bound to PP1 (PDBid:1JK7). A, okadaic acid; B, dinophysistoxin-1; C, dinophysistoxin-2.

4.4.3. PTX2sa-fatty acid esters

Three series of fatty acid esters (1–3) of pectenotoxin-2 seco acid (PTX-2 SA) (4) and 7-epi-PTX-2 SA were detected by LC-MS analysis of extracts from blue mussels (*Mytilus edulis*) from Ireland (Wilkins et al., 2006). The location of the fatty acid ester linkages were identified by a combination of LC-MS/MS in positive- and negative-ion modes, LC-MS analysis of the products from reaction of the esters with sodium periodate, and NMR analysis of purified samples of the two most abundant ester derivatives. The 37-O-acyl esters of PTX-2 SA (1) were the most abundant, followed by the corresponding 11-O-acyl esters (3), accompanied by low levels of the 33-O-acyl esters (2). The most abundant fatty acid esters in the fractionated sample were, in order, the 16:0-, 22:6-, 14:0-, 16:1-, 18:4-, and 20:5-fatty acids, although a wide array of other PTX-2 SA fatty acid esters were also present at low levels (Fig. 4.3.1).

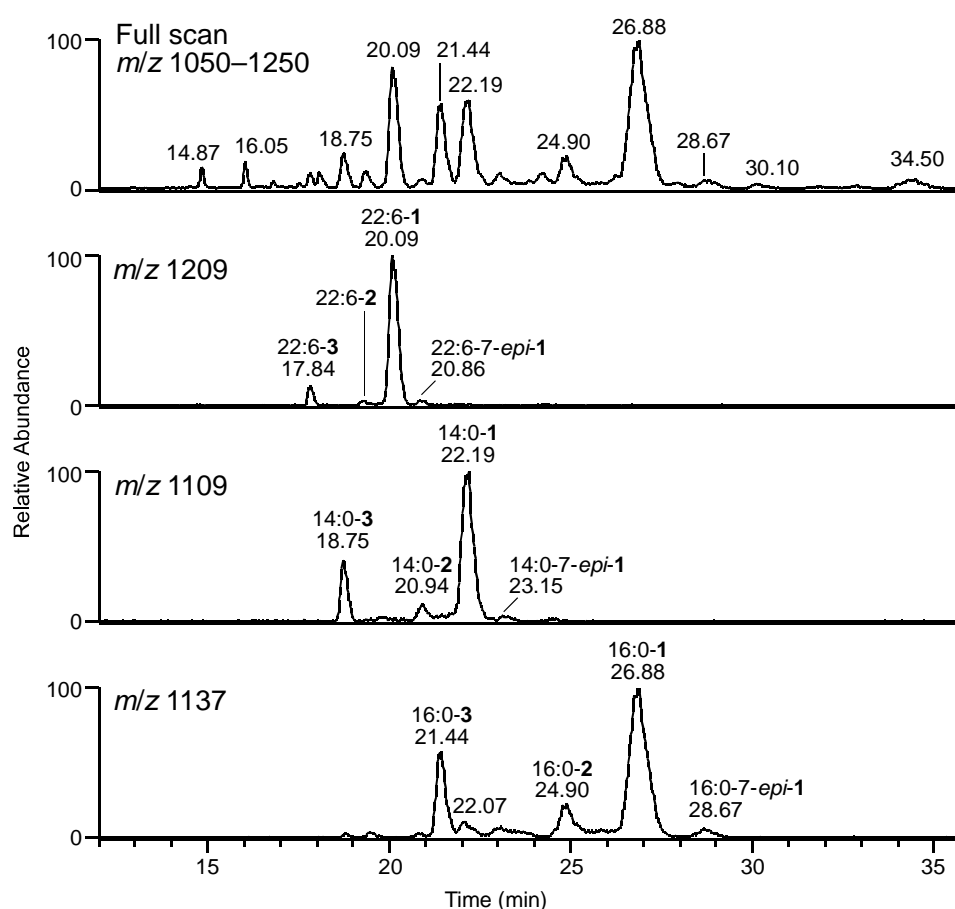


Figure 4.3.1. Extracted selected ion profiles from a typical LC-MS analysis (full scan mode, m/z 500–1500) of acetone fraction from Irish mussels. Extracted profiles correspond to: fatty acid esters of PTX-2 SA and isomers (m/z 1050–1250); 22:6 fatty acid esters of PTX-2 SAs ($[M + Na]^+$, m/z 1209); 14:0 fatty acid esters of PTX-2 SAs ($[M + Na]^+$, m/z 1109); and 16:0 fatty acid esters of PTX-2 SAs ($[M + Na]^+$, m/z 1137).

The existence of these esters shows that the esterification observed for OA-group toxins is also found for PTX-derivatives. In addition, the esterification also demonstrates again that mussels metabolise PTXs very effectively to derivatives in which the macrocycle is opened. Thus, shellfish contribute to a natural detoxification in the case of PTXs.

4.5. Quality control and the performance of methods

4.5.1. Reference materials

4.5.1.1. Classification of reference materials

The International Organisation for Standardisation (ISO) defines a RM as a “material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials” (ISO, 1993). The same document also gives a definition of certified reference materials (CRMs) as a “reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence”. These terms are derived from the metrological requirements RMs and CRMs have to fulfil and do not give a description of the typical use of the material in practice.

Hierarchies and nomenclature of RMs have been discussed and reviewed recently (Emons et al., 2004, Emons, 2005). Similar to suggestions from these reviews, we classify RMs into the following categories according to their intended use: laboratory reference materials (LRMs), interlaboratory reference materials (ILRMs) and certified reference materials (CRMs). While RMs and CRMs in the ISO-terminology also comprise calibrants, this section is concerned mainly with tissue or matrix RMs and the abbreviations LRM, ILRM and CRM used herein will refer to matrix RMs, unless otherwise stated. The following sections give an outline of the authors’ interpretation of the terms LRM, ILRM and CRM.

Laboratory Reference Materials (LRMs)

LRMs are shellfish tissues intended for quality control (QC) purposes in the day-to-day analysis of samples. These materials should be analysed alongside routine samples in every batch, and thus serve as one of the primary tools for accepting the results determined for the samples. They can also be used in the development and in-house validation of a method, in particular for validation of parameters such as precision and ruggedness of the method. LRMs must be homogenous and stable. The stability may be acceptable if it can be guaranteed for frozen storage since these materials do not generally need to be shipped. Due to their frequent use and their main purpose being in initial and ongoing validation of an in-house method, LRMs should be as representative of a real sample as much as possible. In the case of shellfish tissues, this means they should be a wet shellfish tissue with major constituents (e.g. water and lipid contents) close to the mass fraction of fresh shellfish. Depending on the species, raw bivalve tissues typically contain between 75-90 % water and between 1.5 - 3 % lipids (Hess et al., 2007a).

Interlaboratory Reference Materials (ILRMs)

ILRMs are shellfish samples intended for the validation of one method or to check the comparability of several methods through interlaboratory exercises, including collaborative trials, or proficiency testing. Good homogeneity is an obvious prerequisite. Additionally, these materials need to be fit for international dispatch, hence, short-term stability at relatively high temperatures should be tested, typically up to 40 °C. The requirement for increased stability leads generally to the need for some stabilisation techniques to be considered, including heat treatments

and/or the use of additives such as anti-oxidants or antibiotics. If heat treatment is used as a stabilisation technique, the resulting matrix is less reflective of a fresh shellfish sample, however, water and lipid contents can still be matched and commutability studies are not generally necessary, unless the portion size exceeds the prescribed range of acceptable sample size for analysis.

Certified Reference Materials (CRMs)

As outlined in the above-mentioned ISO-definition, the main requirement for CRMs, in addition to homogeneity and long-term stability, is a statement of analyte concentration with uncertainty and traceability to the primary unit or standard. This characteristic allows the use of a CRM in determining the accuracy of a method, which is not possible with a simple LRM. The regular use of a CRM, e.g. monthly, can also facilitate proof of accuracy of a method long-term, thus providing valuable information to laboratory managers and accreditation bodies alike concerning the performance of methods. However, the efforts undertaken in such a certification can only be justified if it can be proven that the material will be stable over a long period. Therefore, a detailed study on feasibility and long-term stability of candidate CRMs must be carried out, often leading to further research if any degradation is detected in long-term or in accelerated stability studies.

4.5.1.2. Certified calibrants

The efforts in the preparative isolation of AZAs have been described above. The certification of the purified materials is an exercise requiring strict quality control in the certifying laboratory. Such quality control allows to trace the certified values back to internationally accepted standards. In the case of AZA, the certification relies on quantification by NMR, a method with external calibration against a reference standard of caffeine (Burton et al., 2005). While certification of AZA1 has already been completed in 2007 (NRCC, 2007), work on AZA2 and -3 is still ongoing (Perez et al., 2010).

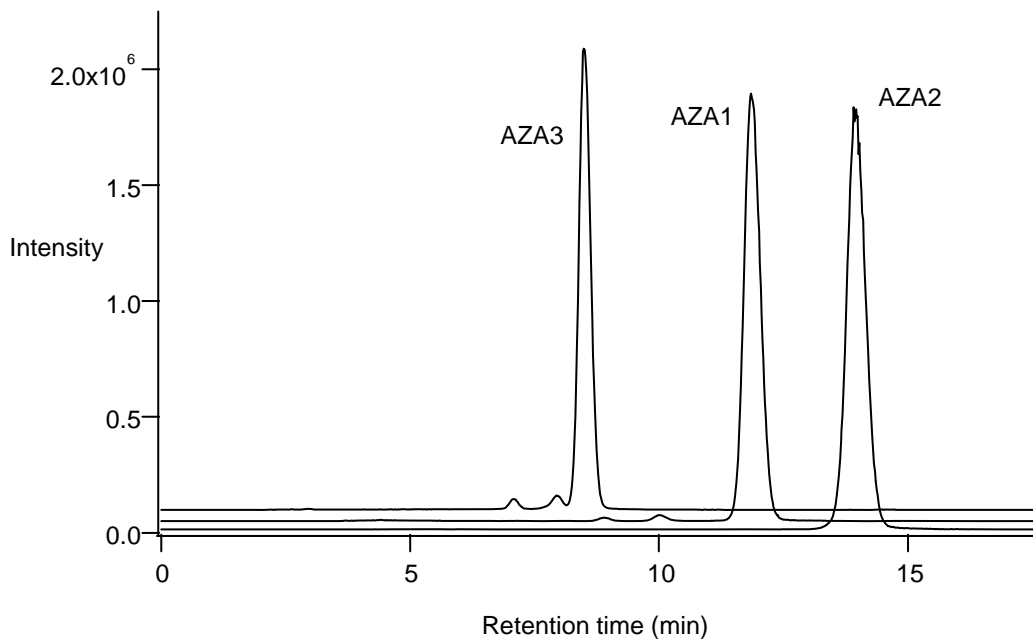


Figure 5.1.1. Purified AZA1, -2 and -3 in a certification exercise (adapted from Perez et al., 2010).

The stability of the purified compounds in solution is another important parameter that needs to be established during certification (Fig. 5.1.2); it not only important for the storage of the certified material but also for the shipping at international level.

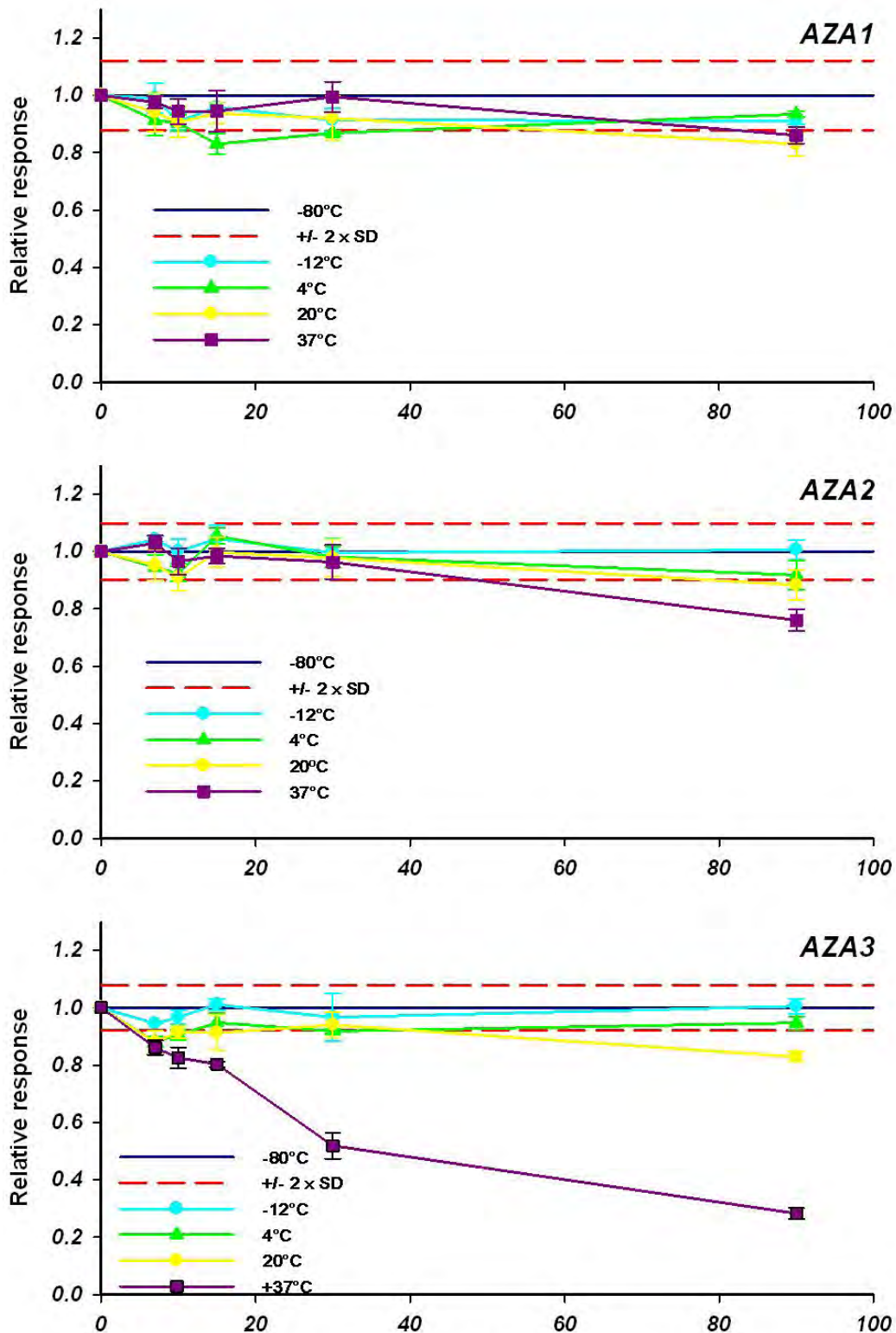


Figure 5.1.2. Short term stability study on the certified calibrants AZA1, -2 and -3 (adapted from Perez et al., 2010); note that degradation of AZAs in methanol only occurs at temperatures above 0 °C and is particularly strong for AZA3 at 37 °C.

4.5.1.3. Matrix reference materials

Requirements for matrix reference materials were described by Hess et al., 2007a. Three studies are described here as they constituted milestones in the production of shellfish tissue reference materials for phycotoxins:

- a) Addition of stabilisers to shellfish matrix
- b) Irradiation of shellfish matrix as a stabilising technique
- c) Freeze-drying of shellfish tissues as a stabilising technique

All three were carried out as part of the PhD of Pearse McCarron, a systematic study investigating the causes of instability of toxins and possible remedies in the production of homogeneous and stable reference materials.

- a) Addition of stabilisers to shellfish matrix

Five separate materials were prepared from a mussel (*Mytilus edulis*) tissue containing domoic acid (DA) from scallop hepatopancreas (*Pecten maximus*) (McCarron et al., 2007a). Homogenates were separately spiked with antibiotics, an antioxidant, or a combination of both. Control materials did not contain any additives and were prepared from lightly cooked mussels and from mussel tissues that had been autoclaved prior to homogenisation. Stability studies were run over a 148 day period at 3 different temperature conditions; -20°C, +4°C and +40°C. All materials were characterised by HPLC-UV. Homogeneities were demonstrated at the beginning of the study, with coefficients of variance less than 4 % (n=9), Table 5.1.1.

Table 5.1.1. Homogeneity of prepared materials characterised on day zero of stability study (n=9), adapted from McCarron et al., 2007a.

Material	Average DA conc. (µg/g)	SD	%CV
Un-stabilised	11.1	0.4	3.5
No Additives	10.9	0.3	3.1
Antioxidant	11.7	0.5	3.9
Antibiotics	11.0	0.2	2.1
Antibiotics and Antioxidant	11.6	0.3	2.5
ASP – LRM*	21.9	0.4	1.7

* N =26

DA was stable at -20°C in all materials. The control materials showed significant degradation after 2 days at +40°C, and after 8 days at +4°C. Each of the materials containing additives demonstrated better stability during the initial period of the study. In addition there was no significant degradation in any of the materials with additives stored at +4°C over the duration of the study. The antibiotic material was more stable than the antioxidant material over the long term at +40°C (Table 5.1.2).

The material containing a combination of the antibiotics and the antioxidant displayed the best stability of all the materials. There was no significant reduction in DA concentration at all temperature conditions after 8 days, and after 32 days the decrease at +40°C was still <20 %.

Table 5.1.2. Degradation of Domoic Acid in the reference materials stored for 148 days at 40 °C (harshest study condition) adapted from McCarron et al., 2007a.

Material	% DA degraded	%CV
Un-stabilised without additives	64.0	4.9
Heat-stabilised without additives	54.2	20.7
Heat-stabilised with antioxidant	56.4	8.9
Heat-stabilised with antibiotics	41.6	0.8
Heat-stabilised with antioxidant + antibiotics	39.0	3.0

Subsequently, a DA laboratory reference material (LRM) was prepared from mussels both naturally contaminated and fortified with DA and, based on previous results, spiked with both the antioxidant and antibiotics. A short-term stability study on this material gave similar results as the corresponding material in the additives study (Fig. 5.1.3).

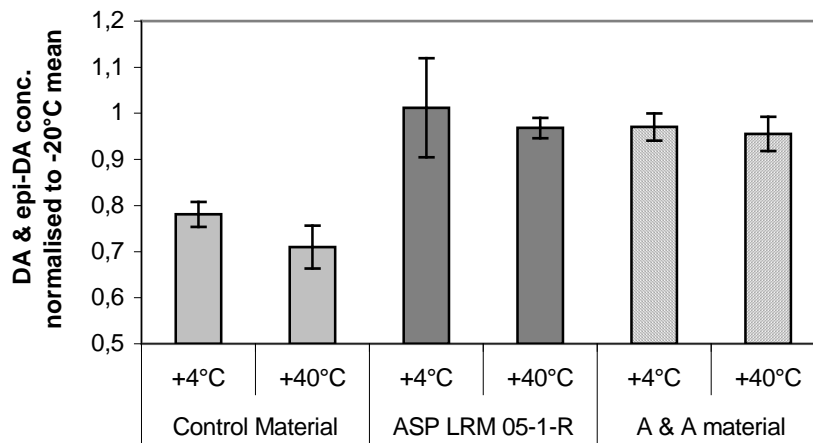


Figure 5.1.3. Normalised DA concentrations in control material, ASP LRM 05-1 and combination material after 8 days at +4 °C and +40 °C. Error bars represent ± 1 stdev ($n=3$), adapted from McCarron et al., 2007a.

This study shows that a combined use of the additives investigated, in the preparation of a mussel tissue reference material for DA ensures analyte stability for a period of up to 8 days at temperatures up to +40°C, a condition particularly important for shipping of test materials globally.

b) Irradiation of shellfish matrix as a stabilising technique

The effect of γ -irradiation on concentrations of hydrophilic and lipophilic phycotoxins was investigated using HPLC-UV and LC-MS techniques (McCarron et al., 2007c). Pure toxins in organic solvents and toxins in mussel (*Mytilus edulis*) tissues were irradiated at three different doses.

The effect of irradiation on the viability of microbial activity in shellfish tissues was assessed using total viable counting techniques. Microbial activity depended on the type of shellfish and on the pre-treatment of the shellfish tissues, with or without heat treatment (Table 5.1.3).

Table 5.1.3. CFU/g measured by TVC for fresh mussel and oyster samples given various treatments, adapted from McCarron et al., 2007c.

	Treatment	24 hours	5 days incubation	7 weeks incubation
Oyster	Control	95667	-	-
	Heated	14	-	-
	6 kGy	<LOD	-	-
	12 kGy	<LOD	31*	-
	24 kGy	-	<LOD	<LOD
Mussel	Control	14667	-	-
	Heated	<LOD	-	-
	6 kGy	<LOD	-	-
	12 kGy	<LOD	<LOD	-
	24 kGy	-	<LOD	<LOD

* 1 sample out of 3 gave a positive count after 5 days incubation

All toxin concentrations were reduced to some extent in solution. Most severe decreases were observed for DA (Fig. 5.1.4) and YTX, where the smallest dose of irradiation led to almost complete destruction of the toxin. PTX2 showed a less severe but still continuous decrease of concentration with increasing dose, while Azaspiracid-1 and Okadaic acid were the least affected in solution.

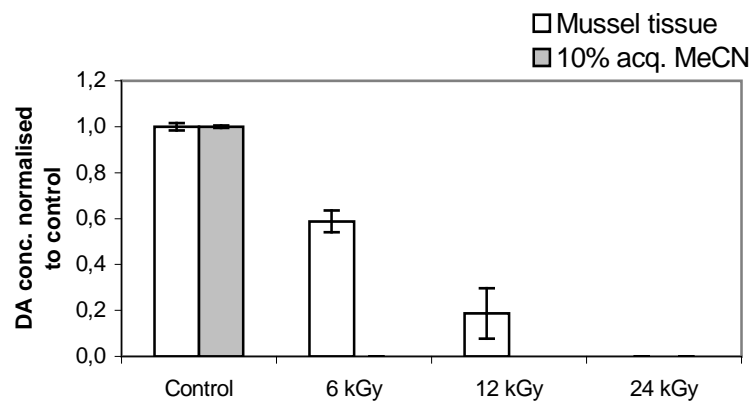


Figure 5.1.4. DA concentrations measured in a tissue matrix and in solution after being subjected to increasing doses of gamma irradiation. Error bars shown represent ± 1 SD ($n=5$), adapted from McCarron et al., 2007c.

In shellfish tissue the decrease of toxin concentrations was much reduced compared to the effect in solution. Following irradiation at the highest dose, reductions in concentrations were between ca. 5 and 20 % for the lipophilic toxins. In particular, there was no statistical difference between control and irradiated samples for AZAs in tissue (Fig. 5.1.5). Irradiation of shellfish tissues contaminated with DA led to a more continuous decrease of the toxin with increasing dose.

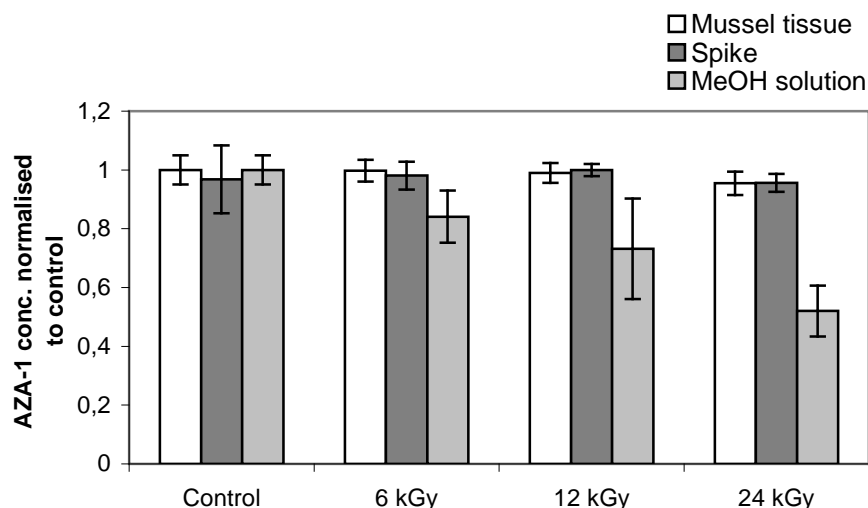


Figure 5.1.5. AZA-1 concentrations measured in MeOH, naturally incurred tissue and spiked tissue after increasing doses of gamma irradiation. Error bars shown represent ± 1 SD ($n=5$), adapted from McCarron et al., 2007c.

To our knowledge this is the first investigation of the effectiveness of irradiation as a technique to stabilise tissue reference materials for the determination of phycotoxins. Our results suggest that this technique is not effective for materials containing Domoic Acid. However, it merits further investigation as a stabilisation procedure for preparation of shellfish tissue materials for some lipophilic toxins, in particular azaspiracids.

c) Freeze-drying as a technique

Wet and freeze-dried reference materials were prepared from each of the two samples for a commutability study (McCarron et al., 2007b). Two samples of mussels (*Mytilus edulis*) were collected from the Southwest of Ireland. One sample contained DA, the other sample contained OA, DTX2 and AZA1, -2 and -3. Wet materials were homogenised, aliquoted and hermetically sealed under argon and subsequently frozen at -80 °C. Dry materials were similarly homogenised but frozen in flat cakes prior to freeze drying. After grinding, sieving and further homogenisation, the resulting powder was aliquoted and hermetically sealed under argon. Domoic acid materials were characterised using HPLC-UV, while LC-MS was used for the determination of lipophilic toxins. Extractability of all phycotoxins studied was comparable for wet and lyophilised materials, once a sonication step was carried out for reconstitution prior to extraction. Homogeneity was assessed through replicate analysis of the phycotoxins ($n=10$), and found to be similar for wet and lyophilised materials, both in the case of hydrophilic and lipophilic toxins (Table 5.1.4). Water contents were determined for both wet and freeze-dried materials (Table 5.1.5), and particle size was determined for the freeze-dried materials. Stability was evaluated isochronously over 8 months at 4 temperatures (-20 , $+4$, $+20$ and $+40$ °C) using the aliquots stored at -80 °C as reference samples.

The freeze-dried material containing domoic acid was stable over the whole duration at all temperature conditions, while the wet material showed some degradation of domoic acid at $+20$ and $+40$ °C (Fig. 5.1.6). In freeze-dried and wet materials containing lipophilic toxins, OA, DTX2, AZA1 and -2 were stable over the whole duration at all conditions, while concentrations of AZA3 changed significantly in both materials at some storage temperatures.

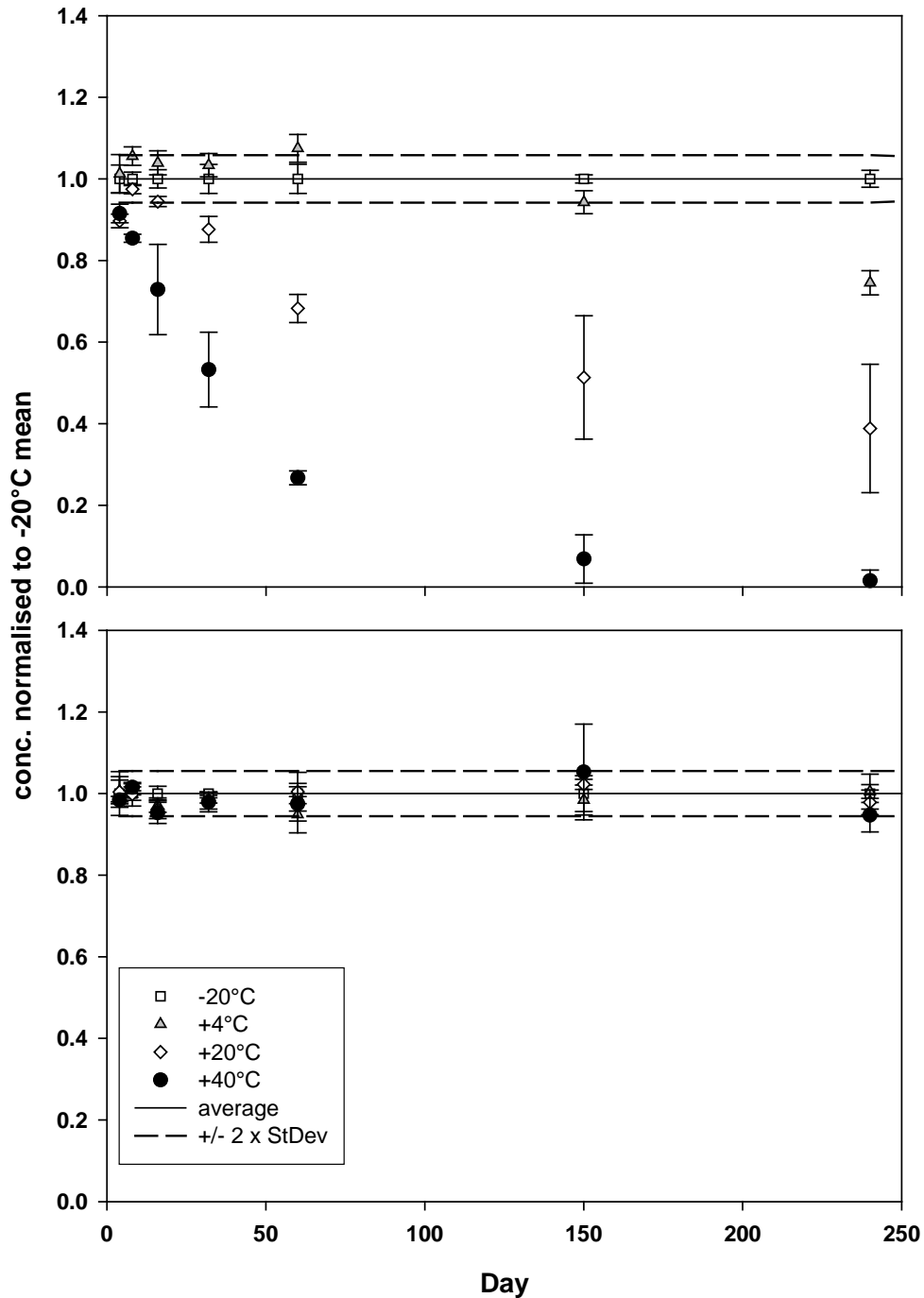


Figure 5.1.6. Stability graphs of DA. A) Wet frozen homogenate. B) Freeze-dried material. Error bars represent $\pm 1SD$ ($n=3$).

These studies were also a driving force in the decision making for the preparation of certified reference materials. The study on additives and the study on irradiation have led to the preparation of a standard reference material for AZAs in a wet mussel tissue. The material has been prepared as a collaboration of the Marine Institute and the NRCC, and is currently undergoing certification at the NRCC. The freeze-drying study had been a particular success, and a multitoxin material has been prepared as a collaborative study between six institutes (MI, NRCC, NSVS, NVI, IRMM, AgResearch), and is currently undergoing certification at the NRCC. The multitoxin material contains toxins from the following six groups: AZA, DA, OA, PTX, SPX and YTX.

Table 5.1.4. Within-bottle homogeneity testing of multi-shot DA and OA/AZA wet and freeze-dried (FD) materials ($n=5$)

	DA				OA sum				AZA sum			
	Wet		FD		Wet		FD		Wet		FD	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Bottle 1	9.92	0.08	50.50	0.32	0.36	0.01	2.08	0.02	0.26	0.01	1.26	0.05
Bottle 2	9.77	0.17	51.69	0.66	0.37	0.02	2.04	0.05	0.25	0.01	1.22	0.02
Bottle 3	9.95	0.11	52.36	2.44	0.38	0.02	2.10	0.08	0.26	0.01	1.31	0.04
Bottle 4	10.04	0.15	51.50	0.39	0.36	0.00	2.20	0.05	0.26	0.01	1.25	0.02
Bottle 5	9.78	0.14	53.07	0.91	0.38	0.01	2.12	0.07	0.25	0.00	1.25	0.01
Average (mg/kg)	9.89		51.82		0.37		2.11		0.25		1.26	
SD (mg/kg)	0.12		0.97		0.01		0.06		0.01		0.03	
%CV	1.2		1.9		2.5		2.8		2.8		2.6	

Table 5.1.5. Water content analysis of wet and freeze-dried DA and OA/AZA materials.

	Average Water Content (%)	SD	%RSD	Method
DA Freeze-dried	5.0	0.2	4.2	KFT
OA/AZA Freeze-dried	5.3	0.1	2.7	KFT
DA Wet	85.1	0.0	0.0	Oven
OA/AZA Wet	84.5	0.1	0.1	Oven

4.5.2. Proficiency testing

Participation in proficiency testing is a means of external quality control for laboratories. As there was very few validated methods for shellfish toxins at the beginning of the millennium, official control laboratories were generally obliged to in-house validate their methods. In addition, certified reference materials were very rare for reasons discussed above, and thus, the role of external quality control became even more important to demonstrate the appropriateness of methods used in official control.

Since the existence of the European network of National Reference Laboratories (NRLs) in 1996, the Community Reference Laboratory for Marine Biotoxins, Vigo (CRL-MB) has been responsible for the provision of proficiency tests for toxins in shellfish. However, due to insufficient funds, this obligation could not be fulfilled adequately. In addition, the obligation of the CRL to carry out proficiency testing only extends to NRLs, and does not include any other official control laboratories, as these are under the responsibility of the respective NRL. Worldwide, there was no other provider for proficiency testing of shellfish toxins in 2003. As NRL for Ireland, the Marine Institute had only one additional official control laboratory; thus it was not possible to carry out sensible proficiency testing. In addition, we had prepared shellfish reference materials for internal quality control (iLRMs), and we had demonstrated sufficient homogeneity and stability. Thus, we decided to also prepare test materials on a larger scale for regular proficiency testing, open to any laboratory. In this way, other official control laboratories in Europe (or globally) could participate and demonstrate the adequate performance of their in-house developed methods.

Several proficiency test providers could have accommodated the shellfish toxin work; in particular 2 providers in UK showed some interest: FAPAS (Food Analysis Proficiency Assessment Scheme) and QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe), both operating on a commercial basis, ie all exercises, including material preparation, need to be financed through the contribution of participants. After discussion with these proficiency test providers with regards to their statistical approaches, and thanks to the previous experience with QUASIMEME, I opted to collaborate with QUASIMEME on this work. Apart from the financial feasibility of the project, the advanced statistics and objective assignment of z-scores practised by QUASIMEME provided particularly interesting tools for the analysis of shellfish toxins. Also, this proficiency test provider had a system set up whereby the introduction of new compound group / matrix combinations into their perimeter was carried out as a development exercise, clearly indicating that the proficiency test provider was in a learning phase. The Marine Institute has provided the test materials for all shellfish toxin exercises since 2003, under my guidance from 2003 to 2008.

Similar to the NRCC's toolbox approach for certified reference materials (pure calibration standards and shellfish tissue materials), we also decided to provide materials of increasing degrees of difficulty to the participants of proficiency tests. We prepared standard solutions of pure toxins (generally as dilutions of the NRCC certified calibrant solutions), crude extracts of naturally contaminated shellfish, and homogenate tissues of naturally contaminated shellfish. In this way, the review of the proficiency test can be exploited to point towards errors in calibration, extraction and instrumental analysis. The Marine Institute provides data on homogeneity and stability of the materials prepared, however, assigned values are derived through quantum statistical tools analysing the data provided by participants. The assignment of z-scores does not depend on the variability between laboratories in the exercise but is objectively pre-determined as a function of the concentration and the degree of difficulty of the analysis.

4.5.2.1. Homogeneity and stability of test materials for proficiency testing

An overview of the first 3.5 years of proficiency testing for shellfish toxins in QUASIMEME was given by Hess and McCarron, 2007. Homogeneity and stability of the materials prepared for DA- and OA-group studies were satisfactory (Table 5.2.1).

Table 5.2.1 Homogeneity and stability of test materials prepared for proficiency testing

	Material	Matrix	Analyte	Homogeneity	Stability			
				CV (%)	Frozen (-20°C)	+4 °C	+20 °C	+40 °C
DA	1	Scallop Adductor	DA	2,1	30 days	30 days	n/a	4 days *
	2	Whole Scallop	DA	3,7	30 days	30 days	16 days	4 days
	3	Whole Mussel	DA	2,7	30 days	30 days	n/a	8 days
	4	Oyster	DA	2,1	30 days	30 days	n/a	30 days
	5	Clam	DA	2,2	30 days	30 days	n/a	4 days *
OA group	6	Whole Scallop	OA	6,2	30 days	30 days	n/a	30 days
	7	Oyster	DTX1	6,2	30 days	30 days	16 days	16 days
	8	Mussel	DTX2	4,6	30 days	30 days	30 days	n/a
	9	Clam	DTX2	6,3	30 days	30 days	n/a	30 days

n/a: no data was acquired for this condition

In 2007, we started evaluating the feasibility for proficiency testing in the area of PSP, as part of an undergraduate thesis (Sandra Noel). The concentration of individual PSP toxins ranged from 4 µgSTXdiHCleq/kg for GTX-5 to 755 µgSTXdiHCleq/kg for GTX-1,4, yielding a total of ca. 2200 µgSTXdiHCleq/kg (Table 5.2.2). The analysis of 13 individual portions showed that there was acceptable PSP homogeneity obtained by the Lawrence method as the CV of 2.6% was less than 8%. GTX-5 was observed to have the lowest concentration of all toxins detected for the saxitoxin group, however, this was still above the limit of quantification. The GTX-5 response was between these one of the 2 lowest levels of the calibration curve. Due to sensitivity problems and lack of time for further optimisation, dcSTX was the only compound evaluated by LC-MS. The mean concentration for dcSTX was found to be 39.9µgSTXdiHCleq/kg with a CV of 8.8%

Table 5.2.2. PSP homogeneity results obtained by the Lawrence method.

	GTX-1,4	GTX-2,3	GTX-5	STX	dc-STX	NEO	Sum
mean concentration (µgSTXdiHCleq/kg)	755	422	4	301	326	370	2177
SD	3.0	2,4	0.0	1.6	1.1	1.2	5.6
CV (%)	4.0	5.6	5.9	5.2	3.5	3.1	2.6
Number of samples	13	13	13	13	13	13	13

A PSP LRM was successfully prepared at the Marine Institute. It was proven to be homogeneous through the DA, PSP and moisture content analysis. For ease of use the LCMS method was the best, as there was no need to perform any oxidation reactions, however, this method seriously lacks in sensitivity, therefore results were only available for 1 out of the 8 main toxins.

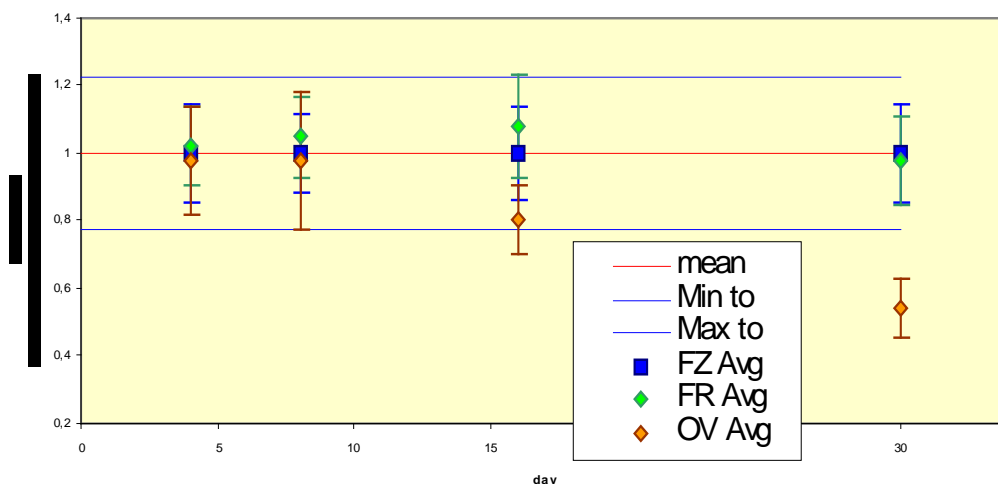


Figure 5.2.1: STX stability study data normalised to daily freezer mean concentration including error bars of $\pm 2sd$.

4.5.2.2. Proficiency testing for domoic acid

Proficiency testing for DA was introduced as the first exercise for shellfish toxins in 2003, as the methodology for this group had been well developed and shown to be rugged in previous interlaboratory exercises (Hess et al., 2005b). Despite numerous methodological differences between laboratories (with or without clean-up, LC-UV or LC-MS detection, numerous chromatographic columns etc.), good comparability between laboratories was already obtained during the first year of the development exercise (DE) (Fig. 5.2.2).

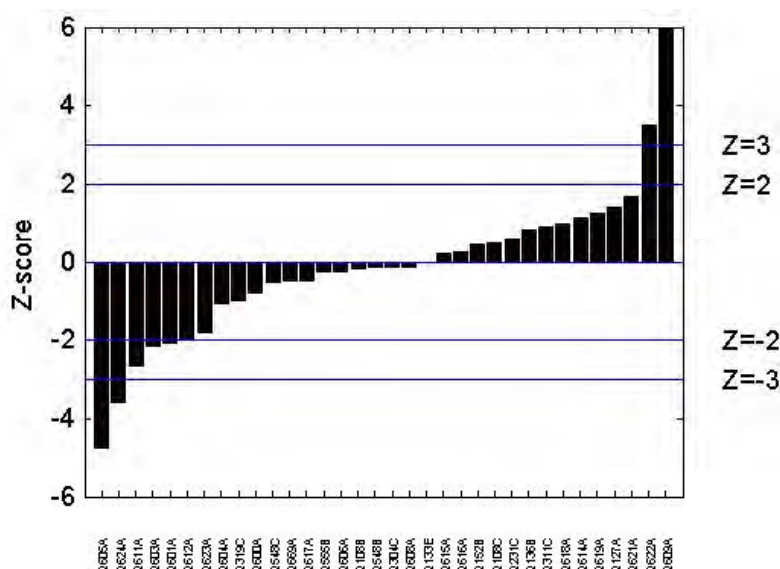


Figure 5.2.2 Proficiency testing for domoic acid (QUASIMEME, DE9 - Round 36). 20 of 34 laboratories achieve 100% correct results (59 % success rate).

This performance has continued in year two of the development exercise (Table 5.2.3), hence, it was decided to continue this combination of matrix and analyte as a routine exercise from the third year. Small improvements were still noticed between year 1 and year 3, as the percentage of laboratories that achieved completely satisfactory z-scores increased from 58.5% on average in year 1 to 74.5% in year 3.

Table 5.2.3 Three years (6 rounds) of proficiency testing for domoic acid. Note the slight trend of increasing success rate from 58.5% in year 1 to 74.5% success in year 3.

DA	Year	Participants	Returns	100%	>75%	% 100%
DE9-R34	2003	40	36	21	28	58
DE9-R36	2003/4	42	34	20	27	59
DE9-R39	2004/5	38	33	22	25	67
DE9-R41	2005	41	38	29	33	76
BT7-R43	2005/6	34	31	20	20	65
BT7-R45	2006	35	32	27	27	84

DE = development exercise, BT = biological tissue (routine proficiency testing round), 100% designates the number of laboratories that have obtained 100% correct z-score; %100% dotes the percentage of laboratories that have obtained 100% correct z-scores.

This exercise also encouraged QUASIMEME to go ahead with the direly needed development exercise for lipophilic shellfish toxins in 2005 (advances on reference material preparation in this area had been ongoing in my laboratory since 2003).

4.5.2.3. Proficiency testing for lipophilic toxins

Introducing proficiency testing for lipophilic toxins was a much bigger challenge, due to a number of factors. First of all, it should be noted that proficiency testing using the official method, the mouse bioassay, was not possible as the obtention of sufficient quantities of test material would have been prohibitive compared to the self-financing obligation of the exercises. Secondly, there is many more toxin groups in the lipophilic class of toxins, as there is already 4 groups regulated in Europe (AZA, OA, PTX, YTX). Furthermore, the LC-MS techniques used in the instrumental analysis of lipophilic toxins were significantly less well studied than the HPLC-UV methods for DA.

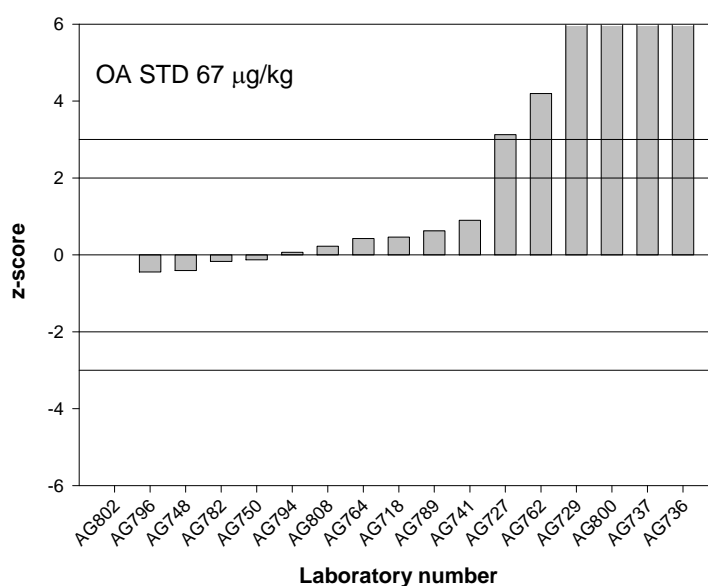


Figure 5.2.3 Proficiency test for lipophilic shellfish toxins (QUASIMEME DE10 Round 43, 2005/6). Note large number of outliers (6/16). The between laboratory CV of the ten laboratories with acceptable z-scores is 9.8 %.

In this context, it was decided to focus on the OA group of toxins as these are a problem worldwide and a sufficient number of laboratories may be interested enough in advanced methodological developments beyond regulatory tests. The relatively poor control of methods, and resulting lack of confidence of laboratories in their methodology showed directly in the first year of proficiency testing, both in the small number of participants (16) and the slightly poorer performance of the laboratories (only 10 out of 16, ie 63%) showed satisfactory performance in the analysis of an OA standard (Fig. 5.2.3), while virtually all laboratories had analysed the standard correctly for DA.

In the following round (DE10 R45, 2006), a standard was distributed for DTX2, a compound for which no certified calibrant had been available at the time. Even though 11 out of 15 laboratories achieved acceptable z-scores, their between laboratory CV was 29%. Therefore, the requirement for certified calibrants is not negligible, in particular in areas where laboratories are still in the learning phase.

