5th Irish Shellfish Safety Scientific Workshop

Rosscarbery Co. Cork 28th October 2004

Organised by the Marine Institute, Food Safety Authority of Ireland and Bord Iascaigh Mhara

CONTENTS

Page

Foreword
Dr. John O'Brien, Chief Executive, FSAI 1

Session 1: Developments in 2004, Monitoring and Reviews
(Chair Dr. John O'Brien, CEO, FSAI)

Introduction and Objectives of the 5th Irish Shellfish Safety Workshop
Micheál Ó Cinneide, Director, Marine Institute 2

Review of Phytoplankton and Environmental Monitoring 2004
Siobhan Moran, Joe Silke, Rafael Salas, Tara Chamberlan, Josephine Lyons, John Flannery, Dave Clarke, Leon Devilly, Terry McMahon, Micheál O Cinneide, Marine Institute 5

The Management Cell, 2004
Tim Coakley, Irish Shellfish Association 11

A Review of Shellfish Toxicity Monitoring in Ireland for 2004
Dave Clarke, Leon Devilly, Billy Gibbons, John Flannery, Philipp Hess, Josephine Lyons, Myra Keogh, Jane Kilcoyne, Maria McCarron, Niamh Mulcahy, Jenny Ronan, Barry Rourke, Rafael Gallardo Salas, Joe Silke, David Swords 15

Session 2: Irish Biotoxin/HAB Research
(Chair Dr. Terry McMahon, Marine Institute)

Biotoxin Chemical and Toxicological Research
Philipp Hess[1], Nils Rehmann[1], Jane Kilcoyne[1], Pearse McCarron[1], Katrin Bender[1] Gavin Ryan[2], Michael Ryan[2]
[1] Marine Institute, Galway Technology Park, Parkmore West, Galway
[2] Dept. Pharmacology – Conway Institute, University College Dublin, Belfield, Dublin 4 23

The Biological Oceanography of Harmful Algal Blooms (BOHAB) Programme, 2004.
Caroline Cusack[1], Joe Silke[2], Georgina McDermott[1], Glenn Nolan[2],
Maeve Gilmartin[2] & Robin Raine[1].
[1]Martin Ryan Institute, National University of Ireland, Galway
[2]Marine Institute, Galway Technology Park, Galway, Ireland 31

The Dynamics of *Alexandrium* Blooms
Aoife Ní Rathaille and Robin Raine
The Martin Ryan Institute, National University of Ireland, Galway 35

Amnesic Shellfish Poisoning Research at Letterkenny Institute of Technology
Yvonne Bogan[1] Anne Louise Harkin[1], Clare Greenwood[1], John Gillespie[1], Philipp Hess[2], John Slater[1]
1. Letterkenny Institute of Technology, Letterkenny, Ireland
2. Marine Institute, Galway, Ireland 40

Session 3: Focus on Shellfish Microbiology
(Chair: Dr. Ronnie Russell, Trinity College Dublin)

Microbiological and Viral contamination of Shellfish: Work of the Irish National Reference Laboratory and Current Progress in Virus Detection Methods
Bill Doré, Marine Institute, Galway 47

Microbial Dispersion in Bannow Bay, Co. Wexford.
Bartholomew Masterson, Department of Biochemistry, University College Dublin 53

Session 4: International and EU research Developments
(Chair: Dr Terence O' Carroll, BIM)

Microbiological Vulnerability: A new approach for shellfish quality assessment and management
Sébastien Chantereau (Comité National de la Conchylicultre, France)
Richie Flynn (Executive Secretary, Irish Shellfish Association) 61

ATTENDEES **67**

FOREWORD

Dr. John O'Brien, Chief Executive, Food Safety Authority of Ireland

The evolution of the science of shellfish safety in Ireland is reflected in the change in title of the 2004 workshop, which addressed the broader aspects of shellfish safety rather than focusing uniquely on biotoxins. Biotoxin monitoring in Ireland is a major success story. It is the fruit of successful collaboration among the shellfish industry, the Marine Institute, The Department of Communications, Marine and Natural Resources, and the Food Safety Authority of Ireland. I am particularly pleased to congratulate Micheal O'Cinneide and his team at the Marine Institute for all they have achieved. Scientifically, it has been facilitated by the Molluscan Shellfish Safety Committee. The progress of recent years is evocative of Lord Kelvin's adage that "If it cannot be expressed with number, we don't know much about it". Analytical advances have provided us with the numbers and we can say with confidence that we know much more than we used to know. Kelvin's related philosophy is even more important from a food safety perspective: "What gets measured, gets managed". This is true also for the Irish shellfish industry and what is getting managed is not only the safety of shellfish but also the business risk for producers.

I was impressed to hear that 7000 tests were conducted in the integrated monitoring programme in 2004. The year 2004 was a year of low toxicity with less than 4% of samples positive for DSP. There was good news too on the commercial front. There is continuing strong growth in the molluscan shellfish industry.

Nor is the biotoxin research field standing still. Almost €1.3 million has been secured in Irish and European research funding for 4 research projects (ASTOX to look at azaspiracid toxicology; BIOTOX to explore alternatives to the mouse bioassay; BOHAB to study the bio-oceanography of harmful algal blooms; and HABBUOY which addressed in situ phytoplankton monitoring). Continued investment in biotoxin research is necessary to detect and quantify toxins more precisely and more quickly – permitting better operational risk management. Investment in toxicology is necessary to ensure that measures taken in response to analytical results are proportionate to the human risk. As chemical methods become the norm ultimately replacing the mouse bioassay, such toxicology data will be indispensable.

Progress in shellfish safety is not limited to the biological sciences, but a pool of expertise from many disciplines has made the Irish approach to risk management truly world class. For the consumer, one of the attractions of shellfish, apart from the taste, is the fact that they come from the wild, growing on what the sea can provide them. Biotoxins are also a natural phenomenon delivered by the sea, with the potential to accumulate in molluscs. Measures such as telemetry stations can give advance warning when ocean currents and temperatures might have potential to result in elevated biotoxin levels. Telecommunications technology can also assist producers to react quickly by relaying SMS messages instantly and to the production sites.

INTRODUCTION AND OBJECTIVES OF THE 5TH IRISH SHELLFISH SAFETY WORKSHOP

Micheal Ó Cinneide,
Director, Marine Environment & Food Safety Services, Marine Institute

On behalf of the Marine Institute and our co-sponsors, BIM and the Food Safety Authority of Ireland (FSAI), I would like to welcome the participants to this, our 5th annual Shellfish Safety Workshop.

The workshop is part of the Marine Institute's role as the National Reference Laboratory for Marine Biotoxins in Ireland. Since 2000, this workshop has become an annual event, where scientists, regulators and shellfish farmers meet to review developments in the monitoring and research of shellfish safety issues in Ireland and internationally.

Given the gale force winds and floods on the way to West Cork, we are delighted that up to eighty participants have braved the elements to join us!

Objectives. Our specific objectives for the 2004 Workshop are:

- Review the Irish shellfish monitoring system and to assess the trends in toxicity during 2004
- Summarise current Irish research work in Harmful Algal Blooms (HAB's), phytoplankton and toxicology
- Take stock of developments since the 2003 workshop and provide a forum for debate/feedback
- Strengthen our focus on shellfish microbiology and virology.