In a following round (QUASIMEME DE10, R47), 10 laboratories obtained between laboratory CVs of 17.9 and 20.7% for materials contaminated at 372 and 104 µg/kg (sum of OA, DTX1 and -2 following hydrolysis), respectively. This trend also indicates overall slight improvements, however, a smaller number of laboratories participated, thus the comparison is somewhat skewed.

In 2007/8, we also started to provide AZA-containing matrices for the lipophilic exercise, and there is likely going to be an increasing interest in these exercises due to the recent legislative developments in Europe suggesting a stronger need for instrumental analysis in this area.

4.5.2.4. Proficiency testing for saxitoxin group toxins

Proficiency testing in the area of the STX group of toxins is also very challenging since there is not even any certified shellfish tissue reference material available. This lack of certified materials results in reduced competence of official control laboratories since their in-house quality control procedures are limited to cumbersome in-house validation trials regards recovery.

Thanks to our progress at the Marine Institute on STX-contaminated shellfish tissue materials in 2007, we were able to introduce an exercise with QUASIMEME in 2008/9. Similar to the start of lipophilic toxin exercises, only 13 laboratories provided results for this toxin group (Table 5.2.4).

Table 5.2.4. Results of the first QUASIMEME proficiency testing round for STX-group toxins (2009). Two shellfish tissues were analysed by 13 labs. Note that concentrations were both below and above the regulatory limit of 800 µg STX-diHCleq kg⁻¹ shellfish meat.

Matrix/ Determinand	Assigned Value	Units	Total Error%	NObs Numerical	Model Between Lab CV%	Model percentage in PMF1	DIN38402 Mean	DIN38402 Between Lab CV%
QST075BT								
Total STX-equiv	452,297	µg/kg	12,72	13	32,54	73,17	473,83	37,00
QST076BT								
Total STX-equiv	947,253	µg/kg	12,61	13	45,34	79,80	1000,54	48,82

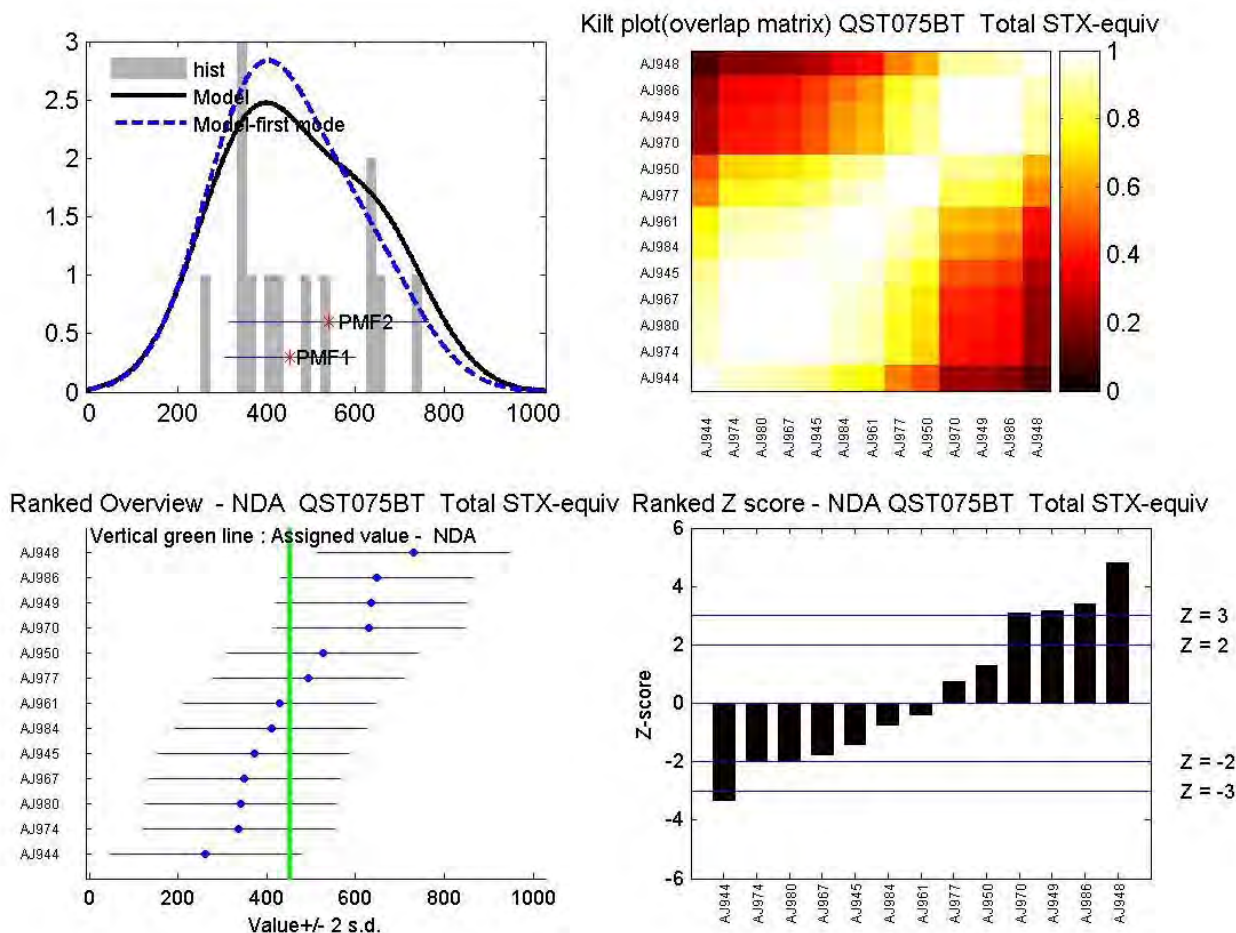


Figure 5.2.4. Different representations of the same proficiency testing round. Top left: Cofino-model based on quantum statistics, top right: kilt plot (overlap matrix), bottom left: individual laboratories' results with averaged within-laboratory standard deviation, bottom right: z-score plot.

Similar to the initial results in the OA-group, there is also relatively large between laboratory variability (Table 5.2.4 and Fig. 5.2.4).

Contrary to an exercise previously organised by FAPAS, the results are in the same order of magnitude, suggesting that there was some progress in the analysis of STX-group toxins. However, there appears to be a need for significant improvements in the performance of laboratories.

Such improvement could be made through provision of certified tissue reference materials, and the NRCC is currently working on the certification of a material for this purpose.

Additional exercises by the CRL and other organisations, including the AOAC Phycotoxin Task Force, may assist in advancing the state of the art.

4.5.3. Method comparison, evaluation and fitness for purpose

In order to evaluate a method completely, it is interesting to know its performance in comparison to other methods. Once these characteristics have been established, it is possible to make recommendations to risk managers, with a view to provide a maximum of information relevant to evaluating the risks posed by marine biotoxins. Thus, we have compared different analytical techniques and detection approaches amongst themselves. In this section, I present a comparison between analytical techniques for the determination of DA (LC-UV vs. LC-MS), a comparison between chromatographic techniques and immunological methods (LC-UV vs. ELISA for DA and LC-MS vs. biosensor for OA), and finally I also describe a comparison between LC-MS and MBA at the example of AZAs.

4.5.3.1 LC-UV, -MS and antibody-based methods for DA & OA

Under European legislation, domoic acid (DA), the main constituent of amnesic shellfish poisoning is monitored to protect the shellfish consumer. To ensure comparability amongst analytical data, it was deemed necessary to undertake performance assessments of the methods conducted by monitoring laboratories of the United Kingdom and Ireland (Hess et al., 2005b). In phase I of a two-phase inter-comparison, three laboratories used HPLC-UV. Concentration data for a DA standard solution, a crude extract of whole scallops and a scallop homogenate fell within internationally accepted limits, demonstrating good agreement for these matrices. Between laboratory analyses of a scallop gonad showed a higher (>16 %) variation. In phase II, a second gonad homogenate containing DA one order of magnitude higher in concentration gave results acceptable to internationally set criteria. The efficiency of the strong anion exchange cartridges used in sample extract clean up should be monitored as part of a laboratory quality control system. From a recovery study, it is suggested that recovery correction should also be applied (Table 5.3.1).

Table 5.3.1. Recovery of DA from the CRM (MUS1-B) in relation to the elution volumes applied during clean up.

Laboratory	DA recovered (%)	Elution volume (mL)
LAB-1 (phase I)	89.4	2
LAB-1 (phase II)	98.4	5
LAB-2	90.4	2
LAB-3	87.8	2
LAB-4	79.0	3

The recovery losses could be traced back to losses on SPE cartridges, at least in the organisers laboratory (Fig. 5.3.1). There was no difference in the quantitation of DA in standard solutions or shellfish using either LC-UV or LC with mass spectrometric (MS) detection, and between-laboratory MS data for a gonad homogenate was also equivalent.

Despite numerous variations of the published method practiced by the monitoring laboratories (Tables 5.3.2 and 5.3.3), results were found not to be compromised, thus demonstrating an acceptable degree of ruggedness, as well as comparability between the participants (Table 5.3.4 and Fig. 5.3.2).

Very similar results have also been obtained in proficiency testing studies for DA (QUASIMEME, see also section 4.5.2.2). These method inter-comparisons raise the question whether proficiency testing in general is not a similarly effective tool as method validation. Method validation demonstrates the validity of a given method as prescribed at a given time. However, often the participants during a method validation study may use chromatography tools available at the time, even if these are not specified in the validated protocol. Subsequently, nothing guarantees per se that the validated protocol will be carried out with the same tools and the same performance.

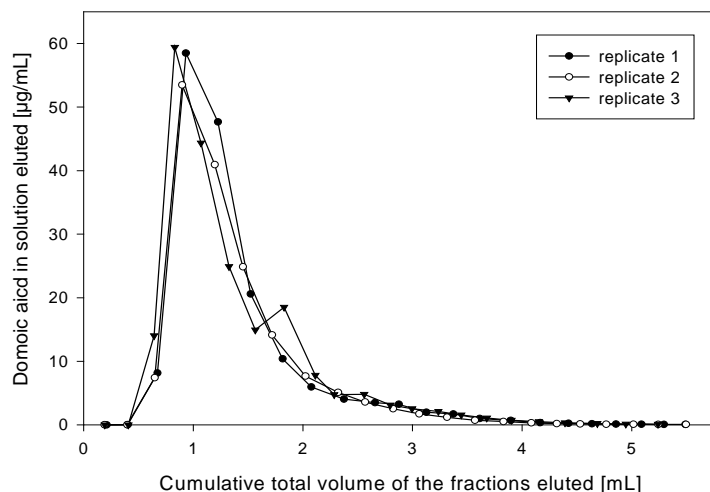


Figure 5.3.1. Elution profile of a DA standard solution using a Waters QMA SAX cartridge.

Table 5.3.4. Observed mean DA concentrations and coefficients of variation (c.v.) for standard solutions, and the scallop gonad homogenate analysed during phase II of the trial.

Laboratory	Nominal DA concentration ($\mu\text{g/mL}$)									
	5		2		1		DA+ <i>epi</i> -DA in gonad [mg/kg]		DA+ <i>epi</i> -DA in gonad [mg/kg], corrected*	
	Observed DA concentration ($\mu\text{g/mL}$)									
	mean	CV (%)	mean	CV (%)	mean	CV (%)	mean	CV (%)	Corrected	CV (%)
LAB-1-1	5.02	1.53	1.98	1.14	0.98	1.83	19.6	5.87	19.9	5.88
LAB-2	5.15	0.64	2.08	1.95	1.02	2.98	19.2	0.89	21.3	0.85
LAB-3	5.10	0.51	2.17	0.53	1.19	0.59	17.0	5.12	19.3	5.18
LAB-4 (MS)	4.90	0.36	1.84	0.82	0.92	0.91	18.5	7.35	23.4	7.35
LAB-1 (MS)	5.03	1.72	2.01	0.92	0.98	0.96	19.4	8.30	19.7	8.17
LAB-1-2	N/A	N/A	N/A	N/A	N/A	N/A	20.7	8.84	21.0	8.86
Overall	5.04	1.9	2.01	6.0	1.02	10.0	19.1	6.5	20.8	7.2

corrected for recovery of DA as determined for the Certified Reference Material MUS-1B (NRC, Canada); N/A not analysed

Table 5.3.2. Overview of extraction and clean up procedures for the analysis of DA in shellfish by the four participants.

Laboratory	LLOD* ($\mu\text{g/g}$)	Extraction			Clean-up					
		Blender	Sample weight (g)	Solvent amount (mL)	SAX cartridge type		Conditioning	Sample load (mL)	Wash	Elution
					size	manufacturer				
LAB-1	2.5	Ultra Turrax	4 ± 1	16 ± 1 (MeOH:H ₂ O, 1:1; v:v)	0.5 g	Waters QMA®	3 mL MeOH, 6 mL MeOH:H ₂ O (1:1; v:v)	5 (Phase I)	5 mL 10 % ACN; 0.3 mL CAB	2 mL CAB (Phase I)
								2.5 (Phase II)		5 mL (Phase II)
LAB-2	1.0	Silverson SL2	4 ± 0.1	16 ± 0.1 (MeOH:H ₂ O, 1:1; v:v)	0.5 g	IST Isolute®	6 mL MeOH 3 mL H ₂ O 3 mL MeOH:H ₂ O (1:1; v:v)	5	5 mL 10 % ACN; 0.3 mL CAB	2 mL CAB
LAB-3	1.0	Ultra Turrax	4 ± 1	16 ± 1 (MeOH:H ₂ O, 1:1; v:v)	3 mL	Supelclean LC-SAX®	6 mL MeOH 3 mL H ₂ O 3 mL MeOH:H ₂ O (1:1; v:v)	5	5 mL 10 % ACN; 0.5 mL CAB	2 mL CAB
LAB-4	0.6	Vortex mixer	4 ± 1	20 ± 1 (MeOH:H ₂ O; 2:1; v:v)	0.5 g	IST Isolute®	6 mL MeOH 3 mL H ₂ O 3 mL MeOH:H ₂ O (1:1; v:v)	3 x 1	3 mL 10 % ACN	3 mL ACN:H ₂ O:FA (10:88:2; v:v:v)

CAB (citric acid buffer), ACN (acetonitrile), FA (formic acid), SAX (strong anion exchange).

*LLOD = lower limit of determination of DA.

Table 5.3.3. HPLC details for the analysis of DA as carried out by each participating laboratory.

Laboratory (detection system)	Mobile Phase		HPLC Column Type		Calibration		DA & <i>epi</i> -DA separation
	ACN (%)	Additive ^{‡‡}	Guard	Analytical	Levels [‡]	Range ($\mu\text{g}/\text{mL}^{-1}$)	
LAB-1 phase I (LC-UV)	10	0.1 % TFA	10 x 4.6 mm, 5 μm Purosphere	250 x 4.6 mm, 5 μm Spherisorb ODS2	6	0.25-10	Yes
LAB-1 phase II (LC-UV)	10	0.1 % TFA	10 x 4.6 mm, 5 μm Vydac 201GK54T	250 x 4.6 mm, 5 μm Vydac 201TP54	6	0.25-10	Yes
LAB-1 phase II (LC-MS)	9.5	2mM AF + 50 mM FA	10 x 2 mm, 3 μm BDS Hypersil C8	50 x 2 mm, 3 μm BDS Hypersil C8	6	0.25-10	Yes
LAB-2 (LC-UV)	15	0.1 % TFA	4 x 4mm, 5 μm Licrochart RP18e	250 x 4.0 mm, 5 μm Lichrosphere RP18e	1	10	Yes
LAB-3 (LC-UV)	10	0.1 % TFA	None	250 x 4.6 mm, 10 μm Vydac 201TP104	5	1-50	Yes
LAB-4 phase II (LC-MS)	12.5	0.1 % FA	30 x 4.6 mm, 5 μm Sphereclone ODS2	250 x 4.6 mm, 5 μm Sphereclone ODS2	7	0.1-9	Yes

[‡] Number of DA calibration standards used to calibrate the instrument for each batch of analyses

^{‡‡} AF (ammonium formate), FA (formic acid), TFA (trifluoroacetic acid)

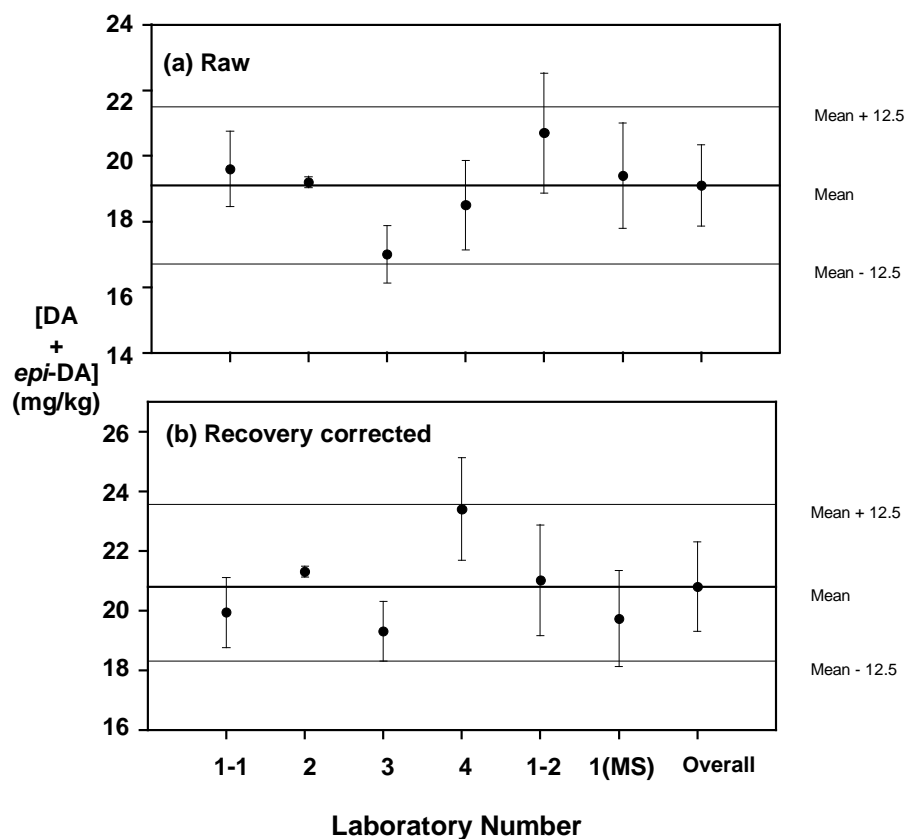


Figure 5.3.2. Phase II of study: analysis of a scallop gonad homogenate (a) raw data, and (b) recovery corrected data.

Method comparison can also be extended from different variations with physicochemical techniques based on chromatography to the comparison of chromatographic techniques to immunological tools. As an example, I would mention a study carried out with the help of an undergraduate student (Katrin Bender), in which we participated in the intercomparison for an ELISA on DA, and where we also compared the performance of the ELISA to HPLC.

HPLC analysis detected concentrations below 20mg kg⁻¹ DA in 18 stations (28.1%) and above 20mg kg⁻¹ DA in 46 stations (71.9%). ELISA analysis detected concentrations below 20mg kg⁻¹ DA in 15 stations (23.4%) and above 20mg kg⁻¹ DA in 49 stations (76.6%). Calculating from the average result of ELISA and HPLC, 15 stations (23.4%) were below 20mg kg⁻¹ DA and 49 stations (76.6%) were above 20mg kg⁻¹ DA in total.

Examining the outliers in the ELISA results only at # 45 a possible spectroscopic error could be detected in the replicates.

At 10 stations (15.6%) the ELISA gave lower results than the HPLC method. At 54 stations (84.4%) the ELISA gave higher results than the HPLC method. At 12 stations (18.8%) the difference in results between both methods fell below 2mg kg⁻¹.

The total tissue results of ELISA and HPLC gave a good correlation with $r^2 = 0.741$ and a 2% higher recovery in the ELISA (see Fig. 5.3.3). The ELISA showed an offset of 7.14mg kg⁻¹. After one outlying replicate from the ELISA analysis was removed the correlation and the recovery both improved to $r^2 = 0.781$ and 1% over-recovery. After outlier analysis the correlation still improved to $r^2 = 0.899$ but the over-recovery of the ELISA increased to 15%.

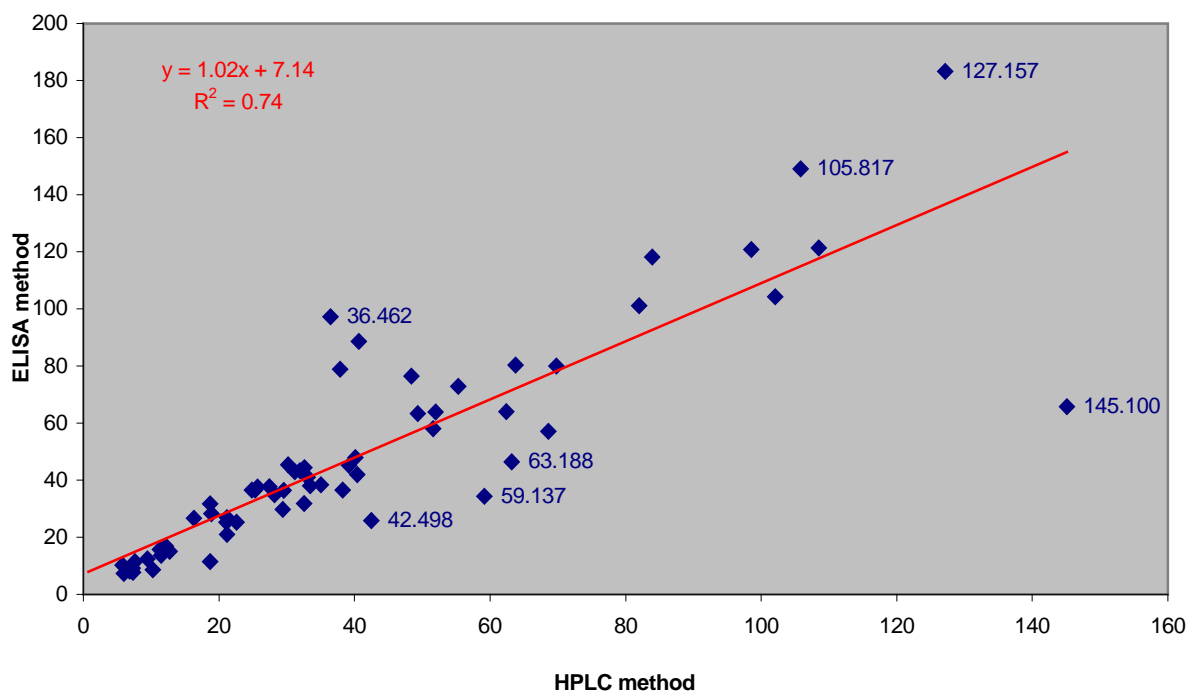


Figure 5.3.3. Bias plot HPLC method vs. ELISA method (entire tissue concentration), adapted from Bender, 2004.

These results were very encouraging and were also confirmed later again during the BIOSSENSE validation study where 4 laboratories (including the Marine Institute) also found very good agreement with the ELISA results. Subsequently, BIOSSENSE obtained AOAC approval of the ELISA method for DA and the method was allowed for official control in the EU legislation (Anonymous, 2006b).

In a recent collaborative study between the Marine Institute and Queen's University Belfast, a rapid analytical optical biosensor based immunoassay was developed and validated for the detection of okadaic acid and its structurally related toxins from shellfish matrix (Stewart et al., 2009). The assay utilises a monoclonal antibody which not only binds to OA and related toxins but also reflects their differential toxicities, resulting in a pseudo-functional assay. Single laboratory validation of the assay for quantitative detection of OA determined that it has an action limit of 120 $\mu\text{g}/\text{kg}$, a limit of detection of 31 $\mu\text{g}/\text{kg}$, and a working range of 31-174 $\mu\text{g}/\text{kg}$. The midpoint on the standard matrix calibration curve is 80 $\mu\text{g}/\text{kg}$, well below the current regulatory limit of 160 $\mu\text{g}/\text{kg}$. Inter- and intra-assay studies of negative mussel samples spiked with various OA concentrations produced average CV and SD values of 7.9 and 10.1 respectively. The assay was also validated to confirm the ability to accurately co-detect and quantify DTX-1, DTX-2 and DTX-3 without the need to perform alkaline hydrolysis for the detection of DTX-3 from shellfish matrix, resulting in a quick and easy to perform extraction procedure. Excellent correlations with the data generated by the biosensor method and LC/MS/MS were obtained using a certified reference material ($r^2 = 0.99$). When a laboratory reference material from naturally contaminated shellfish tissues and naturally contaminated mussel samples were analysed a correlation coefficient of $r^2 = 0.97$ was obtained (Fig. 5.3.4). The new procedure could be used to screen and monitor toxin levels and assist in the development of better risk management strategies for the shellfish industry and regulatory authorities.

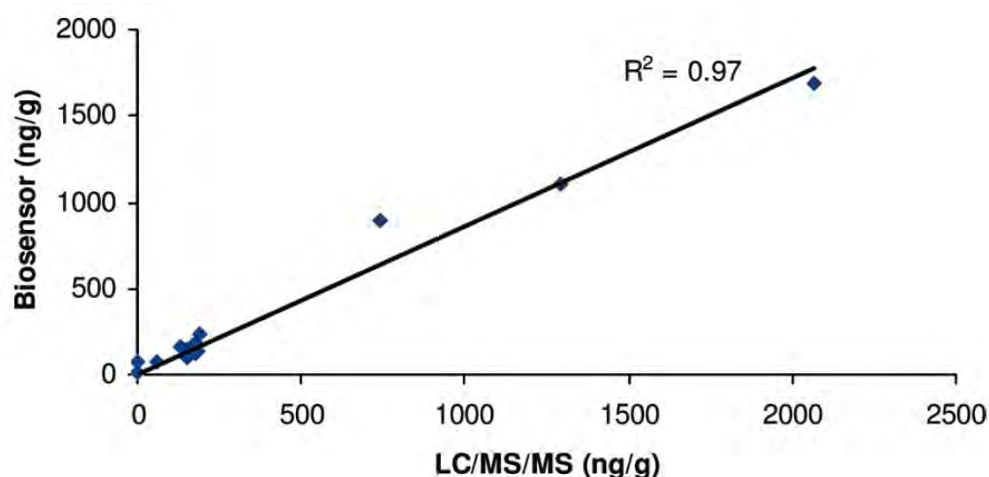


Figure 5.3.4. Comparison of biosensor and LC/MS/MS results of all naturally contaminated samples (LRMs and Norwegian samples)

4.5.3.2 LC-MS versus MBA for lipophilic toxins

Shellfish toxins pose particular problems to public health protection due to a number of differences compared to other contaminants. In particular, the lack of prediction capability of the occurrence of shellfish toxins is a major complicating factor. In combination with the requirement to produce live bivalve molluscs, this results in the need for continued monitoring of shellfish harvesting areas. Since the 1980s, official control in many countries has been carried out using mouse bioassays (MBA) for lipophilic toxins (azaspiracids, okadaic acid group toxins, pectenotoxin and yessotoxin group toxins). More recently, in some countries, e.g. Germany and Sweden, LC-MS based methodology has been used as the primary detection tool; in other countries, e.g. Norway, Ireland and the Netherlands, the bioassays have been complemented by LC-MS in the official monitoring programs.

Our study examining the comparability of the EU harmonised mouse bioassay (MBA) for lipophilic toxins with an LC-MS based method (Hess et al., 2009) was carried out at the example of a well-characterised set of blue mussel tissues. The tissues had been prepared from naturally contaminated mussels which had been shown to have no other known toxins present but AZAs. Different levels (7) of contamination were prepared by mixing the tissues with uncontaminated hepatopancreas, their homogeneity is shown in (Table 5.3.5).

Table 5.3.5. Homogeneity study. Relative standard deviations (RSDs) in [%], n=5.

Level	1	2	3	4	5	6	7
RSD	n/a*	3.5	1.8	5.2	6.6	4.0	4.6

*n/a = RSD is not applicable where concentrations were not quantifiable

This study has shown that the MBA can detect azaspiracids at the current regulatory limit of 160 µg/kg. At the same time, the study also clearly demonstrates that the assay can not reliably detect any concentration below this level. Comparison of these data with Aune et al. (2007) allows for the assumption that dose-response curves of shellfish toxins are similar in the MBA, with the only difference consisting in the LC50 of each compound (LC50 being the concentration lethal to 50 % of the animals).

Table 5.3.6. Average concentrations measured by LC-MS-MS for individual Azaspiracid analogues in hepatopancreas tissue and toxicity weighted AZA1-equivalents in hepatopancreas (HP) and calculated whole flesh (WF) equivalents. All concentrations in [mg/kg], n=3. The TEFs for AZA2 and -3 were 1.8 and 1.4, respectively (from Ofuji et al., 1999).

Level	1	2	3	4	5	6	7
AZA1 (in HP)	<LOQ	0.16	0.31	0.50	0.65	0.80	0.90
AZA2 (in HP)	<LOQ	0.07	0.13	0.20	0.26	0.33	0.36
AZA3 (in HP)	<LOQ	0.09	0.17	0.27	0.34	0.43	0.50
AZA1-Equivalence in Hepatopancreas	<LOQ	0.41	0.78	1.24	1.59	1.99	2.24
AZA1-Equivalence in Whole flesh*	<LOQ	0.06	0.12	0.19	0.24	0.30	0.34
Estimated WF _{min} *	n/a	0.05	0.10	0.16	0.20	0.25	0.28
Estimated WF _{max} *	n/a	0.07	0.14	0.22	0.29	0.36	0.40

* The transformation of AZA1-equivalents from HP into WF equivalents is based on the estimate of the HP being 15.25 % of WF weight on average in fresh mussels. The minimum WF equivalent (WF_{min}) is based on the minimum HP content observed in raw mussels in the authors' laboratory (12.5%), while WF_{max} is based on a maximum of 18% HP in WF.

Table 5.3.7. Mouse bioassay results for seven assays (3 mice each) carried out on independent days (over a 6-week period) for each of the seven concentration levels. Note, although there were three survivors at level 4 and 5, these mice survived in three separate assays, and hence did not lead to any negative assay outcome in any of the 7 independent assays at these two levels.

Level	AZA1-equiv. in WF(µg/g)*	AZA1-equiv. in HP*	Assay Neg / Pos	No. mice alive	No. mice dead	% survival	% death
1	< LOQ	<LOQ	7/0	21	0	100	0.0
2	0.06	0.41	7/0	21	0	100	0.0
3	0.12	0.78	3/4	7	14	33.3	66.7
4	0.19	1.24	0/7	3	18	14.3	85.7
5	0.24	1.59	0/7	3	18	14.3	85.7
6	0.3	1.99	0/7	1	20	4.8	95.2
7	0.34	2.24	0/7	1	20	4.8	95.2

* AZA1-equivalents in WF are calculated and indicative, those in HP are measured and adjusted for RRFs and TEFs.

Thus, it also becomes apparent that the detection capability of the MBA for OA-group toxins is significantly poorer than for azaspiracids. In fact, the probability to detect a positive result at the current regulatory limit has been estimated to be less than 50 % for OA (EFSA, 2008a). In practise, this lack of detection capability at the regulatory limit has for instance led to ca. 10% of samples above 160 µg/kg OA-equivalents not being detected by the MBA in France, over the period from 2003 to 2008 (Belin et al., 2009).

My recent review (Hess, 2010) gave several examples of shellfish toxin occurrences in the field that were examined using data from the Irish and French official monitoring programmes to illustrate the difficulties encountered in protecting the consumer from the risk of shellfish toxins. In either monitoring system, the accumulation of toxins in blue mussels has been followed using the MBA and LC-MS based methods in parallel. The first case (Fig.1.2.1) could be described as a

classical event, as it involves toxins that have been reported to make shellfish consumers sick at levels incurred during the event (in this case, no sickness occurred as monitoring results were known to regulatory authorities immediately, and closure of the harvest areas prevented any risk to the public).

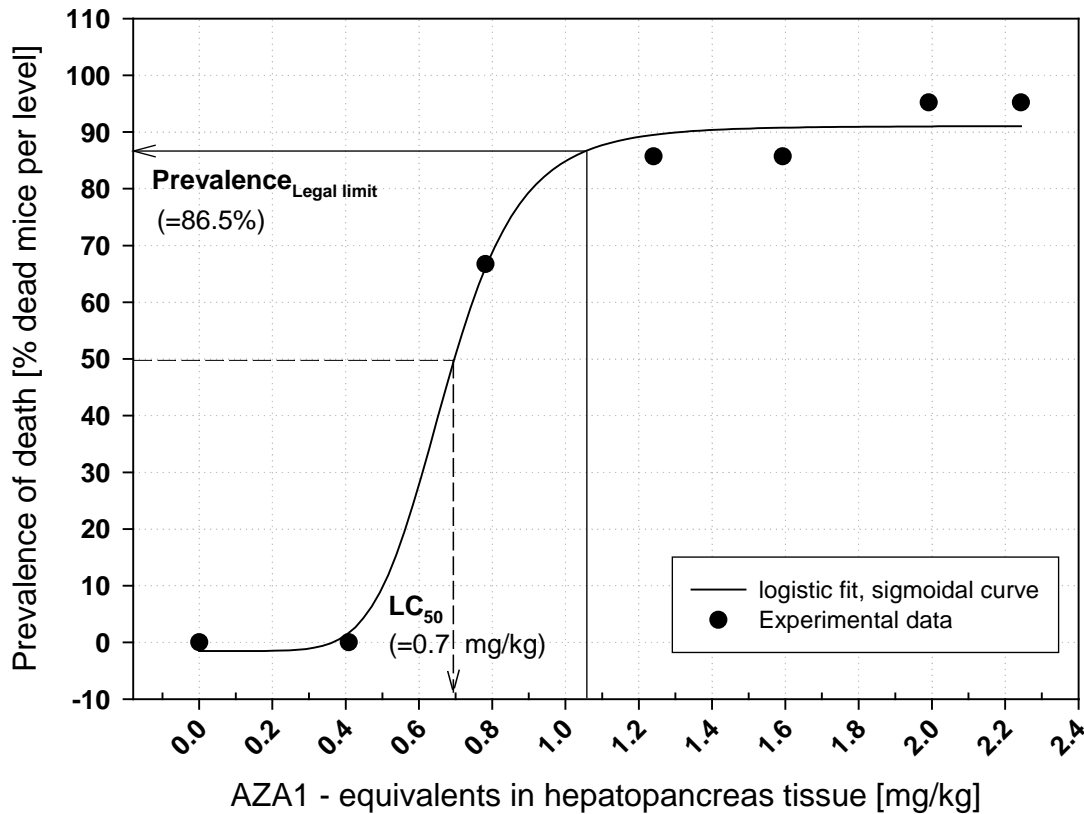


Figure 5.3.5. Prevalence of death in mice as a function of AZA1-equivalents in hepatopancreas (HP) tissues. Each experimental data point was determined using 3 mice in each of 7 independent assays (21 mice per level, i.e. total of 147 mice).

Part c of Fig.1.2.1 outlines the results of the MBA, which is the regulatory test in many countries, including Ireland. It is apparent that using the MBA, the toxicity appears without warning, i.e. from the week of 9th to 16th of July 2001. If the chemical monitoring which was ongoing in parallel had not already indicated low levels of toxins of the AZA-group (see part a) of Fig. 1.2.1), the area may only have been closed on the 18th of July 2001, i.e. 9 days from the last “non-toxic” sample date (weekly sampling plus 48 h from the sample taken to the result obtained). This would have resulted in harvesting of the area for probably 3-5 days with high toxicity present in shellfish which may have led to illness if these shellfish had been marketed. The rapid accumulation of shellfish toxins is a phenomenon which has often been underestimated and may lead to severe public health problems as well as to significant economic losses if end-product testing is carried out efficiently and timely.

In the same graph, it is also apparent that two shellfish toxin groups may co-occur independently; in this case, OA-group toxins and AZA-group toxins. This co-occurrence may be governed by hydrographic and environmental conditions but it is not necessarily reproducible, as shown by comparison of Fig.1.2.1 with Fig. 1.2.2 (2001/2 versus 2008/9). Due to the possible co-occurrence of several toxin groups, the methods used in official control of harvesting areas must be comprehensive (Table 4). The MBA is capable of detecting both OA and AZA-group toxins, as are many of the in-house validated LC-MS based methods (Fux et al., 2007; Gerssen et al., 2009;

These et al., 2009). If a (hypothetical) AZA-specific ELISA had been the only technique used to detect toxins, the toxicity of the OA-group could have been neglected, and the shellfish growing area could have been reopened prematurely in January 2002 for the 2001 event (Fig.1.2.1). Similarly, if only a protein phosphatase assay (specific for OA-group toxins) had been used in official control of the harvesting area in Ireland in 2008, the area would have remained open in the month of June; yet, serious illness would have befallen the consumers of the shellfish due to the presence of AZA-group toxins (Fig. 1.2.2). Therefore, shellfish producers and official control authorities need to know all the agents potentially causing hazards in specific areas such that methods appropriate for public health protection can be implemented. Another aspect of shellfish area management is also illustrated in Figures 1.2.1 and 1.2.2: the natural detoxification of shellfish in the growing area is significantly slower than the accumulation period. Thus, although the presence of (potentially) toxic algae may only last several days or weeks, the toxicity may persist in shellfish for many months after the algal bloom has disappeared. In this case, toxicity was still above threshold for 6 months after the algal appearance. These prolonged closure periods are potentially a problem for public health protection authorities, as they involve much effort in risk communication; many consumers in shellfish producing countries would be aware of the occurrence of shellfish toxicity during summer months, however, prolonged toxicity into winter months is a less known phenomenon and requires additional efforts in managing the risks. In addition, it has been noted that detoxification rates are higher in the beginning of the detoxification (Morono et al., 1998), and the very slow detoxification during later periods causes many problems to shellfish producers. Therefore, competent authorities also frequently face further difficulties in effectively implementing closure of production areas over these long periods. The prolonged retention of toxins also means that it is difficult for importing countries to keep track of the contamination levels in other locations, a complication which results in the need for detection of many toxin groups in imported shellfish.

Apart from the abovementioned economic boundaries in which shellfish producers operate, there are also challenges associated with emerging toxins and the type of testing used in official control. As mentioned above, official control has historically relied upon animal testing to assess the toxicity present in shellfish samples. Over the years, the compound groups responsible for causing shellfish poisoning have been identified (Hess, 2008), yet due to lack of pure compounds toxin-group specific methods have not been implemented as official methods for most toxin groups (apart from DA and STX). The event shown in Fig. 1.2.3 exemplifies differences in the interpretation of toxic events, depending on the method used in the official control of harvesting areas. In this area, okadaic acid-group toxins known to cause human poisoning exceed the regulatory limit only during one week during the summer of 2005. Other toxin groups have also been monitored, including pectenotoxins, which occurred at very low levels, and always below regulatory levels. Additionally, all other regulated lipophilic toxins (YTXs and AZAs) and known non-regulated bioactive compounds (gymnodimines and spirolides) were either totally absent or present at levels more than 10-fold lower than the regulatory limits. Yet, the MBA as reference test repeatedly gave positive results for the area over the whole summer period. In fact, the area could not be opened during summer 2005 due to the sporadic occurrence of positive results.

These positive results of the MBA may be related to yet unknown toxins of public health relevance, or they may be due to interference from bioactive compounds which are not relevant to public health. Thus, in a regime which had been based on chemical analysis (by LC-MS), production would have continued after a 3-week closure period (one week toxin levels exceeded regulatory limits and 2 consecutive clear tests are required to re-open an area). The following year showed an even more dramatic picture where MBA results were again sporadically positive between May and September, while all lipophilic toxins known to occur in this area (OA, AZA, PTX, GYM, SPX, YTX) were well below the threshold expected to result in positive results in MBAs (Fig. 1.2.4). The situation in Arcachon in 2006 had been further complicated by the fact

that anecdotal evidence provided by oyster producers from the area suggested that consumption of these oysters did not result in acute human illness. Additionally, there has also been no epidemiological evidence from official health surveillance bodies suggesting a problem of public health in the area over the period from 2005 to 2009. Further research will be required to establish whether these positive MBA results are indicative of significant risks to the consumers of shellfish.

The above considerations demonstrate that the MBA for lipophilic toxins can not be effectively used to follow quantitatively the uptake of toxins in shellfish and their detoxification. This limitation brings a number of difficulties with it, as it means that the producers are not able to evaluate the official monitoring results to plan ahead their production activities.

Overall, the studies examining the performance of the MBA also suggest that the sensitivity of the assay is not sufficient enough to detect toxins of the okadaic acid group at the regulatory limit. The increase of toxins in heat treatment similar to commercial processing of shellfish has been previously shown for both the azaspiracid and the okadaic acid group (Hess et al., 2005; McCarron et al., 2008; McCarron et al., 2009), and it has been recognised that the prediction of toxin levels in processed product is impossible using the MBA assay (EFSA, 2009b). Therefore, the lack in detection capability of the MBA results in serious deficiencies for its use for commercial pre-harvest and end product testing. This limitation also leads to difficulties for the shellfish industry to use results of official control conducted with the MBA.

4.6. Characterisation of hazards from marine biotoxins

As outlined in the introduction, initial information on toxins must include their identity and quantity. Preparative isolation and structural studies by LC-MS and NMR are necessary to confirm the identity of marine toxins. Method development, validation and definition of critical parameters in quality assurance are subsequent requirements in the quantitative determination of such contaminants.

This chapter will discuss further chemical characteristics such as lipophilicity and reactivity of marine biotoxins. These parameters are discussed in the context of the environmental distribution and persistence as well as the pharmacokinetic behaviour of marine toxins.

In addition, there will be some sections presenting collaborative work on the toxicology of the compounds which provide answers to specific questions in risk assessment and management.

4.6.1. Physico-chemical characteristics and metabolic transformations of shellfish toxins

4.6.1.1. Lipophilicity of marine biotoxins

Lipophilicity expressed as $\log P_{ow}$ is an important physico-chemical parameter used in quantitative structure/activity relationship, drug and pesticide design and toxicology studies. Lipophilicity measurement is required by the EU regulation for any new chemical available commercially above a certain tonnage produced. Two methods are described in the Organisation for Economic Co-operation and Developments (OECD) guidelines for the measurement of $\log P_{ow}$: the shake flask method (OECD, 1995) and chromatographic methods (OECD, 2004). NMR spectroscopy can also be used for the determination of the pKa. This technique was used for the determination of the pKa of the ASP toxin DA by investigating the 1H NMR and ^{13}C NMR spectra as a function of pH and pD (Walter et al., 1992).

The partitioning behaviour of compounds is most heavily affected by their ability to dissociate (Fig. 6.1.1).

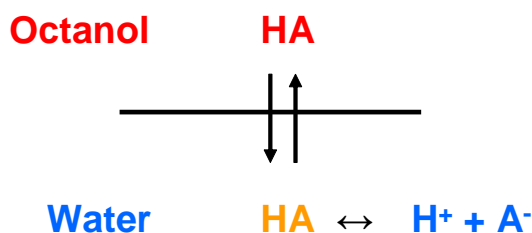


Figure 6.1.1. Illustration of a solvent water system and the species partition for a weak acid. Reproduced from (Gocan et al., 2006)

An alternative to the shake flask method for the evaluation of lipophilicity can be achieved using liquid chromatography (OECD, 2004). The retention of a compound in reversed-phase high performance liquid chromatography is governed by its lipophilicity/ hydrophobicity, and thus shows correlation with an octanol-water partition coefficient (Valko, 2004).

The chemicals are retained in proportion to their hydrocarbon-water partition coefficient. The retention time is described by the capacity factor k which can be calculated from the retention time and the void time of the system. The void time is the time taken for an unretained substance to elute. The chromatographic method relies on the production of a calibration curve with reference compounds of known lipophilicity that are chosen to cover a range of different lipophilicities.

Acidity constant, K_a , is also known as acid dissociation constant. “The term K_a is the ionisation constant of a chemical reaction in solution in which a molecule accepts or loses a hydrogen ion, in response to change in pH”. Equation 1 and 2 show the ionisation of an acid and the simplified calculation of the dissociation constant of an acid.



Equation 1: Chemical equilibrium and of an acid.

$$K_a = \frac{[H^+][A^-]}{[HA]} \leftrightarrow pK_a = -\log_{10} K_a$$

Equation 2: Simplified dissociation constant and pK_a of an acid.

Since values for K_a vary over a large magnitude, it is common to take the negative logarithm to the base ten of the value, pK_a as shown in equation 2.

The simplified equation was used for the dissociation constant in order to avoid complications involved in using activity. The ionic strength of the medium is therefore not taken into account in the calculation of the pK_a .

The determination of the lipophilicity relies on the relationship shown in equation 3. The constants a and b are determined by injection of reference compounds of known $\log P_{ow}$.

$$\log P = a \log k_w + b$$

Equation 3: Relationship between $\log P_{ow}$ and $\log k_w$, where a and b are the slope and intercept, respectively.

Where $\log k_w$ represents the retention time of a compound in a chromatographic environment where only buffered water is used as a mobile phase. The value of $\log k_w$ can not be measured directly and need to be calculated by extrapolation of the equation 4 expressing the capacity factor k as a function of the fraction of organic modifier φ .

$$\log k = c + d\varphi + e\varphi^2$$

Equation 4 : Quadratic relationship between $\log k$ and the fraction of organic modifier in the aqueous phase (φ).

The capacity factor (k), is determined from the retention time (t_r) and the dead time (t_0).

The retention time (t_r), also known as peak elution time, is the time (min) it takes for a compound to elute and is usually the peak maximum.

The dead time (t_0), also known as hold-up time or void time, is the time that is required to elute a component that is not retained by the stationary phase. The dead time includes any volumes contributed by the sample injector, the detector and connectors. The dead time (min) can be calculated by dividing the dead volume (ml) by the flow rate (ml/min).

The adjusted retention time (t'_r) is shown in equation 5.

$$t'_r = t_r - t_0$$

Equation 5 : Adjusted retention time is equal to the retention time minus the dead time.

The capacity factor (k) is a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase. As shown in equation 6 the capacity factor can be calculated by dividing the adjusted retention time by the void time.

$$k = \frac{t_r - t_0}{t_0}$$

Equation 6 : Capacity factor is equal to the adjusted retention time divided by the void time.

The capacity factor, k , is established from the retention time of each compound analysed (at each mobile phase composition, in each buffered mobile phase). The percentage of organic phase present in the mobile phase is plotted (x-axis) against the logarithm of the capacity factor ($\log k$) (y-axis). At 0 % organic phase, $\log k = \log k_w$ and equation 4 reduces to $\log k_w = c$.

The distribution coefficient has the expression:

$$\log D = \log P + \log[H^+] - \log K_a$$

Equation 7: The logarithm of the distribution coefficient as a function of the logarithm of the partition coefficient, the logarithm of the concentration of H^+ in solution and the logarithm of the dissociation constant.

This may also be written as:

$$\log D = \log P + pK_a - pH$$

Equation 8: Relationship between the $\log D$, $\log P$, pK_a and pH .

Equation 7 and 8 both display the mathematic relationship between $\log D$, $\log P$, pK_a and pH . As shown in equation 8, $\log D$ is dependant on pH while $\log P_{ow}$ is not. Therefore, where a value for $\log D$ is stated, a pH value must also be given (Fig. 6.1.2).

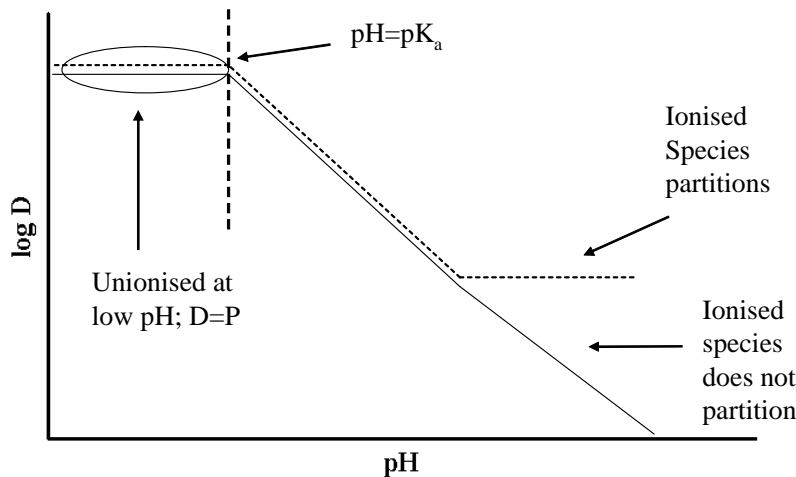


Figure 6.1.2. Illustration of the relationship between $\log D$, $\log P$, pK_a and pH . Reproduced from (Gocan et al., 2006)

Fig. 6.1.2 shows the plot for an acid where a negative slope is obtained. Bases yield positive slopes (mirror image). The end of the plateau just before the slope decreases indicates the point where the pK_a of the compound is equal to the pH . The whole area of the plateau gives a value for

the distribution coefficient, which at this pH is unionised and is therefore equal to the partition coefficient. The pH nomenclature used in the following sections was chosen according to the IUPAC definition (IUPAC, 2007). The w_pH scale refers to pH standardisation with aqueous reference buffers and pH measurement in water while s_pH refers the pH scale refers to pH standardization with aqueous reference buffers and pH measurement in the organic solvent. The pH of a buffer solution can be calculated from equation 9, where $[A^-]$ and $[HA]$ are the concentrations of the basic and acid components of the buffer. By definition the buffering capacity of a buffer solution is limited to ratios of the concentration $[Base]/[Acid]$ from 0.1 to 10. Therefore, an acid/base couple solution is considered as a buffer if the pH is within 1 pH unit of the pK_a .

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

Equation 9 : Relationship between pH, pK_a and concentration of buffer components

During the PhD thesis of Elie Fux, we have chromatographically determined the pK_a of a number of marine biotoxins to derive their $\log P_{ow}$ (Table 6.1.1), software-predicted values are also given.

Table 6.1.1. Predicted and experimental pK_a values (\pm SD) for AZA1, AZA2, OA, DTX1, DTX2 and YTX.

Toxin	Predicted pK_a	Experimental pK_a
AZA1	4.90, 9.20	5.8 ± 0.2
AZA2	4.90, 9.20	5.8 ± 0.2
OA	3.80	4.9 ± 0.5
DTX1	3.80	4.9 ± 0.5
DTX2	3.80	4.9 ± 0.5
PTX2	No acidic function	No apparent pK_a observed
YTX	na	6.9 ± 0.5

na: not available

The results obtained allowed for the evaluation of the pK_a of some of the marine toxins. OA, DTX1 and DTX2 were found to have the same pK_a 's (4.9 ± 0.5) and were more acidic than AZA1 and AZA2 which also were found to have the same pK_a (5.8 ± 0.2). PTX2 appeared as a neutral molecule. The determination of the pK_a of YTX was attempted and only a limited number of measurements allowed for the calculation of a pK_a value. The pK_a values determined experimentally are coherent with those predicted by literature, if somewhat higher. In particular, there was no difference observed between AZA1 and -2, as would be expected from computer-prediction. Similarly, there was no difference in acidity between OA, DTX1 and -2. However, both AZAs and OA-group toxins showed higher experimental pK_a -values than predicted, probably related to experimental error for OA-group toxins and due to the basic cyclic amine functionality in the AZA-molecule interacting with the carboxylic acid group.

Table 6.1.2. Predicted and experimental $\log P$ values (\pm SD) for AZA1, AZA2, OA, DTX1, DTX2 and PTX2.

Toxin	Predicted $\log P_{ow}$	Experimental $\log P_{ow}$
AZA1	4.06 ± 2.20	7.54 ± 1.01
AZA2	4.32 ± 2.33	8.18 ± 1.28
OA	4.45 ± 2.60	5.05 ± 0.72
DTX1	4.79 ± 2.67	6.88 ± 1.41
DTX2	4.46 ± 2.55	5.61 ± 0.81
PTX2	4.45 ± 1.99	6.47 ± 2.37

The determination of log P values deviated significantly from predicted or published values. The results obtained using the predicting software were associated with a relatively large error. The program (VCCLab, 2008) provided an average value of the logPow from 10 prediction softwares (details are available on-line). The high standard deviation was a consequence of the wide range of results obtained by the different methods. For example, the logPow of OA ranged from 1.98 to 9.56 (obtained with MLOGP and KOWWIN softwares, respectively).

The evaluation of the lipophilicities of the toxins was calculated from calibration curves obtained from reference compounds of known logPow obtained at pHs below the pKa of the toxins (apart for PTX2 for which all pH conditions were used in the calculation). The results obtained were surprisingly high (logPow > 5 for OA, DTX1, DTX2, PTX2 and logPow > 6 for AZA1 and AZA2). Limited confidence can be attributed to the results due to the lack of reference compounds with logPow > 5. The predictions of the logPow using computational predictions were systematically lower than those obtained experimentally (logPow ranging from 4.06 to 4.79) but were associated with large variability.

The limited calibration range with reference compounds not exceeding logPow of 4.5 led to a relatively large uncertainty in the logPow values established. Thus, the determination of the lipophilicity by LC-MS proved to be a challenging task and further development is required to establish if chromatographic methods are suitable for the determination of the logPow of lipophilic marine toxins.

4.6.1.2. Chemical stability of azaspiracids

As mentioned in the introduction, the chemical stability of a toxin will determine its persistence in the marine environment as well as in biological systems (Rehmann, 2008; Alfonso et al., 2008).

Initial results presented here come from studies on the isolation of AZAs. While previous work by the Yasumoto group had shown the instability of AZA in strong alkaline base, little was known on the behaviour of AZAs with respect to acids.

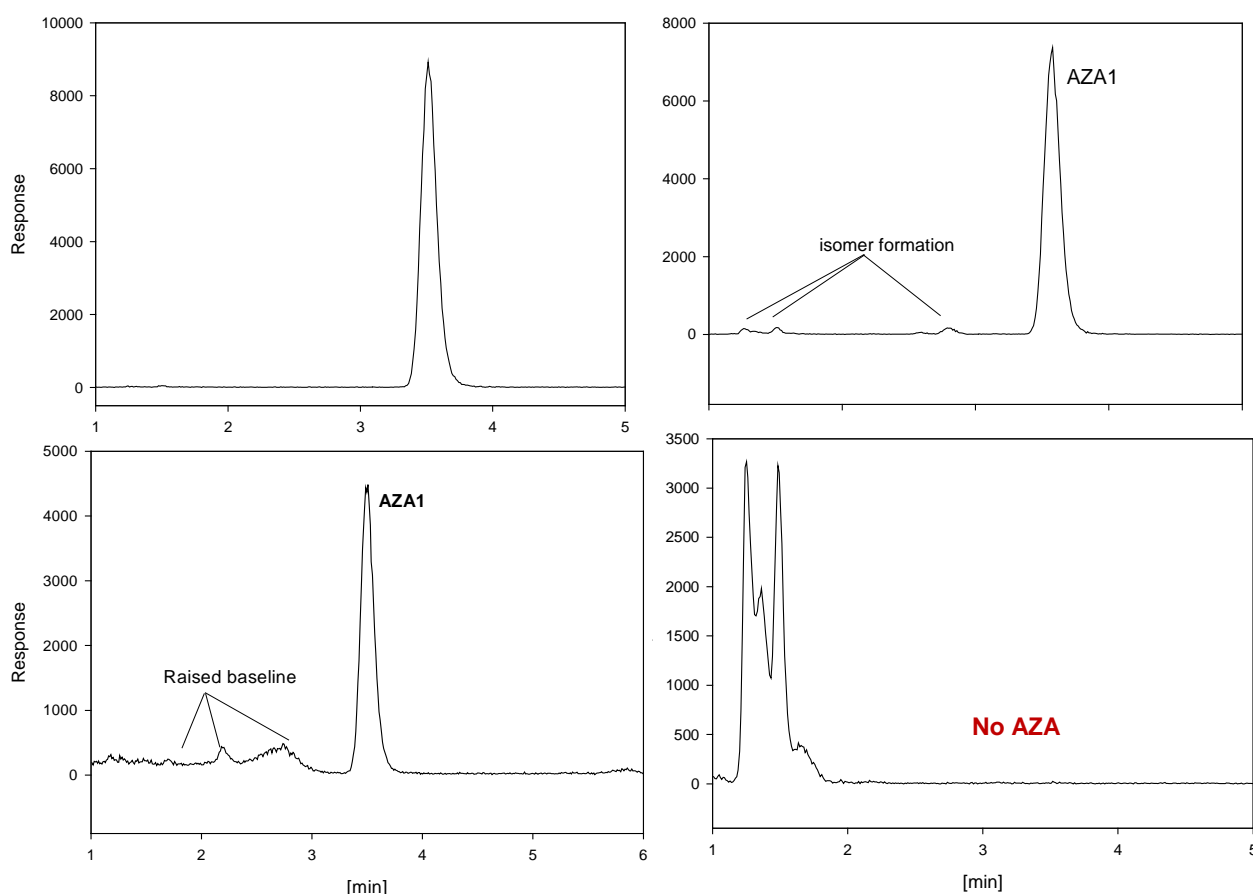


Figure 6.1.3. Degradation of AZA1 under different pH conditions: pure standard in MeOH kept for 48 h at 45°C (top left), standard in aqueous acetonitrile exposed to acetic acid (0.1 %) for 48 h at 45°C (top right), standard in aqueous acetonitrile exposed to NaOH (50 mM) for 24 h at room temperature (bottom left), standard in aqueous acetonitrile exposed to formic acid for 48 h at 45°C (bottom right).

Exposed to acetic acid, AZA shows slow degradation over 48 h (Figure 6.1.3), even at 45°C. With formic acid this degradation is much more rapid at 45°C and complete at 48 h; at room temperature the degradation with formic acid can be monitored over 48 h. Sodium hydroxide has also a similar effect: at room temperature the degradation is slow enough to be followed, while the degradation at 45°C is rapid and complete after 24 h. Ammonium formate or acetate have no effect, even 50 mM ammonium hydroxide in aqueous acetonitrile leads to very minor degradation at 45°C over 48 h.

The observations from isolation studies have led us to wonder whether AZAs effectively withstand human stomach conditions. In attempt to mimic potential degradation of AZA in

conditions of the human stomach, its stability against HCl was tested. Strong acid, such as HCl, leads to very rapid degradation during relatively short periods (Fig. 6.1.4).

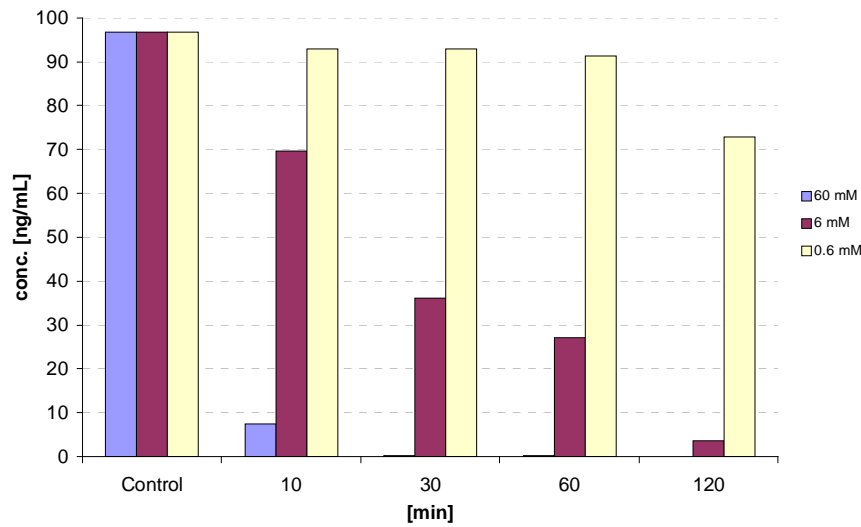


Figure 6.1.4 Concentration changes of AZA1 in solution with HCl in methanol at 37°C (n=1). Even a moderate pH of 3.5 still shows to affect the toxin concentration after a longer period of time (> 60 min).

However, mussel tissue has a strong buffering effect on the degradation of AZAs by strong acid (Fig. 6.1.5), and the pH of the acid must be lowered to 0.4 (1 M HCl) to observe significant degradation during typical stomach residence times (Fig. 6.1.6).

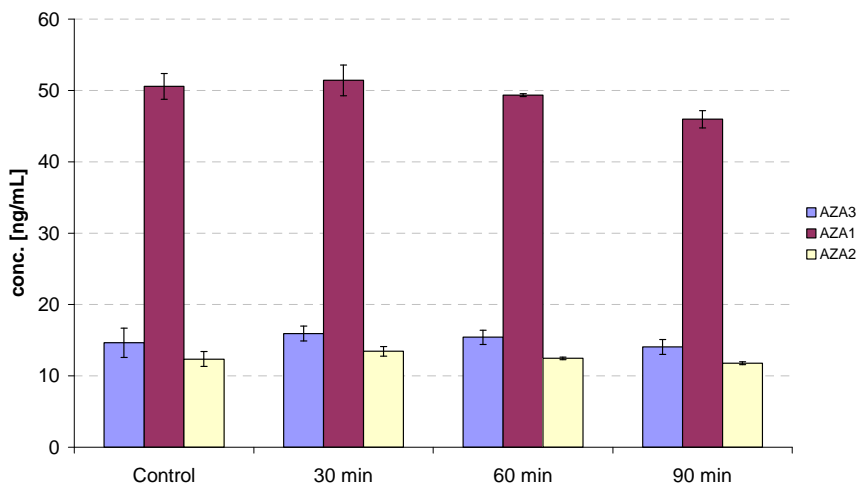


Figure 6.1.5. Concentration of AZAs in mussel tissue with addition of 0.1 M HCl (pH=1.4) (error bars = 1 SD, n=4) at 37°C. Again no significant difference in concentration of AZA was observed ($t=1.17$, $\alpha_{0.05}=2.45$ at 90 min)

As the human stomach does not typically achieve such low pH value, this last observation suggests that AZAs in shellfish tissues would not be degraded in the human stomach. However, as the stomach also contains enzymes aiding digestion, we investigated the influence of pepsin on this process. Contrary to the expectation, the addition of pepsin did not facilitate degradation of AZAs but led to an increase in concentration (Fig. 6.1.7). This phenomenon needs to be further investigated and suggests that some AZA could have been strongly bound to proteins in the

shellfish matrix and not be effectively extracted during the standard extraction procedure using 100% MeOH. Such binding phenomena may be indicative of poor bioavailability and should be further investigated in vivo.

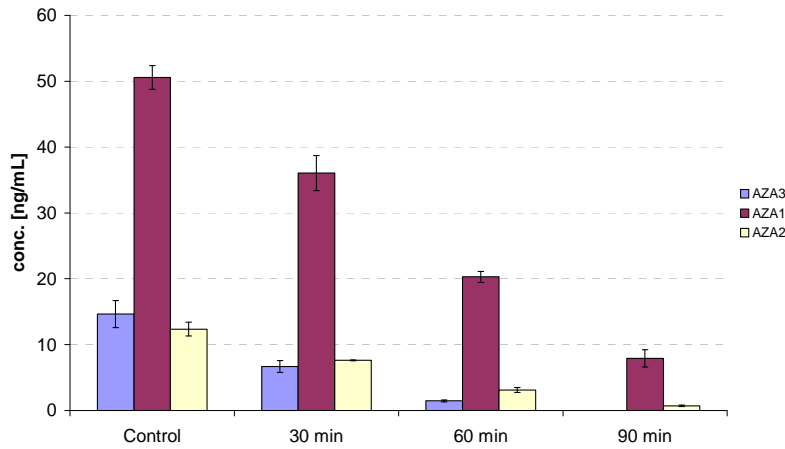


Figure 6.1.6. Concentration of AZAs in mussel tissue with addition of 1.0 M HCl (pH=0.4) (error bars = 1 SD, n=4) at 37°C. A significant decrease of toxin concentration was observed that nearly destroyed all AZA within 90 min. Even after 30 min reaction time, the decrease in concentration of AZA observed showed to be significant ($t=11.53$, $\alpha_{0.05}=2.45$)

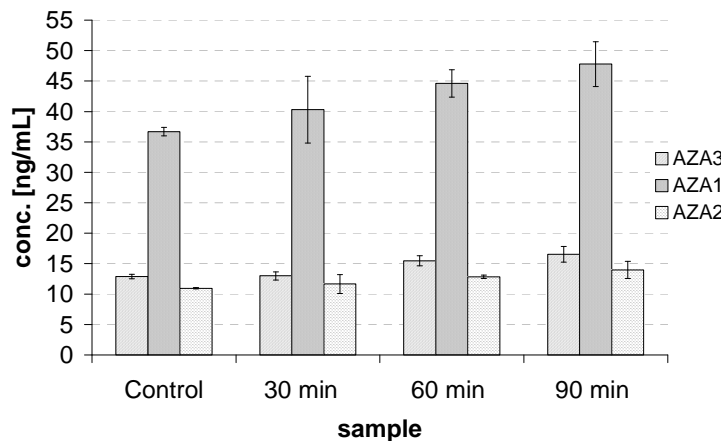
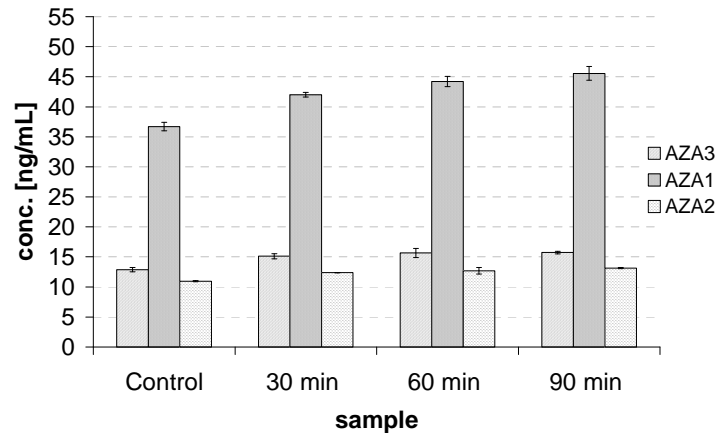


Figure 6.1.7. Simulation of mussel tissue digestion with the digestive enzyme pepsin at pH 5.1 (top) or at pH 2.3 (bottom). Note that AZA concentration increase in both cases with treatment time; however, error bars are greater for samples treated with acid as well as pepsin.

The mechanistic pathway of degradation of AZAs has not been examined yet, we proposed a potential degradation route through acid-catalyzed opening of the A-ring (Figure 4.6.4; Alfonso et al., 2008).

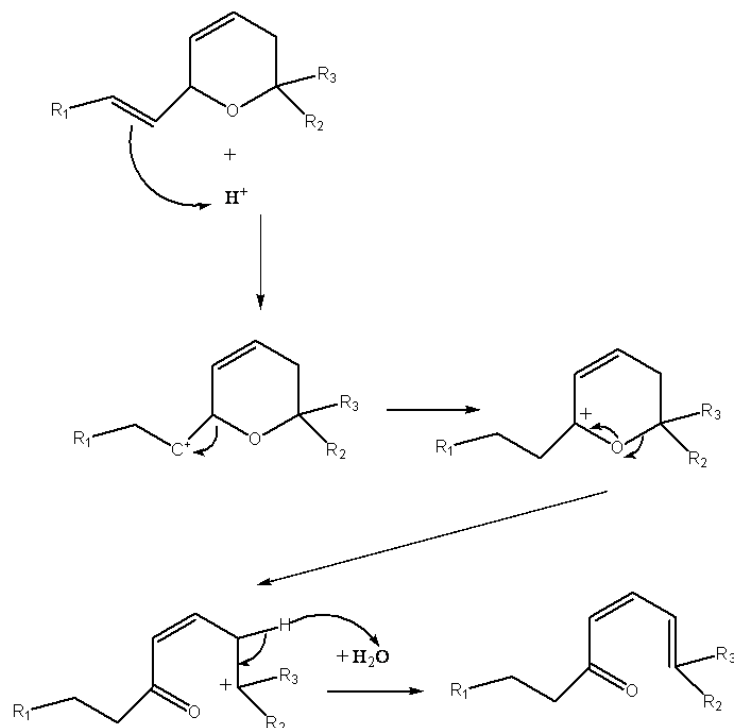


Figure 6.1.8. Acid catalysed degradation of the A-ring of AZA (postulated pathway, adapted from Alfonso et al., 2008).

4.6.1.3. Transformation of azaspiracids in shellfish

The observation of an increase in AZA-concentration upon heat treatment of mussels (Hess et al., 2005a) was initially attributed to water loss of the shellfish without loss of toxin in the cooking fluids.

Later studies on the preparation of reference materials revealed that there was a larger increase in concentration than could be explained from the water loss. McCarron (2007) noted that it was specifically an increase in AZA3 that accounted for the increase in concentration of AZAs (since these were generally calculated as a sum of the toxicologically weighted concentrations of AZA1, -2 and -3 (Fig. 6.1.9).

Spontaneous transformation of AZA17 and -19 into AZA3 and -6, accelerated by heat-treatment, could be shown through isotopic labelling reactions to be due to decarboxylation (McCarron et al., 2009), Fig. 6.1.10 and 6.1.11.

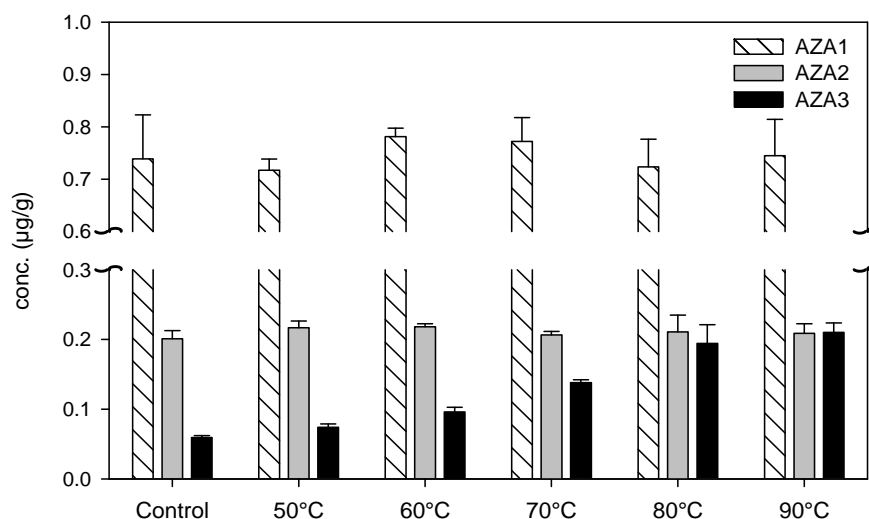


Figure 6.1.9. Levels of AZA1, -2 and -3 extracted from aliquots ($n = 3$) of uncooked mussel tissue from Bruckless heated at increasing temperatures. Error bars shown represent ± 1 SD.

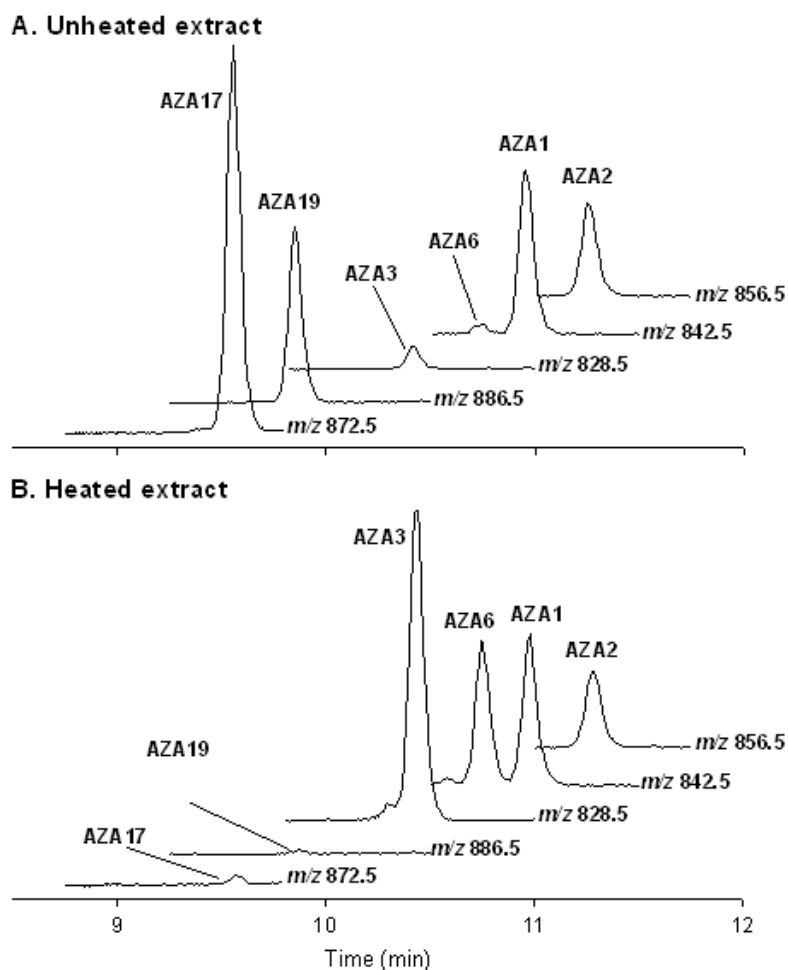


Figure 6.1.10. LC-MS (method B) chromatograms of unheated (A) and heated (B) aliquots of fraction 17 from LH20 fractionation of raw HP extract. Traces show peaks for AZA17 and -19 in unheated sample, with subsequent absence in heated sample, with a corresponding increase in AZA3 and AZA6 upon heating.

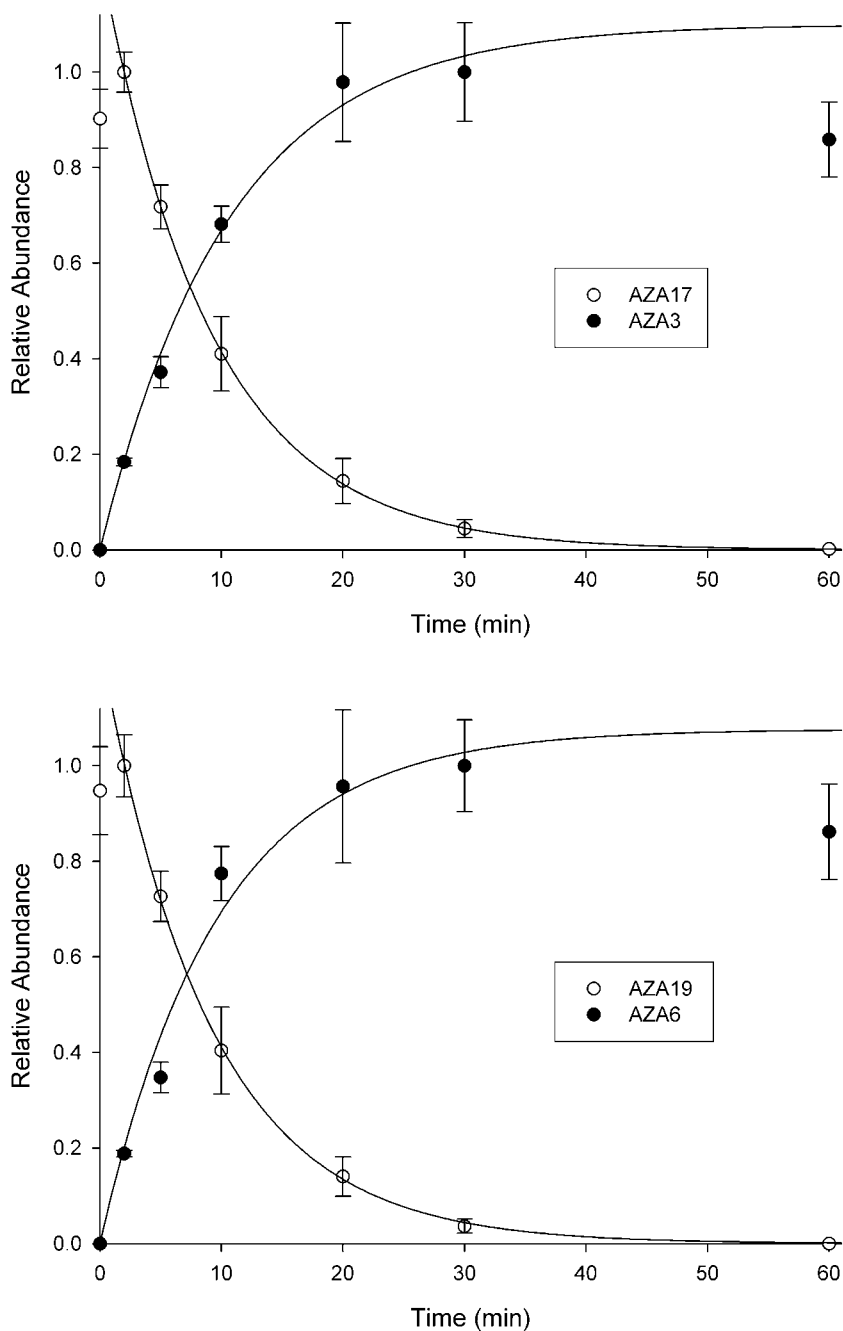


Figure 6.1.11. Change in concentration with time of AZA17 and AZA3 (top), and AZA19 and AZA6 (bottom) in Bruckless mussel HP extracts upon heating at 70 °C. Error bars represent ± 1 SD. Analysis carried out using LC-MS method B. Exponential curves were fitted to the first 30 min for AZA3 and -6 due to degradation with prolonged heating, and to 2–30 min for AZA17 and -19.

In parallel, we had been made aware by Krock (personal communication, 2008) that the organism producing AZAs, *Azadinium spinosum*, only produced AZA1 and AZA2. Therefore, we postulated that the metabolism in shellfish must include oxidation of AZA1 and -2 to AZA17 and -19, which upon storage and heating is transformed into AZA3 and -6 (Fig. 6.1.12).

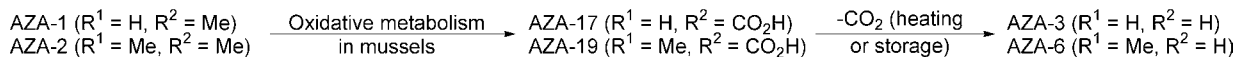
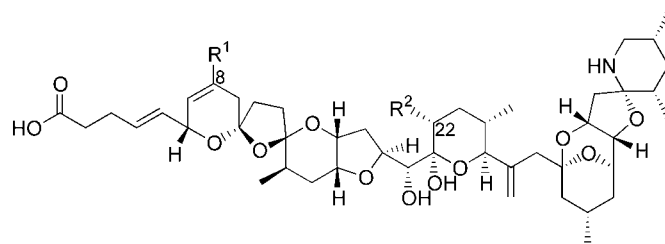


Figure 6.1.12. Proposed oxidative metabolism of AZA1 and -2 in shellfish to form 22-carboxylated metabolites (AZA17 and -19), which undergo decarboxylation when heated to form AZA3 and -6, respectively, adapted from McCarron et al., 2009.

This postulate remains obviously to be proven in experiments but appears to be consistent with all observations so far.

4.6.1.4. Chemical stability of okadaic acid and analogues

During systematic studies of the BIOTOX project on methodology for lipophilic toxins, we examined the sources of variability in the analysis of OA. Particular attention was paid to the hydrolysis step as it involves a chemical reaction with strong base, and neutralisation with strong acid, also resulting in some dilution of the extract.

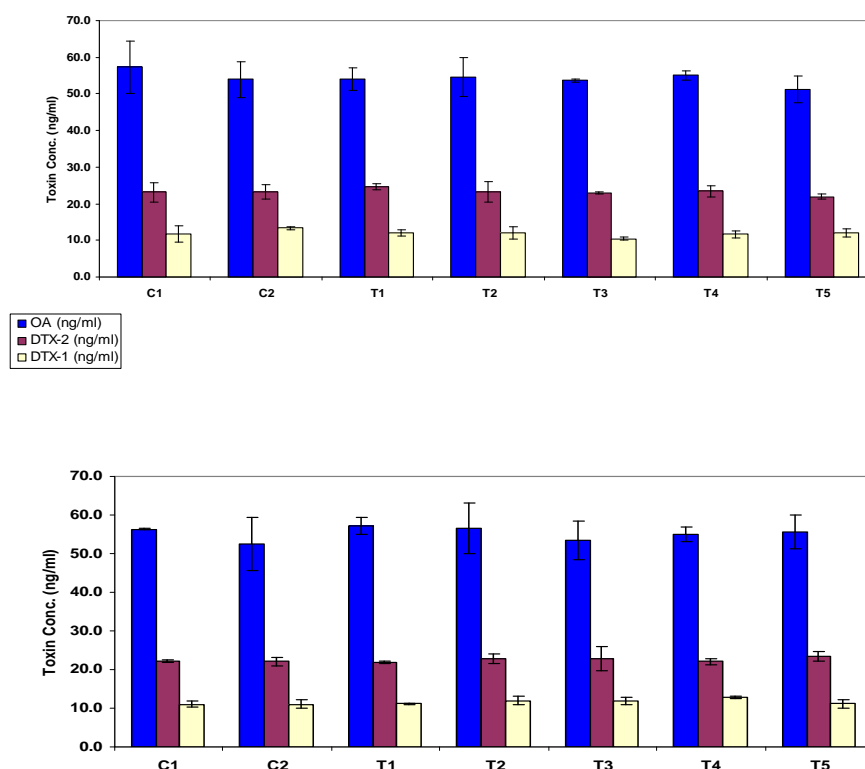


Figure 6.1.13. A: Stability of OA, DTX-2 and DTX-1 in MeOH under alkaline hydrolysis conditions B: Stability of OA, DTX-2 and DTX-1 in matrix solution under alkaline hydrolysis Conditions. C1-control, C2-dilution control, T1- 10 min hydrolysis, T2- 20 min hydrolysis, T3- 40 min hydrolysis, T4- 60 min hydrolysis, T5- 120 min hydrolysis. Error bars represent ± 1 SD (n = 3). Unpublished data (BIOTOX-project, Barry Rourke, Marine Institute).

These steps obviously increase the potential for error through additional handling of the crude extract prior to LC-MS analysis. Initially, we examined the stability of OA, DTX1 and –2 during treatment with sodium hydroxide (Fig. 6.1.13). No degradation was observed for either standards of matrix solutions, even when the samples were heated to 76°C for up to 2 h.

However, when similar treatment was carried out on a matrix extract exposed to HCl both OA and DTX1 very rapidly degraded (Fig. 6.1.14).

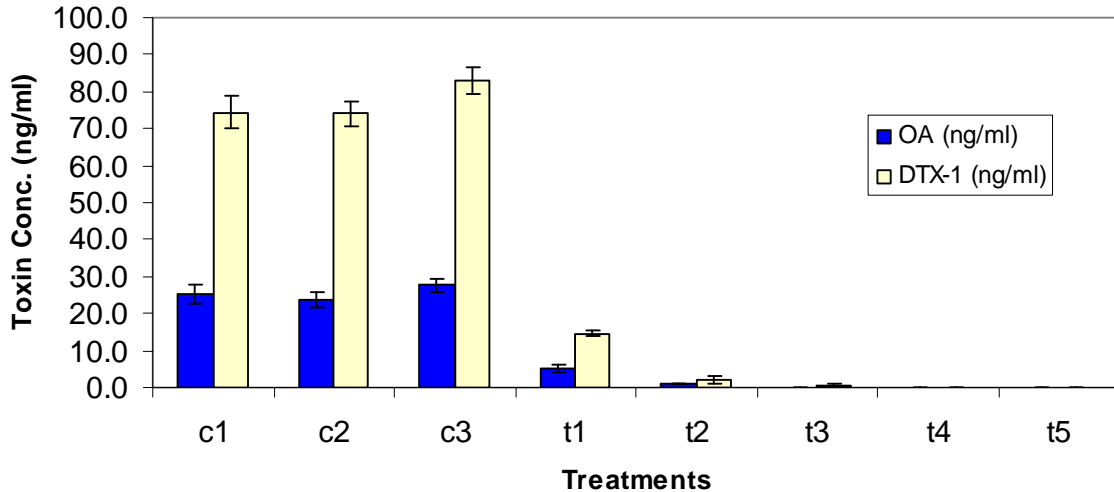


Figure 6.1.14. Stability of OA and DTX-1 in matrix solution under acidic hydrolysis conditions. C1-control, C2-dilution control, C3-hydrolysis dilution control, T1- 10 min hydrolysis, T2- 20 min hydrolysis, T3- 40 min hydrolysis, T4- 60 min hydrolysis, T5- 120 min hydrolysis. Error bars represent ± 1 SD ($n = 3$). Unpublished data (BIOTOX-project, Barry Rourke, Marine Institute).

This rapid degradation could also mean that if the strong acid and base used in the hydrolysis procedure are not prepared to exact equimolar concentration, there may well be an excess of strong acid in the neutralised extract. If in addition, the extract had not been completely cooled down after the hydrolysis reaction, there could be easily degradation of OA-group toxins by any excess acid.

Therefore, it is recommended that the cooling of extracts needs to be verified in each laboratory due to different glassware which may require different cooling times. In addition, it may interesting to change the neutralisation agent from the strong acid to acetic acid, thereby further reducing the risk of degradation of OA and its analogues.

4.6.2. Influence of industrial processing and heat treatment procedures of shellfish on toxin concentration

4.6.2.1. Evisceration of scallops to reduce domoic acid content

As outlined in section 4.3.4, the contamination of domoic acid in scallops had been shown to be an EU-wide problem in 1998 – 1999. Thus, measures to value this resource were of adamant importance to the shellfish industry. The physiology of scallops allows easy visual distinction of its internal organs (Fig. 6.2.1).

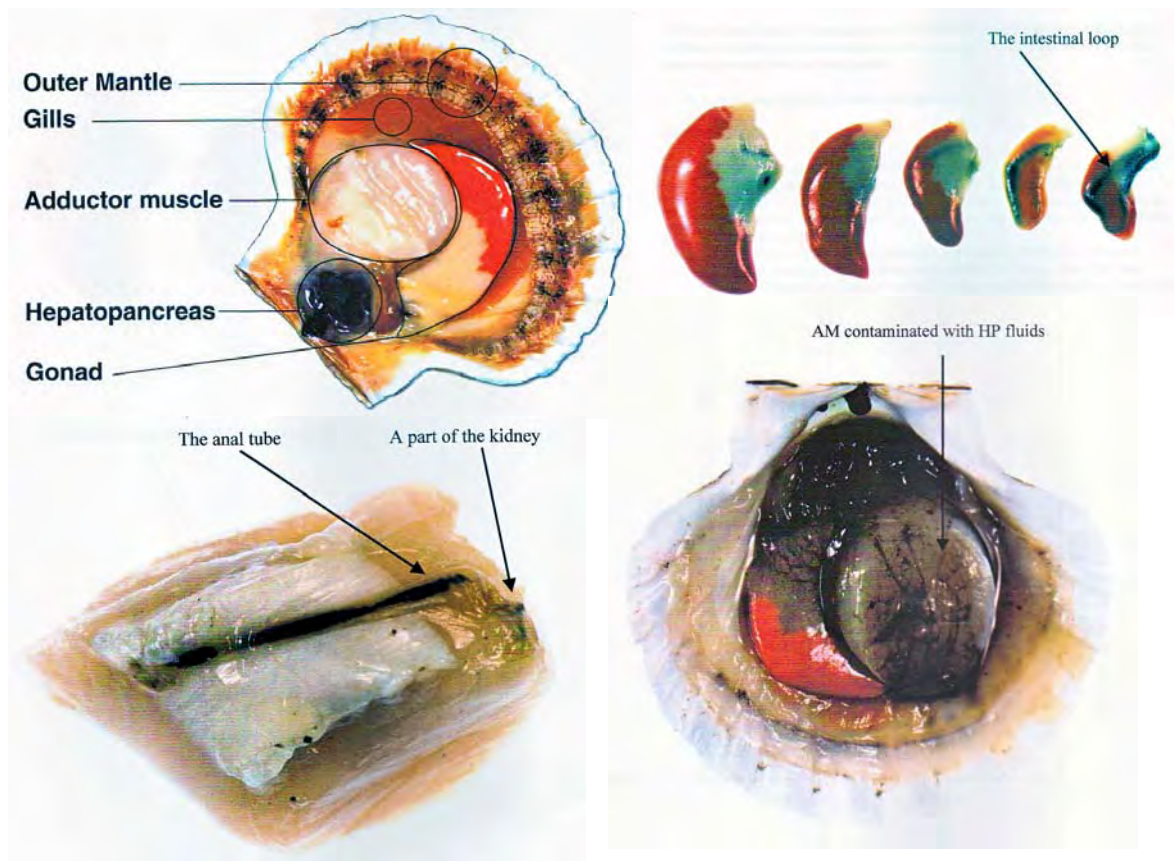


Figure 6.2.1. Scallop physiology and contamination mechanisms. Top left: internal organs of scallop (mantle, gills, adductor muscle, hepatopancreas and gonads); top right: gonads of decreasing size indicating different states of sexual reproduction cycle (small gonads contain little sperm and are more translucent, the intestinal loop becomes visible as a dark outline); bottom left: poorly dissected adductor muscle with anal tube and remains of kidney left on muscle); bottom right: adductor muscle had been exposed to hepatopancreas fluids for 1 min, staining does not wash off easily and contains high concentrations of DA), adapted from UK-NRL, 2001.

During a study in 2001, we examined the possibility that scallop adductor muscles become contaminated during dissection of scallops for evisceration (Fig. 6.2.1). It was noted that fluids from scallop hepatopancreas could indeed contribute significantly to the contamination of the remaining adductor muscle (UK-NRL, 2001). The dissection of scallops may also remove more or

less efficiently all of the digestive organs from the adductor muscle. Therefore, we investigated the dissection procedure in much detail and observed that remaining parts on the adductor muscle (such as the anal tube and the kidney) may also contribute significantly to contamination of the adductor muscle. A follow-up study showed that there was no difference between dissection as carried out in a laboratory setting at the Marine Laboratory, Scotland, and a commercial processing plant for scallop evisceration in Scotland (Hess et al., 2000).

4.6.2.2. Influence of heat treatment on content of domoic acid in mussels

As part of the PhD thesis of Pearse McCarron, we investigated systematically the influence of heat-treatment on different toxins in shellfish. As DA does not frequently occur in mussels (*M. edulis*) in Europe, there has been no study to our knowledge on the effects of heat treatment on DA in mussels prior to our study (McCarron and Hess, 2006). We found that the heat treatments applied (cooking and autoclaving) had very little influence on the concentration of DA in the tissue compared to observations with other toxins (Table 6.2.1 and Fig. 6.2.2).

Table 6.2.1. Toxin budget of DA (mg) in whole flesh and collected fluids from the steamed and autoclaved sub-samples of the Snave sample. Differences shown (%) are those between the sum of measured DA and the expected DA. The DA amount expected was calculated from the DA concentration in the whole flesh of the uncooked sub-sample.

DA amounts	Steamed (mg)	Autoclaved (mg)
DA in flesh	6.96	5.53
DA in cooking fluids	2.49	4.29
Sum of measured DA	9.45	9.82
DA expected	10.2	11.3
% difference	7.5%	12.9%

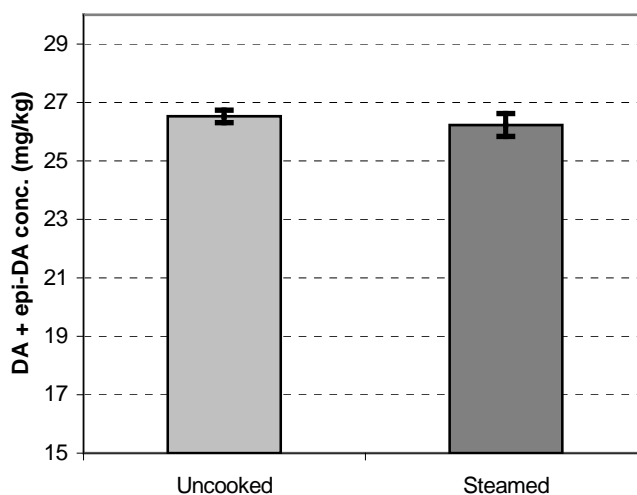


Figure 6.2.2. Mean DA + epi-DA concentrations in cooked and uncooked whole tissue from Ardgroom mussels. Error bars represent +/- 1 SD (n = 6).

We noted that DA passed significantly into the fluids secreted from tissues during the heat treatment, and this phenomenon equilibrated largely the concentration effect due to loss of fluid.