Key Irish Developments in 2004

- The Marine Institute is now managing an integrated monitoring programme with over 7,000 samples tested per annum
- The phytoplankton, bioassay and chemistry results are now available on the Marine Institute website **(www.marine.ie)**
- The Molluscan Shellfish Safety Committee (MSSC) and the Management Cell are working effectively (43 decisions, year to date)
- Toxicity has again been low in 2004 (**3.2%** of shellfish samples were positive)
- We have made further progress in laboratory accreditation and quality systems in the areas of bioassay, phytoplankton and chemical testing (LC-MS methods)
- The MI, FSAI and BIM presented the developments the Irish shellfish monitoring system to the French shellfish importers group (SNCE) in Bantry, March 2004
- The **ICMSS** international Conference on Molluscan Shellfish Safety was held in NUI Galway in June 2004 and attracted over 300 delegates
- The EU Food & Veterinary Office (FVO) carried out a week long audit of the Irish bivalve mollusc control system in October; while their report is not yet available, we expect the feedback on the monitoring programme to reflect the positive impact of investments since 2000
- MI has recruited staff for the National Reference Lab in Shellfish Microbiology, and Bill Doré (Team Leader) will present the work programme at this workshop

Collaborative Projects in Harmful Algal Events (HAE's) Research
The Workshop will give us an update on some key research projects, which are underway in the areas of Biotoxins and Harmful Algal Events (HAE's). Many of these are funded by the Marine Research Measure (2000- 2006). These include:

- **ASTOX** (Budget €400,000) **-** Marine Institute staff are working with scientists from the Conway Institute, University College Dublin, the NOAA laboratory in Charleston, USA and Japanese colleagues on the Chemistry and toxicology of Azaspiracids
- **BOHAB** (Budget €400,000) **-** NUI Galway, the Marine Institute and Woods Hole Oceanography Institution (WHOI) and Dartmouth College, USA are collaborating in a project on the biological oceanography of Harmful Algal Blooms in Irish waters
- **HAB BUOY** (Budget €70,000) – a CRAFT funded project with partners from Ireland, UK and Spain on developing new technologies on *in situ* phytoplankton identification.
- NUI Galway and Bioresearch Ireland, Galway are continuing research work on *Alexandrium* lifecycles and the development of immuno-assays, with funding from the Higher Education Authority and the PRTLI (Programme of Research in Third Level Education))
- Letterkenny Institute of Technology, Donegal has built up its capability in shellfish research

Biotoxins and HAB's are a global issue. The scale of the processes underlying marine toxins means that international cooperation is essential. The Marine Institute continued to work closely in 2004 with partners from Europe, the USA, New Zealand and Japan and we look forward to building on these links in the years ahead.

Key partners for MI include the network of National Reference labs for Biotoxins in the EU and Cawthron Institute, Nelson, New Zealand in the validation work on the LCMS tests for a range of biotoxins and in phytoplankton

Communications with Stakeholders.
The Marine Institute is committed to open communications with our stakeholders, especially with industry, regulators and scientists. As an integral part of the MI Shellfish safety programme, we seek to promote communications through the following channels:

- Collaboration with the Food Safety Authority (FSAI) in the successful development of the online HAB database.
- Weekly Reports by fax or email
- SMS Text message service by mobile phone re changes in bay status to over 90 industry and regulators
- Daily phone contact with samplers and industry members
- Participation at the MSSC meetings and its subcommittees
- Participation and advice to the Management Cell, which includes representatives of the FSAI, Department of Marine, MI and Irish Shellfish Association
- Arranging conferences, workshops and regional meetings

- Active participation in the meetings and working groups of EU Network of National Reference Laboratories for Biotoxins and Shellfish Microbiology
- Issuing the Proceedings of the annual Shellfish Safety Workshop to 500+ interested parties

Future Evolution.
The Marine Institute will continue to work with our partners to support the development of the Irish Shellfish industry by promoting shellfish safety and by building the best Shellfish Safety system in the Northern hemisphere.

The future evolution of the Irish Shellfish safety programme will include:

- Validation and development of LC-MS methods for multi toxin analysis
- Reduced use of mouse bioassay and gradual increase of alternative methods
- Research and liaison work with other EU member states, the EU Commission, the WHO and CODEX to draft a regulatory framework for alternative test methods
- Development of HAB models that will provide an early warning system for industry and regulators

Irish Molluscan Shellfish Output, 1990 – 2003
Ireland's investment in shellfish safety has supported the strong growth of the molluscan shellfish industry. BIM survey figures indicate that the value of farmed shellfish production in 2003 was €41.8 million and the export value was €39 million (Figure 1).

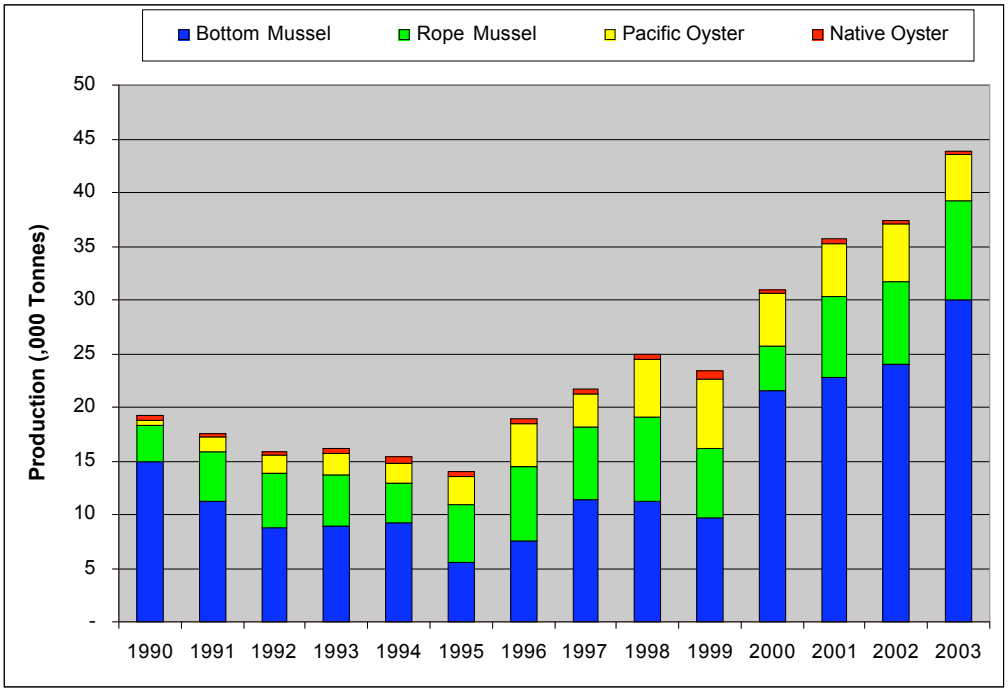

Figure 1. Annual production (tonnage) of the principal farmed shellfish species for the period 1990-2003. (Source – ***Status of Irish Aquaculture Report***, 2004)

REVIEW OF PHYTOPLANKTON AND ENVIRONMENTAL MONITORING 2004

Siobhan Moran[1], Joe Silke[1], Rafael Salas[1], Tara Chamberlan[2], Josephine Lyons[1], John Flannery[1], Dave Clarke[1], Leon Devilly[1], Terry McMahon[3], Micheal O Cinneide[1] .

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The following paper provides an overview of phytoplankton sampling, analysis and reporting in 2004 to date (Weeks 1 to Week 42). Comparisons are made with the same time period in 2003. The occurrence of potentially toxic and harmful phytoplankton found in Irish coastal and shelf waters in 2004 is also reviewed, general improvements in reporting are discussed, and the quality scheme and environmental data for 2004 are outlined. The results given are derived from data collected by the Marine Institute as part of the National Phytoplankton and Biotoxin Monitoring Programme.