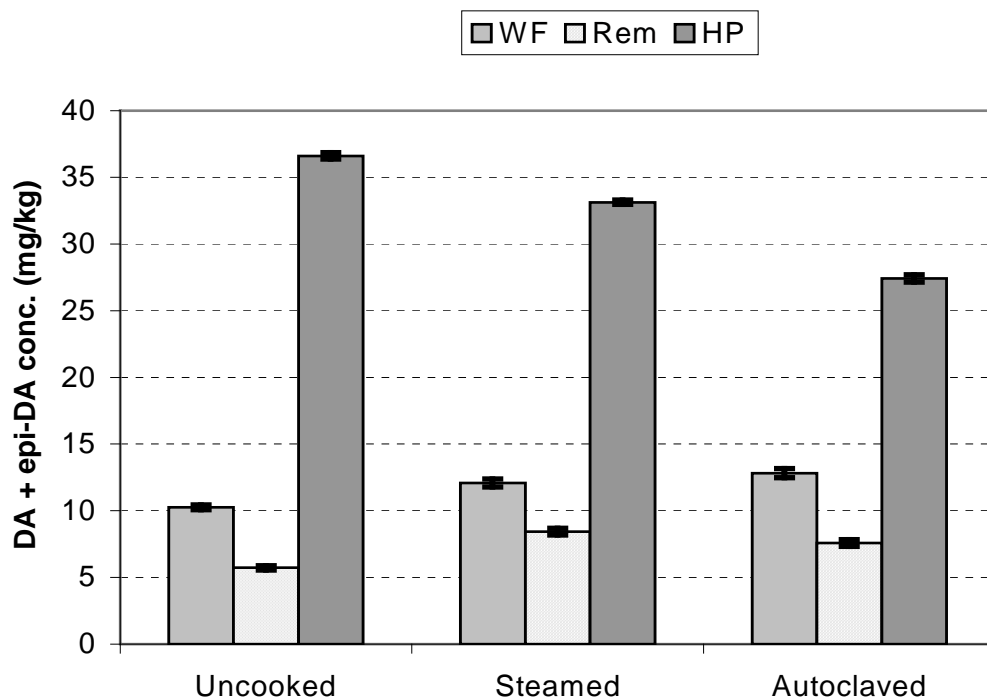


Figure 6.2.3. Mean DA + epi-DA concentrations in uncooked, steamed and autoclaved whole flesh (WF) tissues from Snave mussels. Also shown is the tissue distribution of DA between hepatopancreas (HP) and remainder tissues (Rem) for the various sub-samples. Error bars represent +/- 1 SD (n = 3).

In a second sample during the same study (Fig. 6.2.3), we did observe a decrease, however, this decrease was still not as significant as those observed for OA- and AZA-group toxins, despite very similar losses of fluid (6.2.4).

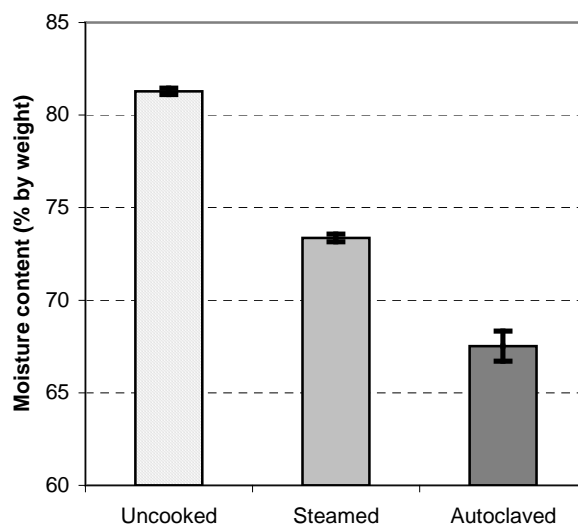


Figure 6.2.4. Mean moisture contents for the uncooked, steamed and autoclaved whole flesh tissues from the Snave sample. Error bars represent +/- 1 SD (n = 3).

When investigating the effect of heat on the DA concentration in tissues without water loss, it could be shown that the degradation of DA at 90°C during short time periods (e.g. cooking for 15 min) did barely lead to degradation, never exceeding 10% (Fig. 6.2.5). Therefore, DA has been shown to be quite resistant to typical heat treatments.

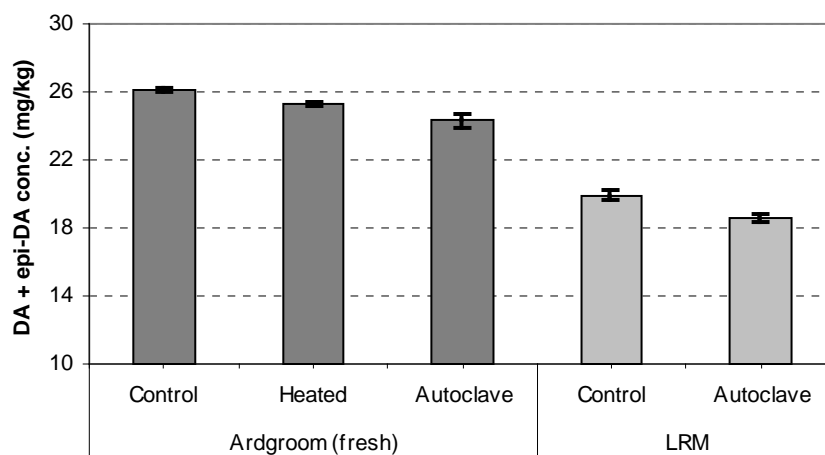


Figure 6.2.5. Mean DA + epi-DA concentrations in fresh mussel (untreated, heated at 90 °C and autoclaved for 15 min), and LRM aliquots (untreated and autoclaved for 15 min). Error bars represent +/- 1 SD (n = 3).

4.6.2.3. Influence of heat treatment on content of okadaic acid in mussels

In the paper of McCarron et al. (2008), the influence of conventional steaming (over boiling water for 10 minutes) and autoclaving (121°C for 15 minutes) on the level of okadaic acid and DTX2 in mussels was investigated.

We investigated the effect of processing on whole flesh, the digestive glands and remainder tissue (remaining after careful dissection of the digestive glands). After steaming, an increase in the concentration of OA group toxins in whole flesh of 30% and 70% was found for the two samples studied. After autoclaving, an increase in the concentration of OA equivalents of 70% and 84 % was observed for the two samples (Fig. 6.2.6 and Table 6.2.2). Measurements of the moisture content indicated that these increases are caused by water loss during processing. Although not consistent for both samples, the results of the study suggested that redistribution from the digestive gland to the remainder tissue might occur during processing. We concluded that this suggests that analysis of whole shellfish flesh, opposed to the digestive gland, is more appropriate for regulatory purposes, particularly when processed shellfish are analysed.

Table 6.2.2. Whole flesh concentrations of OA and DTX2 measured in the various treatment lots of both samples. Also shown are the theoretical concentrations of both toxins based on the water loss as a result of the steaming and autoclaving treatments.

		Fresh sample		Steamed sample		Autoclaved sample	
		Measured (mg/kg)	Theory (mg/kg)	Measured (mg/kg)	Theory (mg/kg)	Measured (mg/kg)	Theory (mg/kg)
1st sample	OA	0.90	na	1.17	1.14	1.57	1.35
	DTX2	0.11	na	0.12	0.14	0.15	0.17
2nd sample	OA	0.43	na	0.75	0.54	0.81	0.67
	DTX2	0.08	na	0.12	0.10	0.12	0.13

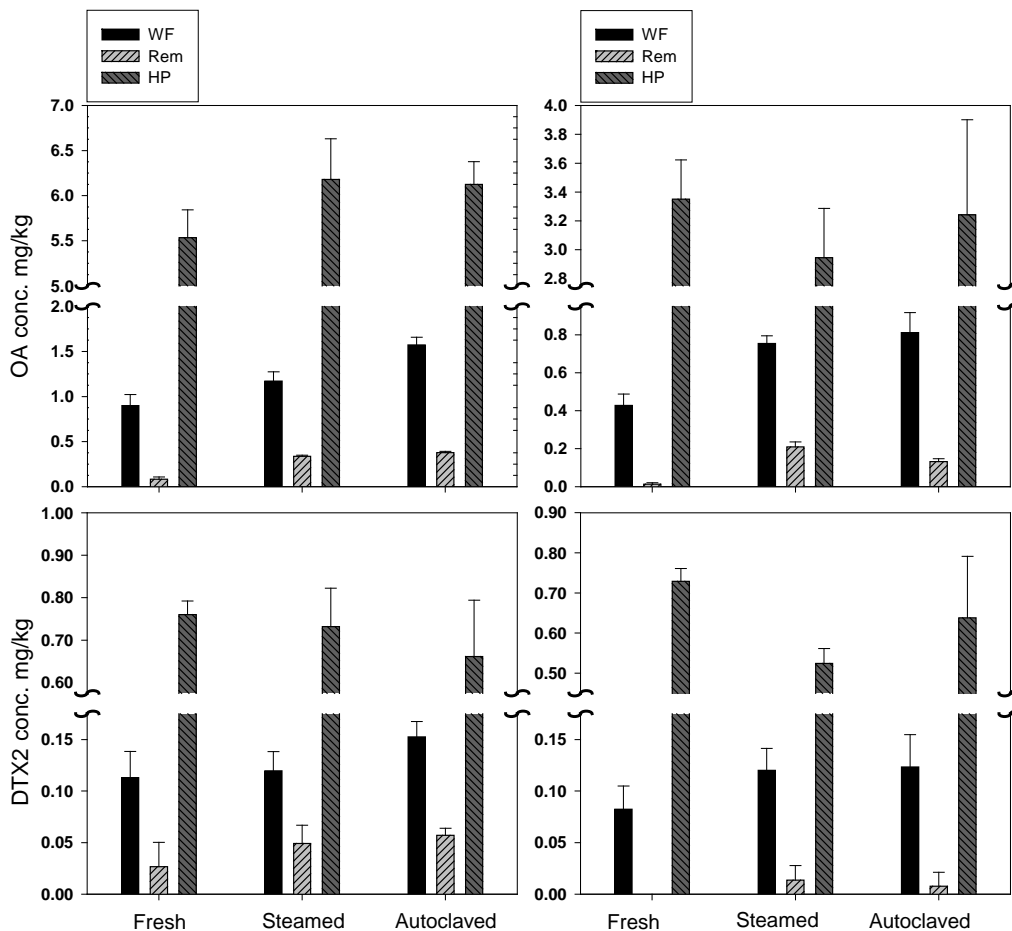


Figure 6.2.6. OA and DTX2 concentrations in fresh, steamed and autoclaved lots of two mussel samples collected from Bruckless (WF, HP and Rem tissues). Error bars shown represent $\pm 1SD$ ($n=3$). Note: Y-axis breaks have been included.

When examining the effects of heat treatment on OA group toxin concentrations in shellfish tissues without the possibility of water losses, we confirmed that OA is heat stable for normal cooking temperatures, ie up to 100°C (Mc Carron et al., 2008). After exposure to high temperature for 10 minutes the concentration of OA decreased significantly at temperatures of 130°C and higher (Fig. 6.2.7). At 150°C the degradation was about 40% compared to the control situation. Degradation of DTX2 appeared to start at 100°C, and was about 60% at 150°C.

As autoclaving is used in the process of reference material preparation, we also investigated this procedure in more detail (Fig. 6.2.8). Corresponding to the previous results, it was found that DTX2 decreases to a larger extent than OA and the overall decrease in concentration can be in the region of 20 – 30 %.

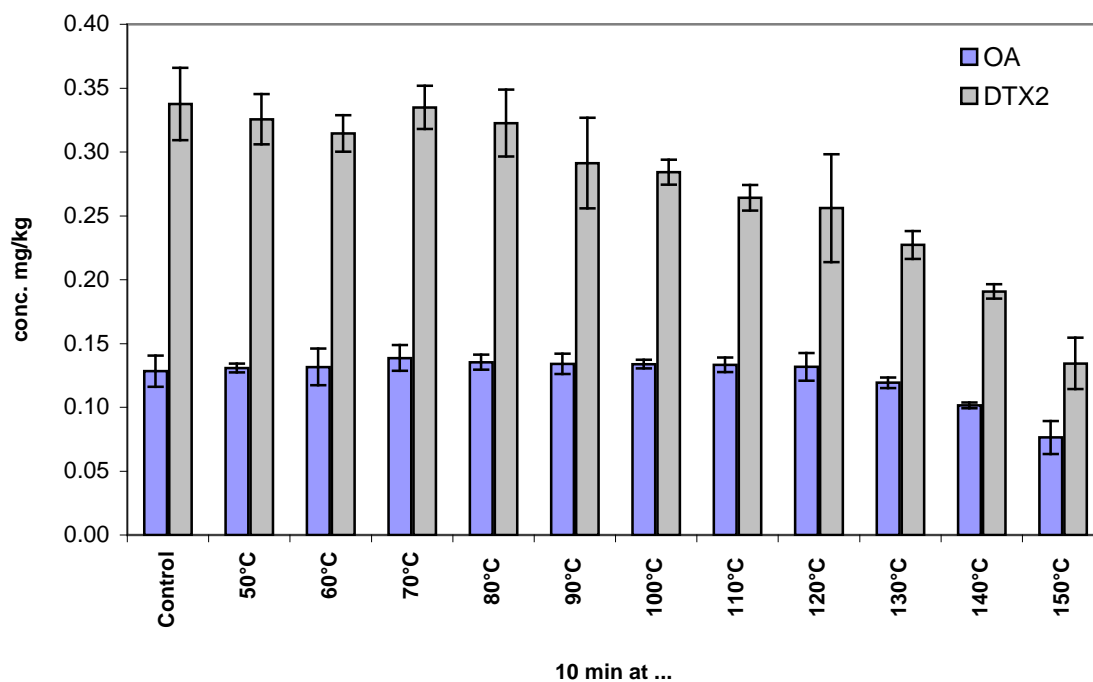


Figure 6.2.7. Levels of OA and DTX2 extracted from aliquots (n=3) of uncooked mussel tissue heated at increasing temperatures. Error bars shown represent ± 1 SD

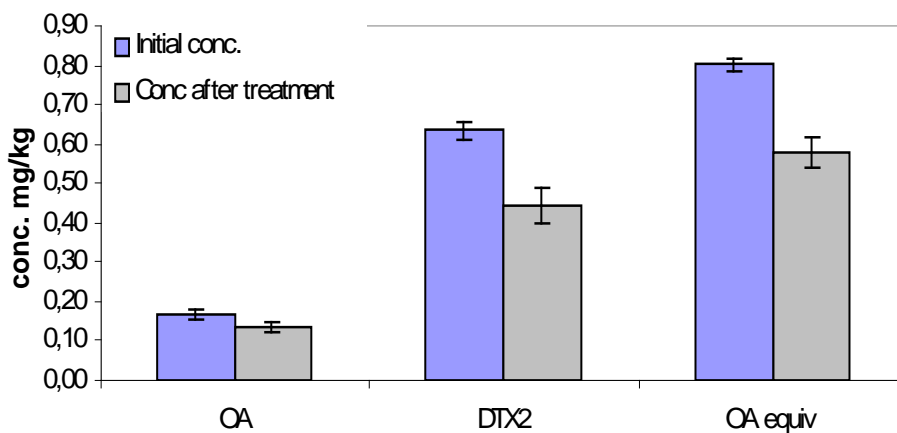


Figure 6.2.8. OA and DTX2 concentrations in control and autoclaved aliquots of an LRM. Error bars represent ± 1 SD (n=3)

4.6.2.4. Influence of heat treatment on content of azaspiracids in mussels

Similar to DA and OA-group toxins, AZAs have also been evaluated for their resistance to heat treatments. In addition to the studies presented under sections 4.5.1.3 and 4.6.1.2, outlining some reactions specific to AZA-analogues, we observed that AZAs have differing degrees of stability in heat treatments. In autoclaving procedures for instance, AZA3 is completely destroyed in shellfish tissues, while AZA1 and -2 are only partly degraded (Fig. 6.2.9). In a systematic experiment, AZA1 and -2 start to be destroyed from ca. 120°C, while AZA3 is already significantly affected at 100 to 110°C.

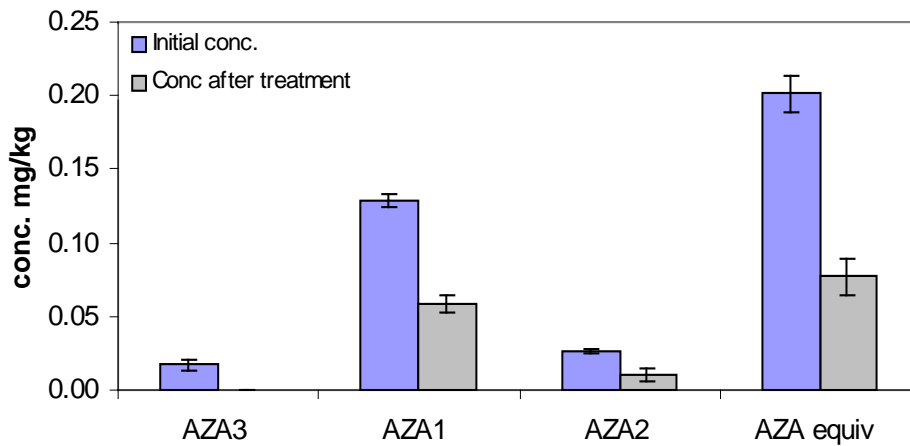


Figure 6.2.9. AZA concentrations in control and autoclaved aliquots of an LRM. Error bars represent ± 1 SD ($n=3$), adapted from McCarron et al., 2007 (PhD thesis).

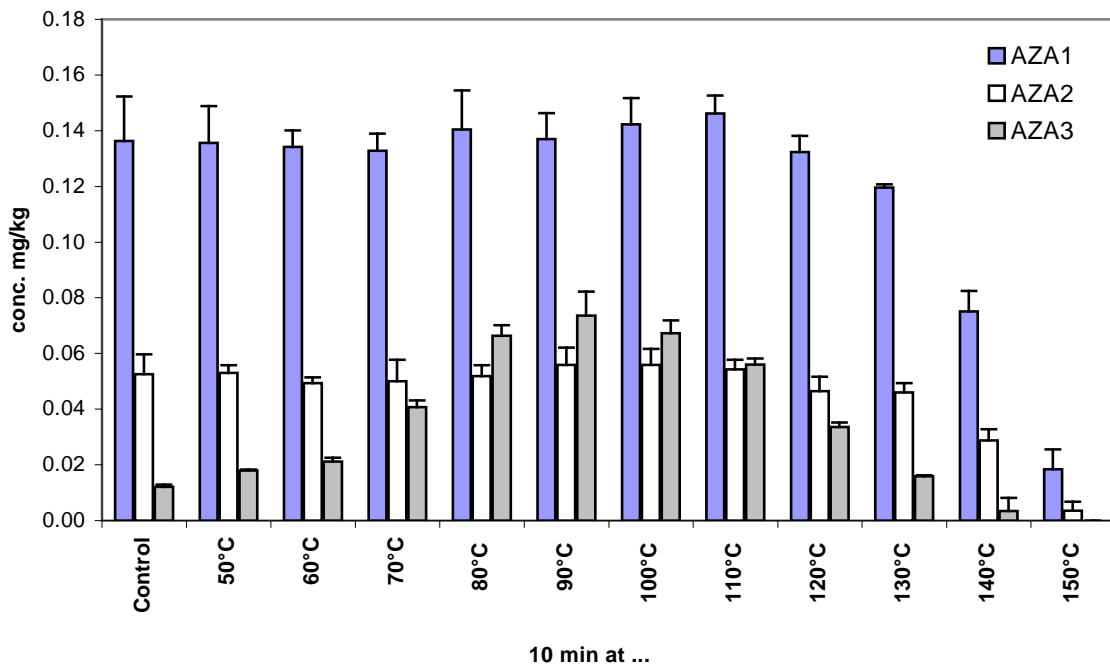


Figure 6.2.10. Levels of AZA1, -2 and -3 extracted from aliquots ($n=3$) of uncooked mussel tissue from Bantry Bay heated at increasing temperatures. Error bars represent ± 1 SD, adapted from McCarron et al., 2007 (PhD thesis)

4.6.3. Interaction of toxins with biological systems

4.6.3.1. Relative toxicity of DTX2

DTX2 is a toxin that was discovered in Ireland in 1992, and, similar to AZAs, neither a certified pure standard nor a CRM was available for this compound prior to the ASTOX project. DTX2 is in many ways related to AZAs: firstly, DTX2 may co-occur with AZAs in Irish shellfish as repeatedly observed since 2001, secondly, both give similar symptoms including diarrhea upon oral exposure in mammals, and lastly, these compounds would traditionally have been detected by the global toxicity assays using rats or mice. Furthermore, toxicological information on DTX2 was only indirectly available from mouse bioassays in the routine monitoring. Hence isolation of this compound was also written into the aims of the project. While the isolation is described under section 4.2.8 of this report, I describe here the progress in addition to the planned work under ASTOX, progress that was only possible thanks to the additional collaboration with the NSVS.

Detailed comparison of the mouse bioassay with data obtained from LC-MS analysis suggested, independently in Ireland and more recently Norway, that DTX2 may be less toxic than OA. Therefore, a study was designed to compare the toxicity of DTX2 to that of OA, using both the intraperitoneal route of exposure in mice and the functional PP2a assay. DTX2 was isolated at the MI as described under 4.2.8 of this report and toxicological studies were conducted at the NSVS.

The intraperitoneal route of exposure was chosen as it would save on toxin consumption (LD₅₀ ip typically lower than LD₅₀ by oral exposure), and because of the success of ip comparison with functional assays in the field of paralytic shellfish toxins. The animal study suggested that DTX2 is only half as toxic as OA as illustrated by the prevalence of death in mice injected with varying doses of OA and DTX2 (Fig. 6.3.1).

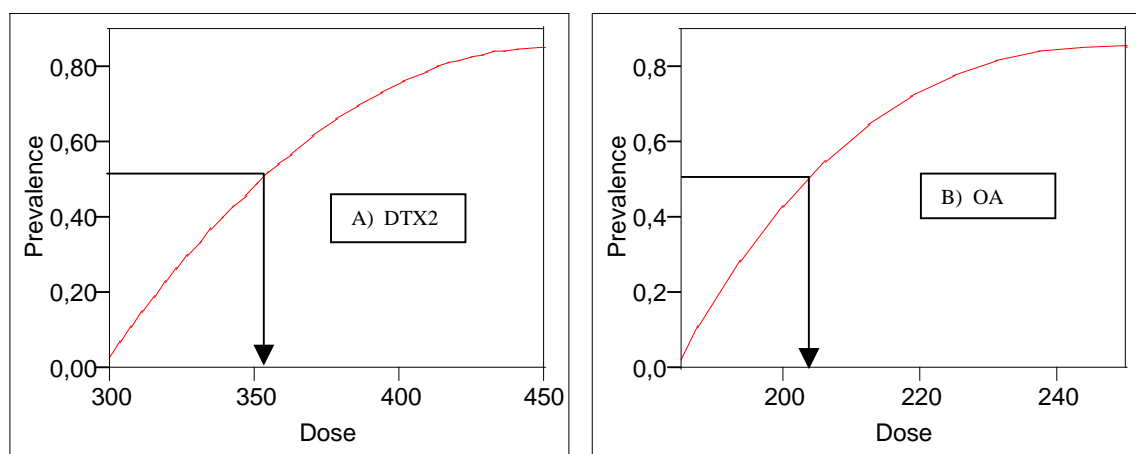


Figure 6.3.1. The prevalence of death as a function of A) DTX2 dose and B) OA dose [μg], as predicted by the use of a second degree polynome.

The functional PP2a assay confirmed these results within the error of measurement, since a relative effective dose of 0.6 was found for DTX2 compared to OA. Results were published by Aune et al., 2007. Overall, this reduced toxicity of DTX2 compared to OA is important when implementing methods which are not based on detecting toxicity, as is the case for LC-MS. Hence, the results of this study facilitate the use of LC-MS for official monitoring of shellfish toxins.

4.6.3.2. Toxicity of azaspiracids and mechanism of action

While the gastro-intestinal effects in humans following consumption of AZA-contaminated mussels has been reported on several occasions (McMahon and Silke 1996; FSAI, 2006), the mechanism of action of azaspiracid had not been elucidated as of yet. An extensive review of the study of toxicology related to AZAs is provided by Twiner et al., 2008. The toxicity of azaspiracids had initially been studied as part of the original isolation work (Satake et al., 1998a, Ofuji et al., 1999). These studies clarified that AZA1 to -3 are toxins of similar potency to OA in terms of acute effects following intraperitoneal injection in mice; AZA4 and -5 were found to be somewhat less toxic than the other 3 analogues (Ofuji et al., 2001). Subsequently, studies by Ito et al., 2000, investigated effects of oral exposure, suggesting AZAs were taken up into the blood stream following oral gavage as they caused multiple organ damage in mice. Furthermore, studies at sublethal doses indicated that there may be chronic effects, and that further studies were required to fully demonstrate these phenomena (Ito et al., 2002). The rudimentary structure activity relationship of the first 3 studies (Satake et al., 1998; Ofuji et al., 1999 and 2001) were followed by the investigation of Ito et al., 2006, on synthetic AZA1 and haptens of AZA which showed that the stereochemistry is an important aspect in the toxic mechanism of AZA. This study also demonstrated that it is not a specific part of the AZA molecule which interacts with the biological target but that the whole basic skeleton is required for the interaction.

Based on these studies *in vivo*, the ASTOX project attempted to investigate the mechanism of action of AZA. As part of this project, we investigated the cytotoxicity of AZA1 in different mammalian cell lines (Fig. 6.3.3), showing that lymphocytes were particularly sensitive to this toxin (Twiner et al., 2005). The same study also outlined the morphological changes seen in cells exposed to AZA, confirming earlier observations by Flanagan et al., 2001. These observation also strengthened the hypothesis that the cytoskeleton was significantly affected by AZAs (Fig. 6.3.2).

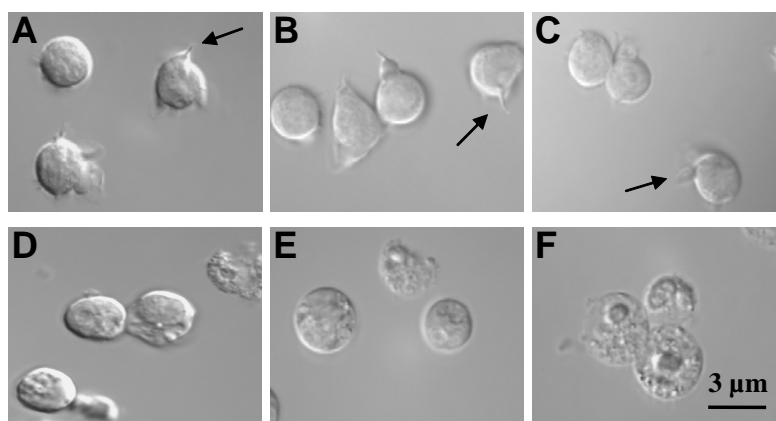


Figure 6.3.2. Photomicrographs of Jurkat T lymphocyte cells following exposure to AZA1. Jurkat cells were exposed to AZA1 for 24, 48, and 72 hr prior to photographs being taken. Panels A, B, and C illustrate control cells 24, 48, and 72 hr after incubation with equivalent volumes of the MeOH vehicle, respectively. Panels D, E, and F illustrate cells exposed to 10 nM AZA1 for 24, 48, and 72 hr, respectively. Arrows indicate presence of pseudopodia.

By way of elimination, Twiner et al., 2005, also demonstrated that PP2a is not inhibited by AZA and, therefore, the study clarified that AZA has a different mechanism of action from OA.

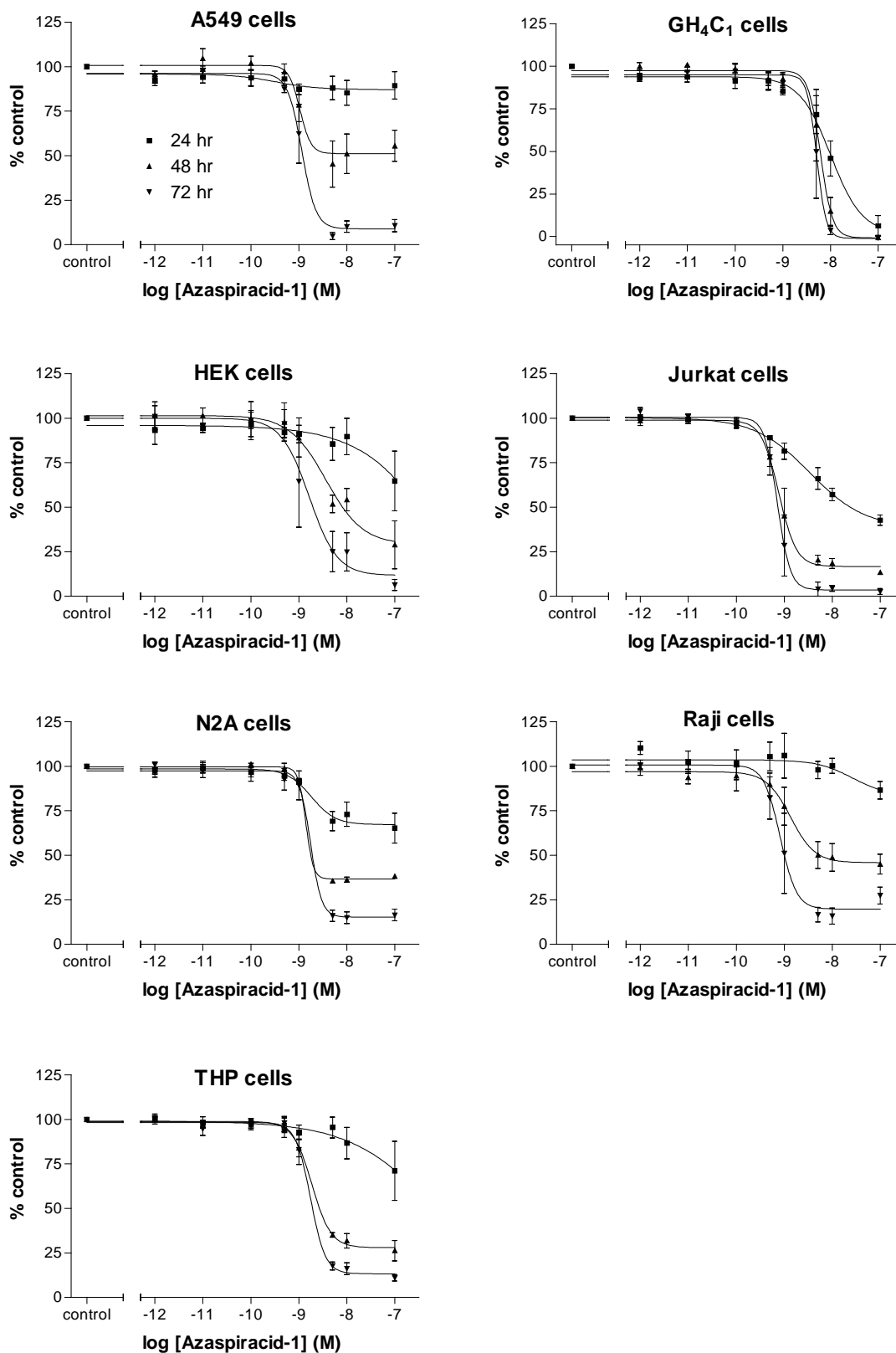


Figure 6.3.3. Effect of AZA1 on the viability of seven cell types as determined using the MTS assay. Cells were continuously exposed for 24, 48, or 72 h

AZAs have been shown to cause relevant alterations in the gastrointestinal tract, including the destruction of villi in the small intestine of orally exposed mice. Studies on the transepithelial electrical resistance (TEER) were thus carried out on the Caco2 cell-line by our partner at University College Dublin using the WPI REMS Autosampler, an automated electrical resistance measurement device (Hess et al., 2007b). After a stable TEER reading was achieved cells were exposed continuously to AZA1 (0.1 – 100 nM) for periods of 24, 48 and 72 hr. No significant change in TEER was observed at any time-point up to a concentration of 2.5 nM. A significant decrease was observed at 24, 48 and 72 hr with 5 nM AZA1 or higher (Fig. 6.3.4). This decrease in TEER correlates with an increase in paracellular permeability. The ability of AZA1 to functionally alter Caco-2 barrier function mimics the in vivo situation. This disruption to the barrier function could in turn enhance antigenic exposure to underlying immune cells, further compromising barrier function (Bruewer et al., 2003). An assessment of tight junction components, e.g. occludin, claudin, was carried out in order to further clarify AZA1's ability to alter paracellular permeability in this model. The robustness of our model was also tested using AZA1 of lower purity. No alteration in sensitivity was observed compared to AZA1 of higher purity, indicating that impurities in the preparation did not alter the responses to AZA (data not shown).

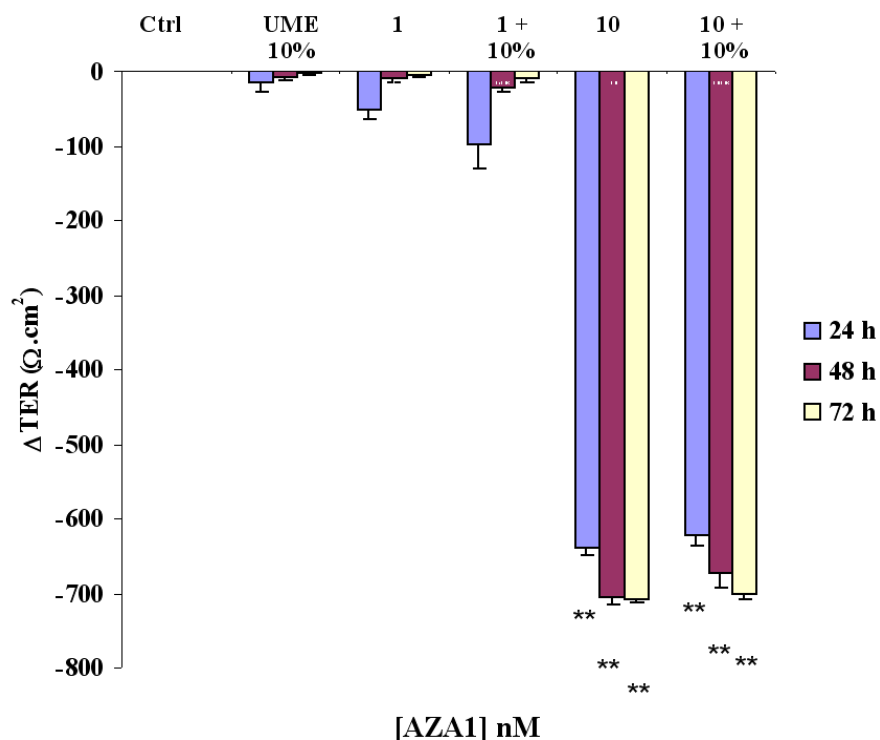


Figure 6.3.4. Effect of AZA1 and unpurified mussel extract (UME) on the TEER across Caco-2 cell monolayers using the REMS autosampler after various concentration and exposure periods. **Indicates statistically different to control: $p < 0.01$.

The integrity of epithelia is based on proper cell-cell adhesive structures, primarily involving the E-cadherin cell-cell adhesion protein, and marine biotoxins often affect this molecular system in epithelial cells, calling our attention to the possibility that the effects induced by AZAs in the small intestine might include alterations of E-cadherin. The effects AZA1 on two epithelial cell lines were investigated, the MCF7 and Caco 2 cells, that have been obtained from human breast and colon cancers, respectively (Ronzitti et al, 2007).

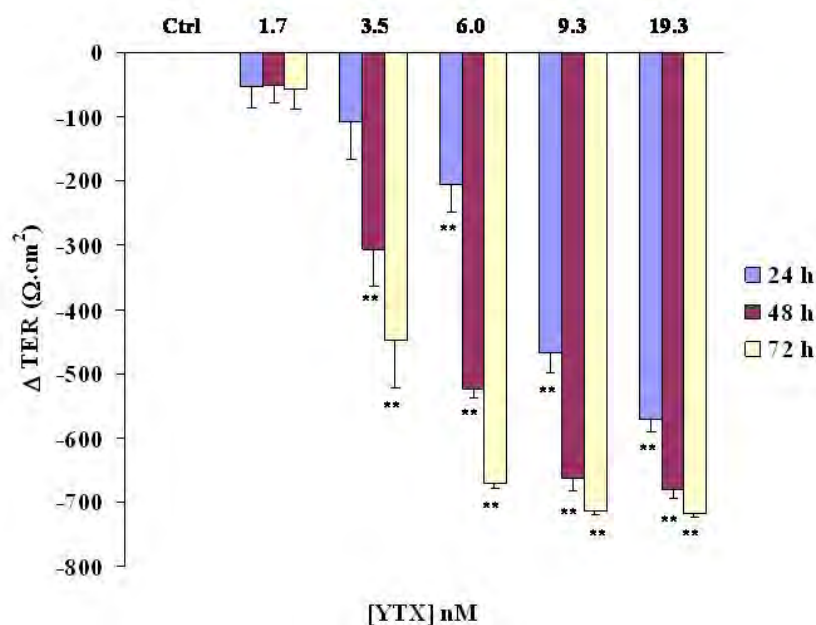


Figure 6.3.5. Effect of YTX on the TEER across Caco-2 cell monolayers using the REMS autosampler. **Indicates statistically different to control : $p < 0.01$.

Nanomolar concentrations of AZA1 reduced MCF7 cell proliferation and impaired cell-cell adhesion. AZA1 altered the cellular pool of E-cadherin by inducing a dose- and time-dependent accumulation of an E-cadherin fragment (ECRA₁₀₀), with an EC₅₀ of 0.47 nM. The immunological characterisation of ECRA₁₀₀ revealed that it consists of an E-cadherin molecule lacking the intracellular domain, and these data showed that the effect induced by AZA1 in MCF-7 cells is undistinguishable from that induced by yessotoxin in the same experimental system. A comparison of toxin effects in MCF-7 and Caco 2 cells confirmed that the effects induced by AZA1 and yessotoxin are undistinguishable in these cells. Treatment of fibroblasts with AZA1 did not affect the cellular pool of N-cadherin, showing that the toxin effect is cadherin-specific. A comparison of the effects induced by AZA1, YTX and OA on F-actin and E-cadherin in MCF7 and Caco 2 cells showed that 1 nM AZA1 did not cause significant changes in F-actin and that accumulation of ECRA₁₀₀ did not correlate with decreased levels of F-actin under our experimental conditions. Matching our results with those available in literature, we notice that, when molecular effects induced by AZA1 and YTX have been studied in the same *in vitro* systems, experimental data show they are undistinguishable in terms of sensitive parameters, effective doses, and kinetics of responses in several cell lines.

Available experimental data then pose the question of whether AZAs and YTXs might share their molecular mechanism(s) of action in some target cells and/or biological settings, and it seems important that future investigations will approach a comparative analysis aimed at clarifying this aspect. Since those two classes of algal toxins display notable structural differences, and have distinct properties in toxicity studies involving oral administration of the toxins in mouse models, it would be very important achieving a better understanding of the molecular mechanism of action of AZAs and YTXs, as a rational basis for a better management of risks related to human ingestion of seafood contaminated by those two classes of compounds. Moreover, the possible agonistic/antagonistic roles reciprocally played by AZAs and YTXs under some biological settings must be clarified, including knowledge of relative activities of different AZA analogues in sensitive systems.

As AZA had also been reported to have neurotoxic effects in mice when injected intraperitoneally, the effects on neuronal cells were investigated (Kulagina et al., 2006). Similar to slow effects in mammalian cell culture, the effects of AZA1 on spinal cord neuronal cells grown on microelectrode arrays also showed delayed onset (ca. 40 min to stable baseline). The effect was significantly enhanced by pre-treatment with bicuculine, a GABA receptor inhibitor (Fig. 6.3.6). Patch-clamp experiments suggested that Na⁺- or Ca²⁺-channels are not involved in the mechanism of action. The two observations are suggestive of the neurotoxic effects of AZA, however, the slow onset indicate that secondary pathways may play a role in this toxicity.

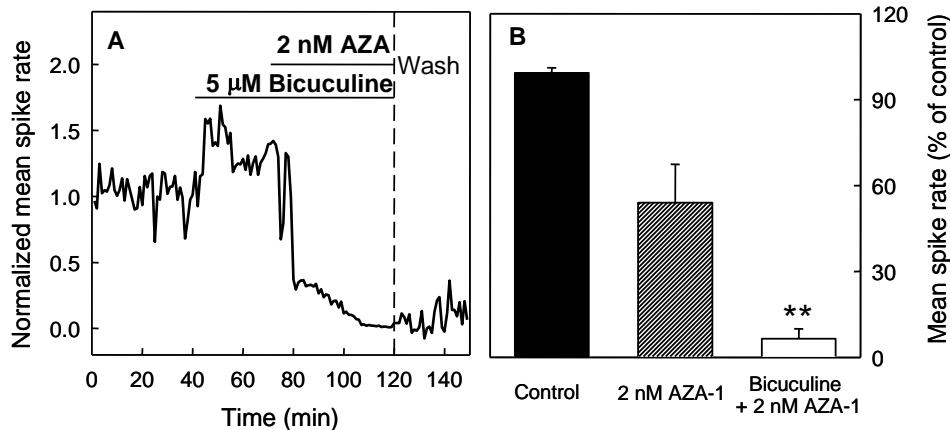


Figure 6.3.6. Effect of pre-treatment with bicuculine, a GABA receptor inhibitor, on AZA1 induced inhibition in spinal cord neuronal networks. (A) Representative recording trace of mean spike rates after introduction of bicuculine (5 μM) and AZA1 (2 nM). Lines represent exposure periods for each exposure treatment. (B) Inhibition of network spike rates (mean ± SD, n ≥ 3) due to exposure of AZA1 alone versus AZA1 exposed following bicuculine pre-treatment.

To aid in the determination of the mechanism of action of AZA1, the effects of AZA1 on Jurkat lymphocyte T cells gene expression was assessed (Twiner et al., 2008). For these experiments, we employed Agilent's whole human genome expression microarray where cells were continuously exposed to AZA1 (10 nM) over a 24 hr period and differential expression for >37000 genes was assessed at three time points (1, 4, 24 hr). Gene expression levels were compared to basal gene expression in control cells exposed to the equivalent amounts of vehicle at the same time points. Rigorous filtering and mining of the data permitted identification and analysis of signalling pathways that were significantly affected by AZA1 exposure. Transcript levels for 20 genes were confirmed via quantitative-PCR.

Of the most significant genes identified for the 4 and 24 hr time points, there are 16 genes in common, many of which are known to be involved in fatty acid and/or cholesterol synthesis. For annotated genes of known function, there are 16 fatty acid/cholesterol synthesis genes up regulated at 4 hr, and 17 similar functioning genes up regulated at 24 hr. This remarkable clustering of genes of similar function and regulation prompted further illustration of this biological pathway in detail (Figure 6.3.7). In a time-dependent trend, at least one isoform of nearly all the genes necessary to synthesize cholesterol are significantly up regulated at 4 and 24 hr. The lone exception is the fourth step of the synthesis where phosphomevalonate kinase (PMVK; NM_006556) catalyses the phosphorylation of mevalonate-5-P into mevalonate-5-PP. Gene expression and enzymatic activity of PMVK has been shown to correspond with cellular sterol levels (Olivier et al., 1999), however, PMVK is not differentially expressed at any of the time points assessed in this study.

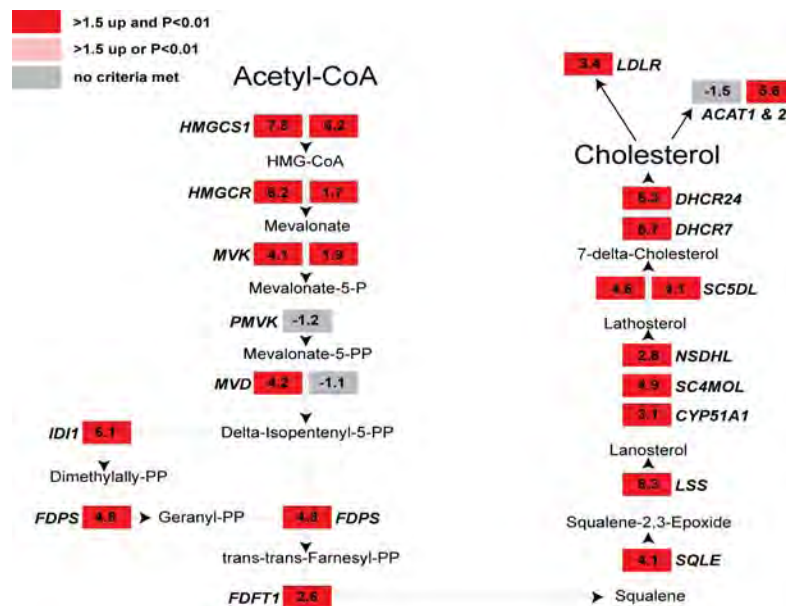


Figure 6.3.7. Differential gene expression of the cholesterol biosynthesis pathway in human lymphocyte T cells exposed to AZA1 (10 nM) for 24 hr. Enzyme names are abbreviated and illustrated in italics and bolded

With continued AZA1 exposure, dramatic and coordinated up regulation of nearly all cholesterol and fatty acid synthesis genes is observed. While this study has not identified the exact mechanism of action, it is clear that in lymphocyte cells, a sensitive cell/tissue type identified from in vivo studies (Ito et al., 2000; Ito et al., 2002; Ito et al., 2006), specific and related, transcriptionally-controlled pathways are differentially expressed by AZA1 in a time-dependent manner. Not only will these data lead future hypothesis driven investigations for determining the exact molecular target of AZA1, but the data can also be used for the development of exposure biomarkers and for an assessment of the potential therapeutic properties of AZA1.

Finally, we also investigated the teratogenic potential of AZA1 in the medaka model (Colman et al., 2005). Micro-injection into embryos of medaka clearly demonstrated retardation of the embryonic development (Fig. 6.3.8), as well as a reduction in the hatching success of the embryos. If there is a mechanism for AZAs to transfer into fish, this could also have significant effects on the reproduction of fish and thus on the aquatic ecosystem as such.