Overview

Staff from the Department of Communications, Marine and Natural Resources (DoCMNR) are responsible for the sample management of the National Monitoring Programme. Throughout the year water samples are collected at shellfish and finfish production sites by DoCMNR and the aquaculture industry. Where depth allows, samples are collected using a Lund tube (5-15 m), otherwise surface samples are collected. At aquaculture production sites sample frequency is weekly during "high toxicity risk" periods (Spring to Autumn) and reduced to monthly in winter when little or no phytoplankton growth occurs. Eleven "sentinel sites", around the coast are sampled on a weekly basis all year round. This provides information on the phytoplankton community structure and species succession throughout the year. Results are reported on a daily basis and over 220 reports were sent to the aquaculture industry and regulators by fax and e-mail in 2004 to date. The turnaround time of phytoplankton results in working days, from time of receipt into the laboratory was 99% within two working days. This is broken down to 88% within one working day with a further 11% within two working days. All results were also posted on the Marine Institute's Web site at www.marine.ie/habsdatabase.

To date in 2004 the number of phytoplankton samples analysed by the Marine Institute is approximately 1,841, of which ~1,700 (91.5%) are directly related to the National Monitoring Programme (NMP). This overall number is directly comparable to the same period in 2003, although NMP samples increased by 5%.

Samples from a total of 61 shellfish sites and 45 finfish sites (including Irish Naval Service samples), were submitted throughout 2004. Samples submitted from south-west to south-east coastal sites were analysed in the MI Bantry laboratory, while all remaining samples submitted were analysed in the MI Galway laboratory.

The Lund Tube sampling method accounted for almost 54% of samples collected in 2004, with 20% sampled from the surface. However, 22% of samples received by the labs gave no information on sampling method. Rejected samples accounted for 9.9% in 2004, a reduction from 12.6% in 2003. The reasons for rejections are detailed in Figure 1.

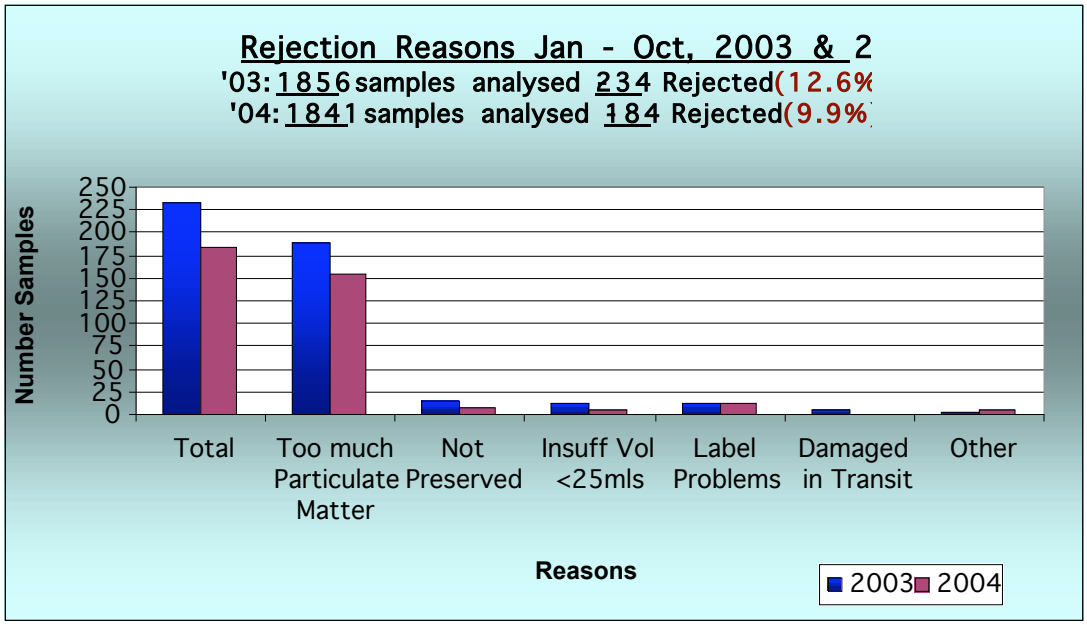

Figure 1. Breakdown of rejection reasons for samples submitted and rejected by the Phytoplankton laboratories in Galway and Bantry, 2003 and 2004.

Toxic Phytoplankton in Irish Waters 2004

The presence of toxic algae in waters can directly affect shellfish harvesting, particularly in the summer when greater levels of toxic algae are present, resulting in biotoxin contamination in the shellfish. Consequently if these toxin levels exceed a regulatory limit, closures of production areas will result. In Irish waters there are four main toxic algal groups that occur. These are the phytoplankton species that produce the toxins that cause Diarrhetic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP), and Azaspiracid Poisoning (AZP), events.

In 2004, closures in shellfish growing areas mainly resulted from DSP and PSP events. Clarke *et al.* (in these proceedings) provide details of closures and toxin concentrations.

*Paralytic Shellfish Poisoning (*PSP) *toxins*

Saxitoxins are produced by the *Alexandrium* spp. Due to the potential severity of the toxin, the presence of this species in water samples triggers the testing of shellfish samples for PSP toxins.

To date the only production area that has experienced closures due to PSP toxins is Cork Harbour, North Channel on the south coast. *Alexandrium* spp. was first observed on 20th Jan (Week 4) and continued until there was a small peak on 04th May (Wk 19) of 320 cells/l. This dropped slightly until 08th June (Wk 24) when no's reached 3700cells/l. The following week – 15th June (Wk 25) - the highest no. of cells were observed at 75,800 cells/litre. **Positive PSP** results for samples of *M. edulis* occurred on 21st June (Wk 26) and 28th June (Wk 27).

Levels of *Alexandrium* sp. decreased to >12,000 cells/litre for week 26, falling to 440 cells for week 27. On 13th July (Wk 29) numbers had risen to 4000 and decreased again until 6th September when another small peak of 3700 cells/l occurred. By 11th Oct the level had dropped to 760cells/l. Samples of *C.gigas* from the area were below the limit of detection for the AOAC bioassay during this time period. Another smaller peak of 3760 cells/l occurred on 6th September but by 11th October numbers were at 760cells/l.

High no's of *Alexandrium* sp. were also observed in Oysterhaven >23,000 cells/litre in week 27 & Wexford Harbour >8,000 cells/litre in week 24.

Also in Clew Bay *Alexandrium* spp. was present. In Clew Bay North, Inishlaughil, numbers peaked at 1240 cells/l on 19th July but by 9th August had dropped to 240 cells/l. In Clew Bay South, Murrisk, *Alexandrium spp*. peaked at 3440 cells/l on 03rd August but by the end of the month had fallen to 80 cells/l.

Diarrhetic Shellfish Poisoning (DSP) toxins
Okadiac Acid and DTX's are produced by the dinoflagellates *Dinophysis* spp. and *Prorocentrum lima*. The majority of closures in Irish production areas occur as a result of these toxins. Toxicity in shellfish can be recorded at very low cell counts (>200cells/l).

Low levels of *Dinophysis acuta & D. acuminata,* and *Phalacroma rotundatum* (Synonym *Dinophysis rotundata*) were first observed off the south coast in May, particularly in Bantry Middle, North & South, at levels of 40 cells/l, and also along the west coast in Aughinish, Killary & Clew bay North, at similar levels.