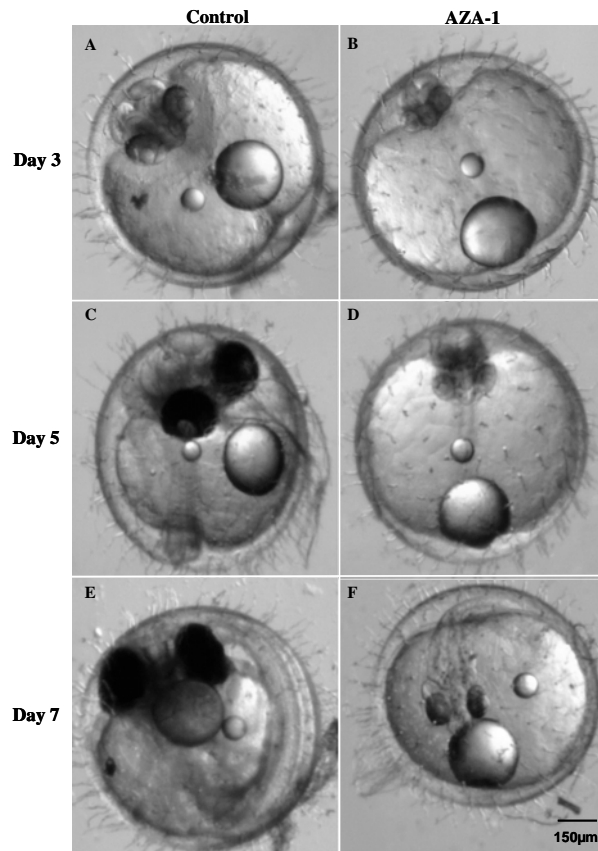


Figure 6.3.8. Photomicrographs (80 x magnification) of developing medaka embryos injected with AZA1. Embryos were injected with 89 pg equivalents of triolein oil (A, C, E) or 89 pg AZA1 (B, D, F) and photomicrographs were taken on day 3 (A, B), day 5 (C,D), and day 7 (E,F). Microinjections occurred on day 0, 6-8 hr following fertilization. In all frames the smaller droplet is the injected oil and the larger droplet is the natural oil.

5. Conclusions and outlook

5.1. *Methods of analysis*

In the area of analytical methodology, I believe to have made significant contributions in the analysis of STX-group toxins as well as in the analysis of lipophilic toxins, both through the development of LC-MS methods and the systematic investigation of parameters affecting the MS detection.

Our studies on the STX-group toxins have shown that HILIC can provide significant advantages over other types of chromatography, and that detection limits could be lowered to levels similar to or below the limit of detection the currently used mouse bioassay for these toxins.

The systematic investigation of the parameters affecting the LC-MS analysis of lipophilic toxins as part of the BIOTOX project has allowed a number of laboratories to familiarize themselves with the critical parameters affecting results. In particular, chromatographic separation could be optimized to suit instruments of different manufacturers. Different MS analysers were compared and the advantages of triple-stage quadrupoles for the quantitative analysis of organic traces could also be demonstrated at the example of shellfish toxins. Matrix effects were demonstrated to be dependant on analyte, shellfish species, instrument design and cleaning procedures. Recommendations have been made to overcome or minimize and control remaining matrix effects to allow effective quality control to be implemented in this complex analysis. Clean-up techniques have been investigated, however, even though SPE showed advances over liquid-liquid partitioning, no definitive recommendation could be made to remove matrix effects completely.

5.2. *Distribution of toxins in the environment*

The distribution of shellfish toxins in the environment was studied at different scales. For OA, the dependance of the toxic potential of a causative organism on the strain could be demonstrated at the example of *Dinophysis acuminata* which seems to produce significantly smaller amounts of toxin per cell in a strain from the American East coast, when compared to European strains.

Our studies have also outlined that AZA-group toxins occur not only in Ireland but also in areas as different as Norway and Portugal. Indirectly, the efforts in providing AZA-calibrants to the wider scientific community have also led to the geographical distribution of these toxins to be described all along the European Atlantic coast, and beyond.

Species differences in the uptake of lipophilic toxins were described since the introduction of LC-MS testing in a routine setting in Ireland. It is clearly shown that OA- and AZA-group toxins are accumulated to a larger extent by mussels compared to oysters.

The factors leading to the contamination of scallops with DA and the variability of this contamination were investigated. The depth at which scallops were cultured did not appear to affect their contamination; this suggests that at relatively low water depths (< 15 m) the influence of benthic diatoms is negligible in the study area (West coast of Ireland). The studies in the South East coast of Ireland and around the Isle of Man suggest that water depth is not a determining factor per se in the contamination of scallops. To the South East of Ireland a correlation could be shown between geographical distribution of Da in scallops and the occurrence of nutrients, thus providing circumstantial evidence that nutrient availability may influence

primary production, including pelagic diatoms such as *Pseudo-nitzschia*, which in turn increases the contamination of scallops with DA.

The use of SPATT-technology for the prediction of the appearance of toxins in shellfish was investigated and could not be confirmed in our study. However, the technique could be used to demonstrate for the first time the presence of a number of toxins in Irish waters (DTX1, YTX, SPX). In addition, the use of SPATT-samplers corroborated the hypothesis that shellfish do not absorb lipophilic toxins directly from the seawater.

5.3. Preparative isolation of bioactive natural products

The greatest effort in the area of preparative isolation was spent on AZAs, mostly as part of the ASTOX-project. The original isolation procedure published by Japanese collaborators has been evaluated, adapted and standardized in such a fashion that current work in this area can continue to progress. The preparative isolation of toxins of this group has also allowed the identification of a large number of analogues, amongst others it has led to the discovery of oxidized and dihydroxylated AZAs. The quality of the purified compounds has been demonstrated to be of very high level. A certified standard has been obtained for AZA1 as a result of these efforts. Also, toxicology studies could progress significantly thanks to the availability of purified AZA1.

DTX2 has been isolated in quantities sufficient to conduct toxicological evaluation and to produce reference standards, certification of a portion is ongoing at NRCC.

An array of derivatives was elucidated for PTXs: acyl esters of the main metabolite of PTX2, PTX2sa were discovered in concentrated extracts of mussels from Ireland. Subsequently these compounds were also found in freshly contaminated mussels from Norway, demonstrating that these compounds occur systematically as part of the contamination of mussels by *Dinophysis*.

Overall, these studies have also contributed to the knowledge of the reactivity of lipophilic shellfish toxins, both in terms of their chemical stability, and in terms of their metabolism.

5.4. Quality control and the performance of methods

A large number of reference materials have been prepared as in-house quality control materials, in systematic studies during the ASTOX project, for method validation in the BIOTOX project, as part of proficiency testing with QUASIMEME, and in multi-partner collaboration for the preparation of certified shellfish tissue reference materials. Two reference materials have been prepared for certification which is still ongoing at the NRCC.

Open proficiency testing has been introduced for the DA-, OA-, AZA- and STX-groups of toxins. These proficiency tests allowed for the evaluation of the performance of laboratories for a much larger number of different types of shellfish matrices than this would have been possible through the preparation of certified reference materials. In this way, we were able to test during three years the performance of > 30 laboratories for the analysis of DA in 5 different type of shellfish tissues, while over an even longer period the only certified material for this toxin was a mussel digestive gland tissue. Proficiency testing for the STX-group has been conducted while there was not even a certified reference material available for this toxin group.

LC-UV has been compared to LC-MS for the detection of DA, demonstrating that either technique can be used reproducibly and accurately, and that SPE clean-up for this toxin group can be eliminated if sufficient chromatographic separation is achieved in the final separation. LC-UV was also compared to an ELISA for the detection of DA, and we established that both could be applied without compromising the accuracy of the results. Similarly, LC-MS was compared as

analytical technique to a recently developed immunoassay for the OA-group of toxins. Satisfactory results were obtained, indicating that the cross-reactivity of this particular ELISA reflects the relative toxicities of individual analogues.

The performance of the mouse bioassay as the existing official technique for the detection of lipophilic toxins was compared to an in-house validated LC-MS method at the example of AZAs. The comparison demonstrated that both techniques are capable of detecting AZA-contamination at the current regulatory limit of $160 \mu\text{g kg}^{-1}$; The comparison also clearly outlined that the MBA is not capable of detecting concentrations any lower than this. Thus, it was demonstrated that detection of toxins by MBA in raw shellfish is not an appropriate tool to control the concentrations of AZAs in heat-treated shellfish.

5.5. Characterisation of hazards from marine biotoxins

The acidity constants (pK_a) and the degree of lipophilicity (P_{ow}) were estimated for lipophilic toxins of the four regulated groups (AZA, OA, PTX and YTX) using a chromatographic approach. PTX2 appeared to be a neutral compound while the remaining three had decreasing acidity in the following order: $\text{OA} > \text{AZA1} > \text{YTX}$. For YTX this acidity certainly refers to the acidity of the second sulphate group, the acidity of the first sulphate group being too low to be determined chromatographically. The lipophilicity of YTX could not be determined due to limited chromatographic data points. The remaining compounds can be ranked in decreasing order of their lipophilicity expressed as $\log\text{P}_{\text{ow}}$: $\text{AZA2} > \text{AZA1} > \text{DTX1} > \text{PTX2} > \text{DTX2} > \text{OA}$.

The chemical stability of AZA- and OA-group toxins has been shown as a function of pH and temperature, in solution and in shellfish matrix. OA-group toxins have been confirmed to withstand strong base treatment in MeOH solution, however, their rapid decomposition was shown under the influence of strong acids at elevated temperature (i.e. 76°C). In solution, AZAs have shown to degrade rapidly under the influence of strong bases and acids, as well as under the influence of weak acids (formic acid and acetic acid) when the temperature is increased somewhat (40°C). In aqueous medium, mussel tissue has largely buffered the influence of acids and even an increase in concentration was observed when digestive enzymes were added to a heated mixture of AZA-contaminated shellfish tissues and HCl. Transformation of AZAs in shellfish appears to be driven by oxidation reactions. The two analogues produced by *Azadinium spinosum* may well be metabolized to 18 further analogues in shellfish, including the spontaneous, heat-catalysed transformations of AZA17 and -19 to AZA3 and -6, respectively.

The influence of heat treatments and industrial processing has been evaluated for both hydrophilic and lipophilic toxins: DA, AZA and OA. The influence of processing procedures in the evisceration of scallops on the DA contamination of the edible parts, in particular the adductor muscle was investigated. Both the remaining parts of digestive organs (anal tube and kidneys) and the spillage of fluids from the hepatopancreas were identified as major contributors. The field variability and the dissection parameters together have influenced the legislation for DA in pectinidae. The concentrations of DA in mussels (*M. edulis*) were shown not to be influenced heavily by heat treatment of this shellfish. On the contrary, the heat-treatments of AZA- and OA-contaminated mussels were clearly shown to increase the concentration of these analytes in the matrix, both due to water loss from the matrix and, for AZAs, also through the heat-catalysed transformation of AZAs. These studies have clarified the need for fully quantitative methods in the detection of shellfish toxins during official control.

The interactions of shellfish toxins with biological systems were evaluated for live animals, in cellular models and in biochemical terms for AZA1 and DTX2. The relative toxicity of DTX2 compared to OA was determined by intraperitoneal injection in mice. DTX2 appears to be only about half as toxic as OA; this was also confirmed by analysis of the purified compounds using the

functional assay of PP2a inhibition. The toxicity of AZA1 was evaluated in vitro in many different models: the cytotoxicity was shown in a number of cell lines, with lymphocytes exhibiting the highest sensitivity at low nanomolar concentration. The transepithelial electrical resistance of Caco2 cells was drastically reduced at similarly low concentrations, and cell adhesion proteins were affected by exposure of Caco2 cells in two independent studies (claudins and e-cadherin). Gene-chip studies pointed to lipid-synthesis and wound-healing pathways being affected. Further studies will be required to fully evaluate the mechanism of action of AZAs.

5.6. Looking forward

The conscientious reader will have noticed without problem that as a chemist I have focused initially on bringing up the field of chemical analysis towards the state-of-the-art through the development of methods, evaluation of critical parameters affecting their performance and through the provision of quality control tools, including certified standard and reference materials. Even though I have centered my work so far only on already regulated compounds in this area, much remains to be done: the full profile of reference compounds is not yet available and the reactivity of all compounds is far from being completely understood. I will try to continue to work in this area and encourage others to contribute to our understanding in a systematic fashion, which should allow us to rationalize resources that are scarce, even at international scale.

I am intrigued by the recent progress in the area of algal culture, i.e. the discovery of the producer of azaspiracids and the possibility to culture *Dinophysis* species. As the availability of toxins for method development and validation and for toxicological work is still a major pitfall of toxinology, our efforts should focus most in this area. In this context, I am very keen to continue the research started in the ASTOX project through its successor-project: ASTOX2. In this enlarged consortium, we have access to *Azadinium* species to study their behaviour and potential use for sustainable production of azaspiracids, we also have already access to larger amounts of toxin from the previous isolation studies on shellfish which should allow us to progress our understanding of the toxicity of azaspiracids. The culture of toxic algae, such as *Dinophysis* will also complete our knowledge on the complex profiles of metabolites produced by this organism which has been a challenge at global scale. I have a feeling that we have only scratched the tip of the iceberg.

While the oncoming changes of official control towards the use of chemical testing for lipophilic toxins may allow us to focus regulatory efforts on the “known problems”, i.e. toxins of which we know people get sick, I believe that the challenge of bio-active compounds in the marine environment remains. Recent studies have shown that more than 40,000 bioactive compounds have already been recorded in the marine environment. While live animal assays have helped in the discovery of a few dozen compound groups, the potential of the ocean for biological activity is far from being explored completely. We have seen the difficulty in elucidating the mechanism of action for novel bioactive compounds when starting from scratch, which may take years if not decades. The pharma-industry is taking an opposite route in their drug discovery approach: the biological target is validated first, and then large libraries of compounds are screened, first virtually and then in actual bioactivity screening. Currently the process of drug discovery and development lasts similar periods as the elucidation of a novel structure and its mechanism of biological action, however, if the two approaches could be cross-linked, a potentially very productive novel discipline could be instilled. In invited lectures at the Marine Natural Products conference (2007) and the AOAC Annual Meeting (2008) I have pinpointed to such approaches but I am sure that further efforts will be necessary to constructively achieve any progress in this

area. As a start, I have engaged in a collaborative effort with Nantes University to search the coastal environment for bioactive natural products combining classical and novel approaches to investigate our living filters of the seas: bivalve molluscs.

The avid reader would have noted that most of the research presented here has been carried out in a very applied context: science at service of the production of safe shellfish. Some of the studies allow for a more precise measure of the compounds present, others clarify the behaviour of the toxins in commercial processing and others again pinpoint to the extent of toxicity of the toxins. When I joined Ifremer, I took on the responsibility of leading a research department with the central theme of shellfish safety, covering both the threat of phycotoxins and the microbiological threat to the consumer of shellfish. The two topics are not only related through shellfish as a commodity but also through environmental interactions of toxic algae and the marine microbial community. One of the great challenges in this area remains the understanding of the ecological interactions of microscopic organisms, such as toxic phytoplankton and marine bacteria, in their chemical and biological environments. While global change with anthropogenic variants such as nutrients and pesticides from agricultural run-off affects the chemical environment of (toxic) micro-organisms, the biological web of grazers, parasites, symbionts and competitors is also still poorly understood. It is one of my personal ambitions to build interactions with colleagues in this area which would allow us to better answer our respective questions in this area. Other links between the microbiological safety of shellfish and the phytoplankton threat are the improvement of tools used the genomic and metabolomic areas: the way in which shellfish bind and digest microbes needs to be examined closely to understand their retention and the metabolism of the chemicals involved.

6. References

- Aasen JAB, Hardstaff W, Aune T, Quilliam M (2006) Discovery of fatty acid ester metabolites of spirolide toxins in mussels from Norway using liquid chromatography/tandem mass spectrometry. *Rapid Comm. Mass Spectrom.* 20: 1531-1537.
- Abraham A, Plakas SM, Wang ZH, Jester ELE, El Said KR, Granade HR, Henry MS, Blum PC, Pierce RH, Dickey RW (2006) Characterization of polar brevetoxin derivatives isolated from *Karenia brevis* cultures and natural blooms. *Toxicon* 48: 104-115.
- Adachi R, Fukuyo Y (1979) The thecal structure of a toxic marine dinoflagellate *Gambierdiscus toxicus* gen. et spec. nov. collected in a ciguatera-endemic area. *Bull. Jap. Soc. Sci. Fish.* 45: 67-71.
- Alam M, Sanduja R, Hossain MB, van der Helm D (1982) Gymnodinium breve toxins. I. isolation and X-ray structure of O,O-dipropyl (E)-2-(1-methyl-2-oxopropylidene) phosphorohydrazidothioate (E)-oxime from the red tide dinoflagellate *Gymnodinium breve*. *J. Am. Chem. Soc.* 104: 5232-5234.
- Alfonso C, Rehmann N, Hess P, Alfonso A, Wandscheer C, Abuin M, Vale C, Otero P, Vieytes M, Botana LM (2008) Evaluation of various pH and temperature conditions on the stability of azaspiracids, and their importance in preparative isolation and toxicological studies. *Anal. Chem.* 80: 9672-9680.
- Aligizaki K, Katikou P, Nikolaidis G, Panou A (2008) First episode of shellfish contamination by palytoxin-like compounds from *Ostreopsis* species (Aegean Sea, Greece). *Toxicon* 51: 418-427.
- Amzil, Z., Sibat M., Royer F., Savar V. (2008) First report on azaspiracids and yessotoxins groups detection in French shellfish. *Toxicon*, 52, 39-48.
- Anonymous (1986) Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animal used for experimental and other scientific purposes. *OJ L 358*, 18.12.1986, p. 1–28.
- Anonymous (1991) European Commission, Council Directive 91/492/EEC of 15 July, *Off. J. L 268* (1991) 1.
- Anonymous (1997) Council Directive 97/61/EC of 20 October 1997. *Official Journal L 295*, 29/10/1997 p. 35 – 36.
- Anonymous (2002) 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. *Official Journal L75*, pp. 62–64. (repealed by (2006/765/EC)).
- Anonymous (2002) Commission Decision 2002/226/EC of 15 March 2002 establishing special health checks for the harvesting and processing of certain bivalve mollusks with a level of amnesic shellfish poison (ASP) exceeding the limit laid down by Council Directive 91/492/EEC. *Official Journal L75*, pp. 65–66.
- Anonymous (2004) 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. *Official Journal L 139*, 30.4.2004, p. 206–320.
- Anonymous (2005) 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. Official Journal L 338, 22.12.2005, p. 27–59.

Anonymous (2006a) Commission Regulation (EC) No 1664/2006 of 6 November 2006 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. Official Journal L320, p. 13 – 45. (acceptance of Lawrence method for PSP toxins.)

Anonymous (2006b) Commission Regulation (EC) No 1666/2006 of 6 November 2006 amending Regulation (EC) No 2076/2005 laying down transitional arrangements for the implementation of Regulations (EC) No 853/2004, (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council. Official Journal L320, p. 47 – 49. (10% tolerance for B class areas)

Association of Official Analytical Chemists (2005a). Official Method 959.08. Paralytic Shellfish Poison, Biological Method. In: AOAC Official Methods of Analysis, 18th Ed., AOAC Gaithersburg, USA, 79–80.

Association of Official Analytical Chemists (2005b) Off. Method 2005.06. Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish using Prechromato-graphic Oxidation and Liquid Chromatography with Fluorescence Detection. In: AOAC Official Methods of Analysis, 18th Ed., AOAC, Gaithersburg, USA, 81–82.

Aune T, Larsen S, Aasen J, Rehmann N, Satake M, Hess P (2007) Relative toxicity of dinophysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* 49: 1-7.

Aune T, Sorby R, Yasumoto T, Ramstad H, Landsverk T (2002) Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. *Toxicon* 40: 77-82.

Aune, T., Torgersen, T., Aasen, J., Castberg, T., Naustvoll, L.-J. and Woll, A. (2006) Risk assessment of DSP toxins in brown crabs (*Cancer pagurus*). In: *Molluscan Shellfish Safety. Proceedings of the 5th International Conference on Molluscan Shellfish Safety*, Galway, Ireland, June 14th-18th, 2004 (Eds.: K. Henshilwood, B. Deegan, T. McMahon, C. Cusack, S. Keaveney, J. Silke, M. O’Cinneide, D. Lyons and P. Hess), pp. 464-468.

Baden DG, Bourdelais AJ, Jacocks H, Michelliza S, Naar J (2005) Natural and derivative brevetoxins: Historical background, multiplicity, and effects. *Environ. Health Perspect.* 113: 621-625.

Belin C, Soudant D, Amzil Z. (2009) Surveillance des toxines lipophiles dans les coquillages. Analyses statistique et comparaison des résultats obtenus par deux méthodes d’analyse : les bio-essais sur souris et les analyses chimiques par CL-SM/SM. Rapport exécuté dans le cadre de la Convention Etudes DGAL / Ifremer, correspondant à la Subvention pour charges de service public, Programme 206, 94 pp Octobre 2009.

Bialojan C, Takai A (1988) Inhibitory effect of a marine sponge toxin, okadaic acid, on protein phosphatases. *Biochem.J.* 256: 283-290.

BIM (2006) Review of the Irish Rope Mussel Industry, a report jointly commissioned by Board Iascaigh Mhara and Entreprise Ireland. PricewaterhouseCoopers, March, 2006.

BIOTOX (2007) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. Report on transition study, Biré R. and Hess P. 43 pp.

BIOTOX (2008a) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. WP2, deliverable 7: Report on the

MS detectors, MS and LC parameters, and performance characteristics, affecting chromatographic separation of lipophilic marine biotoxins, Biré R. and Hess P. 75 pp.

BIOTOX (2008b) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. WP2, deliverable 15: Report on improved extraction and clean-up procedures, Fux E., Gerssen A., Biré R., Mulder P., Rourke B., McElhinney M., Kotterman M., Hess P. 111 pp.

BIOTOX (2008c) BIOTOX EU FP6 project n°. 514074 Development of cost-effective tools for risk management and traceability systems for marine biotoxins. WP2, deliverable 22 Report on matrix effects, Fux E., Biré R. and Hess P. 70 pp.

Bogan, Y.M., Bender, K., Hervas, A., Kennedy, D.J., Slater, J.W., Hess, P. (2007a) Spatial variability of domoic acid concentration in king scallops *Pecten maximus* off the southeast coast of Ireland. *Harmful Algae*, 6, 1-14.

Bogan, Y.M., Harkin, A.L., Gillespie, J., Kennedy, D., Hess, P., Slater, J.W. (2007b) The influence of size on domoic acid concentration in king scallop, *Pecten maximus* (L.) *Harmful Algae*, 6, 15-28.

Bogan, Y.M., Kennedy, D., Harkin, A.L., Gillespie, J., Hess, P., Slater, J.W. (2006) Comparison of domoic acid concentration in king scallops, *Pecten maximus* from seabed and suspended culture systems. *J. Shellfish Res.* 25 (1), 129-135.

Bogan Y.M., Kennedy D., Harkin A.L., Gillespie J., Vause B.J., Beukers-Stewart B.D., Hess P., Slater J.W. (2007c) Variation in domoic acid concentration in king scallop (*Pecten maximus*) from fishing grounds around the Isle of Man. *Harmful Algae*, 6, 81-92.

Boni, L., Milandri, A., Poletti, R. & Pompei, M., 1993., DSP cases along the coasts of Emilia-Romagna (Northwest Adriatic Sea). In: Smayda, T.J. & Shimizu, Y. (Eds.), *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam, pp. 475–481.

Bourdelaïs AJ, Campbell S, Jacocks H, Naar J, Wright J, Carsi J, Baden DG (2004) Brevetoxin is a natural inhibitor of brevetoxin action in sodium channel receptor binding assays. *Cell. Mol. Neurobiol.* 24: 553-563.

Britton R, Roberge M., Brown C, van Soest R, Andersen RJ (2003) New okadaic acid analogues from the marine sponge *Merriamium oxeatum* and their effect on mitosis. *J. Nat. Prod.* 66, 838-843.

Burton I.W., Quilliam M.A., Walter J.A. (2005) Quantitative ¹H-NMR with External Standards: Use in Preparation of Calibration Solutions for Algal Toxins and Other Natural Products. *Analytical Chemistry* 77:3123-3131

Campbell D.A.; Kelly M.S.; Busman M.; Bolch C.J.; Wiggins E.; Moeller P.D.R.; Morton S.L.; Hess P., and Shumway S.E. (2001) Amnesic shellfish poisoning in the King Scallop, *Pecten maximus*, from the west coast of Scotland. *J. Shellfish Res.* 20(1), 75-84.

Castèle S, Catterall WA (2000) Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 82: 883-892.

Cembella AD, Krock B (2008) Cyclic imine toxins: chemistry, biogeography, biosynthesis and pharmacology. In: LM Botana (ed.): *Seafood and Freshwater Toxins, Pharmacology, Physiology and Detection*, Taylor & Francis Ltd, Boca Raton, 561-580.

Chateau-Degat ML, Chinain M, Cerf N, Gingras S, Hubert B, Dewailly E (2005) Seawater temperature, *Gambierdiscus* spp. variability and incidence of ciguatera poisoning in French Polynesia. *Harmful Algae* 4: 1053-1062.

- Ciminiello P, Fattorusso E, Forino M, Poletti R, Viviani R (2000) Structure determination of carboxyhomoyessotoxin, a new yessotoxin analogue isolated from Adriatic mussels. *Chem Res. Toxicol.* 13: 770-774.
- Ciminiello P, Dell'Aversano C, Fattorusso E, Forino M, Magno S, Guerrini F, Pistocchi R, Boni L (2003) Complex yessotoxins profile in *Protoceratium reticulatum* from north-western Adriatic sea revealed by LC-MS analysis. *Toxicon* 42: 7-14.
- Ciminiello P, Dell'Aversano C, Fattorusso E, Forino M, Tartaglione L, Grillo C, Melchiorre N (2008) Putative palytoxin and its new analogue, ovatoxin-a, in *Ostreopsis ovata* collected along the Ligurian coasts during the 2006 toxic outbreak. *J. Am. Soc. Mass Spectrom.* 19: 111-120.
- Colman J.R., Twiner M.J., Hess P., McMahan T., Doucette G.J., and Ramsdell J.S. (2005) Teratogenic Effects of Azaspiracid-1 Identified by Microinjection of Japanese Medaka *Oryzias Latipes* Embryos. *Toxicon* 45, 881-890.
- Daigo K (1959) Constituents of *Chondria armata*. I. Detection of the anthelmintic constituents. *Yakugaku Zasshi* 79: 350-353.
- Daigo K (1959) Constituents of *Chondria armata*. II. Isolation of an anthelmintic constituent. *Yakugaku Zasshi* 79: 353-356.
- Dechraoui MYB, Wacksman JJ, Ramsdell JS (2006) Species selective resistance of cardiac muscle voltage gated sodium channels: Characterization of brevetoxin and ciguatoxin binding sites in rats and fish. *Toxicon* 48: 702-712.
- Dell'Aversano C, Eaglesham GK, Quilliam MA (2004) Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography-mass spectrometry. *J. Chromatogr. A* 1028: 155-164.
- Dell'Aversano C, Hess P, Quilliam MA (2005) Hydrophilic interaction liquid chromatography-mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. *J. Chromatogr. A* 1081: 190-201.
- Dell'Aversano C, Walter JA, Burton IW, Stirling DJ, Fattorusso E, Quilliam MA (2008) Isolation and structure elucidation of new and unusual saxitoxin analogues from mussels. *J. Nat. Prod.* 71: 1518-1523.
- DG Sanco (EU Directorate General for Consumer Health and Protection). Report of the meeting of the Working Group on Toxicology of DSP and AZP, 21 to 23rd May 2001, Brussels.
- Dickey RW (2008) Ciguatera toxins: chemistry, toxicology and detection. In: LM Botana (ed.): *Seafood and freshwater toxins - Pharmacology, Physiology and Detection*, CRC Press, Taylor & Francis Group, Boca Raton, 479-500.
- Doucette DA, Tasker RA (2008) Domoic acid: detection methods, pharmacology, and toxicology. In: LM Botana (ed.): *Seafood and Freshwater Toxins, Pharmacology, Physiology and Detection*, Taylor & Francis Ltd, Boca Raton, 397-429.
- Draisci R, Ferretti E, Palleschi L, Marchiafava C, Poletti R, Milandri A, Ceredi A, Pompei M (1999) High levels of yessotoxin in mussels and presence of yessotoxin and homoyessotoxin in dinoflagellates of the Adriatic Sea. *Toxicon* 37: 1187-1193.
- Draisci R, Lucentini L, Mascioni A (2000) Pectenotoxins and yessotoxins: chemistry, toxicology, pharmacology, and analysis. In: Botana, L.M. (ed.): *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection*. 2000 Marcel Dekker, Inc, New York, 289-324.

Durando P, Ansaldi F, Oreste P, Moscatelli P, Marensi L, Grillo C, Gasparini R, Icardi G, (2007) Collaborative Group for the Ligurian Syndromic Algal Surveillance. Euro Surveill. 12: E070607.1.

EFSA (European Food Safety Authority) (2009e) Marine biotoxins in shellfish – Summary Opinion, Scientific Opinion of the Panel on Contaminants in the Food chain; adopted on 13 August 2009, EFSA Journal (2009) 1306, 1 - 23.

EFSA (European Food Safety Authority) (2009d) Marine biotoxins in shellfish – Domoic acid group, Scientific Opinion of the Panel on Contaminants in the Food chain; adopted on 2 July 2009, EFSA Journal (2009) 1181, 1-61.

EFSA (European Food Safety Authority) (2009c) Marine biotoxins in shellfish – Pectenotoxin group, Scientific Opinion of the Panel on Contaminants in the Food chain; adopted on 27 May 2009, EFSA Journal (2009) 1109, 1-47.

EFSA (European Food Safety Authority) (2009b) Influence of processing on the levels of lipophilic marine biotoxins in bivalve molluscs, Statement of the Panel on Contaminants in the Food Chain (Question No EFSA-Q-2009-00203), Adopted on 25 March 2009, EFSA Journal (2009) 1016, 1-10.

EFSA (European Food Safety Authority) (2009a) Marine biotoxins in shellfish – Yessotoxin group, Scientific Opinion of the Panel on Contaminants in the Food chain, adopted on the 2 December 2008, EFSA Journal (2009) 907, 1-62.

EFSA (European Food Safety Authority) (2008b) Marine biotoxins in shellfish – Azaspiracid group, Scientific Opinion of the Panel on Contaminants in the Food chain, adopted on 9 June 2008, EFSA Journal (2008) 723, 1-52.

EFSA (European Food Safety Authority) (2008a) Marine biotoxins in shellfish – Okadaic Acid and analogues, Scientific Opinion of the Panel on Contaminants in the Food chain, adopted on 27 November 2007, EFSA Journal (2008) 589, 1-62.

Emons H, Linsinger TPJ, Gawlik BM (2004) Reference materials: terminology and use. Can't one see the woods for the trees? TRAC. 23, 442-449

Emons H. (2005) The 'RM family'-Identification of all of its members. Accreditation and Quality Assurance 10, 690 – 691.

FAO (2004) FAO Food and Nutrition Paper 80, Food and Agriculture Organisation of the United Nations, Rome, 2004. Authors: van Egmond H.P., van Appeldoorn M.E. and Speijers G.J.A.

FAO. Report of the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs, Oslo, Norway, Sept. 26-30, 2004. Available at: ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf (accessed 5 June 2009).

Ferrari S, Ciminiello P, Dell'Aversano C, Forino M, Malaguti C, Tubaro A, Poletti R, Yasumoto T, Fattorusso E, Rossini GP (2004) Structure-activity relationships of yessotoxins in cultured cells. Chem. Res. Toxicol. 17: 1251-1257.

Finch S, Wilkins A, Hawkes A, Jensen D, MacKenzie L, Beuzenberg V, Quilliam M, Olseng C, Samdal I, Aasen J, et al. (2005) Isolation and identification of (44-R,S)-44,55-dihydroxyessotoxin from *Protoceratium reticulatum*, and its occurrence in extracts of shellfish from New Zealand, Norway and Canada Toxicon 46: 160-170.

Flanagan, A.F., Callanan, K.R., Donlon, J., Palmer, R., Forde, A., Kane, M., 2001. A cytotoxicity assay for the detection and differentiation of two families of shellfish toxins. Toxicon 39, 1021-1027.

- Frolova GM, Kuznetsova TA, Mikhailov VV, Elyakov GB (2000) An enzyme linked immunosorbent assay for detecting palytoxin-producing bacteria. *Bioorganicheskaya Khimiya* 26: 315-320.
- FSAI (2006) Risk Assessment of Azaspiracids (AZAs) in Shellfish: A report of the Scientific Committee of the FSAI (Food Safety Authority of Ireland), 2006, 39 pages. <http://www.fsai.ie>.
- Fux E., Biré 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.
- Fux E., McMillan D., Bire R., Hess P., (2007) Development of an Ultra Performance Liquid Chromatography – Mass Spectrometry method for the detection of lipophilic marine toxins. *J. Chromatogr. A* 1157, 273-280.
- Fux E., Marcaillou C., Mondeguer F., Bire R., Hess P. (2008a) 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., Rode D., Bire R., Hess P. (2008b) Approaches to the evaluation of matrix effects in the liquid chromatography-mass spectrometry (LC-MS) analysis of three regulated lipophilic toxin groups in mussel matrix (*Mytilus edulis*). *Food Addit. Contam.* 25 (8), 1024-1032.
- Gallacher S.; MacRae J.; Ferguson C.; Smith E.; Hess P.; Johnstone M.; Glover L.A.; Bates S.S., and Garthwaite I. (2000) Identity of bacteria isolated from the domoic-acid-producing diatom *Pseudo-nitzschia multiseries* and investigations into mechanisms by which they influence toxin production. Poster presented at the 9th Conference on Harmful Algal Blooms, Tasmania 6 - 11 February 2000.
- Gallitelli M, Ungaro N, Addante LM, Gentiloni N, Sabbà C (2005) Respiratory illness as a reaction to tropical algal blooms occurring in a temperate climate. *JAMA* 293: 2599-2600.
- Gerssen A., McElhinney M.A., Mulder P.P.J., Bire R., Hess P., de Boer J. (2009) Solid phase extraction for removal of matrix effects in lipophilic marine toxin analysis by liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* 394 (4) 1213-1226.
- Gessner BD, McLaughlin JB (2008) Epidemiologic impact of toxic episodes: neurotoxic toxins. In: LM Botana (ed.): *Seafood and Freshwater Toxins, Pharmacology, Physiology and Detection*, Taylor & Francis Ltd, Boca Raton, 77-103
- Giacobbe, M.G., Penna, A., Ceredi, A., Milandri, A., Poletti, R. & Yang, X., 2000. Toxicity and ribosomal DNA of the dinoflagellate *Dinophysis sacculus* (Dinophyta). *Phycologia* 39, 177-182.
- Gocan S., Cimpan G., Comer J. (2006) Lipophilicity Measurements by Liquid Chromatography In Grushka, E. and Grinberg, N. (Ed.) *Advances in Chromatography*, Taylor and Francis, CRC Press Florida, USA. pp. 79-176.
- Habermann E, Ahnert-Hilger G, Chhatwal GS, Beress L (1981) Delayed haemolytic action of palytoxin general characteristics. *Biochim. Biophys. Acta* 649: 481-486.
- Hackett J. D., Tong M., Kulis D.M., Fux E., Hess P., Bire R., Anderson D.M. (2009) DSP toxin production de novo in cultures of *Dinophysis acuminata* (Dinophyceae) from North America. *Harmful Algae* 8, 873-879.
- Hall S, Reichardt PB (1984) Cryptic paralytic shellfish toxins. In: Regalis EP (ed.): *Seafood Toxins*. American Chemical Society, Washington DC, 113-123.

Hallegraeff GM (2004) Harmful algal blooms: A global overview. In: GM Hallegraeff, DM Anderson, AD Cembella (eds.): *Manual on Harmful Marine Microalgae*, 2nd ed. UNESCO, Paris, 25-49.

Harada T, Oshima Y, Yasumoto T (1984) Assessment of potential activation of gonyautoxin V in the stomach of mice and rats. *Toxicon*, 22: 476-478.

Hartshorne RP, Catterall WA (1984) The sodium channel from rat brain. Purification and subunit composition. *J. Biol. Chem.* 259: 1667-1675.

Hess P. (2010) Requirements for screening and confirmatory methods for the detection and quantification of marine biotoxins in end-product and official control. *Accepted in Anal. Bioanal. Chem. Jan 2010*; DOI 10.1007/s00216-009-3444-y.

Hess P. (2008) What's new in toxins? Pp 360-370, in "Proceedings of the 12th International Conference on Harmful Algae". (Eds. : Moestrup, Ø., G. Doucette, H. Enevoldsen, A. Godhe, G. Hallegraeff, B. Luckas, N. Lundholm, J. Lewis, K. Rengefors, K. Sellner, K. Steidinger, P. Tester, A. Zingone). International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, 2008 Copenhagen. pp 397.

Hess P (2007) "Bioactive Compounds in Shellfish – Drug Discovery or Food Safety Management?". Invited Plenary Lecture at 5th European Conference on Marine Natural Products, Ischia, Italy, 16-21 Sept. 2007.

Hess P, Aasen JB (2007) Chemistry, origins and distribution of yessotoxin and its analogues. In: LM Botana (ed.): *Chemistry and pharmacology of marine toxins*, Blackwell Publishing, Oxford, 187-202.

Hess P.; Bates L.A.; Brown N.; McEvoy J.D.G.; Kennedy D.G.; Young P.B.; Slattery D.; McGovern E.; McMahon T.; Morris S., and Gallacher S. (2001) Intercomparison of methods for the determination of domoic acid, the amnesic shellfish poisoning toxin, in standard solutions of domoic acid, extracts and homogenates of the scallop *Pecten maximus*. (UK National Reference Laboratory for Marine Biotoxins). Fisheries Research Services - Research Report. 2001 Mar: pp. 102 + appendices.

Hess, P., Brown, N.A., Bates, L.A., Turriff, J.J., Howard, F.G., C F Moffat, C.F. (2000) A comparison of domoic acid concentrations in scallop (*Pecten maximus*) gonads processed by FRS ML with those prepared by a commercial processor. Fisheries Research Services Report No 03/00.

Hess P, Butter T, Petersen A, Silke J, McMahon T (2009) Performance of the EU harmonised mouse bioassay for lipophilic toxins for the detection of azaspiracids in naturally contaminated mussel (*Mytilus edulis*) hepatopancreas tissue homogenates characterised by liquid chromatography coupled to tandem mass spectrometry. *Toxicon* 53, 713-722.

Hess, P., Gallacher, S., Bates, L.A., Brown, N., Quilliam, M.A. (2001) Determination and confirmation of the amnesic shellfish poisoning toxin, domoic acid, in shellfish from Scotland by liquid chromatography and mass spectrometry. *J. Assoc. Off. Anal. Chem. Int.* 84, 1657-1667.

Hess P., Grune B., Anderson D.B., Aune T., Botana L.M., Caricato P., van Egmond H.P., Halder M., Hall S., Lawrence J.F., Moffat C., Poletti R., Richmond J., Rossini G.P., Seamer C. and Serratos Vilageliu J. (2006) Three Rs Approaches in Marine Biotoxin Testing - The Report and Recommendations of a joint ECVAM/DG SANCO Workshop (ECVAM Workshop 55). *Altern. Labor. Anim. (ATLA)* 34, 193-224.

Hess P., McCarron P., Quilliam M.A. (2007a) Fit-for-purpose Shellfish Reference Materials for Phycotoxins in Internal and External Quality Control. *Anal. Bioanal. Chem.* 387, 2463-2474.

Hess P., McCarron P., Rehmann N., Kilcoyne J., McMahon T., Ryan G., Ryan P.M., Twiner M.J., Doucette G.J., Satake M., Ito E., Yasumoto T. (2007b) Isolation and purification of azaspiracids from naturally contaminated materials, and evaluation of their toxicological effects– final project report ASTOX (ST/02/02). Marine Institute – Marine Environment & Health Series – No. 28, ISSN: 1649-0053; 129 pp.

Hess P. and McCarron P. (2007) “Review of Proficiency Testing for Shellfish Toxins”. Invited Plenary Lecture at 6th Intl. Conf. Molluscan Shellfish Safety (ICMSS), March 2007 Blenheim, New Zealand.

Hess P., McCarron P., Rehmann N., Ryan M.P., Ryan G. (2003) “Isolation and purification of azaspiracids from naturally contaminated materials and evaluation of their toxicological effects (ASTOX)” pp. 18-20 in Proc. 4th Irish Marine Biotoxin Science Workshop, Renvyle, November 7th 2003, Ireland.

Hess P., McMahon T., O’Cinneide M. (2003) “Monitoring strategies for DSP and related toxins in shellfish using animal testing and LC-MS techniques, at the example of shellfish monitoring in Ireland since 2001.” pp 71-76 in Proc. 8th Canadian Workshop on Harmful Marine Algae. 28-30 May 2003, Moncton, Canada, 2003

Hess P., McMahon T., Slattery D., Swords D., Dowling G., McCarron M., Clarke D., Gibbons W., Silke J., O’Cinneide M. (2003) Use of LC-MS testing to identify lipophilic toxins, to establish local trends and interspecies differences and to test the comparability of LC-MS testing with the mouse bioassay: an example from the Irish biotoxin monitoring programme 2001. in : Molluscan Shellfish Safety; editors: A. Villalba, B. Reguera, J.L. Romalde, R. Beiras; publishers: Consellería de Pesca e Asuntos Marítimos da Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Santiago de Compostela, Spain. ISBN: 84-453-3638-X.

Hess P, Morris S, Stobo LA, Brown NA, McEvoy JDG, Kennedy G, Young PB, Slattery D, McGovern E, McMahon T, et al. (2005a) LC-UV and LC-MS methods for the determination of Domoic Acid. *TrAC*, 24: 4, 358-367.

Hess P, Nguyen L, Aasen J, Keogh M, Kilcoyne J, McCarron P, Aune T (2005b) Tissue distribution, effects of cooking and parameters affecting the extraction of azaspiracids from mussels, *Mytilus edulis*, prior to analysis by liquid chromatography coupled to mass spectrometry. *Toxicon* 46, 62-71.

Hess P., Rehmann N. (2008) Pharmacological concepts and chemical studies relevant to evaluating the toxicity of azaspiracids. Pp 55 – 62 in “Proceedings of the 8th Irish Shellfish Safety Workshop”, Marine Institute, Rinville, 7th December, 2007. (eds. McMahon T., Deegan B., Silke J., O’Cinneide M.) Marine Environment and Health Series Report No. 33, 2008.

Hille B (1975) The receptor for tetrodotoxin and saxitoxin: a structural hypothesis. *Biophys. J.* 15: 615-619.

Hille B (1968) Pharmacological modifications of the sodium channels of frog nerves. *J. Gen. Physiol.* 51: 199-219.

Holland PT, Selwood AI, Mountford DO, Wilkins AL, McNabb P, Rhodes LL, Doucette GJ, Mikulski CM, King KL (2005) Isodomoic acid C, an unusual amnesic shellfish poisoning toxin from *Pseudo-nitzschia australis*. *Chem. Res. Toxicol.* 18: 814 – 816.

Hu T, Doyle J, Jackson D, Marr J, Nixon E, Pleasance S, Quilliam MA, Walter JA, Wright JLC (1992) Isolation of a new diarrhetic shellfish poison from Irish mussels. *J. Chem. Soc., Chem. Commun.*, 39-41.

- Hu TM, Curtis JM, Oshima Y, Quilliam MA, Walter JA, Watsonwright WM, Wright JLC (1995) Spirolide-B and spirolides-D, 2 novel macrocycles isolated from the digestive glands of shellfish. *J. Chem. Soc. Chem. Comm.*, 2159-2161.
- Hu TM, Curtis JM, Walter JA, Wright JLC (1996) Characterization of biologically inactive spirolides E and F: Identification of the spirolide pharmacophore. *Tetrahedron Lett.* 37: 7671-7674.
- ISO (1993) International vocabulary of basic and general terms in metrology. Geneva, Switzerland, ISO/IEC.
- Ishida, H., Muramatsu, M., Kosuge, T., Tsuji, K. (1996) Study on neurotoxic shellfish poisoning involving New Zealand shellfish *Crassostrea gigas*. In: T. Yasumoto, Y. Oshima and Y. Fukuyo (eds.): *Harmful and Toxic Algal Blooms*, Intergovernmental Oceanographic Commission of UNESCO, 491-494
- Ito E, Frederick MO, Koftis TV, Tang W, Petrovic G, Ling T, Nicolaou KC (2006) Structure toxicity relationships of synthetic azaspiracid-1 and analogs in mice. *Harmful Algae* 5: 586 – 591.
- Ito, E., Satake, M., Ofuji, K., Kurita, N., McMahon, T., James, K., Yasumoto, T., 2000. Multiple organ damage caused by a new toxin azaspiracid, isolated from mussels produced in Ireland. *Toxicon* 38, 917-930.
- Ito, E., Satake, M., Ofuji, K., Higashi, M., Harigaya, K., McMahon, T., Yasumoto, T., 2002. Chronic effects in mice caused by oral administration of sublethal doses of azaspiracid, a new marine toxin isolated from mussels. *Toxicon* 40, 193-203.
- Ito E, Suzuki T, Oshima Y, Yasumoto T (2008) Studies of diarrhetic activity on pectenotoxin-6 in the mouse and rat. *Toxicon* 51: 707-716.
- Jaime E., Hummert C., Hess P., and Luckas B. (2001) Determination of paralytic shellfish poisoning toxins by high-performance ion-exchange chromatography. *J. Chromatogr. A.* 929, 43-49.
- Kamiyama, T., Suzuki, T., 2009. Production of dinophysistoxin-1 and pectenotoxin-2 by a culture of *Dinophysis acuminata* (Dinophyceae). *Harmful Algae* 8, 312-317.
- Katikou P (2008) Palytoxin and analogues: ecobiology and origin, chemistry, metabolism, and chemical analysis. In: LM Botana (ed.): *Seafood and freshwater toxins - Pharmacology, Physiology and Detection*, CRC Press, Taylor & Francis Group, Boca Raton, 631-663.
- Kimura S, Hashimoto Y, Yamazato K (1972) Toxicity of the zoanthid *Palythoa tuberculosa*. *Toxicon* 10: 611-617.
- Kita M, Uemura D (2008) Diverse chemical structures and bioactivities of marine toxins: palytoxin and symbiodinolide. In: LM Botana (ed.): *Seafood and freshwater toxins - Pharmacology, Physiology and Detection*, CRC Press, Taylor & Francis Group, Boca Raton, 665-674.
- 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.
- Kulagina, N.V., Twiner, M.J., Doucette, G.J., Ramsdell, J.S., Hess, P., McMahon, T., O'Shaughnessy, T.J., Ma, W. (2006). Effect of azaspiracid-1 on action potential generation and voltage-gated currents in cultured spinal cord neuronal networks. *Toxicon* 47, 766-773.