Figures 2 to 4 show highest numbers of *Dinophysis spp*. cells/l for the months of June, July and August around the Irish coast with more detailed maps of the south west coast for the same months. In July positive bioassays were observed for Bantry Inner North, Middle and South and Glengarriff. From the maps it can be seen that cell numbers peaked in August in most sites shown. Highest numbers observed were in Bertraghboy Bay (56160 cells/l) and in Killary Outer, Bantry Inner, Middle and South, Bantry Outer, Dunmanus, Wexford and Oysterhaven/Kinsale.

A **Positive DSP** bioassay was observed for Bantry North - Snave, on 12th July, (Wk 29). Snave like the other sites in Bantry North, Middle and South first showed a presence of *D. acuminata* on 9th May (Wk 20) at 40cells/l. A presence of *Dinophysis dens* (40cells/l) in Snave was recorded on 31 May (Wk 23). Over the next five weeks, levels slowly increased to 240cells/l (Wk 28). The levels peaked in Snave on 11th July at 960 cells/litre (Wk 29). The majority of these were *Dinophysis acuminata*.

Levels rose gradually in Bantry North, Middle and South until 9th August (Wk 33) where numbers increased considerably in all three areas, with the total *Dinophysis spp*. count being dominated by *D. acuta*. In Bantry North (Snave) numbers rose to 3260 cells/l with *D. acuta* making up 78% of the total *Dinophysis* count. On 30th Aug (Wk 36) cell numbers had fallen to 2840 cells/l, where *D. acuta* accounted for 89% of the total species count.

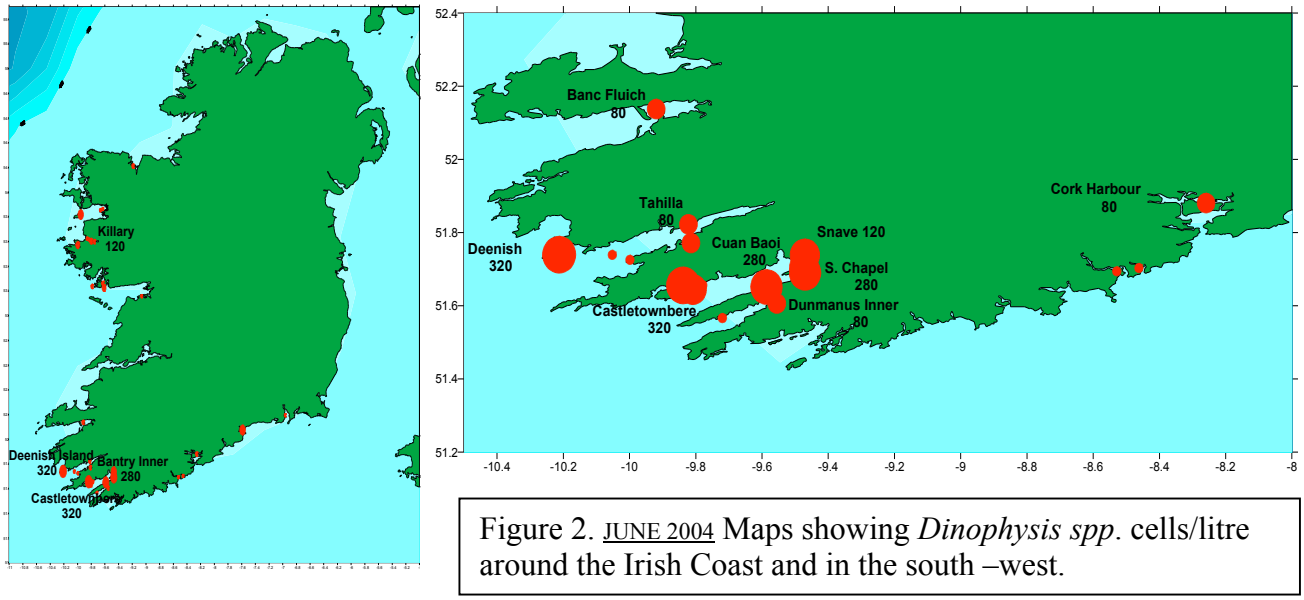

Figure 2. JUNE 2004 Maps showing *Dinophysis spp*. cells/litre around the Irish Coast and in the south –west.

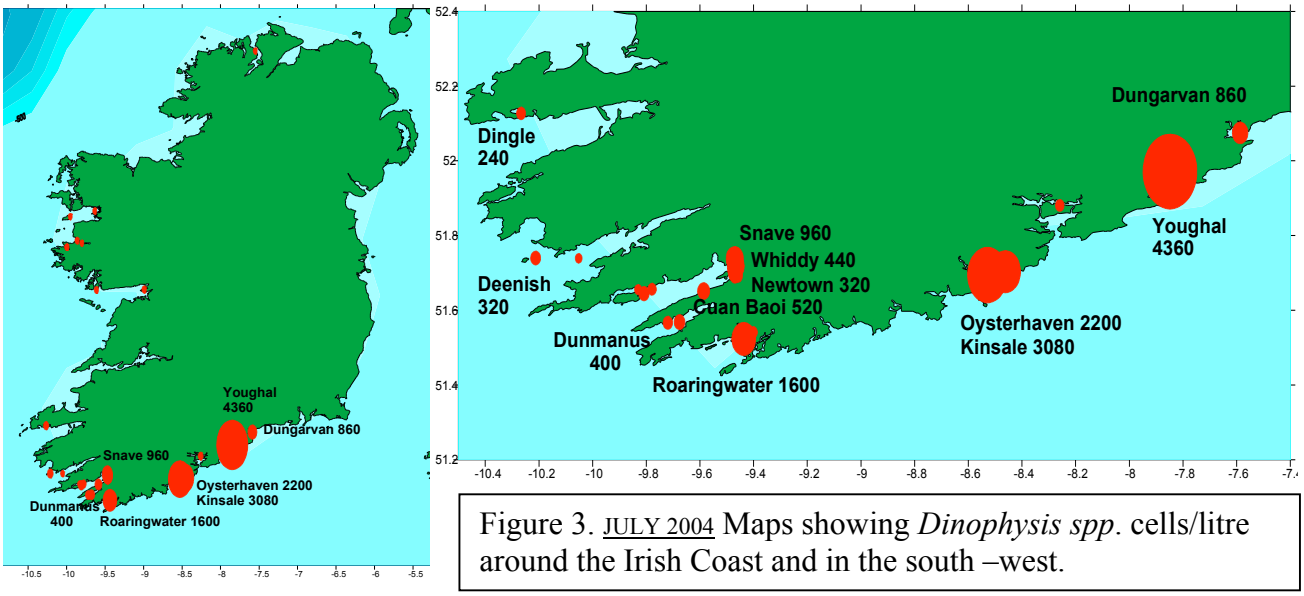

Figure 3. JULY 2004 Maps showing *Dinophysis spp*. cells/litre around the Irish Coast and in the south –west.

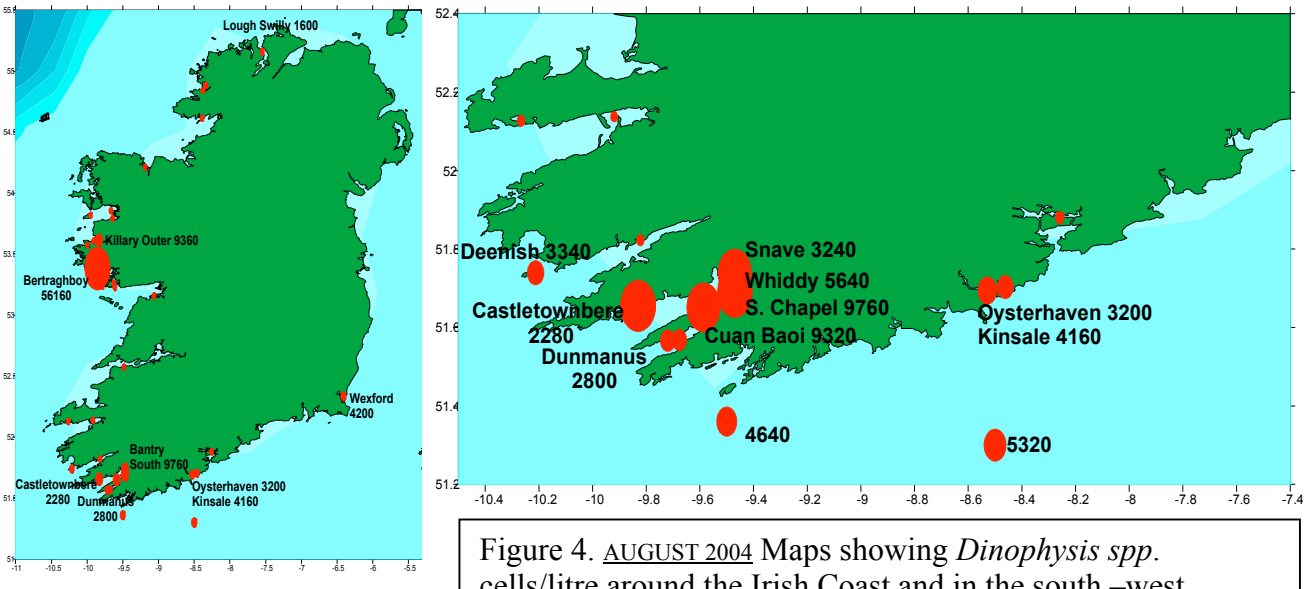

Figure 4. AUGUST 2004 Maps showing *Dinophysis spp*. cells/litre around the Irish Coast and in the south –west.

In Bantry Middle (North Chapel and Whiddy Pt.) peaks of 2280 and 5640 cells/l occurred respectively on 9th Aug. Again *D. acuta* comprised of approx. 80% of the total cell no and despite numbers decreasing gradually over the next couple of weeks, *D. acuta* remained the dominant species at approx 92 – 99% of the total *Dinophysis* count.

A correlation was shown between *Dinophysis* species, *D. acuminata* and *D. acuta* and Okadaic Acid toxins. In these proceedings Clarke *et al.* further explores this relationship, particularly with the OA derivative DTX-2 and the presence of *D. acuta* in the water.

In Castletownbere, a peak for the month of June of 320cells/l on the 18th corresponded to a **positive DSP** bioassay observed in *M.edulis*, sampled on 21st June (Wk 26). This *Phalacroma rotundataum* (Synonym *Dinophysis rotundata*) first appeared on 7th June at 40cells/l, (Wk 24). On 18th June (Wk 25) the highest no. of cells was observed at 320 cells/litre, which dropped to 160 cells/litre on 24th June (Wk 26).

By September *Dinophysis spp*. cell numbers around the coast had dramatically fallen, with Lambs Head showing the highest level of 360 cells/l. Numbers in October had further fallen to a presence level.

Problematic Species in Irish Waters in Irish Waters 2004

Phytoplankton species that are harmful to finfish were also recorded in Irish waters in 2004. These included the heterotrophic dinoflagellate *Notilluca scintillans* which regularly blooms off the east coast in summer, and *Prorocentrum minimum/balticum* which bloomed under ecologically favourable estuarine conditions.

Notilluca scintillans was the main bloom former of 2004, with cells first appearing on 8th June in North Chapel (Bantry Bay) with 80 cells/l. High numbers of these cells were observed between the 18 July to the 31st August, with a presence of cells still in October. Generally the species was observed off the southwest to the southeast coasts, from Castlemaine Harbour to Waterford. The highest count was taken at Howth, Co. Dublin on 13th Aug of 4.25 million cells/l.

Prorocentrum minimum/*balticum* also bloomed off the west coast in Kilrush Co. Clare, although over a very short time period. It's presence was noted between the 20th and 28th July with counts of 336 million cells/l recorded on 23rd and 451 million cells/l on 27th.

Reporting Improvements

Proposed plans for 2005 include modifying the existing daily report layout to be more user-friendly and making available to all interested parties, a quick reference identification card, with information on different phytoplankton species found commonly in Irish Waters. The first draft of this identification card was presented at these proceedings.

Also proposed for the coming year is a potential modification to the reporting structure of phytoplankton information. As well as the daily reports continuing to be issued on the HABS website, a weekly report will be developed showing trends for designated areas around the country. This will present in visual format recent history of an area and describe trends for particular species.

Quality System

In October 2004, the EU Food and Veterinary Office conducted a detailed audit mission on the Biotoxin Monitoring Programmes in Ireland of which the Phytoplankton Unit participated.

Also in October 2004 the Marine Institute Phytoplankton Unit, applied for INAB accreditation for the test method for Marine Phytoplankton Analysis using the Utermöhl cell counting method. Involvement in this scheme includes the adherence to Standard Operating Procedures associated with the test method, daily recording of data and also involves taking part in regular audits - both internal and external – inter-comparison testing and international proficiency testing.
Once accredited the Marine Institute Phytoplankton laboratories will be one of very few laboratories with accreditation for this test method in the world.

Environmental Data

Currently Temperature loggers have been deployed by the Marine Institute in 19 aquaculture sites around the country, including the key BOHAB sites Killary Harbour and Bantry Bay. The benefit of these is the ability to record differences in water temperature from the bottom to the surface. This can be useful in determining the occurrence of cold water subsurface intrusions. (Figure 5.) These can be associated with the advection of offshore populations of phytoplankton into bays, and often occur with the relaxation of onshore wind turning to offshore, thereby causing the surface warmer water to be replaced with deeper colder water from offshore.

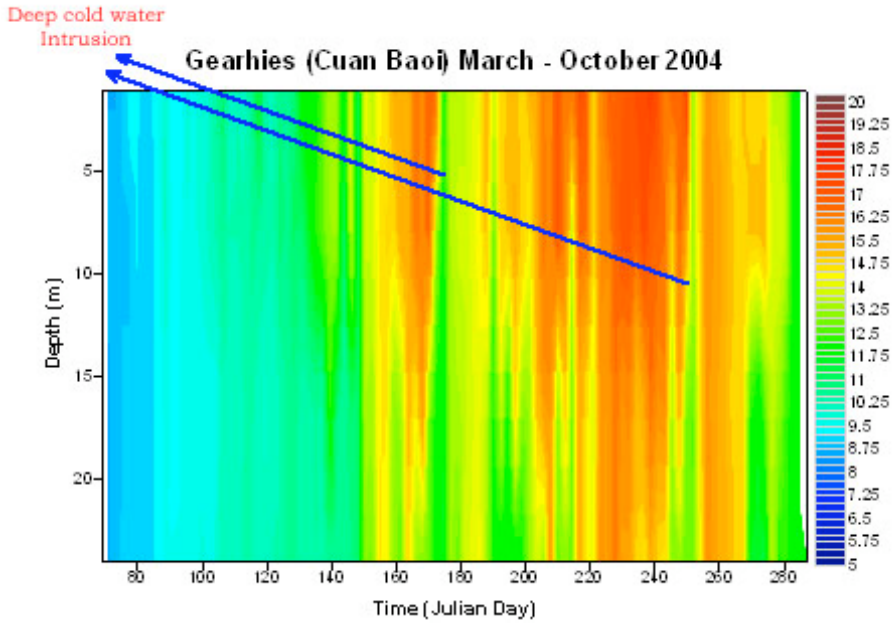

Figure 5. Temperature logger data for Gearhies (Cuan Baoi), March to October 2004, showing water temperature plotted against time (x-axis) and depth (y-axis)

Reference

Clarke, D. Devilly, L., Gibbons, B., Flannery, J., Hess, P., Lyons, J., Keogh, M., Kilcoyne, J., McCarron, M., Mulcahy, N., Ronan, J., Rourke, B., Gallardo Salas, R., Silke, J., and Swords, D. A Review of Shellfish Toxicity Monitoring in Ireland, 2004. (These Proceedings).