Kumagai M, Yanagi T, Murata M, Yasumoto T, Kat M, Lassus P, Rodriguez-Vazquez, JA (1986) Okadaic acid as the causative toxin of diarrhetic shellfish poisoning in Europe. *Agric. Biol. Chem.* 50: 2853-2857.

Landry Y, Gies JP (2008). "Drugs and their molecular targets: an updated overview". *Fundam. Clin. Pharmacol.* 22 (1): 1-18.

Larsen K, Petersen D, Wilkins AL, Samdal IA, Sandvik M, Rundberget T, Goldstone D, Arcus V, Hovgaard P, Rise F, et al. (2007) Clarification of the C-35 Stereochemistries of Dinophysistoxin-1 and Dinophysistoxin-2, and its consequences for binding to protein phosphatase. *Chem. Res. Toxicol.* 20: 868–875.

Lawrence, J. E., Bauder, A. G., Quilliam, M. A. & Cembella, A. D., 1998. *Prorocentrum lima*: a putative link to diarrhetic shellfish poisoning in Nova Scotia, Canada. In: Reguera, B., Blanco, J., Fernandez, M. L. & Wyatt, T. (Eds.) *Harmful Algae*, Santiago del Compostella: Xunta de Galicia and Intergovernmental Oceanographic Commission. pp. 78-79.

Lawrence J F, Niedzwiadek B, Menard C (2004) Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection: Interlaboratory Study. *J. AOAC Int.* 87: 83- 100.

Lee JS, Igarashi T, Fraga S, Dahl E, Hovgaard P, Yasumoto T (1989) Determination of diarrhetic shellfish toxins in various dinoflagellate species. *J. Appl. Physiol.* 1, 147-152.

Legrand AM, Fukui M, Cruchet P, Yasumoto T (1992) Progress on chemical knowledge of ciguatoxins. *Bull. Soc. Pathol. Exot.* 85, 467-469.

Lenoir S, Ten-Hage L, Turquet J, Quod JP, Bernard C, Hennion MC (2004) First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in Southwestern Indian Ocean. *J. Phycol.* 40, 1042-1051.

Lerma J, Paternain AV, Rodríguez-Moreno A, López-García J (2001) Molecular Physiology of kainate receptors. *Physiol. Rev.* 81, 971-998.

Lewis RJ, Sellin M, Poli MA, Norton RS, MacLeod JK, Sheil MM (1991) Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). *Toxicon* 29: 1115-1127.

Lin Y-Y, Risk M, Ray SM, Van Engen D, Clardy J, Golik J, James JC, Nakanishi K (1981) Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). *J. Am. Chem. Soc.* 103, 6773-6775.

Lipinski, C., Lombardo, F., Dominy, B. & Feeney, P. (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* 23, 3–25.

Lombert A, Bidard JN, Lazdunski M (1987) Ciguatoxins and brevetoxin share a common receptor site on the neuronal voltage-dependent Na⁺ channel. *FEBS Lett.* 219, 355-357.

Lu CK, Lee GH, Huang R, Chou HN (2001) Spiro-prorocentrimine, a novel macrocyclic lactone from a benthic *Prorocentrum* sp of Taiwan. *Tetrahedron Lett.* 42, 1713-1716.

MacKenzie, L. , Beuzenberg, V., Holland, P., McNabb, P. & Suzuki, T. S. A., 2005. Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. *Harmful Algae* 4, 75-85.

Maeda M, Kodama T, Tanaka T, Yoshizumi H, Takemoto T, Nomoto K, Fujita T (1986) Structures of isodomoic acids A, B and C, novel insecticidal amino acids from the red alga *Chondria armata*. *Chem. Pharm. Bull* 34, 4892-4895.

- Maeda M, Kodama T, Tanaka T, Yoshizumi H, Takemoto T, Nomoto K, Fujita T (1987) Structures of domoic acid A and B, novel amino acids from the red alga, *Chondria armata*. *Tetrahedron Lett.* 28: 633-636.
- Marcaillou, C., Mondeguer, F. & Gentien, P., 2005. Contribution to toxicity assessment of *Dinophysis acuminata* (Dinophyceae). *J. App. Phycol.* 17, 155-160.
- McCauley JA, Nagasawa K, Lander PA, Mischke SG, Semones MA, Kishi Y (1998) Total synthesis of pinnatoxin A. *J. Am. Chem. Soc.* 120: 7647-7648.
- 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.
- McCarron P, Burrell S, Hess P (2007a) Effect of Addition of Antibiotics and an Antioxidant, on the Stability of Tissue Reference Materials for Domoic Acid, the Amnesic Shellfish Poison. *Anal. Bioanal. Chem.* 387: 2495-2502.
- McCarron P., Emteborg H., Hess P. (2007b) Freeze-drying for the stabilisation of shellfish toxins in mussel tissue (*Mytilus edulis*) reference materials. *Anal. Bioanal. Chem.*, 387, 2475-2486.
- McCarron P., Kotterman M., de Boer J., Rehmann N., Hess P. (2007c) Feasibility of g-Irradiation as a Stabilisation Technique in the Preparation of Tissue Reference Materials for a Range of Shellfish Toxins. *Anal. Bioanal. Chem.* 387, 2487-2493.
- 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, 1081-1089.
- 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.
- 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 (8), 901-918.
- McMahon T, Silke J (1996) Winter toxicity of unknown aetiology in mussels. *Harmful Algal News* 14, 2.
- McMahon T. and Silke J. 1998. Re-occurrence of winter toxicity in Irish mussels. *Harmful Algal News* 17, 12.
- Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J. Nutr.* 130, 1007S-1015S.
- Miles CO, Wilkins AL, Munday R, Dines MH, Hawkes AD, Briggs LR, Sandvik M, Jensen DJ, Cooney JM, Holland PT, et al. (2004) Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. *Toxicon* 43, 1-9.
- Miles CO (2007) Pectenotoxins. In: LM Botana (ed.): *Chemistry and pharmacology of marine toxins*, Blackwell Publishing, Oxford, 159-186.
- Miles CO, Samdal IA, Aasen JAB, Jensen DJ, Quilliam M, Petersen D, Briggs LM, Wilkins AL, Rise F, Cooney JM, et al. (2005) Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. *Harmful Algae* 4, 1075-1091.

- Miles CO, Wilkins AL, Jensen DJ, Cooney JM, Quilliam MA, Aasen J, Mackenzie AL (2004) Isolation of 41a-homoyessotoxin and the identification of 9-methyl-41a-homoyessotoxin and norring A-yessotoxin from *Protoceratium reticulatum*. *Chem. Res. Toxicol.* 17: 1414-1422.
- Miles CO, Wilkins AL, Hawkes AD, Selwood AI, Jensen DJ, Munday R, Cooney, JM, Beuzenberg V (2005) Polyhydroxylated amide analogs of yessotoxin from *Protoceratium reticulatum*. *Toxicon* 45: 61-71.
- Miles CO, Wilkins AL, Selwood AI, Hawkes AD, Jensen DJ, Cooney JM, Beuzenberg V, MacKenzie AL (2006) Isolation of Yessotoxin 32-O-[beta-L-arabinofuranosyl-(5' -> 1')-beta-L-arabinofuranoside] from *Protoceratium reticulatum*. *Toxicon* 47: 510-516.
- Molgo J, Girard E, Benoit E (2007) Cyclic imines: an insight into this emerging group of bioactive marine toxins. In: LM Botana (ed.): *Phycotoxins, Chemistry and Biochemistry*, Blackwell Publishing, Oxford, 319-335.
- Moore RE, Bartolini G (1981) Structure of palytoxin. *J. Am. Chem. Soc.* 103: 2491-2494.
- Moore RE, Bartolini G, Barchi J, Bothnerby AA, Dadok J, Ford J (1982) Absolute stereochemistry of palytoxin. *J. Am. Chem. Soc.* 104: 3776-3779.
- Moore RE, Scheuer PJ (1971) Palytoxin: A New Marine Toxin from a Coelenterate. *Science* 172: 495-498.
- Morono, A., Fernandez, M.L., Franco J.M., Martinez A., Reyero M.I., Miguez A., Cacho E., Blanco, J. (1998) PSP and DSP detoxification kinetics in mussels *Mytilus galloprovincialis* : effect of environmental parameters and body weight. *Harmful Algae*. B. Reguera, J. Blanco, M.L. Fernandez and T. Wyatt (Eds.) Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO.
- Morris P., Campbell D.S., Taylor T.J., Freeman J.I. (1991) Clinical and epidemiological features of neurotoxic shellfish poisoning in North Carolina. *Am. J. Public Health* 81, 471-473.
- Munday R. (2008) Toxicology of cyclic imines: gymnodimine, spirolides, pinnatoxins, pteriatoxins, prorocentrolide, spiro-prorocentrimine and symbioimines. In: LM Botana (ed.): *Seafood and Freshwater Toxins, Pharmacology, Physiology and Detection*, Taylor & Francis Ltd, Boca Raton, 581-594.
- Murata M., Kumagai M., Lee J.S., Yasumoto T. (1987) Isolation and structure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. *Tetrahedron Letters* 28: 5869-5872.
- Murata M., Yasumoto T. (2000) The structure elucidation and biological activities of high molecular weight algal toxins: maitotoxin, prymnesins and zooxanthellatoxins. *Nat. Prod. Rep.* 17: 293-314.
- Murata M., Legrand A.M., Ishibashi Y., Yasumoto T. (1989) Structures of ciguatoxin and its congener. *J. Am. Chem. Soc.* 111: 8929-8931.
- Murata M., Legrand A.M., Ishibashi Y., Fukui M., Yasumoto T. (1990) Structures and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. *J. Am. Chem. Soc.* 112: 4380-4386.
- Nicolaou K., Chen D., Li Y., Qian W., Ling T., Vyskocil S., Koftis T., Govindasamy M., Uesaka N. (2003) Total synthesis of the proposed Azaspiracid-1 Structure, Part 2: Coupling of the C1-C20, C21-C27, and C28-C40 Fragments and completion of the synthesis. *Angew. Chem.* 42: 3649-3653.

- Nicolaou K., Li Y., Uesaka N., Koftis T., Vyskocil S., Ling T., Govindasamy M., Qian W., Bernal F., Chen D. (2003) Total synthesis of the proposed azaspiracid-1 structure, Part 1: Construction of the Enantiomerically pure C1-C20, C21-C27, and C28-C40 fragments. *Angew. Chem.* 42: 3643-3648.
- Nicolaou KC, Vyskocil S, Koftis TV, Yamada YM, Ling T, Chen D, Tang W, Petrovic G, Frederick M, Li Y, Satake M (2004a) Structural Revision and Total Synthesis of Azaspiracid-1, Part 1: Intelligence Gathering and Tentative Proposal. *Angew. Chem. Intl. Ed.* 116: 4412-4418.
- Nicolaou KC, Koftis T, Vyskocil S, Petrovic G, Ling T, Yamada YM, Tang W, Frederick M (2004b) Structural Revision and Total Synthesis of Azaspiracid-1, Part 2: Definition of the ABCD Domain and Total Synthesis. *Angew. Chem. Intl. Ed.* 116: 4418-4424.
- 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.
- Ofuji K., Satake M., McMahon T., Silke J., James K.J., Naoki H., Oshima Y., Yasumoto T. (1999) Two analogs of azaspiracid isolated from mussels, *Mytilus edulis*, involved in human intoxication in Ireland. *Natural Toxins* 7, 99-102.
- Onoue Y, Noguchi T, Maruyama J, Hashimoto K, Seto H (1931) Properties of two toxins newly isolated from oysters. *J. Agric. Food Chem.* 2: 420-423.
- Onuma Y, Satake M, Ukena T, Roux J, Chanteau S, Rasolofonirina N, Ratsimaloto M, Naoki H, Yasumoto T (1999) Identification of putative palytoxin as the cause of clupeotoxism. *Toxicon* 37: 55-65.
- Oshima Y (1995) Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. *J. AOAC Int.* 78: 528-532.
- Ozawa S, Kamiya H, Tsuzuki K (1998) Glutamate receptors in the mammalian central nervous system. *Prog. Neurobiol.* 54: 581-618.
- Park M. G., Kim S., Kim H.S., Myung G., Kang Y.G., Yih W., 2006 First successful culture of the marine dinoflagellate *Dinophysis acuminata*. *Aquat. Microb. Ecol.* 45,101–106.
- Penna A, Vila M, Fraga S, Giacobbe MG, Andreoni F, Riobo P, Vernesi C (2005) Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8s rDNA sequences. *J. Phycol.* 41: 212-225.
- Perez R., Rehmann N., Crain S., LeBlanc P., Craft C., McKinnon S., Reeves K., Burton I.W., Walter J.A., Hess P., Quilliam M.A. and Melanson J.E. (2010) The Preparation of Certified Calibration Solutions for Azaspiracid 1, -2 and -3, Potent Marine Biotoxins found in Shellfish. *Manuscript in preparation for submission to Anal. Bioanal. Chem.*
- Pierce RH, Henry MS, Blum PC, Hamel SL, Kirkpatrick B, Cheng YS, Zhou Y, Irvin CM, Naar J, Weidner A, et al. (2005) Brevetoxin composition in water and marine aerosol along a Florida beach: Assessing potential human exposure to marine biotoxins. *Harmful Algae* 4: 965-972.
- Plakas SM, Wang ZH, El Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW (2004) Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. *Toxicon* 44: 677-685.
- Poli MA, Lewis RJ, Dickey RJ, Musser SM, Buckner CA, Carpenter LG (1997) Identification of Caribbean ciguatoxins as the cause of an outbreak of fish poisoning among U.S. soldiers in Haiti. *Toxicon* 35: 733-741.

- Pulido OM (2008) Domoic acid toxicologic pathology: a review. *Mar. Drugs* 6: 180-219.
- Quilliam MA, Hess P, Dell'Aversano C (2001) Recent developments in the analysis of phycotoxins by liquid chromatography - mass spectrometry. In: WJ De Koe, RA Samson, HP Van Egmond, J Gilbert, M Sabino (Eds.): *Mycotoxins and Phycotoxins in Perspective at the Turn of the Millenium*, W.J. de Koe, 383-391.
- Quilliam, M. A., Hardstaff, W. R., Ishida, N., McLachlan, J. L., Reeves, A. R., Rose, N. W. & Windust, A. J., 1996. Production of Diarrhetic Shellfish Poisoning (DSP) toxins by *Prorocentrum lima* in culture and development of analytical methods. In: Yasumoto, T., Oshima, Y. & Fukuyo, Y. (Eds.) *Harmful and Toxic Algal Blooms*, IOC of UNESCO, Senday, pp. 289–292.
- Quilliam, M. A., Gilgan, M. W., Pleasance, S., Defreitas, A. S. W., Douglas, D., Fritz, L., Hu, T., Marr, J. C., Smyth, C., Wright, J. L. C., 1993. Confirmation of an incident of diarrhetic shellfish poisoning in eastern Canada. In: Smayda, T. J. & Shimizu, Y. (Eds.) *Toxic Phytoplankton Bloom in the Sea*, Newport, RI, USA. Elsevier Sci. Publ., New York, pp. 547–552.
- Quilliam MA, Wright JLC (1989) The amnesic shellfish poisoning mystery. *Anal. Chem.* 61: 1053-1060.
- Quilliam MA, Xie M, Hardstaff WR (1995) A rapid extraction and cleanup procedure for the liquid chromatographic determination of domoic acid in unsalted seafood. *J. AOAC Int.* 78: 543-554.
- Ramsdell JS (2007) The molecular and integrative basis to domoic acid toxicity. In: LM Botana (ed.): *Chemistry and pharmacology of marine toxins*, Blackwell Publishing, Oxford, 223-250.
- Rehmann N, Hess P, Quilliam MA (2008) Discovery of New Analogs of the Marine Biotxin Azaspiracid in Blue Mussels (*Mytilus edulis*) by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 22: 549-558.
- Redondo J, Fiedler B, Scheiner-Bobis G (1996) Palytoxin-induced Na⁺ influx into yeast cells expressing the mammalian sodium pump is due to the formation of a channel within the enzyme. *Mol. Pharmacol* 49: 49-57.
- Rein KS, Lynn B, Gawley RE, Baden DG (1994) Brevetoxin B: chemical modifications, synaptosome binding, toxicity, and an unexpected conformational effect. *J. Org. Chem.* 59: 2107-2113.
- Rhodes L, McNabb P, de Salas M, Briggs L, Beuzenberg V, Gladstone M (2006) Yessotoxin production by *Gonyaulax spinifera*. *Harmful Algae* 5: 148-155.
- Rodrigue DC, Etzel RA, Hall S, de Porras E, Velasquez OH, Tauxe RV, Kilbourne EM and Blake PA. (1990) Lethal paralytic shellfish poisoning in Guatemala. *Am J Trop Med Hyg* 42 (3), 267-271.
- Ronzitti G., Hess P., Rehmann N., Rossini G. P. (2007) Azaspiracid-1 alters the E-cadherin pool in epithelial cells. *Toxicol. Sci.* 95 (2) 427-435.
- Samdal IA, Naustvoll LJ, Olseng CD, Briggs LR, Miles CO (2004) Use of ELISA to identify *Protoceratium reticulatum* as a source of yessotoxin in Norway. *Toxicon* 44: 75-82.
- Satake M, MacKenzie L, Yasumoto T (1997a) Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. *Nat. Toxins* 5: 164-167.
- Satake M, Ichimura T, Sekiguchi K, Yoshimatsu S, Oshima Y (1999) Confirmation of yessotoxin and 45,46,47-trinoryessotoxin production by *Protoceratium reticulatum* collected in Japan. *Nat. Toxins* 7: 147-150.

Satake M, Ofuji K, Naoki H, James KJ, Furey A, McMahon T, Silke J, Yasumoto T (1998a) Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. J. Amer. Chem. Soc. 120: 9967-9968.

Satake M, Tubaro A, Lee JS, Yasumoto T (1997b) Two new analogs of yessotoxin, homoyessotoxin and 4,5-hydroxyhomoyessotoxin, isolated from mussels of the Adriatic Sea. Nat. Toxins 5: 107-110

Satake M, Murata M, Yasumoto T (1993) The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus*. Tetrahedron Lett. 34: 1975-1978.

Satake M, Morohashi A, Oguri H, Oishi T, Hiramama M, Harada N, Yasumoto T (1997c) The absolute configuration of ciguatoxin. J. Am. Chem. Soc. 119: 11325-11326.

Satake M, Fukui M, Legrand AM, Cruchet P, Yasumoto T (1998b) Isolation and structures of new ciguatoxin analogs, 2,3-dihydroxyCTX3C and 51-hydroxyCTX3C, accumulated in tropical reef fish. Tetrahedron Lett. 39: 1197-1198.

Satake M, Shoji M, Oshima Y, Naoki H, Fujita T, Yasumoto T (2002) Gymnocin-A, a cytotoxic polyether from the notorious red tide dinoflagellate, *Gymnodinium mikimotoi*. Tetrahedron Lett. 43: 5829-5832.

Schantz EJ, Mold JD, Stanger DW, Shavel J, Riel FJ, Bowden JP, Lynch JM, Wyler RS, Riegel B, Sommer H (1957) Paralytic shellfish poison. VI. A procedure for the isolation and purification of the poison from toxic clam and mussel tissue. J. Am. Chem. Soc. 79: 5230-5235.

Schantz EJ, McFarren EF, Schafer ML, Lewis KH (1958) Purified shellfish poison for bioassay standardization. J. Assoc. Off. Anal. Chem. 41: 160-168.

De Schrijver K, Maes I, De Man L, Michelet J. (2002) An outbreak of diarrhoeic shellfish poisoning in Antwerp, Belgium. Eurosurveillance 7, 139-141.

Seki T, Satake M, MacKenzie L, Kaspar HF, Yasumoto T (1995) Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. Tetrahedron Lett. 36: 7093-7096.

Shimizu Y, Chou HN, Bando H, Van Duyne G, Clardy JC (1986) Structure of brevetoxin A (GB-1 toxin), the most potent toxin in the Florida red tide organism *Gymnodinium breve* (*Ptychodiscus brevis*). J. Am. Chem. Soc. 108: 515-516.

Sommer H, Meyer KF (1937) Paralytic Shellfish Poisoning. A.M.A. Arch. Path. 24: 560-598.

Suzuki T, Igarashi T, Ichimi K, Watai M, Suzuki M, Ogiso E, Yasumoto T (2005) Kinetics of diarrhetic shellfish poisoning toxins, okadaic acid, dinophysistoxin-1, pectenotoxin-6 and yessotoxin in scallops *Patinopecten yessoensis*. Fish. Sci. 71: 948-955.

Swanson K, Villareal T, Campbell L (2008) The 2008 DSP Event along the Texas Coast: Detection and Observations. Poster presentation at the 122nd AOAC Annual Meeting and Exposition, 21 - 25 September 2008, Dallas, Texas, US.

Tachibana K, Scheuer PJ, Tsukitani Y, Kikuchi H, Van Engen D, Clardy J, Gopichand Y, Schmitz FJ (1981) Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. J. Am. Chem. Soc. 103: 2469-2471.

Takada N., Umemura N., Suenaga K., Uemura D. (2001) Structural determination of pteriatoxins A, B and C, extremely potent toxins from the bivalve *Pteria penguin*. Tetrahedron Lett. 42: 3495-3497.

Takai A., Mieskes G. (1991) Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases. *Biochem. J.* 275: 233-239.

Takemoto T., Daigo K. (1958) Constituents of *Chondria armata*. *Chem. Pharm. Bull.* 6: 578-580

Takemoto T., Daigo K. (1960) Über die Inhaltsstoffe von *Chondria armata* und ihre pharmakologische Wirkung. *Archiv. der Pharmazie* 293: 627-633.

Taleb H., Vale P., Amanhir R., Benhadouch A., Sagou R., Chafik A. (2006) First detection of azaspiracids in North West Africa. *J. Shell. Res.* 25: 1067-1071.

Teitelbaum J.S., Zatorre R.J., carpenter S, Gendron D., Evans A.C., Gjedde A., Cashman N.R. (1990) Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. *N. Engl. J. Med.* 322: 1781-1787.

These A., Scholz J., Preiss-Wiegert 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 (21), 4529-4538.

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.

Torgersen T, Miles CO, Rundberget T, Wilkins AL (2008) New Esters of Okadaic Acid in Seawater and Blue Mussels (*Mytilus edulis*). *J. Agric. Food Chem.* 56: 9628-9635.

Tubaro A, Sosa S, Carbonatto M, Altinier G, Vita F, Melato M, Satake M, Yasumoto T (2003) Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. *Toxicon* 41: 783-792 .

Tubaro A, Sosa S, Altinier G, Soranzo MR, Satake M, Della Loggia R, Yasumoto T (2004) Short-term oral toxicity of homoyessotoxins, yessotoxin and okadaic acid in mice. *Toxicon* 43: 439-445.

Twiner M.J., Hess P., Bottein Dechraoui Y., McMahon T., Ramsdell J.S., Samsons M.S., and Doucette G.J. (2005) Cytotoxic and Cytoskeletal Effects of Azaspiracid-1 on Mammalian Cell Lines. *Toxicon* 45, 891-900.

Twiner MJ, Rehmann N, Hess P, Doucette GJ (2008a) Azaspiracid Shellfish Poisoning: A review on the ecology, chemistry, toxicology and human health impacts. *Marine Drugs* 6: 39-74.

Twiner M.J., Ryan J.C., Morey J.S., Van Dolah F.M, Hess P., McMahon T., Doucette G.J. (2008b) Transcriptional profiling and inhibition of cholesterol biosynthesis in human T lymphocyte cells by the marine toxin azaspiracid. *Genomics* 91, 289-300.

Vale P., Bire R., Hess P. (2008) Confirmation by LC-MS-/MS of azaspiracids in shellfish from the Portuguese northwestern coast. *Toxicon* 51, 1449-1456.

Uemura D, Ueda K, Hirata Y, Naoki H, Iwashita T (1981) Further studies on palytoxin.1. *Tetrahedron Lett.* 22: 1909-1912.

Uemura D, Ueda K, Hirata Y, Naoki H, Iwashita T (1981) Further studies on palytoxin.2. Structure of palytoxin. *Tetrahedron Lett.* 22: 2781-2784.

Uemura D, Chou T, Haino T, Nagatsu A, Fukuzawa S, Zheng S, Chen H (1995) Pinnatoxin A: a toxic amphoteric macrocycle from the Okinawan bivalve *Pinna muricata*. *J. Am. Chem. Soc.* 117: 1155-1156.

Uemura D, Hirata Y, Iwashita T, Naoki H (1985) Studies on palytoxins. *Tetrahedron* 41: 1007-1017.

Ueoka R, Ito A, Izumikawa M, Maeda S, Takagi M, Shin-ya K, Yoshida M, van Zoest RWM, Matsunaga S (2009) Isolation of azaspiracid-2 from a marine sponge *Echinoclathria* sp. as a potent cytotoxin. *Toxicon* 53: 680 – 684.

UK-COT (The Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment), 2006. Statement on risk assessment of marine biotoxins of the okadaic acid, pectenotoxin, azaspiracid and yessotoxin groups in support of human health. Available at URL: <http://www.food.gov.uk/multimedia/pdfs/cotstatementlipophilic200616.pdf> , accessed 14/11/2009.

UK-NRL (2001) “Domoic Acid in the King Scallop *Pecten maximus*”, confidential report prepared for the EU ASP working group by the UK National Reference Laboratory; January 2001, 85pp.

Underdal B, Yndestad M, Aune T. (1985) DSP intoxication in Norway and Sweden, Autumn 1984- Spring 1984. Anderson, D. M., White, A. W., and Baden, D. G. Toxic Dinoflagellates, 489-494. Amsterdam, Elsevier.

Vale P. (2007) Chemistry of diarrhetic shellfish poisoning toxins. In: LM Botana (ed.): Chemistry and pharmacology of marine toxins, Blackwell Publishing, Oxford, 211-221.

Vale P., Bire R., Hess P. (2008) Confirmation by LC-MS-MS of azaspiracids in shellfish from the Portuguese northwestern coast. *Toxicon* 51, 1449-1456.

Valko K. (2004) Application of high-performance liquid chromatography based measurements of lipophilicity to model biological distribution: Estimation of Physicochemical Properties by Chromatographic and Electrophoretic Techniques. *J. Chromatogr. A* 1037, 299-310.

Van Dolah, F. M. (2000) Marine algal toxins: Origins, health effects, and their increased occurrence. *Environ. Health Perspect.*, 108, 133–141.

VCCLab, 2008. Virtual Computational Chemistry Laboratory. Available at www.vcclab.org (last visit in Sept 08)

Virchow R (1885) Über die Vergiftungen durch Miesmuscheln in Wilhelmshaven. *Berliner Klinische Wochenschrift* 48: 1-2.

Walter JA, Falk M, Wright JLC (1994) Chemistry of the shellfish toxin domoic acid: characterization of related compounds. *Can. J. Chem.* 72: 430-436.

Walter JA, Leek DM, Falk M (1992) NMR study of the protonation of domoic acid. *Can. J. Chem.* 70: 1156-1161.

Wang Z, Plakas SM, El Said KR, Jester ELE, Granade HR, Dickey RW (2004) LC/MS analysis of brevetoxin metabolites in the Eastern oyster (*Crassostrea virginica*). *Toxicon*, 43: 455-465.

Wilkins AL, Rehmann N, Torgersen T, Rundberget T, Keogh M, Petersen D, Hess P, Rise F, Miles CO, (2006) Identification of fatty acid esters of pectenotoxin-2 seco acid in blue mussels (*Mytilus edulis*) From Ireland. *J. Agric. Food Chem.* 54: 5672-5678.

Wolff M. (1887) Ueber das erneute Vorkommen von giftigen Miesmuscheln in Wilhelmshaven. *Virchows Arch.* 110: 376-380.

Wong JL, Oesterlin R, Rapoport H (1971) The structure of saxitoxin. *J. Am. Chem. Soc.* 93: 1238-1239.

Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS, Jackson JBC, Lotze HK, Micheli F, Palumbi SR et al. (2006) Impact of biodiversity loss on ocean ecosystem services. *Science* 314: 787-790.

- Wright JLC, Falk M, McInnes AG, Walter JA (1990) Identification of isodomoic acid D and two new geometrical isomers of domoic acid in toxic mussels. *Can. J. Chem.* 68: 22-25.
- Yanagi T, Murata M, Torigoe K, Yasumoto T (1989) Biological activities of semisynthetic analogs of dinophysistoxin-3, the major diarrhetic shellfish toxin. *Agric. Biol. Chem.* 53: 525-529.
- Yasumoto, T., 1990. Recent progress in the chemistry of dinoflagellate toxins. In: Granéli, E. Sündstrom, E. B., Elder, E. & Anderson, D. M. (Eds.), *Toxic Marine Phytoplankton*. pp. 259-27.
- 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.
- Yasumoto T, Oshima Y, Sugarawa W, Fukuyo Y, Oguri H, Igarashi T, Fujita N (1980) Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. *Bull. Japan Soc. Sci. Fish* 46: 1405-1411.
- Yasumoto T, Murata M, Lee JS, Torigoe K (1989) Polyether toxins produced by dinoflagellates. In: *Mycotoxins and Phycotoxins '88* Natori S, Hashimoto K, Ueno Y (eds.), 375-382.
- Yasumoto T, Murata M, Oshima Y, Sano M (1985) Diarrhetic shellfish toxins. *Tetrahedron* 41: 1019-1025.
- Yasumoto T, Nakajima I, Bagnis R, Adachi R (1977) Finding of a dinoflagellate as a likely culprit of ciguatera. *Bull. Jap. Soc. Sci. Fish.* 43: 1021-1026.
- Zaman L, Arakawa O, Shimosu A, Onoue Y, Nishio S, Shida Y, Noguchi T, (1997) Two new isomers of domoic acid from a red alga, *Chondria armata*. *Toxicon* 35: 205-212.

7. French Summary of “HDR”

7.1. Introduction

Les efflorescences d’algues toxiques – un phénomène croissant ?

Le terme phycotoxines décrit des métabolites naturels produits par des algues unicellulaires, donc des protistes. La plupart des phycotoxines sont synthétisées par des dinoflagellés, à l’exception de l’acide domoïque qui est produit par des diatomées du genre *Pseudo-Nitzschia*. Les saxitoxines sont certes synthétisées par des dinoflagellés mais elles le sont également par des cyanobactéries. Initialement, plusieurs phycotoxines ont été associées à d’autres organismes que des micro-algues, par exemple l’acide okadaïque qui a été isolé de l’éponge *Halichondria okadaii* (Tachibana et al., 1981), l’acide domoïque qui elle a été identifiée dans la macro-algue rouge *Chondria armata* (Daigo et al., 1959a et b ; Takemoto et Daigo, 1958 et 1960), ou encore la palytoxine qui a été trouvée dans le zoanthaire colonial corallien *Palythoa toxica* (Moore et Scheuer, 1971).

Par accumulation dans la chaîne alimentaire, ces toxines peuvent se concentrer dans une variété d’organismes marins, tels que des bivalves filtreurs, des organismes fouisseurs et brouteurs (tuniciers et gastéropodes) ainsi que des poissons herbivores ou encore prédateurs. Ce mémoire traitera des toxines hydrophiles et lipophiles, plus particulièrement des toxines appartenant aux groupes de l’acide domoïque, des saxitoxines, de l’acide okadaïque et ses analogues, des azaspiracides, des pecténotoxines et des yessotoxines. Ce sont essentiellement toutes les phycotoxines faisant actuellement l’objet d’une réglementation en Europe avec des seuils sanitaires spécifiques. Une attention particulière est apportée aux azaspiracides grâce au travaux que j’ai effectués concernant ce groupe de toxines qui était peu connu quand j’ai commencé mes recherches dans le domaine des phycotoxines en 1998.

Les empoisonnements dus à l’ingestion des fruits de mer contaminés par des phycotoxines ont été observés depuis longtemps, et les témoignages historiques ainsi que les habitudes de consommation de certaines populations dans les zones côtières et tropicales démontrent bien que les efflorescences d’algues toxiques (harmful algal blooms ou HAB) sont des phénomènes naturels (Hallegraeff, 2004). Durant les trente dernières années, les HAB ont attiré une attention croissante de la société et de la communauté scientifique. Les intoxications humaines dues aux fruits de mer contaminés ont impliqué parfois des dizaines ou même des centaines de personnes, et ont ainsi certainement contribué à un intérêt croissant pour ce problème de santé publique. Cette prise de conscience a alors abouti à plus de recherches dans ce domaine qui ont permis d’enrichir notre compréhension de la prolifération des algues toxiques, des processus de contamination des fruits de mer par les phycotoxines, ainsi que de la chimie et de la toxicologie de ces composés.

L’accumulation d’information dans ce domaine a amené à la conclusion que nous observons une augmentation globale des efflorescences d’algues nuisibles et de la contamination des fruits de mer. En corollaire des mesures plus complexes et effectives ont ainsi été développées et mises en place afin d’éviter des cas d’intoxications humaines par les phycotoxines. Des signalements de plus en plus fréquents d’algues toxiques sont certainement dus en partie à l’attention accrue portée à ces phénomènes. Par contre, d’autres facteurs accèdent le développement croissant des épisodes d’algues toxiques : leur apparition dans des zones où ces algues n’ont pas été signalées auparavant, ainsi que l’augmentation de la durée et de l’intensité de ces épisodes (Hallegraeff, 2004). Ces changements sont illustrés avec la tendance actuelle à l’augmentation d’*Ostreopsis* en

Méditerranée. Ces épisodes sont dans certains cas, comme en Italie en 2005, accompagnés d'effets nuisibles sur la santé de centaines de personnes, (Gallitelli et al., 2005 ; Durando et al., 2007).

Les facteurs susceptibles d'être impliqués dans l'accroissement global des HAB incluent l'eutrophisation des eaux côtières en tant que conséquence d'activités urbaines, aquacoles et agricoles accrues, ainsi que certaines activités économiques reliées à l'urbanisation, le changement climatique, les transferts d'algues toxiques ou de leur kystes dans les eaux de ballast des navires, ou encore les déplacements des stocks de coquillages (Hallegraeff, 2004). De plus, une méta-analyse récente de données publiées révèle qu'une diminution locale de la biodiversité et des équilibres écosystémiques favoriseraient l'apparition des efflorescences algales nuisibles (Worm et al., 2006).

Diversité chimique des toxines d'algue

Il faut d'abord réaliser que les toxines d'algues sont des composés naturels, par conséquent, il est normal qu'il y ait plusieurs systèmes enzymatiques capables de métaboliser les phycotoxines. Cette caractéristique différencie les toxines de manière générale des composés anthropogéniques tels que les biphényles polychlorés (PCB) ou certains pesticides pour lesquels la nature n'a pas prévu de voies métaboliques. Cependant, à l'instar des PCB, dioxines et hydrocarbures aromatiques polycycliques, la plupart de ces groupes de toxines ont également de nombreux analogues. Ainsi, entre les composés produits naturellement par les algues et leurs métabolites présents dans les autres organismes accumulant les toxines, les phycotoxines représentent un vaste assemblage de composés. En tant que produits naturels résultant d'une biosynthèse, ces composés appartiennent aux classes des acides aminés (acide domoïque), des alcaloïdes (saxitoxines et tétrodotoxines) et des polyketides (tous les autres).

Les toxines d'algues sont également souvent classées dans les petites molécules ce qui permet de les distinguer des polymères naturels (protéines, carbohydrates, acides désoxyribonucléiques). Effectivement, les poids moléculaires des phycotoxines varient typiquement entre 300 et 1500 da ; néanmoins, il y a aussi quelques exceptions comme les palytoxines (PITX) et les maitotoxines (MTX) qui sont des molécules de taille considérable de 2677 et 3422, respectivement. La maitotoxine a été décrite comme la plus grande toxine naturelle non-protéinique. De même, les phycotoxines se distinguent du large groupe des venins de serpent, d'araignée ou encore de coquillages (cônes), qui sont des biopolymères de taille moléculaire importante. Un résumé de quelques caractéristiques chimiques de ces composés est donné dans le tableau 1.1.1 (page 37).

La terminologie servant à décrire les différents composés est parfois confuse : le terme *analogue* est utilisé ici pour désigner, au sens le plus large, tous les composés qui ont le même squelette moléculaire de base, sans préjuger de leur origine. Le terme *dérivé* peut également comprendre tous les analogues, qu'ils soient issus du métabolisme ou de réactions chimiques artificielles. Le terme *métabolite* peut comprendre les analogues produits par les algues mêmes (il a alors le sens d'un métabolite primaire ou secondaire) ou bien les analogues formés par la métabolisation des toxines d'algues dans d'autres organismes vivants. Toutefois le terme métabolite reste réservé à des composés formés naturellement. Les *isomères* sont les analogues d'un composé ayant la même masse moléculaire mais présentant des changements de structure. On parlera de stéréo-isomères pour des différences stériques et d'isomères positionnels pour les différences de position des groupements fonctionnels dans la structure chimique.

Les malaises associées à la consommation des coquillages sont multiples et trouvent leur origine dans trois phénomènes principaux : les allergies aux coquillages mêmes, les infections bactériennes ou virales et les empoisonnements. Les symptômes, pour certains de ces phénomènes, peuvent être similaires, ainsi identifier l'origine du mal n'est pas trivial. Toutefois, les allergies peuvent être vérifiées par certaines techniques médicales tandis que le patient peut en apprendre plus en cumulant l'expérience acquise en consommant des coquillages. Les infections bactériennes et virales ont généralement besoin d'une période d'incubation avant de se manifester (12 à 24 h), tandis que les empoisonnements se déclarent au cours de la digestion (2 à 4 h), voire plus rapidement pour certaines toxines.

Contrairement à l'empoisonnement par histamine provenant de la consommation de poissons avariés, les empoisonnements dus aux biotoxines marines ne peuvent pas être anticipés par les consommateurs en regardant l'aspect des coquillages ou des poissons car ceux-ci peuvent être en parfait état de fraîcheur tout en étant hautement contaminés. Selon les symptômes observés, les toxines ont été classées en plusieurs groupes distincts : les toxines amnésiantes, les toxines diarrhéiques, les toxines du complexe ciguatera, les neurotoxines du type brevetoxine et du type palytoxine et les toxines paralysantes. Les toxines diarrhéiques (AZA et OA) engendrent des troubles digestifs multiples, tels que crampes d'estomac, vomissements, diarrhées, ainsi que des symptômes secondaires tels que déshydratations et maux de tête. Les toxines amnésiantes provoquent les mêmes effets que les toxines diarrhéiques mais en plus, elles causent aussi des effets neurologiques qui incluent la perte permanente de la mémoire courte. Les neurotoxines du type brevetoxine (BTX) constituent un double risque : elles peuvent être absorbées par voie respiratoire ou par la peau (lors de l'exposition sur une plage) mais aussi ingérées via la consommation de coquillages. Dans ce dernier cas, ces toxines sont à la fois diarrhéiques et neurotoxiques (paresthésie circumorale et inversion des sensations de chaud et de froid), tandis que les effets subis par l'exposition sur les plages peuvent comprendre des troubles respiratoires, surtout observés chez les personnes sensibles telles que les asthmatiques, ainsi que des troubles dermatologiques (eczémas, irritation des muqueuses etc.). Il semble que les effets des palytoxines peuvent également être engendrés par ces deux voies (exposition respiratoire /dermatologique et voie orale), mais l'étendue des effets est moins bien connue et le mécanisme d'action des palytoxines n'est pas complètement élucidé. Il en est de même pour le complexe ciguatérique (CTX) rencontré suite à la consommation de poissons contaminés dans les zones tropicales et subtropicale qui, en plus des troubles digestifs, a de nombreux effets à long terme mais le périmètre des symptômes est aussi mal défini que les agents qui causent les effets. Les toxines paralysantes (STX et TTX) peuvent être mortelles et l'apparition des symptômes peut être rapide (< 30 min). Ce sont des neurotoxines dont les symptômes d'intoxication varient depuis des picotements des lèvres et de la bouche jusqu'à la paralysie des membres et de l'appareil respiratoire, ce qui entraîne la mort en cas d'intoxication grave.

Il n'existe aucun antidote pour les phycotoxines, bien qu'une piste prometteuse ait été obtenue par Bourdelais et al. (2004) dans le cas des brévétotoxines. Pour les toxines paralysantes, les traitements, suites à de fortes expositions, doivent être immédiats. Ils incluent la vidange gastrique, afin d'éliminer le plus rapidement possible les toxines ainsi que la mise sous respiration artificielle pour éviter la mort par asphyxie. Le médecin généraliste a donc une responsabilité importante dans la reconnaissance des symptômes et joue un rôle clé pour le signalement des toxi-infections alimentaires collectives (TIAC).

Alors que certaines toxines présentent des effets aigus réversibles, en dehors de l'effet létal à fortes doses (STX, TTX), d'autres composés peuvent engendrer des effets à long terme (DA, BTX, CTX). De plus, l'existence d'effets chroniques n'a pas été clarifiée pour plusieurs groupes (AZA, OA, PITX). Le procédé d'évaluation de risque est typiquement déclenché s'il y a un problème de salubrité ou de santé publique persistant ou s'il y a un doute sur d'éventuels effets d'un agent pathogène ou toxique. Le Codex Alimentarius a ainsi défini trois phases dans l'analyse du risque :

1. Evaluation du risque
2. Gestion du risque
3. Communication sur le risque

La gestion préventive ou curative du risque (alimentaire ou environnementale) est généralement basée sur la gravité du risque et sa fréquence. Pour établir ces deux paramètres, la plupart des gestionnaires font appel à un organisme indépendant d'évaluation du risque. En France, à l'échelle locale, les préfets ont un rôle de gestionnaire au quotidien pour ce qui est de la fermeture et de l'ouverture des zones de production de coquillage ou des plages. Ils font typiquement appel à des missions inter-services pour la sécurité alimentaire (MISSA) pour approfondir leurs connaissances des problèmes posés par les coquillages. A l'échelle nationale, le gouvernement est le gestionnaire du risque : Direction Générale de la Santé (DGS), et Direction Générale de l'Alimentation (DGAL). Les organismes d'évaluation des risques sont l'Agence Française de la Sécurité Sanitaire des Aliments (Afssa) et l'Agence Française pour la Sécurité Sanitaire de l'Environnement et du Travail (Afsset) ; ces deux institutions venant de fusionner en 2010. A l'échelle européenne, la Direction Générale de la Santé des Consommateurs (DG Sanco) gère les risques et est aussi l'acteur principal dans la formulation des législations relatives à la salubrité des coquillages. Dans le domaine de l'alimentaire, la plupart des lois sont directement applicables par chaque Etat membre, certains règlements nécessitent toutefois une traduction en loi nationale pour devenir effectifs. Pour se faire assister dans l'évaluation du risque, la DG Sanco peut faire appel à l'Agence Européenne de la Sécurité Alimentaire (European Food Safety Authority, EFSA). La DG Sanco agit aussi à l'échelle internationale pour obtenir des compromis avec les autres nations. L'organe qui fait autorité dans le domaine alimentaire est le *Codex alimentarius*. Le Codex se base sur deux institutions complémentaires : l'organisation mondiale de la santé (OMS ou WHO World Health Organisation) et l'organisation mondiale de du commerce (OMC ou WTO World Trade Organisation). L'OMS peut organiser des groupes de travail pour l'évaluation du risque, comme elle l'a fait pour les biotoxines marine en 2004.

Il faut également rappeler que le procédé d'analyse de risque est par définition de nature itérative. Une première évaluation de risque pour un composé va typiquement aboutir à des recommandations demandant aux gestionnaires d'accumuler le plus d'information possible sur le sujet. Ensuite les mesures de gestion de risque vont être mises en place pour générer ces informations. Après obtention d'informations supplémentaires, l'évaluation de risque entrera dans une deuxième phase quantitative qui peut permettre d'établir des limites maximales de contaminations à ne pas dépasser. Les mesures de gestions peuvent alors être revues afin d'améliorer la salubrité et faciliter le commerce libre. Le plus souvent, il est nécessaire de réaliser plusieurs cycles d'obtention d'information, d'évaluation de risque et de mise en place de mesures de gestion avant qu'un problème puisse être géré de manière efficace.

La méthodologie pour détecter le composé impliqué fait souvent partie de chaque cycle : dans un premier temps les méthodes sont souvent rudimentaires et ni spécifiques, ni quantitatives. Si un danger est avéré, les méthodes de détection doivent être améliorées, des étalons doivent être

obtenus et les méthodes doivent être soumises à des protocoles de validation inter-laboratoires. Une fois que les méthodes mises en place permettent de détecter le ou les composés de manière quantitative et que le danger est établi afin que le risque puisse être géré, la méthodologie aura alors besoin d'un outil de contrôle qualité approfondi, tels que des matériaux de référence certifiés. La caractérisation complète des dangers comprend une description de la stabilité des composés afin d'établir leur persistance dans l'environnement et dans le processus de digestion humaine.

Pour mettre mes différents travaux scientifiques en perspective, j'ai choisi de les présenter selon leur contribution au processus de caractérisation des dangers, pour l'évaluation des risques posés par les biotoxines marines. Ainsi, il y a plusieurs chapitres en relation avec la méthodologie permettant de détecter les toxines, de manière spécifique et quantitative : les développements de méthodes et le contrôle qualité pour ces méthodes. La distribution des toxines dans l'environnement marin influence bien sûr l'étendue des dangers. Les questions posées sont : quelles sont les espèces de coquillage touchées, quelle est l'étendue géographique des algues toxiques et de leurs toxines, quels facteurs influencent leur apparition ? L'isolement des composés bioactifs joue un rôle majeur dans la mise en place des méthodes quantitatives et spécifiques, mais il est également nécessaire d'obtenir des composés purifiés afin de pouvoir tester leur potentiel toxique et leurs toxicités relatives parmi les différents analogues impliqués. Le contrôle qualité a besoin de nombreux outils qui doivent être mis en place de manière efficace pour le contrôle qualité interne: des étalons certifiés et des matériaux de référence. Les essais d'aptitude sont un exemple de contrôle qualité externe. Finalement, j'ai aussi décrit quelques études caractérisant la stabilité et la réactivité chimique des toxines, leur lipophilicité, ainsi que l'investigation de leur toxicité et de leur mécanisme d'action.