THE MANAGEMENT CELL, 2004

Tim Coakley, Irish Shellfish Association

In order to understand the Management Cell (MC) and how it works it is important to understand the history and evolution of the Irish Biotoxin Monitoring Programme.

The monitoring of Biotoxins evolved over time, but by in large, it did not keep pace in terms of innovation and reliability with the increasing requirements for better food safety and the increasing size of the shellfish industry.

Biotoxin monitoring for regulatory purposes suffered from a number of problems throughout the 1980's and 1990's. The program was poorly resourced, it relied on bioassays only, the science was not well developed, plankton surveillance was limited and decision-making was a rigid process. Industry was critical of the program because there was no third party input, constructive criticism was unwelcome and was largely ignored. The programme was defensively run.

In 1999/2000 Irish shellfish caused illness in European markets and the Biotoxins monitoring system was thrown into deep crisis. Consumer protection had been compromise, industry and customers lost confidence in the ability of the system to effectively regulate for Biotoxins. Shellfish companies suffered product recall, destruction and huge financial losses. The survival of the shellfish industry was in doubt.

The incidents of consumer illness in 1999/2000 led to a complete overhaul of the Biotoxin monitoring programme. The Minister for the Marine ordered the overhaul and additional resources were made available. The Food Safety Authority of Ireland (FSAI) took responsibility for the reform process and the Molluscan Shellfish Safety Committee (MSSC) became a vehicle for change.

The Molluscan Shellfish Safety Committee

The MSSC is a national forum comprising representatives of all parties involved in the production and placing bivalve molluscs in the marketplace. Its job is to discuss the safety of the product and the management of the industry, from a consumer protection perspective.

The following are its terms of reference:

1. Protection of consumer health
2. Ensuring consumer confidence in the safety of molluscan shellfish.
3. To support the long term sustainable development of the shellfish industry
4. To maximize the export potential of Irish shellfish

Membership of the MSSC
Food Safety Authority of Ireland (Chair)
Department of Communications Marine & Natural Resources.
Marine Institute
Producers
Processors
Bord Iascaigh Mhara
Laboratories
Health Boards

Following extensive discussions a new and comprehensive written management protocol for regulation of Biotoxins in shellfish was agreed. The key elements of the program are as follows:

- Extensive coastal and oceanic plankton surveillance has been established.
- A 24-hour mouse bioassay based on Di-Ethyl Ether extraction was introduced.
- LCMS chemical testing for a range of toxins (DSP, AZA) HPLC testing for ASP was introduced in 2001 and Jellet Rapid Test for PSP commenced in 2004.
- Laboratories were audited and obtained accreditation.
- An agreed sampling protocol was established and sample tracking and management was assigned to a dedicated sample manager.
- Target turnaround times for analysis and reporting of samples were set.
- Results were immediately reported by mobile phone text, fax and e-mail.

Finally the principle of active management of borderline and out-of-character results was agreed and the Management Cell was established.

The Management Cell (MC)
There was recognition by the MSSC that a Biotoxin programme based entirely on a strict protocol could not cater for all eventualities and that without active management consumer safety could be compromised. It was also recognised that that the absence of active management might lead to the shellfish industry suffering unnecessary losses. The MC was set up by the MSSC with the approval of the Minister for the Marine. Its presence and roll is confirmed in the Biotoxin protocol document. Its decisions are reviewed by the MSSC. The Management Cell is a risk management tool.

Make-up of the Management Cell
The MC comprises one representative of each of the following:

1. Food Safety Authority of Ireland (Chair)
2. DCMNR
3. Marine Institute
4. Shellfish Industry

It is important to understand that the management cell is not a replacement for normal sampling, nor is it a replacement or alternative to the MSSC.
The aim of the Management Cell is to proactively manage the risk presented by marine Biotoxins.

The objective is to facilitate rapid decision making in non-routine situations.

The scope of the Management cell applied initially to rope mussels only but now includes all mussels and other species.

It considers and makes decisions on a range of non standard situations as follows:

- Borderline or out of character Biotoxin results.
- Prolonged borderline toxicity.
- The results from test analyses are inconsistent with local or national trends.
- A single, unexpected negative or positive result occurs.
- Borderline Biotoxin results need consideration.
- Sampling continuity has been interrupted.
- LCMS breakdown.
- A production area has been assigned an incorrect status.

The decisions are reached by consensus and are based on an assessment of the overall risk by reference to the following elements:

- The species of bivalve mollusc.
- The details of the bioassay
- Chemistry result.
- Phytoplankton result.
- Time of year / risk profile.
- Adjacent areas status.
- Relevant historical data and data analysis reports.
- Any other relevant data.

If consensus cannot be reached the view of the FSAI takes precedence.

Management cell options.
Having considered all of the relevant information the Management Cell can decide to implement any of the following options.

1. Change a production area's status.
2. Recommend a voluntary closure to producers.
3 Close adjacent areas within the same bay.
4 Increasing sampling frequency.
5 Reduce sample frequency based on bay profile & season.
6 Other action as appropriate.

Management Cell Decisions 2004

- Total : 49 decisions to date in 2004 (up to October 2004)
- Change sampling frequency for Oysters & Razors (fortnightly to monthly)
- Precautionary advise issued due to rapid increase in toxicity leading to voluntary closures
- First decision involving PSP
- Sites reported on bioassay only due to LCMS problems

Benefits of MC decisions for the shellfish industry.

- Most of the decisions to date have facilitated early harvesting.
- Closed periods have been micro-managed at both ends.
- Crop losses have been reduced
- An opportunity to correct genuine errors in the system.
- Some control over the way the industry is managed.

Is it a success?

To date the management cell is a success for both consumers and industry. Its decisions have withstood the test of time.

The next 12 months and beyond will see continuous monitoring and review of its processes and information. It is envisaged that diminishing gaps in data and phytoplankton monitoring will assist the MC further. Chemical monitoring of DTX3 may reduce the role of the Management Cell and the framework of the Management Cell may have uses in other areas and other sectors.

A REVIEW OF SHELLFISH TOXICITY MONITORING IN IRELAND FOR 2004

Dave Clarke[1], Leon Devilly[1], Billy Gibbons[2], John Flannery[1], Philipp Hess[1], Josephine Lyons[1], Myra Keogh[2], Jane Kilcoyne[1], Maria McCarron[2], Niamh Mulcahy[1], Jenny Ronan[1], Barry Rourke[1], Rafael Gallardo Salas[1], Joe Silke[1], David Swords[2].

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The National Marine Biotoxin Monitoring Programme for shellfish is co-ordinated by the Marine Institute's National Marine Biotoxin Reference Laboratories based in Dublin and Galway. Samples of shellfish species are routinely analysed by bioassay and chemical methods in accordance with EU Directive 91/492 and Council Decisions 2002/225/EC and 2002/226/EC.