7.2. Développement des méthodes

Dans le domaine des méthodes pour les saxitoxines, une avancée majeure a été obtenue par Sommer et Meyer (1937) avec le développement d'un test souris pour les toxines paralysantes. Ce test est basé sur l'extraction des composés hydrophiles avec l'acide chlorhydrique, et l'injection des extraits filtrés et neutralisés dans la cavité intrapéritonéale des souris, qui sont alors observées pendant 15 min afin de déterminer la concentration de toxines présentes. Bien que ces toxines n'aient été caractérisées que durant les années 1960, le test a été validé en 1990 par l'AOAC, quasiment dans sa forme d'origine excepté que l'étalonnage est effectué avec des étalons mieux caractérisés.

De façon analogue pour les toxines lipophiles, des essais sur souris ont été développés durant les années 1950 pour les neurotoxines du type brevetoxine (McFarren, 1959), et ensuite par Yasumoto et al. (1979 et 1985) pour les toxines diarrhéiques, plus particulièrement l'acide okadaïque et ses analogues, les dinophysistoxines. Cette dernière procédure a également été évaluée pour sa capacité à détecter les azaspiracides à partir de certaines concentrations (Hess et al., 2009). De plus, un bioessai sur rat a été développé par Kat et al. (1978) mais ce test ne peut détecter que des toxines diarrhéiques, essentiellement l'acide okadaïque et analogues et les azaspiracides. Il n'a cependant pas été caractérisé en terme de performance (limite de détection, reproductibilité).

Ainsi, quatre groupes de toxines peuvent être détectés par des bio-essais (AZA, BTX, OA, STX). Néanmoins, les bio-essais pour les toxines lipophiles ne sont pas quantitatifs et non pas été validés lors d'exercices inter-laboratoires. Ces tests ne sont pas spécifiques et souffrent également d'interférences par d'autres composés lipophiles bioactifs, dont on ne connaît pas bien l'importance pour la santé publique. Les toxicités des composés lipophiles varient de manière significative, et la limite de détection par les bio-essais ne permet donc pas de déterminer la toxicité en fonction des limites à respecter d'un point de vue salubrité ou réglementaire. Enfin, les bio-essais sur animaux pour les toxines lipophiles sont contestés du point de vue de la protection des animaux : les souris sont soumises à des souffrances pendant 24 h et les rats pendant 16 h. Le bio-essai sur souris pour les toxines paralysantes est également soumis à des questions d'éthique car les animaux souffrent clairement de douleurs. Du fait de ces limites propres aux bio-essais, les scientifiques et les gestionnaires cherchent aujourd'hui des méthodes alternatives aux tests sur animaux (Hess et al., 2006).

En conséquence, mes travaux ont porté en grande partie sur le développement de méthodes physico-chimiques ainsi que sur l'évaluation des paramètres critiques affectant la performance de ces méthodes.

Spectrométrie de masse couplée à la chromatographie liquide pour les saxitoxines

Les méthodes physico-chimiques pour la détection des saxitoxines, telles qu'elles étaient développées jusqu'ici, étaient basées sur la séparation par chromatographie liquide car les saxitoxines sont solubles dans l'eau et non-volatiles. Du fait de leur forte polarité, les STX doivent être accompagnées par des agents formant des couples d'ions pour avoir suffisamment de rétention en chromatographie en phase inverse (Oshima, 1995). Si cette pratique n'est pas appliquée, la chromatographie ne permet pas de séparer les épimères GTX1/4 ou GTX2/3 (Lawrence et al., 2004). Comme les saxitoxines n'ont pas de chromophore, la détection est basée sur une dérivation (généralement par oxydation chimique) suivi par une détection fluorimétrique.

Un des problèmes des agents de couplage d'ions est le fait que ces phases mobiles ne sont pas faciles à évaporer et que les couple d'ions ne s'ionisent pas si facilement pour la détection par spectrométrie de masse, ce qui entraînerait que ces méthodes SM auraient une faible sensibilité.

Un premier travail, en collaboration avec un groupe en Allemagne, a consisté à développer un réacteur électrochimique pour l'oxydation en ligne des saxitoxines, suivi par une détection soit fluorimétrique soit par spectrométrie de masse (Jaime et al., 2001). Nous avons évalué une séparation basée sur la chromatographie ionique qui permet d'éviter les agents de couplage d'ions et de laisser espérer obtenir des limites de détection plus basses. De plus, nous avons utilisé un gradient qui permettait de séparer tous les types de saxitoxines en une fois, sans respect de leur charge (dérivés carbamoyles, sulfocarbamoyles et décarbamoyles). Les épimères ont également pu être séparés. Néanmoins la méthode présentait encore des inconvénients majeurs : la séparation était longue (> 60 min) et la sensibilité n'était pas suffisante dans un contexte d'analyse réglementaire si le spectromètre de masse était utilisée comme détecteur. Ce manque de sensibilité était probablement lié au fait qu'un détecteur quadripôle simple avait été utilisé durant l'étude, et que la ligne de base était perturbée du fait d'un ratio masse/charge (m/z) faible des saxitoxines (300 à 500) soit dans une région de spectre où il y a beaucoup d'ions.

Dans une collaboration tripartite (avec NRCC et Université Federico II, Naples), nous avons pu améliorer la méthodologie en utilisant une chromatographie développée plus récemment : la chromatographie à interactions hydrophiles et lipophiles, ou en anglais Hydrophilic Lipophilic Interaction Chromatography, HILIC (Dell'Aversano et al., 2005). Cette chromatographie est basée sur des phases stationnaires greffées à la fois avec des molécules lipophiles et avec des molécules hydrophiles. Pour avoir des gradients équivalents aux gradients en phases inverses il faut alors diminuer la partie organique durant le gradient mais la séparation optimale a été obtenue en mode isocratique. Afin d'optimiser cette chromatographie, une étude systématique a été conduite sur l'influence du pH, de la température, de la concentration en tampon et en pourcentage organique sur la sélectivité, la rétention et la sensibilité. Dans les conditions optimales, tous les analogues peuvent être suffisamment séparés en 30 min, soit un avantage majeur pour cette chromatographie. De plus, en utilisant la spectrométrie de masse en tandem (API-4000), la sensibilité de la méthode a pu être améliorée de manière à obtenir une sensibilité équivalente ou meilleure que la détection par fluorimétrie, dépendant de l'analogue en question. Certains paramètres critiques sont intéressants pour mettre en place ce type de méthode dans le cadre de l'analyse de routine pratiquée pour les contrôles officiels. Néanmoins, les temps de rétention sont légèrement affectés par la matrice coquillage et la méthode n'a pas encore été évaluée de manière quantitative pour les effets de matrice des différents espèces de coquillage. En effet, le nombre de laboratoires à l'échelle internationale disposant d'appareils de sensibilité suffisante est faible dans le domaine des phycotoxines, c'est pourquoi une étude inter-laboratoire pour la validation de cette méthode n'a pas pu être menée à terme.

Spectrométrie de masse couplée à la chromatographie liquide pour les toxines lipophiles

Contrairement aux saxitoxines, la sensibilité des détecteurs de spectrométrie de masse est généralement considérée comme suffisante pour la détection des toxines lipophiles à des concentrations inférieures aux limites réglementaires actuelles. De plus, les chromatogrammes obtenus par la spectrométrie de masse tandem (LC-MS-MS) ne montrent pratiquement pas d'interférence, donc l'interprétation est simple par rapport aux chromatogrammes obtenus par la détection en fluorescence. Grâce à ces avantages, la LC-MS-MS a été reconnue comme étant « la technique par excellence » pour les toxines lipophiles.

Un première méthode par détection simple quadripôle a pu être développée en collaboration avec le NRCC (Quilliam et al., 2001). Cette méthode a été conçue pour la détection rapide de plusieurs groupes de toxines. Ce développement a permis au laboratoire national de référence en Grande-Bretagne de se familiariser avec la technologie et de mettre en place une aide à l'interprétation pour la surveillance de routine par le test souris. Ensuite, j'ai changé de poste pour mettre en place cette technologie en Irlande, où le besoin de connaître les profils toxiques était encore plus élevé, du fait des rejets massifs de coquillages exportés en 1999 vers la France. Comme à l'époque, il n'y avait pas de méthode validée par des essais inter-laboratoires pour l'analyse des biotoxines marines par LC-MS, la seule possibilité d'obtenir une accréditation pour cette méthode était une validation en intra-laboratoire. Durant 18 mois, la méthode mise en place en Irlande a donc été validée selon les critères de l'ISO 17025 et ceux de l'organisme d'accréditation national Irlandais (Irish National Accreditation Board ou INAB) pour les paramètres : périmètre, spécificité, sélectivité, LOD, LOQ, gamme linéaire, robustesse, justesse et fiabilité (répétabilité et reproductibilité). Un périmètre réduit à été choisi au départ (OA, DTX2, AZA1, -2 et -3), puis, par la suite, la DTX1 et les esters des DTX ont été rajoutés. Certains des paramètres ont été décrits rapidement (périmètre, spécificité, sélectivité, LOD, LOQ, gamme linéaire, robustesse) tandis que d'autres paramètres (justesse, fiabilité et comparabilité entre laboratoires) ont nécessité des travaux supplémentaires. L'étude initiale de la robustesse a clarifié les conditions optimales concernant certains facteurs tel que la quantité et la nature du solvant d'extraction, la masse de d'échantillon à extraire, la durée et la vitesse d'extraction et de centrifugation. Ces paramètres avaient peu d'influence sur le résultat aussi longtemps qu'ils étaient maintenus dans des limites établies. Toutefois, l'évaluation de la robustesse a nécessité de vérifier de nombreux facteurs techniques spécifiques au modèle de spectromètre utilisé (température de source, flux des gaz, voltages etc.) et à la chromatographie liquide (type et configuration des colonnes, âge de la colonne, utilisation de pré-colonne etc.). Finalement, le paramètre décrivant le mieux la robustesse a été choisi comme étant la stabilité de la réponse à travers un lot d'analyse, mesurée en tant que pente des courbes d'étalonnage de parenthèse (avant et après le lot d'analyse). Des différences de pente inférieures à 20% ont été considérées acceptables pour une bonne utilisation de la méthode. La performance a également été vérifiée par l'analyse d'un matériau de référence produit en intralaboratoire pour chaque lot d'analyse. La justesse de la méthode ainsi que la comparabilité entre laboratoires ont été vérifiées dans des études séparées sur les matériaux de référence et les essais d'aptitude (voir les sections 4.5 et 7.5 pour plus de détails).

Dans le cadre du projet BIOTOX, j'ai pu entreprendre des études systématiques sur les paramètres critiques affectant l'analyse des toxines lipophiles par LC-MS avec plusieurs laboratoires. Certains des résultats n'ont pas encore été publiés dans des revues à comité de lecture mais ils sont présentés ici pour avoir une image plus complète des travaux menés. Des rapports de projet ont été préparés et fournis aux partenaires et à la commission européenne (BIOTOX, 2007, 2008a, b et c).

Parmi les conditions chromatographiques, les paramètres évalués les plus importants ont été le mode (gradient ou isocratique), l'acidité de la phase mobile et la pression (HPLC, UHPLC).

Comme pour les toxines hydrophiles, le pH est un facteur primordial pour la sélectivité de la séparation. La PTX2 qui peut être ionisée en mode positif ou négatif co-élue à un pH acide typiquement avec une autre toxine, par exemple la DTX1. Si l'on change le pH de la phase mobile en alcalin, cette toxine peut être retenue jusqu'après l'élution des AZAs, optimisant ainsi la sélectivité de la séparation. Ce différentiel de sélectivité peut être utile dans l'utilisation des

instruments qui ne sont pas adaptés aux changements rapides de la polarité de l'ionisation, tels les instruments d'Applied Biosystems.

Un autre facteur important dans l'analyse par spectrométrie de masse est l'ionisation. Dans la source d'ions qui lie la chromatographie avec le spectromètre de masse, la phase mobile doit être évaporée et les composés d'intérêt doivent être ionisés afin de pouvoir entrer dans le spectromètre de masse. L'évaporation et l'ionisation dépendent fortement de la totalité des composés dissous dans la phase mobile ainsi que des analytes eux-mêmes. Plus il y a de composés dissous en solution, plus il sera difficile d'évaporer le solvant, en particulier si la phase mobile contient des lipides non-volatiles. Comme les coquillages contiennent souvent jusqu'à 2 % de lipides qui sont co-extraits avec les toxines lipophiles, l'espèce de coquillage et la concentration de matrice dans l'extrait vont affecter l'évaporation et donc l'ionisation.

Dans l'étude des transitions (BIOTOX, 2007), nous avons étudié d'une part l'effet du mode d'ionisation et du choix des transitions sur le résultat, et d'autre part l'effet de la concentration en matrice « moule » dans l'extrait sur le résultat. Six laboratoires ont participé à cette étude, permettant ainsi d'obtenir une idée de la variabilité des effets étudiés selon les laboratoires et selon différents instruments. Nous avons étudié deux concentrations de matrice : un ratio solvant/matrice de 5 et de 10 (5 ou 10 mL d'extrait / g de matrice). Pour OA et PTX2, nous avons utilisé le mode d'ionisation positif et le mode négatif, pour AZA que le mode positif et pour YTX que le mode négatif (section 4.2.2.1). Pour l'OA en mode positif nous avons trouvé qu'il y avait toujours plus d'effets de matrice en MS simple qu'en MS tandem, l'effet de matrice se traduisant par une augmentation du signal. Cette surestimation des concentration en OA en mode positif a été confirmée en mode de double transition. La matrice plus concentrée (ratio solvant/matrice 5) a aussi montré plus de variabilité parmi les résultats des différents laboratoires, confirmant ainsi que le paramètre est important à standardiser dans une méthode validée en inter-laboratoire. Des différences extrêmes ont été observées pour la quantification de la DTX2, l'isomère de l'OA en utilisant le mode MS simple en positif. Ainsi nous avons pu recommander l'analyse en mode négatif, utilisant des extraits dilués et de la MS tandem avec deux transitions. Des tendances similaires ont pu être constatées pour les AZA et la YTX en ce qui concerne l'utilisation de la MS simple quadripôle. La PTX2 a montré une augmentation du signal et une forte variabilité entre laboratoire, indépendamment du mode d'ionisation. Cette étude a également permis d'évaluer la performance des différents types de spectromètre de masse, et nous en avons conclu que la spectrométrie basée sur les trappes d'ions classiques n'est pas appropriée pour la quantification des toxines lipophiles, notamment à cause de la sensibilité faible pour l'OA en mode négatif, relié à la règle du tiers pour les trappes d'ions classiques.

Une thèse de doctorat (Elie Fux, 2008), un diplôme de master (Mairead McElhinney, 2008) et un diplôme d'ingénieur (Daniela Rode, 2007), tous financés par BIOTOX, ont également permis d'évaluer différentes approches pour l'estimation des effets de matrice de plusieurs espèces de coquillages. Ces travaux ont été publiés en partie dans des revues à comité de lecture (Fux et al., 2007 et 2008).

Les trois approches étudiées sont : i) l'infusion de l'analyte en post-colonne, ii) l'ajout d'étalons à concentration fixe dans des extraits bruts à concentration en matrice variable et iii) l'ajout d'étalons à concentration variable dans des extraits bruts à concentrations en matrice fixes, aussi appelé méthodes des ajouts dosés ou encore courbe d'étalonnage en matrice. A l'exemple de l'azaspiracide-1, nous avons pu montrer les limites de la technique d'infusion post-colonne. D'abord, nous avons dû constater qu'on ne peut pas utiliser les conditions chromatographiques optimales car la ligne de base est affectée par l'ajout post-colonne, donc

l'évaluation reste limitée à des conditions de chromatographie en mode isocratique. Ensuite, nous avons remarqué que l'interprétation quantitative des effets observés en infusion post-colonne est impossible : nous n'avons pas vu d'interférence quelconque en infusion post-colonne tandis que les deux autres approches d'évaluation d'effets de matrice ont montré qu'il y avait bien un effet de suppression de signal de l'ordre de 20 %. Aussi, nous avons abandonné cette approche pour les études ultérieures.

Comme mentionné ci-dessus, les deux approches ont donné des estimations d'effet de matrice très consistantes, avec les mêmes tendances observées par les deux techniques. Par exemple, l'augmentation de signal pour l'OA a été observée et dans les différentes concentrations de matrices et dans les étalons en matrice. Même si les effets de matrice varient avec l'espèce ou le traitement de la matrice (extraits de coquillages crus ou cuits), les tendances observées étaient toujours les mêmes avec les deux techniques : OA et les DTX ainsi que la PTX2 subissent toujours une augmentation de signal en présence de matrice, tandis que les AZA montrent toujours une diminution du signal, indépendamment des conditions.

Nous avons pu réduire les effets de matrice observés pour OA, AZA1 et PTX2, en utilisant la chromatographie à ultra-haute performance (UHPLC), mais cette amélioration n'était possible qu'avec une colonne C₈ et reste à vérifier par d'autres laboratoires (Fux et al., 2008). Il faut également noter que pour les AZA, les effets étaient quasiment éliminés alors que la même séparation n'apportait qu'une diminution d'un facteur 2 des effets de matrice pour les composés des groupes OA et PTX. Néanmoins, pour le groupe OA, la dilution des extraits a amélioré encore les effets observés pour donner moins de 10% de changement de signal, un pourcentage qui peut être maîtrisé et inclus dans la méthode standardisée si nécessaire. Nous avons également pu estimer la variabilité journalière des effets de matrice qui peut atteindre 15% de différence du signal.

Deux techniques de purification des extraits bruts ont été testées dans le cadre du projet BIOTOX : la purification par extraction sur cartouche (SPE) et la partition liquide-liquide (BIOTOX, 2008b ; Fux et al., 2008 ; Gerssen et al., 2009 ; McElhinney, 2007). La partition liquide-liquide a été examinée en premier avec des solvants non miscibles avec l'eau (dichlorométhane et isopropyl acétate). Le premier résultat clair est que la YTX et ses analogues ne peuvent être récupérés de manière quantitative dans la même fraction éluée que les autres toxines. Ainsi, cette purification complique l'analyse si l'on vise une analyse multitoxines. De plus, nous avons constaté des pertes significatives de toxines dans les procédés d'évaporation. Nous avons donc conclu que la partition n'est pas adaptée à l'analyse des toxines lipophiles. Les cartouches SPE ont également été examinées. Une méthode basée sur la purification avec des cartouches à phases mixtes a pu être validée en intra-laboratoire (Gerssen et al., 2009). Néanmoins, cette méthode n'a pas éliminé les effets de matrice pour l'analyse des toxines lipophiles réalisée dans un deuxième laboratoire. Il reste donc difficile de développer une purification pour un ensemble de composés avec une gamme de lipophilicité aussi large que les toxines appartenant aux groupes AZA, OA, PTX et YTX.

En somme, il faut constater que les effets de matrice restent difficiles à contrôler et dépendent de beaucoup de facteurs, y compris la séparation chromatographique, la phase stationnaire, l'instrument, l'espèce de coquillage et son traitement et la dilution de la matrice. La purification sur cartouche SPE et par partition liquide-liquide contribuent à la variabilité inter-laboratoire de telle façon que l'effort supplémentaire consenti durant l'analyse n'est pas valorisé. C'est pourquoi, de mon point de vue, la validation d'une méthode dans ce domaine sera un défi dans l'avenir et l'importance des essais d'aptitude jouera un rôle majeur dans la vérification de la performance des laboratoires, même si une méthode validée en inter-laboratoire peut être standardisée.

7.3. *Distribution des toxines dans l'environnement*

La distribution des toxines d'algues peut être décrite à différentes échelles : la distribution géographique et temporelle et la distribution à travers les niches de l'écosystème. Comme noté dans l'introduction, la distribution géographique peut être modifiée en fonction des changements globaux, néanmoins il y a aussi des situations qui permettent de détecter des distributions stables. Les données sur la distribution spatio-temporelle et géographique sont primordiales pour l'optimisation des programmes de contrôles officiels. A partir de connaissances approfondies sur les périodes d'apparition et sur la distribution géographique des algues toxiques et de leurs toxines, les programmes de surveillance peuvent être recentrés sur les zones et les périodes à risque. De cette façon, les moyens disponibles peuvent être optimisés afin de maximiser la productivité des zones conchylicoles. Ainsi, la surveillance réglementaire a permis de montrer que la plupart des toxines apparaissent en Europe durant l'été et l'automne. Une exception à cette règle est l'acide domoïque qui a tendance à apparaître plus tôt dans l'année, avec une efflorescence algale de printemps (mars à mai) le plus souvent dominée par les petites diatomées, y compris *Pseudo-nitzschia*. Mais il y a aussi d'autres exceptions telles que la présence hivernale de l'acide okadaïque en méditerranée (données REPHY, Ifremer), ou encore, l'apparition des azaspiracides en hiver (McMahon and Silke, 1996 et 1998).

L'approche écologique va contribuer à notre compréhension des transferts et des transformations à travers le réseau trophique. Dans ce domaine il est bien sûr important d'obtenir des réponses aux questions suivantes :

- Est-ce que les organismes producteurs de toxines sont invariablement directement ingérés par les coquillages ?
- Est-ce qu'il y a des différences dans l'accumulation des toxines selon les espèces de bivalves ?
- Est-ce que les biotransformations et la dégradation des toxines jouent un rôle majeur ?
- Est-ce que les toxines persistent dans l'eau de mer après l'efflorescence algale ?
- Est-ce que l'accumulation et la détoxification dépendent significativement de la disponibilité des toxines dissoutes dans l'eau de mer après les efflorescences ?

L'étude du genre *Dinophysis* est importante car cet organisme a affecté la production conchylicole presque partout dans le monde. Une exception à ce phénomène a été observée aux Etats-Unis où *Dinophysis acuminata* est observé sur la côte Est du pays mais ne provoque pas la toxicité dans les coquillages élevés à proximité. La question s'était alors posée de savoir si la souche américaine de *Dinophysis* n'était tout simplement pas capable de produire ces toxines ou bien si les conditions environnementales sur zones n'étaient pas réunies pour que l'organisme prolifère. Nous avons pu contribuer à une étude de nos collègues nord-américains par la mise en place d'une méthode analytique capable de détecter des faibles quantités de toxines. Les collaborateurs américains ont mis en place la culture du *D. acuminata* américain et ont produit de nombreuses cellules (> 100,000 cellules par échantillon) afin que leur contenu toxinique puisse être déterminé. Nous avons réussi à trouver des concentrations très faibles en toxines diarrhéiques dans cette souche. Pour l'acide okadaïque, la valeur la plus élevée était de 18 fg/cellule tandis que pour la DTX1, elle était de 203 fg/cellule. La plupart des valeurs rapportées par d'autres auteurs dans des souches prélevées sur le terrain ont été plus élevées que ces concentrations. Toutefois, nous avons ainsi pu établir que la souche américaine n'était pas complètement incapable de produire ces toxines bien que des questions restaient à élucider en ce qui concerne les conditions de culture et les conditions environnementales. Il est également intéressant de voir qu'un diol-ester de l'OA a pu être détecté ; une estimation de sa concentration basée sur l'aire sous le pic suggère que la concentration est

similaire à celle de la DTX1, tandis qu'aucun ester diol de la DTX1 n'a pu être détecté. D'autres travaux avec ce groupe de recherche sont en cours pour évaluer l'influence des paramètres de culture sur la production toxinique.

Des études sur l'occurrence des azaspiracides ont été menées en collaboration avec des groupes de recherche en Norvège et au Portugal (Hess et al., 2005 ; Vale et al., 2009). Nous avons ainsi pu établir que les AZA peuvent s'accumuler dans des coquillages provenant de zones variées concernant aussi bien l'Europe du Nord que l'Europe du Sud. D'autres auteurs ont également contribué à établir cette représentation (Taleb et al., 2006) mais l'étendue de la répartition des AZA avait jusque-là été principalement limitée à la côte Atlantique de l'Europe (Twiner et al., 2008a). Par contre, l'AZA2 (dérivé méthylé de l'AZA1) a aussi été trouvé dans une éponge échantillonnée au Japon (Ueoka et al., 2009). Il serait donc intéressant de savoir si c'est bien le dinoflagellé *Azadinium spinosum*, organisme producteur d'AZA trouvé en mer du Nord (Krock et al., 2009 ; Tillmann et al., 2009), qui est responsable de cette répartition mondiale ou si d'autres organismes ont aussi la capacité de produire ces toxines.

Peu de temps après l'introduction de l'analyse chimique en Irlande, comme méthode de routine pour la surveillance des mollusques bivalves dans le cadre des contrôles officiels, nous avons pu établir des tendances en ce qui concerne l'accumulation des azaspiracides et de l'acide okadaïque et ses analogues dans les coquillages élevés en Irlande : principalement les moules (*Mytilus edulis*) et les huîtres (*Crassostrea gigas*). En 2001, sur le plan national, les moules ont été nettement plus affectées par ces toxines lipophiles que les huîtres (Hess et al., 2003). Plus précisément, la limite légale a été dépassée dans 36 % des cas concernant les DTX dans les moules tandis que le dépassement était de 2 % pour les huîtres échantillonnées. De la même façon, pour les azaspiracides, le dépassement a été de 8,5 % pour les moules tandis que seulement 1,6 % des huîtres dépassait la limite légale. De même, les concentrations maximales étaient trois fois plus élevées dans les moules que dans les huîtres pour les azaspiracides, et six fois plus élevées dans les moules que dans les huîtres pour l'acide okadaïque et ses analogues. Ces tendances à l'échelle nationale ont aussi été confirmées à l'échelle locale sur les sites où les deux espèces sont co-cultivées. Ce différentiel entre les deux espèces pose une autre question : est-ce que la différence est seulement due à une différence de filtration (sélectivité plus élevée chez les huîtres) ou est-ce que le métabolisme est significativement différent entre ces deux espèces, ou encore est-ce que ce sont les deux facteurs ensemble sont responsables de la différence ?

Bien qu'il n'y ait pas de témoignages sur des intoxications de consommateurs par l'acide domoïque en Europe, cette toxine est plutôt répandue dans nos eaux. Au début de mes travaux sur les phycotoxines, j'ai contribué à l'évaluation de la distribution géographique de l'acide domoïque. Pour les eaux écossaises, nous avons pu démontrer en 1999 que la contamination des coquilles St. Jacques, *Pecten maximus* s'étendait sur 8 000 lieues², en particulier dans les « Minches », cette mer côtière comprise entre la principale île écossaise et les Hébrides intérieures (Gallacher et al., 2000 ; Hess et al., 2001a). Durant ces études nous avons également pu confirmer que l'acide domoïque s'accumulait quasi-exclusivement dans l'hépatopancréas de la coquille St. Jacques. Du fait d'une large variabilité des concentrations en toxines dans les parties comestibles des coquilles St. Jacques, nous avons également examiné de près les procédés de dissection et nous avons constaté que la contamination du muscle adducteur des coquilles St. Jacques pouvait venir d'une mauvaise dissection, soit d'une contamination par des fluides intestinaux de la coquille St. Jacques soit par la présence de restes d'organes non-séparés du muscle adducteur, tels que l'anus ou le rein (UK-FSA, 2001; Hess et al., 2000). En fonction de ces études, la législation européenne a été changée pour mettre en place des seuils réglementaires dans les parties comestibles des coquilles St. Jacques, et de même des limites maximales dans l'animal entier.

Cette réglementation devrait permettre d'utiliser la ressource des coquilles St. Jacques pour le mieux, et de ne pas mettre en marché des produits toxiques (Anonymous, 2002b).

Les mécanismes d'accumulation de l'acide domoïque dans les coquilles St. Jacques ne sont pas très clairs : les principaux organismes producteurs de l'acide domoïque sont certaines diatomées du genre *Pseudo-nitzschia*. Ces espèces sont pélagiques et ont besoin de lumière pour proliférer. D'autre part, les coquilles St. Jacques vivent sur des fonds marins à de grandes profondeurs d'eau, c.à.d. souvent au delà de 100 m. Dès lors la question se pose de savoir si les coquilles St. Jacques sont contaminées par des algues mortes qui tombent sur le fond (il y a des organismes qui vivent à partir d'une telle « neige marine »), ou sont contaminées par l'acide domoïque dissoute dans l'eau, ou bien si elles sont contaminées par un mécanisme tout autre tel que, par exemple, des diatomées benthiques qui seraient également capables de produire l'acide domoïque. En co-supervisant une étudiante en thèse de doctorat en Irlande (Yvonne Bogan), j'ai pu examiner la question d'un peu plus près. Durant cette étude, nous avons étudié la variabilité de l'acide domoïque en fonction de différents paramètres (profondeur, éloignement de la côte, taille de la coquille, emplacement). Ensuite, l'hypothèse de l'accumulation de l'acide domoïque à partir de la phase dissoute a aussi été testée. L'influence de l'âge ou de la taille des coquilles St. Jacques sur la concentration en acide domoïque ont été examinés. Les corrélations entre la taille des coquillages et la contamination avec l'acide domoïque sont complexes et semblent être plus reliées au taux de filtration qu'à d'autres facteurs (Bogan et al., 2007b). Néanmoins, d'après nos précédents résultats, l'âge des coquilles St. Jacques contribue clairement à la variabilité des concentrations observées sur un même site. Dans une baie de profondeur relativement faible (Clew Bay, côte ouest de l'Irlande), nous avons pu examiner s'il y avait des différences entre des CSJ élevées en surface et en profondeur (Bogan et al., 2006). Aucune différence n'a pu être détectée durant une année, et il y avait bien accumulation et décontamination observée aux deux profondeurs durant cette période. Néanmoins, on pourrait argumenter que la baie était si peu profonde que les eaux étaient continuellement brassées et qu'ainsi l'acide domoïque provenant des *Pseudo-nitzschia* pouvait contaminer les CSJ. Toutefois, des diatomées benthiques n'auraient pas proliféré dans la colonne d'eau et elles n'ont donc pas pu jouer un rôle majeur dans ce cas précis. Une autre étude, au sud-est de l'Irlande et dans des eaux plus profondes, a pu établir une relation inverse entre la profondeur et la contamination en acide domoïque (Bogan et al., 2007a). Néanmoins la zone d'étude était beaucoup plus large, et le gradient de toxicité dans les coquilles St. Jacques coïncidait avec un gradient en sels nutritifs. Ainsi nous avons conclu que ce n'était pas la profondeur mais probablement d'avantage l'éloignement de la côte qui se traduisait par une absence d'algues, et donc, par extension, d'algues toxiques. Cette hypothèse est également corroborée par l'étude de la variabilité de l'acide domoïque dans les coquilles St. Jacques de différents bancs de pêche autour l'Isle du Man (Bogan et al., 2007c). Les coquilles St. Jacques artificiellement exposées à l'acide domoïque dans de l'eau de mer ont fini par accumuler de très faibles concentrations, malheureusement, les conditions d'élevage ne semblent pas avoir été optimales ce qui s'est raduit par des mortalités et des coquillages endommagés à la fin de l'expérience. D'autres études sont donc nécessaires pour étayer cette observation.

La question de l'accumulation des toxines à partir de la phase dissoute a aussi été examinée dans une étude de terrain concernant les toxines lipophiles (Fux et al., 2009). Cette étude a été initialement conçue pour tester si des résines lipophiles ne pourraient pas être utilisées pour anticiper la présence des toxines dans les coquillages, partant de l'hypothèse que des concentrations très basses pouvaient être détectées dans ces résines (McKenzie et al., 2004). Cette hypothèse n'a pas pu être vérifiée dans notre étude car les changements de concentration observés ont été simultanément observés dans les échantillonneurs passifs, les moules indigènes et les moules placées toutes les semaines dans la zone d'étude. Néanmoins, une observation intéressante a été faite en ce qui concerne les toxines lipophiles dissoutes. Après une apparition de *Dinophysis*

acuta en mi-août 2005, les concentrations d'acide okadaïque et de DTX2 ont augmenté dans les trois matrices examinées (coquillages indigènes, coquillages placés et échantillonneurs passifs). La phase de décontamination a été rapide pour les deux types de moules, c.à.d. une semaine, malgré la persistance des toxines dans l'eau qui a perduré plus de trois semaines. A partir de ce résultat, nous proposons que les résines soient utilisées dans l'avenir plutôt pour préciser la répartition spatiale des toxines, en vue, par exemple, d'établir si de nouveaux sites sont appropriés pour la culture conchylicole ou non.

7.4. Isolement préparatif de composés bioactifs naturels

Bien que les effets des biotoxines marine soient connus depuis des siècles, les principes actifs impliqués n'ont pas été découverts avant le 20^{ième} siècle. L'identification et la caractérisation des toxines impliquées reste un processus long et coûteux. Par exemple, à la fin du 19^{ième} siècle, des rapports ont décrit l'empoisonnement par les PSP comme étant causé par la consommation de moules (Virchow, 1885) Cependant, les principes actifs apparaissaient également dans les étoiles de mer (Wolff, 1887), sans que l'identité des principes ait pu être révélée. Ce sont Onoue et al. (1931) qui ont commencé les premiers travaux d'isolement préparatif des saxitoxines. Se sont joints à ces investigations Schantz et al. (1957 et 1958) et enfin, en 1971, Wong et al. qui ont réussi à isoler la saxitoxine et à identifier sa structure chimique. Une caractérisation plus approfondie n'a pas pu être achevée en raison de l'absence de toxine purifiée. Cette absence de toxine purifiée peut s'expliquer par le fait que les organismes micro-algaux ne sont pas faciles à cultiver et que les scientifiques doivent se contenter le plus souvent des contaminations naturelles de coquillages pour extraire et purifier les toxines. De plus, la caractérisation structurale des molécules à cette époque était basée en grande partie sur des réactions chimiques effectuées sur le composé isolé. L'arrivée de techniques non-destructives telles que la spectroscopie par résonance magnétique nucléaire (RMN) a permis la caractérisation de quantités nettement plus petites tandis que, durant les années 1950/60, des centaines de mg étaient nécessaires pour identifier un composé. De nos jours, 10 à 100 µg peuvent parfois suffire pour ce travail. Ainsi, la découverte de l'acide domoïque en tant que phycotoxine a pu être réalisée quelques semaines après des empoisonnements désastreux observés en 1987 au Canada (Quilliam et Wright, 1989). Plus typiquement, l'identification d'un nouvel agent toxique et l'élucidation de sa structure prennent plusieurs années à partir de l'évènement initial d'empoisonnement. Ce fut le cas pour l'acide okadaïque et l'azaspiracide (Satake et al., 1998 ; Yasumoto et al., 1979).

Depuis 1999, il y a eu de nombreuses raisons pour justifier la mise en place de l'analyse chimique en Irlande. Tout d'abord, de nombreux lots de moules produits en Irlande et exportés vers la France avaient été rejetés sur la base de bioessais positifs en France, suggérant que les systèmes de test n'avaient pas été suffisamment harmonisés à travers l'Europe. L'analyse chimique permettait d'envisager la quantification des lots présentant des concentrations proches de la limite de détection du test souris, et ainsi on s'attendait à ce que cette analyse évite la mise en marché des produits toxiques. De plus, des données quantitatives spécifiques aux composés en question étaient nécessaires pour évaluer le risque lié aux azaspiracides afin de pouvoir consolider la législation nationale et européenne. Par ailleurs, l'obtention d'un composé pur pour l'étalonnage est un point critique pour la mise en place d'une méthode physico-chimique quantitative. Pour cela, la mise à disposition d'azaspiracide purifié est d'une importance primordiale. Le plus souvent, la synthèse chimique est trop coûteuse et laborieuse pour produire les quantités de phycotoxines nécessaires aux analyses de routine et à la validation des méthodes. De toute façon en 2001, quand la décision avait été prise de mettre en place l'analyse chimique de routine, la synthèse de l'azaspiracide qui n'était pas encore possible, ne pouvait donc pas devenir une option. L'organisme producteur des azaspiracides n'était pas connu non plus. Alors, la seule possibilité de se procurer des azaspiracides purifiés était l'isolement et la purification à partir de coquillages contaminés, des moules dans ce cas précis. Une demande auprès des collaborateurs Japonais qui avait réussi cet isolement pour les AZA1 à 5 (Satake et al., 1998 ; Ofuji et al., 1999 et 2001), avait permis d'obtenir en 2001 de l'AZA1 pour servir d'étalon de base.

Comme la quantité de cet étalon était limitée, il était nécessaire d'en produire plus ; pour ce faire, j'ai pu me rendre au Japon afin d'apprendre les techniques d'isolement préparatif telles que mises en place par les collaborateurs japonais. Avec un dernier lot de moules contaminées

provenant d'Irlande, nous avons réalisé l'isolement de 2 mg d'AZA1 d'une pureté > 95% avec des impuretés d'analogues < 1%. Les quantités d'impuretés d'analogues ont été mesurées par LC-MS, la pureté même par la comparaison des spectres RMN avec les résultats gravimétriques. Cette quantité s'était révélée suffisante pour permettre l'analyse de routine au quotidien des coquillages par LC-MS entre 2001 et 2007, ainsi que la validation interne de la méthode, et quelques études préliminaires sur la toxicité d'AZA1 *in vitro*. Pour produire plus d'AZA purifié et de manière durable, nous avons mis en place la technique d'isolement en Irlande dans le cadre d'un pré-projet (2001). Ce pré-projet a permis d'acquérir l'équipement nécessaire, tandis que le projet ASTOX (2003 – 2007) a permis de financer une thèse sur ce sujet. Par la suite la procédure développée par les collaborateurs japonais a été adaptée et légèrement modifiée (Rehmann et al., 2008 ; Fig. 4.1.1). Entre autres, des améliorations significatives ont été apportées sur certaines des étapes chromatographiques, notamment la chromatographie en phase normale sur silice à basse pression (Fig. 4.1.2). Cette étape de purification avait montrée des taux de récupération de toxine faibles après transfert initial de méthode (35%), et l'amélioration a permis d'obtenir un taux de récupération moyen de plus de 70%. En 2007, cette procédure a été adaptée puis utilisée, en collaboration avec le NRCC, pour produire un standard certifié de référence pour l'AZA1 (Fig. 5.1.1 et 5.1.2). Des étalons purifiés pour l'AZA2 et -3 sont également en cours d'être certifiés au NRCC grâce à cette collaboration. En plus des isolement d'analogues connus d'azaspiracide, de nouveaux dérivés azaspiracides ont aussi pu être identifiés grâce aux développements d'étapes de purification et d'enrichissement (Rehmann et al., 2008), ainsi des hypothèses sur le métabolisme des AZA dans les moules ont pu être étayées (McCarron et al., 2009).

De la même façon des procédures similaires à celles développées pour l'isolement préparatif des azaspiracides ont été développées et appliquées pour l'isolement préparatif de la DTX2 (Rehmann, 2008). Une collaboration avec des collègues néo-zélandais et norvégiens a permis de clarifier la stéréochimie de la DTX2 (Larsen et al., 2007), en particulier la configuration des groupes fonctionnels du Carbone 35 (Fig. 1.1.4). Ces nouvelles connaissances sur les différences structurales entre l'OA, la DTX1 et la DTX2 ont également contribué à notre compréhension des interactions de ces composés avec les phosphoprotéine-phosphatases (Fig. 4.2.1), ce qui confirme l'observation d'une liaison plus faible entre la DTX2 et la PP2a et une toxicité *in vivo* plus faible de la DTX2 par rapport à l'OA (Aune et al., 2007).

Enfin, nos travaux sur l'isolement préparatif des toxines lipophiles ont également amené à la découverte d'un nouveau groupe de composés : les esters d'acides gras du seco-acide de la PTX2 (Wilkins et al., 2006). Il avait été démontrée que la PTX2 était métabolisée rapidement dans les moules pour former un seco-acide (ouverture du macrocycle). Ce qui n'était pas connu avant nos travaux était le fait que ce seco-acide était ensuite lui-même métabolisé en esters d'acide gras. Les composés les plus abondants étaient les esters 37-O-acylés suivi par les esters 11-O-acylés et ensuite 33-O-acylés. Les groupements acyles observés en ordre décroissant étaient les suivants : 16:0, 22:6, 14:0, 16:1, 18:4 et 20:5, mais d'autres analogues ont aussi été répertoriés (Fig. 4.3.1). Ainsi, la collaboration avec les américains sur la culture du *Dinophysis* ainsi que la collaboration avec les norvégiens et néo-zélandais sur les métabolites de la PTX2 se rejoignent thématiquement pour que de nouveaux travaux puissent maintenant estimer la production toxinique totale en PTX2 lors des épisodes d'apparence d'algue du genre *Dinophysis*.

7.5. Contrôle qualité

La qualité d'une évaluation des risques posés par un groupe de phycotoxines dépend naturellement de la qualité des éléments fournis pour cette évaluation. L'analyse des composés contaminant les coquillages en question doit être quantitative et spécifique. De même, les données toxicologiques nécessaires pour l'interprétation du risque posé par les toxines, ne sont fiables que si les quantités de toxines utilisées pour ces études sont suffisantes et si les toxines sont suffisamment pures pour attribuer les effets observés uniquement aux toxines en question. Par exemple, une évaluation de risque a dû être revue pour les pecténotoxines. En effet, dans un premier temps une intoxication de consommateurs de coquillage en Australie avait été attribuée aux pecténotoxines alors qu'après des études approfondies, il a pu être démontré que les coquillages contenaient des esters de l'acide okadaïque et ses analogues. Ainsi, les effets n'ont pas pu être uniquement attribués aux PTXs présentes comme initialement supposé. En fait, aucune intoxication humaine n'a pu être associée de manière claire uniquement aux PTXs.

Pour vérifier et démontrer la justesse et la fiabilité d'une méthode analytique, l'analyste a besoin d'outils de contrôle internes et externes. Les outils de contrôle qualité en intra-laboratoire sont essentiellement des étalons de calibration et des matériaux de référence bien caractérisés ou certifiés pour leur teneur en toxines. Pour avoir une confirmation que la méthode opérée par un laboratoire est appropriée pour une application, les outils de contrôle internes doivent être validés aussi par des organismes externes au laboratoire. Ainsi, l'accréditation, les essais d'aptitude et la validation de méthode en inter-laboratoire peuvent constituer des contrôles externes. En l'absence de méthodes validées en inter-laboratoire, il peut être utile de vérifier les résultats d'une méthode avec ceux d'une autre méthode appliquée aux mêmes échantillons. Je présente donc ici les études que j'ai menées, souvent en collaboration avec des partenaires internationaux, pour fournir des outils de contrôle qualité interne et externe. Il a déjà été fait mention de la procédure de préparation d'étalon certifié lors de la présentation des travaux relatifs à l'isolement préparatif des toxines lipophiles. Quelques compléments seront apportés par rapport à la certification. La préparation des tissus des coquillages contaminés par les phycotoxines, leur homogénéité et leur stabilité seront aussi traités dans ce chapitre. J'ai également mis en œuvre des essais d'aptitude pour les phycotoxines dont les principaux résultats seront expliqués ici. Enfin, plusieurs exemples seront donnés en ce qui concerne la comparabilité entre différentes méthodes ceci afin de permettre d'évaluer si une méthode est appropriée ou non pour l'application prévue.

Etalons et matériaux de référence

La certification de l'étalon AZA1 représentait un point majeur pour la mise en place d'une méthode d'analyse chromatographique pour cette classe de composés (NRCC, 2007). Cela a nécessité de déterminer la concentration exacte et la pureté du composé isolé et purifié dans la préparation. Pour établir la concentration exacte de l'étalon, une méthode de quantification par RMN récemment développée au NRCC, basée sur un étalonnage externe avec la caféine, a été utilisée (Burton et al., 2005). Ensuite, la stabilité du composé en solution a été établie (Fig. 5.1.2). Enfin, cette même méthode a aussi été utilisée depuis pour la caractérisation des analogues AZA2 et -3. Les études de stabilité ont montré que les AZA1 et -2 sont très stables en solution méthanolique de - 80 °C jusqu'à + 20 °C, et que seulement pour AZA2 on note à partir de + 37 °C une baisse de concentration significative après 3 mois de stockage. Pour AZA3, la concentration est affectée après 8 jours à + 37 °C, et même à + 20 °C on note une baisse significative de la concentration après 3 mois. Ces données confirment que les trois composés peuvent être stockés pendant des années à - 20 °C sans problème, et que le transport à une température inférieure à

+ 4 °C ne pose pas de problème pendant une semaine. Ainsi, nos travaux ont contribué de manière significative à résoudre la question du manque d'étalon pour les analogues AZA réglementés et leur quantification peut maintenant être réalisée par les laboratoires de contrôle officiel.

Le prochain outil de contrôle qualité pour les laboratoires soucieux de la performance de leurs méthodes sont les matériaux de référence réalisés avec des tissus de coquillage contenant des toxines. Selon une revue récente (Hess et al., 2007a), on peut distinguer trois types de tissus de référence, selon leur utilisation : les matériaux de référence d'un laboratoire (Laboratory Reference Material ou LRM), les matériaux de référence interlaboratoire (iLRM) et les matériaux de référence certifiés (CRM). Ces trois matériaux présentent chacun des caractéristiques d'utilisation spécifiques. Essentiellement, pour un LRM, le déterminant le plus important est l'homogénéité car la stabilité peut être garantie pour la plupart des composés en stockant le matériau dans un congélateur (- 20 ou - 80 °C). Pour un iLRM, la stabilité à des températures plus élevées doit au moins être garantie pour la durée d'un transport international, c.-à-d. au moins pendant une semaine. Ensuite, pour un CRM, la stabilité doit être garantie pour du long terme car les efforts de production nécessitent un stockage d'au moins plusieurs années afin de rentabiliser la démarche. Par contre, la caractéristique la plus importante d'un CRM est la justesse de la teneur de la toxine contenue dans le matériau. Durant la thèse de doctorat de Pearse McCarron, que j'ai encadré, nous avons pu examiner de manière systématique les facteurs affectant l'homogénéité et la stabilité des toxines dans les matériaux de référence « coquillages ».

Nous avons étudié majoritairement les matériaux pour lesquels les tissus du coquillage contiennent un pourcentage d'eau reflétant les taux naturels, c.-à-d. entre 85 et 90%, comme la plupart des tissus biologiques animaux. Dans ces tissus, ont lieu des réactions de dégradation qui incluent, typiquement, les oxydations catalysées par la présence d'eau. Ces réactions sont ralenties par un abaissement de la température. Selon la loi d'Arrhenius le ralentissement peut varier entre un facteur 2 et 4 pour un abaissement de la température de 10 C. Comme prévu, et de façon générale, nous n'avons pas trouvé une quelconque dégradation des toxines pendant le stockage des matériaux, au moins jusqu'à trois mois si les tissus étaient congelés à - 20 °C. En conséquence, nous avons souvent utilisé cette condition de stockage comme condition de référence, sauf pour les matériaux destinés à la certification pour lesquels des durées de stockage plus longues sont doivent être anticipées. La congélation réduit également les dégradations microbiologiques qui sont une autre cause éventuelle d'instabilité des toxines dans un matériau.