Diarrhetic Shellfish Poisoning (DSP)

For 2004 (to beginning of October 04) 1835 samples (2200 samples projected year end 04 compared to 2756 samples submitted for 2003) were submitted for DSP Bioassay analysis and chemical confirmatory analysis for the presence of Okadaic Acid equivalents (OA, DTX-1, & DTX-2) and Azaspiracids (AZA 1, 2, 3). The decrease in the number of samples submitted was primarily due to changes in the sample frequency of some species. Blue Mussels (*Mytilus edulis*) & Cockle (*Cerastoderma edule*) samples were submitted on a weekly basis, while Oyster, Razor and Clam species (*Crassostrea gigas*, *Ostrea edulis, Ensis siliqua, Spisula solida, Tapes philippinarum*) were submitted on a monthly basis during winter months and a fortnightly basis during the summer months.

Overall for 2004, (to beginning of October 04) the number of all samples testing positive under DSP Mouse Bioassay was 3.2% (1835 samples analysed) compared to 3.6% over the same time period for 2002, & 3.4% for 2001. A breakdown of percentage positives by species for *M.edulis* reveals 5.8% of samples tested positive (of 1122 samples) compared to the same period for 2003, 6.5% samples tested positive (of 1226). No oyster, cockle, clam samples submitted and analysed were positive for DSP/AZA Toxicity during the same time period.

For the first time, DSP toxicity (mainly in the form of OA esters) was detected in samples of *Aequipecten opercularis* (Queen Scallop) from Donegal in August, highest concentrations observed was 1.5µg/g total tissue (post hydrolysis).

Figure 1 illustrates the percentage of positive samples analysed via DSP Bioassay for 2004. In January and April, three samples analysed tested positive via bioassay. During February, March and May, no samples tested positive for the presence of DSP toxins. Towards the end of June the presence of DSP toxins was observed (0.5% of total samples analysed were positive). DSP toxicity was observed to continue to increase throughout July – August with the highest number of positives (predominantly in the South West) observed in August (13.7% of samples compared to 8.54% in 2003) falling to 8.1% in September (compared to 9.68% in 2003). The level of toxicity was observed to decrease further in October with a number of areas re-opening. High numbers of *Dinophysis* species were observed to be present in these areas during this period (primarily *Dinophysis acuta* in August).

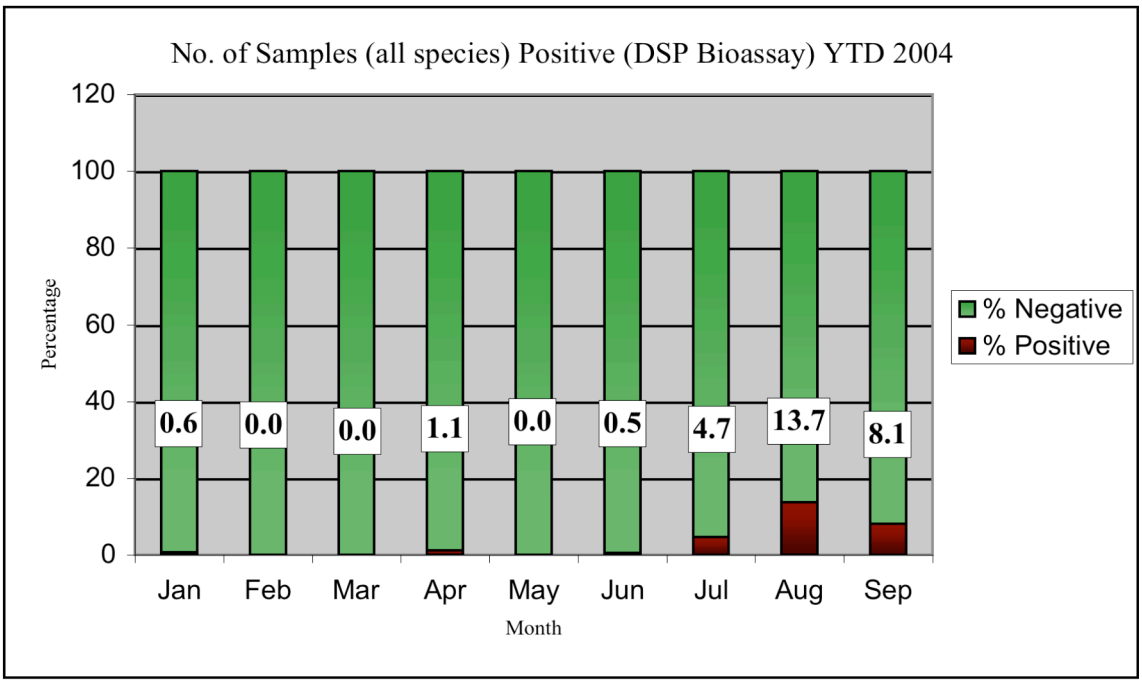

Figure 1. Percentage of Samples Positive for 2004

The predominant toxin observed in positive samples from July to Mid-August was Okadaic Acid, whereas from Mid-August to October the predominant toxin in samples was DTX-2. Where observed, the predominant toxic phytoplanktonic species was *Dinophysis acuta* in these affected areas at this time. Figure 2 illustrates an example of this observation where the occurrence of DTX-2 appears in samples of *M.edulis* two weeks after high numbers of *D.acuta* were observed in the area.

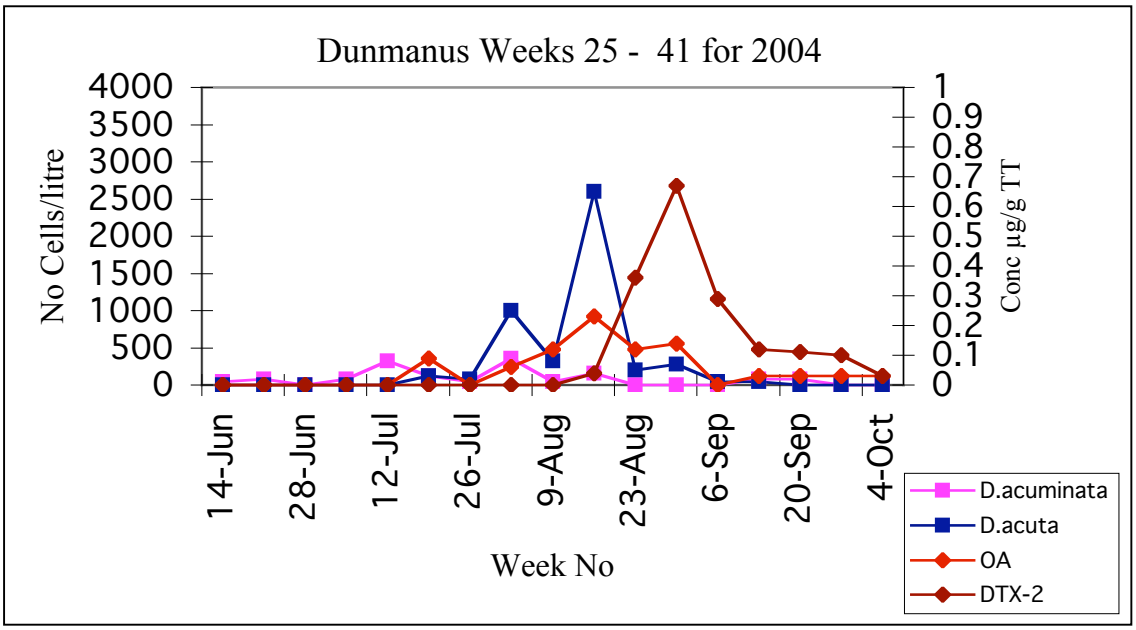

Figure 2. Levels of OA & DTX-2 vs. numbers of *Dinophysis acuta* and *acuminata*

Whilst DTX-2 was observed to be in very high levels in a number of samples from the South West during August, these levels were observed to decrease rapidly in samples from these areas over the following subsequent weeks. Figure 3 illustrates an example of this observation.