Une première étude sur les teneurs en acide domoïque des tissus de coquillage a examiné l'influence des composés stabilisant la matrice (McCarron et al., 2007a). Nous avons considéré deux types de stabilisants, les anti-oxydants et les antibiotiques. Chacune de ces classes de composé pouvant surmonter une des dégradations typiquement engendrées dans les tissus à forte teneur en eau. Les deux types de composés ont été ajoutés séparément ou en combinaison. L'ajout séparé de chaque composé a légèrement amélioré la stabilité de l'acide domoïque mais la combinaison d'ajout a donné le meilleur résultat de stabilité. Les matériaux ainsi dosés ont pu être stockés pendant 8 jours à 40 °C sans dégradation significative de l'acide domoïque (Fig. 5.1.3), ce qui permettait donc l'expédition des tissus à l'échelle mondiale.

Une deuxième étude a évalué l'applicabilité de la technique d'irradiation aux rayons gamma pour la stabilisation des matériaux (McCarron et al., 2007c). Cette technique nécessitant une énergie importante, il y avait deux points à examiner : l'effets de l'irradiation sur les toxines elles-mêmes et l'efficacité de l'irradiation pour détruire les micro-organismes vivant dans la matrice. Les effets de l'irradiation sur les toxines ont été examinés pour des toxines en solution ainsi que pour des tissus de coquillages contaminés. L'efficacité de l'irradiation pour l'abatement de la

concentration en micro-organismes est sans égale. Les tissus d'huîtres ou de moules n'ont pas montré d'activité microbienne après incubation pendant 24 h, indépendamment de la dose d'irradiation utilisée. Même après 5 jours d'incubation, un seul échantillon d'huître irradié a montré une activité microbiologique à une dose de 12 kGy. Aucun tissu n'a montré d'activité microbiologique à la dose de 24 kGy. En revanche, les toxines en solution sont toutes affectées par l'irradiation. L'acide domoïque et la YTX sont dégradés quasiment complètement dès la plus faible dose appliquée (6 kGy). La PTX2 a été moins affectée mais dès la plus faible dose. L'acide okadaïque et l'azaspiracide-1 ont été les moins affectés mais il y avait quand-même une dégradation significative (environ 20%) pour AZA1 à la dose la plus faible. Par contre, la matrice coquillage a un effet protecteur sur les toxines : l'AZA1 contenu dans les tissus était particulièrement stable, ce n'est qu'à la plus haute dose (24 kGy) qu'une légère baisse de la concentration a été observée. Cependant, cette diminution de la concentration n'était pas significative, statistiquement parlant. En fonction de ces résultats prometteurs, un matériau de référence a été préparé en collaboration avec le NRCC pour les azaspiracides et une combinaison de traitements a été appliquée comprenant l'irradiation et l'ajout de stabilisant. La certification de ce matériau est en cours au NRCC.

En collaboration avec l'institut européen des matériaux de référence et de la mesure (Institute for Reference Materials and Measurement - IRMM), nous avons également étudié l'influence du séchage des tissus sur la stabilité des toxines dans la matrice coquillage (McCarron et al., 2007b). Ce traitement s'est montré le plus efficace de tous les traitements étudiés. Pour l'acide domoïque la comparaison d'un tissu de moule à teneur normale en eau (85%) avec le même matériau séché est convainquant. L'acide domoïque est totalement dégradé dans le matériau humide après six mois de stockage à 40 °C tandis que l'acide domoïque dans le matériau séché stocké dans les mêmes conditions n'est pas du tout affecté (Fig. 5.1.6). Pour les toxines lipophiles, la stabilité était déjà meilleure pour les tissus humides. Néanmoins, la dégradation de l'AZA3 a pu être ralentie dans le tissu séché. Ces études ont été déterminantes pour la décision de préparer un matériau de moules séché contaminé avec de multiples groupes de toxines : AZA, DA, OA, PTX, SPX, YTX. La préparation de ce matériau n'a été possible qu'à travers la collaboration de six instituts : Marine Institute, IRMM, NRCC, NSVS, NVI et AgResearch.

Essais d'aptitude

Comme il y avait très peu de CRM et de méthodes validées disponibles pour les phycotoxines au début des années 2000, les laboratoires de contrôle officiel étaient généralement obligés de valider leurs méthodes en interne, et la participation à des essais d'aptitude était un moyen de démontrer la performance du laboratoire vis-à-vis des organismes d'accréditation ou d'agrément. Depuis son existence en 1996, le laboratoire communautaire de référence pour les biotoxines marines (CRLMB) a une obligation légale de fournir des essais d'aptitude, mais cette obligation ne s'étend qu'aux méthodes de référence et aux laboratoires nationaux de référence (LNR). Comme la méthode de référence pour les biotoxines marines lipophiles était le test souris, initialement il n'y avait donc pas d'essai d'aptitude qui aurait pu permettre aux laboratoires de prouver la validité de leurs méthodes d'analyse physico-chimiques pour ces groupes de toxines. Pour tous les autres laboratoires de contrôle officiel, et mis à part certains laboratoires pour lesquels les LNR organisaient ces essais, il manquait une structure d'organisation pour ces essais d'aptitude. Dans la mesure où, en Irlande, il n'y avait qu'un autre laboratoire de contrôle officiel en dehors du LNR, et grâce à l'expérience que nous avons acquise dans la préparation de matériaux de référence homogènes et stables, nous avons décidé d'inciter un autre organisme à inclure les phycotoxines dans son programme d'essais d'aptitude. Plusieurs organismes auraient pu organiser ces essais ;

nous en avons approché plus particulièrement deux: FAPAS et QUASIMEME. Ces deux organismes travaillent sur une base commerciale, c.-à-d. que tous les coûts d'exercice doivent être couverts par les frais de participation. Après négociation, il s'est avéré que l'organisation par QUASIMEME était avantageuse, non seulement du point de vue de la faisabilité financière, mais également sur le plan de l'exploitation statistique des essais. QUASIMEME utilise des statistiques robustes et avancées basées sur la théorie d'incertitude de la mécanique quantique, ce qui permet de décrire de manière plus efficace plusieurs types de distribution des résultats. De plus, les z-scores attribués aux laboratoires sont calculés de manière indépendantes des résultats des laboratoires participants. Par ailleurs, cet organisateur possédait un système opérationnel pour l'introduction de nouveaux couples analyte/matrice, indiquant ainsi aux participants que l'organisme même est encore en phase d'apprentissage sur un groupe de composé. Par analogie avec l'approche du NRCC consistant à fournir des « boîte à outils » aux laboratoires de contrôle officiel, nous avons décidé de distribuer des matériaux correspondant à différents degrés de difficulté d'analyse : des étalons, des extraits bruts et des tissus de coquillage contaminés. De ce fait, nous avons examiné les résultats des laboratoires participants pour détecter les erreurs provenant de l'étalonnage, de l'extraction ou encore de l'analyse instrumentale elle-même. Sous ma supervision, le Marine Institute a fourni les matériaux pour les exercices QUASIMEME de 2003 à 2008, d'abord pour l'analyse de l'acide domoïque, ensuite pour les toxines lipophiles, et tout récemment pour les saxitoxines.

Les exercices pour la détection de l'acide domoïque ont eu un succès quasiment immédiat. Au cours de l'exercice de développement (les 2 premières années), il y avait déjà à peu près 60% des 40 à 42 laboratoires participants qui obtenaient 100% de z-scores corrects. Ainsi, QUASIMEME a décidé de transformer les exercices DA en mode routine pour la troisième année et la performance des laboratoires a encore augmentée jusqu'à une moyenne de 75% des laboratoires avec 100% de z-scores corrects (Tableau 5.2.3).

Les exercices pour les toxines lipophiles ont commencé avec le groupe acide okadaïque qui présentait un certain défi du fait du manque d'expérience des laboratoires. Seulement 10 des 16 laboratoires participants ont réussi à quantifier correctement un étalon d'OA dans un des premiers exercices (avec un coefficient de variation inter-laboratoire de 9.8%). Durant l'exercice suivant, 11 des 15 participants ont quantifié un étalon de la DTX2 correctement, mais avec un CV interlaboratoire de 29%, ce qui suggère que la quantification d'une toxine n'est pas si facile en absence d'étalon certifié pour ce composé. Ce fait nous a aussi encouragé à pousser notre collaboration avec le NRCC pour y inclure la production d'un étalon certifié pour la DTX2. L'étalon de la DTX2 est produit et actuellement en cours de certification. Dans la troisième étape de l'exercice pour les toxines du groupe OA, 10 laboratoires ont obtenu des z-scores acceptables pour deux matériaux contaminés aux concentrations de 104 et 372 µg/kg pour la somme de l'OA, et des DTX1 et -2 suite à l'hydrolyse, avec des CV interlaboratoires de 20,7 et 17,9%, respectivement. Cette performance indique une tendance positive pour la performance des laboratoires, néanmoins il n'y a pas beaucoup de participants et la comparaison entre exercices reste biaisée. Durant l'exercice 2007/2008, nous avons également introduit des matrices contaminées avec des azaspiracides, et je prévois un intérêt croissant pour ces essais d'aptitude grâce aux récents développements de la réglementation en Europe.

Les essais d'aptitude pour le groupe des saxitoxines est également un challenge non-négligeable, à la fois du fait de la complexité de l'analyse de ces composés et du fait du manque de matériaux certifiés de référence, le tout se traduisant par un manque d'expérience des laboratoires. Par ailleurs, le test souris pour les saxitoxines est quantitatif et, malgré les problèmes d'éthique qu'il pose, il est bien accepté parmi les laboratoires de contrôle officiel en raison de la

facilité de sa mise en place (résultats rapides et faciles à interpréter). Grâce à nos progrès dans la préparation de matériaux à base de coquillages contaminés aux saxitoxines en 2007, nous avons pu inciter QUASIMEME à inclure les saxitoxines dans le programme de leurs essais d'aptitude à partir de 2008/2009. Le premier exercice a permis à 8 laboratoires (sur 13 participants) d'obtenir des z-scores acceptables et ainsi d'affirmer leur capacité à analyser les tissus de coquillages contaminés à 452 et 947 µg/kg en équivalent diHCL-STX. Malgré le faible nombre des participants, cet exercice est encourageant car il n'y a pas de résultats extrêmes ($z > 6$) malgré le profil complexe des échantillons fournis.

Dans leur totalité les essais interlaboratoires ont permis aux laboratoires participants d'évaluer un grand nombre de coquillages de différentes espèces, un aspect qui n'aurait jamais pu être réalisé uniquement par le CRM. Ainsi, non seulement des moules et des huîtres ont été testées, mais aussi des coquilles St. Jacques (chair totale, muscle adducteur et corail), des couteaux et des palourdes.

Evaluation de méthode

Pour évaluer la performance d'une méthode dans sa totalité, il est intéressant de comparer ses caractéristiques à celles d'autres méthodes. A partir de là, des recommandations peuvent être faites aux gestionnaires de risque concernant la capacité d'une méthode à analyser un critère réglementaire (paramètre mesuré et norme fixée) en terme de justesse et fidélité, pour l'obtention des informations les plus appropriées à l'évaluation et la gestion des risques. Ainsi nous avons comparé différentes techniques analytiques et différentes approches de détection. Dans ce chapitre, je présente une comparaison entre méthodes d'analyse chromatographique pour la quantification de l'acide domoïque (LC-UV et LC-MS), ensuite une comparaison entre des méthodes chromatographiques et immunologiques (LC-UV vs. ELISA pour DA et LC-MS vs. biocapteur pour OA), et finalement je compare également une méthode LC-MS avec le test souris à l'exemple des azaspiracides.

Dans la législation européenne, on prescrit d'utiliser la méthode « HPLC » pour l'analyse de l'acide domoïque. Comme il n'y a pas de référence dans la législation concernant la méthode à utiliser, il a été nécessaire de comparer les différentes méthodes validées par de nombreux laboratoires afin d'établir les paramètres caractéristiques de ces méthodes. Ainsi, nous avons organisé une comparaison inter-laboratoires en utilisant des méthodes basées sur la détection par LC-UV et par LC-MS (Hess et al., 2005b). Cette étude comparative a permis d'établir qu'il n'y avait effectivement pas de différence entre la technique UV et MS pour la détection de l'acide domoïque si certains paramètres clés étaient respectés pour l'analyse par détection UV. L'un des paramètres critiques est une chromatographie suffisamment pertinente pour séparer l'acide domoïque du tryptophane. L'étude a également permis de mettre en évidence un paramètre clé pour la purification de l'acide domoïque par cartouche SPE. Il s'agit du volume d'élution qui doit être déterminé par chaque laboratoire et vérifié pour chaque modèle de cartouche. Effectivement, la purification SPE ne semble pas nécessaire si les paramètres chromatographique de séparation sont respectés (Tableaux 5.3.2, 5.3.3 et 5.3.4). Par la suite, l'existence de nombreuses variations mineures mises en évidence lors de plusieurs études intra-laboratoires pour l'analyse de l'acide domoïque par des méthodes validées a aussi été confirmée plus tard lors d'exercices d'essais d'aptitude, organisés dans le cadre de notre collaboration avec QUASIMEME.

Un travail effectué pendant une thèse de diplôme (Katrin Bender, 2004) nous a permis d'évaluer la performance de la méthode ELISA développée par BIOSENSE pour la détermination

de l'acide domoïque par rapport à l'analyse par LC-UV. La comparaison avec les résultats d'une étude de terrain (76 sites échantillonnés) a donné une très bonne corrélation ($r^2 = 0,781$; pente = 1,02). Ces résultats encourageants ont aussi été confirmés par l'étude interlaboratoire de BIOSENSE (12 laboratoires ELISA et 4 LC, le Marine Institute avait participé avec les deux méthodes). Par la suite, BIOSENSE a obtenu l'approbation de l'AOAC pour sa méthode ELISA, ce qui a amené la Commission européenne à adopter cette méthode en tant que méthode alternative pour le contrôle officiel (Anonymous, 2006b).

Dans une étude avec un groupe de recherche de Belfast, nous avons pu comparer les résultats de la méthode LC-MS avec ceux obtenus par un biocapteur basé sur un anticorps récemment développé pour l'acide okadaïque. Une très bonne corrélation a été observée entre les deux méthodes pour l'analyse d'échantillons naturellement contaminés provenant d'Irlande et de Norvège ($r^2 = 0.97$) et aussi pour des matériaux de référence (LRM et CRM), avec un $r^2 = 0.99$ (Stewart et al., 2009). Si l'on considère les différents profils étudiés, il est clair que le biocapteur développé est capable de détecter tous les analogues, y compris les esters (DTX3) sans hydrolyse, et que la DTX2 est quantifiée avec une affinité équivalente à sa toxicité relative. Ainsi, cette méthode pourrait être utilisée pour un criblage dans le cadre de l'analyse de routine des toxines du groupe OA.

Ni la méthode LC-MS, ni le test souris pour les toxines lipophiles n'ont été validés par des études inter-laboratoires. Les laboratoires nationaux de référence se sont intéressés aux différences de performance entre ces deux méthodes, parfois pour des raisons scientifiques, parfois pour des raisons éthiques ou encore commerciales. Ainsi, il était devenu impératif d'étudier d'un point de vue scientifique la performance de ces deux approches. Nous l'avons fait à l'exemple des azaspiracides (Hess et al., 2009). Pour cette étude nous avons préparé des matériaux de référence de tissus de moule (*Mytilus edulis*), 7 tissus d'hépatopancréas et 7 tissus de chair totale. Les niveaux de concentrations étaient compris entre zéro et deux fois la limite légale actuelle de 160 $\mu\text{g}/\text{kg}$ (3 niveaux en dessous et 4 niveaux en dessus de la limite). Trois conclusions majeures ont pu être tirées de cette étude. Premièrement, les deux méthodes sont capables de détecter des concentrations supérieures ou égales à la limite légale actuelle. Le test souris a en particulier une probabilité d'environ 95% de détecter un résultat positif à la limite légale actuelle. Deuxièmement, l'étude a permis de clarifier l'origine de la difficulté à comparer les résultats d'une méthode basée sur l'analyse de la chair totale avec une méthode basée sur l'analyse de l'hépatopancréas, c'est à dire la variabilité naturelle du ratio en hépatopancréas et chair totale. Ensuite, il est clair que le test souris ne peut pas détecter des concentrations significativement en dessous de la limite légale actuelle. Par exemple, une concentration de 80 $\mu\text{g}/\text{kg}$ ne serait détectée qu'avec une probabilité de 5%. Si l'on prend en compte les difficultés engendrées par l'augmentation de la concentration en équivalents d'azaspiracide-1 durant le traitement par la chaleur, tel qu'il est appliqué dans les procédés commerciaux ou dans la cuisson domestique des moules (Hess et al., 2005a ; McCarron et al., 2008 et 2009), il devient clair que le test souris n'est pas un outil adapté pour la surveillance dans le cadre des contrôles officiels, en particulier du fait de ses difficultés à détecter des concentrations en dessous de la limite légale. Cet aspect a été validé par une évaluation à l'échelle européenne (EFSA, 2009b).

7.6. *Caractérisation des dangers*

Les chapitres précédents ont décrit la partie de mes travaux concernant deux aspects principaux dans la caractérisation des dangers, d'une part, la nature du danger, c.-à-d. les composés impliqués dans la toxicité et, d'autre part, la concentration et la distribution des composés dans l'environnement marin, plus particulièrement le compartiment coquillage. Cette chapitre décrit les aspects relatifs au potentiel toxique des composés. Dans le domaine de la toxicologie, nous pouvons classer nos études selon les concepts de la toxico-cinétique et la toxico-dynamique. La toxico-cinétique est concernée avec la bio-disponibilité, l'absorption, la distribution, le métabolisme et l'excrétion des composés toxiques, tandis que la toxico-dynamique implique la compréhension des mécanismes de toxicité. Donc, pour la toxico-cinétique nous devons connaître la lipophilicité des composés pour prédire leur bio-disponibilité et leur capacité d'absorption à travers les barrières intestinale et cérébrale, et la réactivité des composés pour pouvoir prédire les concentrations susceptibles d'apparaître à différents endroits dans le corps humain. Ainsi, une première partie décrit mes travaux sur le degré de lipophilicité des phycotoxines et sur leur réactivité. Ensuite, j'ai intercalé un résumé de nos études relatives aux transformations des phycotoxines durant les processus de dissection et de traitement par la chaleur pour compléter la description d'éventuels phénomènes affectant les estimations d'exposition. Ensuite, quelques travaux sont décrits pour souligner les avancées apportés à la compréhension des mécanismes d'action de certaines toxines lipophiles.

Lipophilicité, réactivité et biotransformation des phycotoxines

La lipophilicité d'un composé consiste dans son affinité pour les matières grasses. Généralement, le descripteur classique pour cette caractéristique d'un composé est exprimé comme le coefficient de partage entre l'eau et l'octanole (P_{ow}), ou encore par son logarithme de base 10 ($\log P_{ow}$). Plus le $\log P_{ow}$ est élevé, plus le composé est lipophile. La chimie médicinale a développé une règle de pouce pour l'absorption la plus efficace dans le corps humains. Le plus souvent cette règle est exprimée sous la forme de la loi de « cinq » de Lipinski, selon laquelle les composés idéaux ne dépassent pas un $\log P_{ow}$ de 5, n'ont pas plus de 5 donneurs de proton et n'ont pas un poids moléculaire supérieur à 500 da (Lipinski et al., 1997). Selon l'OECD, il y a deux méthodes reconnues pour la détermination du $\log P_{ow}$, la méthode « shake flask » (partition) et la méthode chromatographique. Comme pour les phycotoxines, il n'y a en général jamais suffisamment de composé pour la méthode de partition, nous avons tenté de déterminer les $\log P_{ow}$ pour plusieurs toxines lipophiles, en utilisant la méthode chromatographique. Pour les composés ionisables, le $\log P_{ow}$ dépend du coefficient d'acidité (pK_a), ainsi nous avons également examiné ce paramètre. Les $\log P_{ow}$ déterminés par chromatographie sont plus élevés que les estimations par modélisation ou provenant de la littérature. Tandis que pour l'acide okadaïque et ses analogues, la différence est dans l'incertitude de la mesure, pour les azaspiracides et la pecténotoxines, les valeurs mesurées sont significativement plus élevées que les valeurs calculées. Une des limites de nos expériences a été la disponibilité limitée de composés avec des $\log P_{ow}$ élevés connus qui peuvent s'analyser par LC-MS. Ainsi, le modèle pourra être affiné une fois que d'autres composés seront décrits pour permettre un étalonnage satisfaisant (Fux, 2008).

La stabilité chimique des phycotoxines lipophiles est un paramètre intéressant pour comprendre leur comportement durant l'analyse chimique, l'isolement préparatif et aussi la toxico-cinétique. Pour l'acide okadaïque et ses analogues, la stabilité envers la soude a été décrite. Nous avons pu confirmer cette stabilité dans les solutions méthanoliques et dans des extraits bruts

de coquillages jusqu'à 2 h à 76 °C. Par contre, l'instabilité de l'OA et ses analogues dans le milieu acide n'a pas été largement divulgué. Nous avons pu établir que l'acide okadaïque est rapidement détruit en milieu acide (HCl) en solution méthanolique, aussitôt que la température est élevée à 76 °C. Cette observation n'est pas seulement caractéristique de l'analyse chimique mais concerne aussi la digestion des tissus de coquillages car ils seront exposés à l'acide chlorhydrique dans l'estomac humain, avant de passer en milieu alcalin dans les intestins. Durant l'hydrolyse, il est donc très important d'assurer un refroidissement de l'extrait avant de le neutraliser avec l'acide.

De manière similaire à l'acide okadaïque, les azaspiracides se dégradent en milieu acide, même si l'on utilise des acides organiques faibles telle que l'acide acétique. Pour l'exposition à l'acide formique, la dégradation à 45 °C est complète après 48 h. Nous avons proposé un mécanisme de réaction pour cette dégradation (Alfonso et al., 2008). Ces observations nous ont permis d'affiner les protocoles d'isolement préparatif pour les azaspiracides (Rehmann, 2008). De plus, cette instabilité en milieu acide pose également la question de la bio-disponibilité des azaspiracides durant la digestion humaine. Les AZA ne peuvent pas être libérés durant la digestion dans l'estomac, sinon ils seraient dégradés rapidement. Comme nous savons que les AZA causent des troubles digestifs chez les humains suite à la consommation de coquillages contaminés, la question se pose de savoir si les effets observés sont causés par des interactions à de très faibles doses d'AZA ou à travers des contacts de surface avec les tissus de coquillages en voie de digestion. Une libération d'AZA a aussi été observée quand nous avons rajouté de la pepsine au milieu simulant la digestion. Ainsi nous nous demandons si l'extraction au méthanol pur est efficace. La biotransformation des AZA dans les coquillages semble créer toute une panoplie de métabolites à partir de l'AZA1 et -2, qui sont les métabolites principaux présents dans l'algue *Azadinium spinosum* (Krock et al., 2009). Jusqu'à 18 dérivés supplémentaires ont été détectés (Rehmann et al., 2008). Les traitements par la chaleur engendrent apparemment des transformations spontanées de décarboxylation de certains analogues et les transforment ainsi en analogues réglementés tel que l'AZA3 (McCarron et al., 2009).

Influence des traitements de coquillages (dissection et cuisson)

Dans les procédés industriels ou domestiques de transformation des coquillages, j'ai examiné surtout la dissection des coquilles St Jacques et son influence sur la teneur en acide domoïque des parties comestibles restantes. De plus, j'ai eu occasion d'examiner les effets des traitement par la chaleur sur DA, OA et AZA.

Comme souligné en section 4.3.4, l'acide domoïque est une toxine abondante dans les coquilles St. Jacques en Europe. Les concentrations peuvent facilement atteindre 20 fois la limite légale dans l'animal entier, alors la dissection par organe comestible s'impose. Néanmoins, même dans les organes comestibles (muscle adducteur, corail ou gonade), des concentrations en dessus de la limite légale sont parfois détectées. Nous avons pu identifier la localisation de certaines sources de ces contaminations dans les tissus du coquillage à travers nos diverses études. D'abord, nous avons constaté que la dissection des coquilles St Jacques peut être incomplète, ainsi des organes très contaminés tel que l'anus et le rein de la coquille St Jacques peuvent rester accrochés sur les muscles adducteurs augmentant ainsi la contamination. De plus, il y a une boucle des intestins qui passe à travers les gonades qui sont ainsi toujours contaminées si l'animal a été exposé à de fortes doses d'acide domoïque. Cet effet est multiplié si les gonades sont vidées après la ponte du fait de la perte de poids qui n'entraîne pas une perte de toxine.

La cuisson des moules n'affecte que très peu les concentrations d'acide domoïque (McCarron et Hess, 2006). Nous avons également observé la présence de l'acide domoïque dans les jus de cuisson. Ainsi, nous interprétons les concentrations égales avant et après la cuisson par le fait que la perte d'eau de la matrice est équilibrée par la perte en acide domoïque que l'on retrouve dans les jus de cuisson, car le composé est soluble dans l'eau. Ceci semble en cohérence avec les mêmes expériences réalisées sur les toxines lipophiles, surtout l'acide okadaïque et ses analogues qui ne migrent pas de la chair vers les jus de cuisson (McCarron et al., 2008). Pour l'OA et ses analogues nous avons donc observé une augmentation d'entre 30 et 70% après cuisson légère (à la vapeur) et entre 70 et 84% d'augmentation après traitement en autoclave. Ces augmentations sont plus importantes que d'éventuelles dégradations observées pour la DTX2 qui, légèrement moins stable que l'OA, commence à se dégrader dès 90 °C après 10 min de cuisson. Ces variations de concentration sont source de difficultés pour l'estimation des doses qui peuvent rendre des personnes malades à la suite d'une consommation de moules, car il n'y a pas de précision sur le poids des portions consommées, ni sur le choix des mesures exprimées en poids de coquillages crus ou cuits. De plus, ces variations de concentration de toxines peuvent être source de rejet de produits si des moules contaminées juste en-dessous de la norme, par exemple à 120 µg/kg, sont cuites à une échelle commerciale avant d'être mises en sachet ou en boîte. En effet, ce produit pourrait facilement contenir 180 µg/kg après le traitement (cuisson) et donc ne pas satisfaire un contrôle du produit final. Ainsi, ces effets observés sont importants pour l'évaluation du risque autant que pour la méthode utilisée durant les contrôles officiels. Un test souris qui ne peut pas détecter des concentrations plus faibles que la limite légale va occasionner des pertes de coquillages et des moins-values chez les professionnels qui ne pourront pas commercialiser leurs produits.

Du fait des transformations de l'AZA17 en AZA3 durant la cuisson des moules (McCarron et al., 2009), les augmentations en équivalent AZA1 pour les trois analogues réglementés (AZA1, -2 et -3) peuvent être d'un facteur 2. Les phénomènes décrits pour l'acide okadaïque et ses analogues ci-dessus sont donc renforcés, et une commercialisation de moules potentiellement contaminées aux AZA est encore plus dangereuse si les contrôles officiels ne se font qu'avec le test souris, dont la probabilité de détection de toxines à la moitié de la limite réglementaire n'est que de 5%. Comme les AZA ont été trouvés tout le long de la côte atlantique de l'Europe, il est clair que ce phénomène n'est pas unique à l'Irlande mais concerne aussi les autres pays producteurs de moules.

Interactions des toxines avec les systèmes biologiques

Mes études sur la toxicité des phycotoxines sont toutes en collaboration avec des toxicologistes. Elles portent presque exclusivement sur les azaspiracides, à l'exception d'une étude de toxicité relative à la DTX2.

L'étude de la toxicité relative à la DTX2 a ses origines dans un besoin d'une meilleure compréhension des résultats de routine en Irlande et en Norvège. Dans ces deux pays, il y avait depuis le début du millénaire une surveillance des toxines lipophiles, comprenant des contrôles officiels, qui faisait appel aux deux techniques complémentaires : le test souris et la LC-MS. Durant la période de 2002 à 2004, il n'y avait pratiquement pas d'apparition d'AZA et en conséquence les seules contaminations par des phycotoxines observées étaient dues aux toxines du groupe d'acide okadaïque. Durant ces années, la surveillance en double technique a servi de comparaison entre les deux techniques. En fonction de ces résultats, nous avons remarqué une prévalence de la DTX2 en Irlande typiquement à partir de mi-août, suite aux apparitions de

Dinophysis acuta. Soit la LC-MS surestimait les concentrations en équivalent OA, soit le test souris n'était pas efficace pour les discerner. Après discussion avec d'autres laboratoires nationaux de référence, les norvégiens ont confirmé des observations similaires. Ainsi, il y avait une motivation pour les deux Etats membres à clarifier la situation. Comme en Irlande le projet ASTOX avait permis au Marine Institute de se former aux procédures d'isolement préparatif pour les toxines lipophiles à l'exemple des AZA, nous avons utilisé des moules contaminées pour isoler la DTX2. Nos collègues japonais ont pu confirmer que nous avons obtenu une toxine suffisamment pure pour l'évaluation toxicologique, et nous avons ainsi procédé à cette évaluation. La toxicité par voie intra-péritonéale a été évaluée et celle-ci correspond à environ 0,6 fois la toxicité de l'acide okadaïque. Un test d'inhibition de la phosphoprotéine-phosphatase-2 a confirmé que ce différentiel est également visible sur ce modèle fonctionnel. Ainsi, nous avons fourni cette donnée aux évaluateurs de risque de l'EFSA, et la toxicité spécifique de la DTX2 peut être dorénavant prise en compte à l'échelle européenne. En parallèle, la préparation d'un étalon certifié a été entrepris en collaboration avec le NRCC. L'étalon a été préparé et la certification est actuellement en cours au NRCC.

Pour les azaspiracides, la nécessité de caractériser la toxicité de ce composé a été émise en Irlande dès 1999, dès lors que des lots de moules avaient été rejetés par des importateurs français. Ainsi, le projet ASTOX sur la production d'un étalon pour les AZA et pour mieux connaître leur toxicité a été bien classé dans le domaine du développement durable de la conchyliculture à l'échelle nationale. Dans ce projet, nous avons des collaborateurs irlandais, japonais et américains pour travailler sur la toxicologie des azaspiracides. Malheureusement, l'organisme producteur des AZA, *Azadinium spinosum*, n'a été découvert qu'après ce projet, destiné à les isoler et qui dépendait de l'apparition de ces toxines dans les coquillages. Ainsi, nous n'avons pu travailler sur la toxicologie qu'avec les stocks d'AZA obtenu, auparavant, au Japon. Ceci ne permettait pas de conduire d'expérience *in vivo* (souris ou rat) mais seulement d'étudier les effets des AZA *in vitro*, techniques nécessitant nettement moins de composé.

Les approches de toxicologie *in vitro* ont été de nature systématique et étaient basées sur les connaissances préalables des effets des AZA observés chez l'homme et les mammifères rongeurs. Une première étude sur la cytotoxicité de l'AZA1 a montré que de nombreuses lignées de cellule étaient sensibles à l'exposition à l'AZA1, et que des lymphocytes (Jurkat) étaient les plus sensibles, avec une concentration effective à 50% (CE₅₀) de l'ordre nanomolaire (Twiner et al., 2005). Cette observation était cohérente avec le caractère neurologique de l'AZA, rapporté par des observations initiales dans les souris exposées à de fortes doses d'AZA. Une deuxième étude dans le domaine de la neurotoxicité a été conduite avec une collaboratrice américaine. Cette étude a pu montrer que les canaux sodique et calcique ne sont pas la cible directe des AZA, mais qu'il y a toutefois également des effets prononcés sur les neurones (Kulagina et al., 2006). Des essais sur cette lignée de cellule ont aussi permis de confirmer les observations de Flanagan et al. (2001) sur les effets morphologiques de l'AZA1 à l'échelle cellulaire. La dégradation des pseudopodes sur ces cellules suggérait aussi que le cytosquelette est affecté. Cette observation a pu être confirmée plus tard à l'échelle moléculaire. En parallèle, nos collaborateurs irlandais ont évalué les effets de l'AZA sur les cellules Caco-2, lignée modèle de l'intestin, en cohérence avec les effets diarrhéiques observés chez l'homme, suite à la consommation de moules contaminées. Effectivement, une diminution complète de la résistance électrique trans-épithéliale (Trans-epithelial Electrical Resistance ou TEER) a été observée dès l'exposition des cellules à des concentrations nanomolaires (Hess et al., 2007b). Ces effets sont similaires mais plus prononcés que ceux de l'acide okadaïque dans le même modèle. Néanmoins, Twiner et al. (2005) ont aussi pu démontrer que l'AZA1 n'affectait pas les phosphoprotéines-phosphatases et que le mécanisme d'action toxique des AZA diffère donc définitivement de celui de l'acide okadaïque et de ses

analogues. Les cellules Caco-2 ainsi qu'une autre lignée de cellule épithéliale (MCF7, cancer du sein) ont pu être examinées à travers une collaboration avec le groupe de Rossini (Université de Modena) pour confirmer qu'une protéine de l'adhérence cellulaire est affectée, l'E-cadhérine (Ronzitti et al., 2007). Des études systématiques sur les effets de l'AZA1 à l'échelle du génomique ont pu être réalisées par deux groupes de recherche, le groupe de Doucette en Amérique et celui de Ryan en Irlande. Le groupe américain a poursuivi la piste des cellules Jurkat (lymphocytes T) en utilisant des puces génétiques humaines. Ils ont découvert qu'il y avait un différentiel dans l'expression des gènes impliqués dans la biosynthèse du cholestérol, lipide membranaire des cellules mammifères (Twiner et al., 2008). Avec des techniques similaires, les irlandais ont poursuivi la piste de la lignée de cellule Caco-2 et ont trouvé que des gènes impliqués dans la réparation de blessures étaient sur-exprimés (Hess et al., 2007b). Enfin, une étude parallèle sur le modèle *medaka* a examiné le potentiel tératogène d'AZA1. Un sous-développement grossier a été détecté dans des embryons des *medakas* exposés à l'AZA1. Ainsi, des effets des AZA sur la reproduction des poissons ne peuvent pas être exclus, si effectivement, il y a transfert à travers la chaîne alimentaire marine. En somme, nos études n'ont pas pu élucider de manière claire et définitive le mécanisme d'action des AZA, néanmoins nous avons pu exclure certains pistes (canaux ioniques et PP2a) et émettre d'autres hypothèses de travail.

7.7. Perspectives

Le lecteur consciencieux aura sans doute noté, qu'en tant que chimiste, j'ai initialement focalisé sur la mise à jour de l'analyse chimique pour atteindre l'état de l'art à travers le développement de méthode, l'évaluation des paramètres critiques affectant leur performance et par la provision d'outil de contrôle qualité, y compris les étalons et des matériaux matrice de référence. Bien que j'ai restreint mon travail jusqu'ici uniquement aux composés déjà réglementés dans ce domaine, il y reste beaucoup à faire. Des étalons ne sont toujours pas disponibles pour tous les composés réglementés et la réactivité des toxines en question est loin d'être clarifiée dans sa totalité. Je continuerai mon travail dans ce domaine, et j'encouragerai d'autres à contribuer à nos travaux d'une manière plus systématique, ce qui devrait nous permettre de rationaliser les ressources disponibles, mais limitées, à l'échelle internationale.

Je suis intrigué par le progrès récent dans le domaine de la culture d'algue, notamment de la découverte du producteur des azaspiracides, *Azadinium spinosum*, et de la possibilité de cultiver *Dinophysis*. Comme la disponibilité des toxines pour le développement et la validation de méthode ainsi que pour les études toxicologiques est toujours insuffisante, nos efforts doivent rester focalisés sur cet aspect. Dans ce contexte, je suis curieux de continuer la recherche que j'ai commencée dans le cadre du projet ASTOX et qui se poursuit dans ASTOX2. Dans ce consortium élargi, nous avons accès à des espèces d'*Azadinium* pour étudier son comportement et son potentiel pour la production durable des azaspiracides. La culture d'algues toxiques va nous permettre de compléter nos connaissances des profils complexes des métabolites produits, spécialement ceux des espèces du genre *Dinophysis*, organisme qui a été un défi international. J'ai le sentiment que nous ne venons que de toucher la pointe d'un iceberg.

Bien que les changements dans le contrôle officiel des phycotoxines vont vers l'analyse chimique, et pourront éventuellement nous aider à focaliser les efforts réglementaires sur les problèmes connus, c.-à-d. les toxines connues pour rendre malades les consommateurs de coquillages contaminés, je pense que le défi des composés bio-actifs dans le milieu marin persistera. Des études récentes ont révélé que plus de 40 000 composés bio-actifs naturels ont déjà été répertoriés dans l'environnement marin. Tandis que les essais sur animaux pour l'analyse des coquillages nous a aidé à découvrir quelques douzaines de composés, le potentiel de nos océans pour l'activité biologique est loin d'être exploré. Nous avons vu la difficulté pour élucider le mécanisme d'action des composés bioactifs récemment découverts. En partant des effets sur les souris, les études d'élucidation de mécanismes d'action peuvent durer des années sinon des décennies. L'industrie pharmaceutique prend l'approche opposée, en validant d'abord la cible biologique prévue, puis en criblant de grands assemblages de composés, dans un premier temps de manière virtuelle et ensuite par criblage biologique à haut débit. Actuellement, le procédé de découverte de nouveaux médicaments et de leur validation prend un temps similaire à nos recherches sur les mécanisme d'action. Néanmoins, si nous pouvions croiser nos deux approches, une nouvelle discipline scientifique très productive pourrait naître. Au cours des congrès internationaux Marine Natural Products (2007) et l'AOAC Annual Meeting (2008), j'ai suggéré ces rapprochements de disciplines. Mais je suis sûr que d'autres efforts seront nécessaires avant de voir un progrès constructif dans cette direction. Pour commencer, je me suis engagé dans une collaboration avec l'université de Nantes pour cribler l'environnement côtier marin pour les composés bio-actifs naturels lipophiles, en utilisant des approches classiques et novatrices afin d'évaluer de plus près les filtres vivants de nos océans que sont les mollusques bivalves.

Enfin, le lecteur intéressé aura remarqué également que la plus grande partie des recherches présentées dans ce mémoire sont de nature appliquée : la science au service de la production de

coquillages salubres pour la consommation humaine. Quelques-unes des études permettront de mesurer plus précisément les toxines présentes, d'autres de clarifier le comportement de ces toxines dans les procédés commerciaux et d'autres encore souligneront l'étendue de la toxicité des composés. Lorsque j'ai rejoint l'Ifremer, je me suis engagé à diriger un département de recherche qui a pour thème central la salubrité des coquillages et cela concerne, à la fois, les contaminations par les phycotoxines et les contaminations microbiologiques qui menacent la santé du consommateur de coquillages. Ces deux sujets ne sont pas uniquement reliés par le thème coquillage, mais ils le sont également par les interactions dans le milieu marin entre les algues toxiques et la communauté microbienne marine. Un des grands défis à surmonter dans ce domaine est notre compréhension des interactions écologiques entre les organismes microscopiques, tels que le phytoplancton et les bactéries marines, dans leurs environnements chimique et biologique. Tandis que les changements globaux, avec toutes les influences anthropogéniques telles que les sels nutritifs et les pesticides venant de l'agriculture et de l'aquaculture, affectent l'environnement chimique des micro-organismes, le réseau trophique des brouteurs, parasites, organismes symbiotiques et compétiteurs est toujours à peine appréhendé. C'est une de mes ambitions personnelles de construire des collaborations avec les collègues de ce domaine pour mieux répondre à nos questions respectives. Un autre lien entre la salubrité microbiologique des coquillages et la menace phytoplanctonique est la récente amélioration des outils génomiques et protéomiques. La manière dont les coquillages accumulent et digèrent les microbes doit être explorée de près afin de mieux comprendre le phénomène de rétention des microorganismes et de leurs toxines, ainsi que leur métabolisme dans les coquillages.

7.8. *Résumé final*

Les algues toxiques qui produisent un ensemble de composés bioactifs, peuvent s'accumuler dans les coquillages et être alors la cause d'empoisonnement chez l'homme, suite à des consommations de coquillages contaminés. La chimie, la distribution et la toxicité des toxines impliquées ont été revues de manière systématique. Ensuite, les procédures d'analyse et de gestion du risque ont été décrites pour les phycotoxines. Les cinq éléments auxquels je pense avoir contribué significativement sont les suivants : i) les méthodes d'analyse, ii) la distribution des biotoxines marines, iii) l'isolement préparatif, iv) le contrôle qualité et v) la caractérisation des dangers posés par ces produits chimiques en terme de leur lipophilicité, stabilité, réactivité et toxicité.

Dans les développements méthodologiques, j'ai souligné les travaux réalisés sur la spectrométrie de masse couplée à la chromatographie en phase liquide pour la détection des toxines à la fois hydrophiles et lipophiles, en portant une attention particulière sur l'identification des paramètres critiques. Pour les toxines du groupe saxitoxines, nous avons introduit la chromatographie à interaction hydrophile et lipophile ce qui nous a permis de séparer la quasi-totalité des analogues de la saxitoxine, tout en obtenant des limites de détection similaires à celles du test souris réglementaire. La détermination des toxines lipophiles a été examinée de manière critique. Les effets de matrice observés ont été décrits systématiquement, et des recommandations ont pu être données pour éliminer ces effets ou, tout au moins, pour les réduire.

La distribution géographique des azaspiracides tout le long de la côte atlantique de l'Europe a été démontrée, et des différences biogéographiques entre les souches européennes et américaines de *Dinophysis* ont été signalées. En Irlande, l'accumulation de toxines lipophiles des groupes de l'acide okadaïque et des azaspiracides a été démontrée avoir des taux plus élevés dans les moules (*Mytilus edulis*) que dans les huîtres (*Crassostrea gigas*), grâce à l'introduction des tests par LC-MS pour la surveillance de routine. La contamination des coquilles St Jacques (*Pecten maximus*) par l'acide domoïque ainsi que les causes de sa variabilité ont été étudiées dans les eaux écossaises et irlandaises. Les résultats des études ont entraîné des changements législatifs concernant la surveillance et la gestion des zones de production. La distribution des phycotoxines a également été examinée à l'aide d'échantillonneurs passifs. Grâce à ces techniques, l'existence de toxines précédemment inconnues en Irlande a pu être démontrée (dinophysistoxine-1, yessotoxine, desméthyle-spirolide C). Nous n'avons pas pu progresser sur la capacité de prédiction en utilisant des échantillonneurs passifs.

Des composés appartenant au trois groupes de toxines suivants ont été isolés : azaspiracide, acide okadaïque et pecténotoxine. Des efforts spécifiques ont été dédiés aux azaspiracides. Ces efforts ont culminé avec la certification d'un étalon pour l'azaspiracide-1 et avec la découverte de huit analogues qui n'ont pas été répertoriés auparavant. Cette découverte résulte dans un nombre total de vingt analogues connus pour ce groupe de toxines. La dinophysistoxine-2 a été isolée avec une pureté suffisamment satisfaisante pour permettre l'approfondissement des connaissances toxicologiques ainsi que pour la production d'un étalon actuellement en voie de certification au Conseil National de la Recherche Scientifique du Canada. L'isolement préparatif des acyl-esters de la seco-acide de la pecténotoxine-2 a nettement clarifié le métabolisme de cette toxine dans les moules (*M. edulis*) ainsi que la complexité des dérivés naturels.

Des matériaux de référence basés sur les tissus de coquillages ont été préparés et les facteurs affectant leur homogénéité ainsi que leur stabilité ont été élucidés. Ces matériaux se sont avérés appropriés pour une utilisation dans la validation intra- et inter-laboratoire, pour des essais d'aptitude ainsi que pour la certification selon les guides de l'organisation internationale de

standardisation. Deux matériaux ont été produits pour la certification : un tissu humide naturellement contaminé avec les azaspiracides ainsi qu'un tissu de moules lyophilisé contaminé avec de multiples groupes de toxine. Les essais d'aptitude ont été introduit pour l'acide domoïque, l'acide okadaïque, les azaspiracides et les saxitoxines. L'utilisation des techniques de chromatographie liquide couplée aux détecteurs à ultraviolet et à spectrométrie de masse a montré que ces deux techniques sont équivalentes pour l'acide domoïque. Des essais immunologiques ont été comparés à la chromatographie liquide couplée aux détecteurs à ultraviolet et à spectrométrie de masse pour l'acide domoïque et l'acide okadaïque. La performance du test souris lipophile a été évaluée et comparée à une méthode validée en interne et basée sur la chromatographie liquide couplée à la spectrométrie de masse, prouvant ainsi que ces deux techniques peuvent être utilisées pour la détection des azaspiracides à la limite de la norme actuelle de $160 \mu\text{g kg}^{-1}$. Néanmoins, le test souris a ses limites et il ne peut détecter des concentrations plus faibles. Ainsi il n'est pas approprié à une utilisation dans le cadre d'un contrôle officiel des produits car il n'est pas capable de prendre en compte les changements de concentration en azaspiracides durant les traitements par la chaleur, tel que la cuisson des moules.

Les constantes d'acidité et de lipophilicité de certains composés lipophiles ont été déterminées en utilisant l'approche chromatographique. De plus, la stabilité et la réactivité de certains composés ont pu être élucidées. Les azaspiracides apparaissent plus sensibles aux traitements alcalins et acides. L'azaspiracide-3 s'est également montré plus sensible à la chaleur que deux de ses analogues (l'azaspiracide-1 et -2). L'acide okadaïque s'est rapidement dégradé sous l'influence d'acides forts si des solutions méthanoliques ont été chauffées. Il apparaît que les traitements des moules par la chaleur engendrent des augmentations très significatives des concentrations de toxines lipophiles, ce qui suggère que les producteurs de coquillages doivent prendre en compte ces effets dans leurs plans de contrôle des points critiques en identifiant les dangers et en effectuant des autocontrôles durant la production. La toxicité de la dinophysistoxine-2 s'est avérée moindre d'un facteur deux par rapport à la toxicité de l'acide okadaïque. La toxicité de l'azaspiracide-1 a été examinée à l'échelle cellulaire et moléculaire. La cytotoxicité, le potentiel tératogène et les effets sur la régulation des gènes ont pu être examinés. Le mécanisme d'action n'a pas été élucidé de manière définitive, néanmoins des résultats préliminaires suggèrent que la biosynthèse des lipides ainsi que les voies de guérison des plaies sont affectées par ce groupe de toxines.