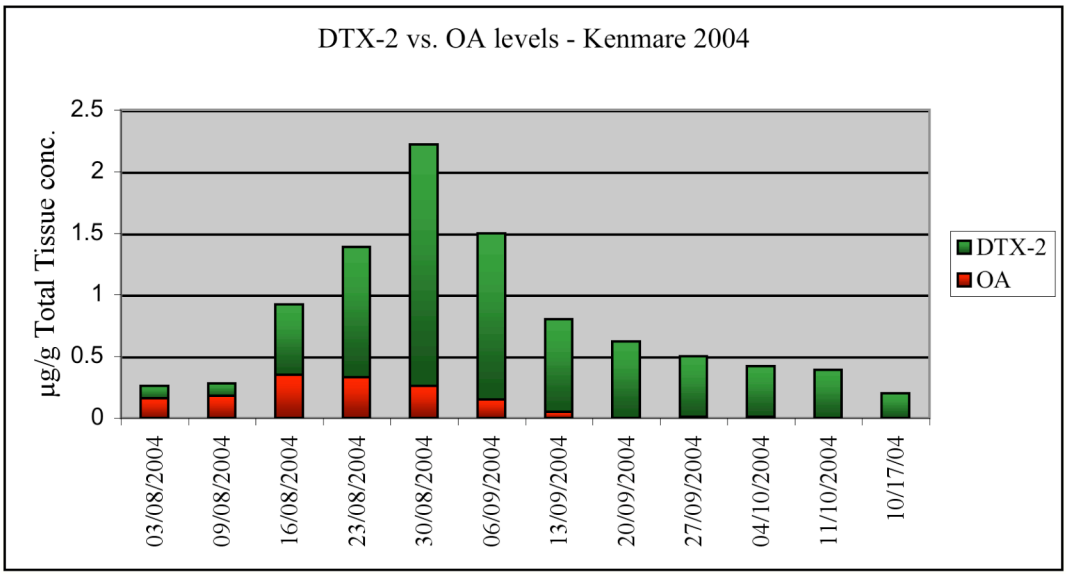

Figure 3. Increasing & Decreasing rates of DTX-2 in Kenmare

Figuress 4.1 – 4.6 illustrate the highest levels of OA equivalents (Pre-Hydrolysis) observed in samples of mussels from June – October 2004, >0.03µg/g whole flesh. All other areas analysed were <0.03µg/g whole flesh. Highest levels of OA equivalents were observed predominantly in the South West.

Figure 4.1 OA levels June

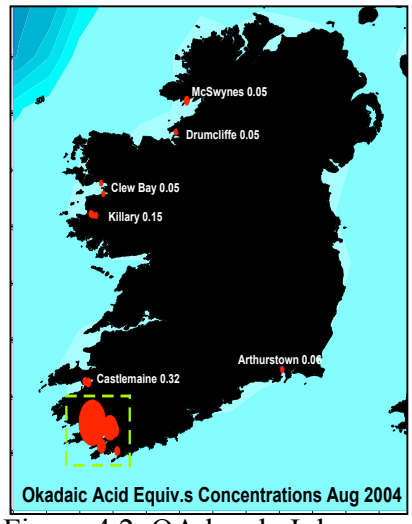

Figure 4.2. OA levels July

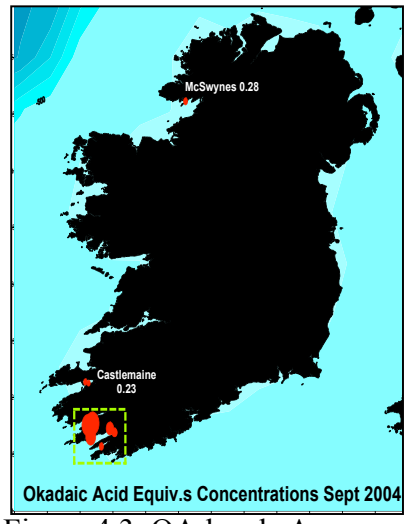

Figure 4.3. OA levels Aug

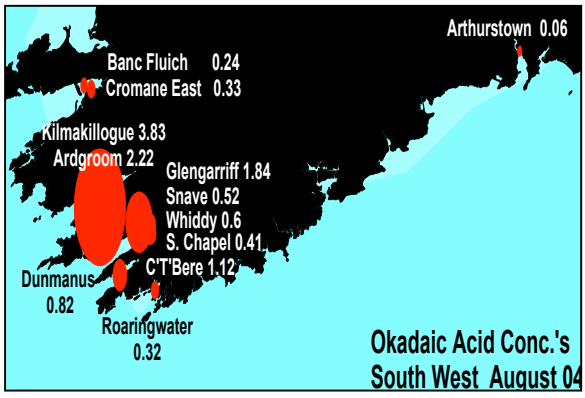

Figure 4.4. OA levels SW August

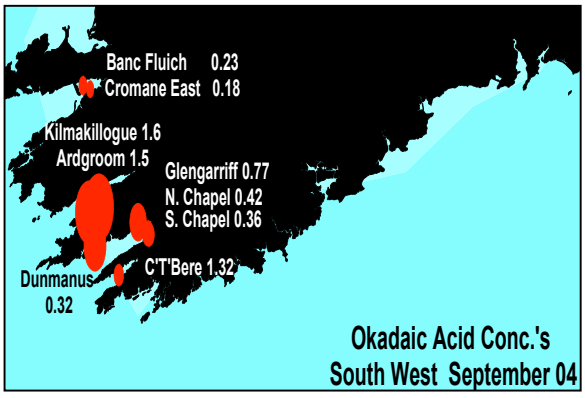

Figure 4.5. OA levels SW September

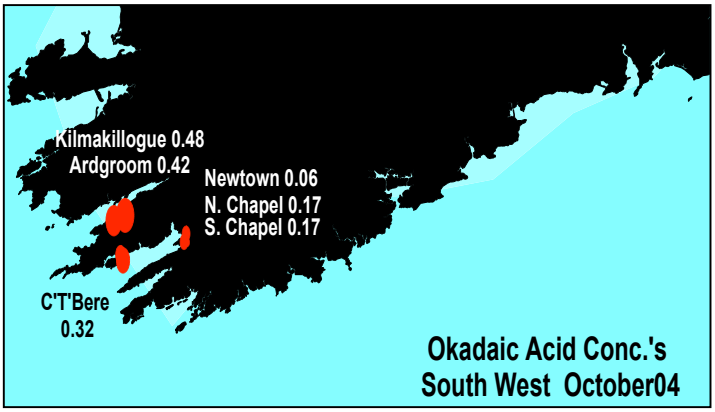

Figure 4.6. OA levels SW October

Comparison: Bioassay & Chemical Analysis

The bioassay results were compared to the chemical results for OA equivalents (including Post Hydrolysis) & AZP (n= 1835 samples Jan – Mid Oct 04), and are illustrated in Figure 5. Overall, a 99.2% correlation was obtained between the two methods (compared to 98.4% in 2003 and 98.8% for 2002).

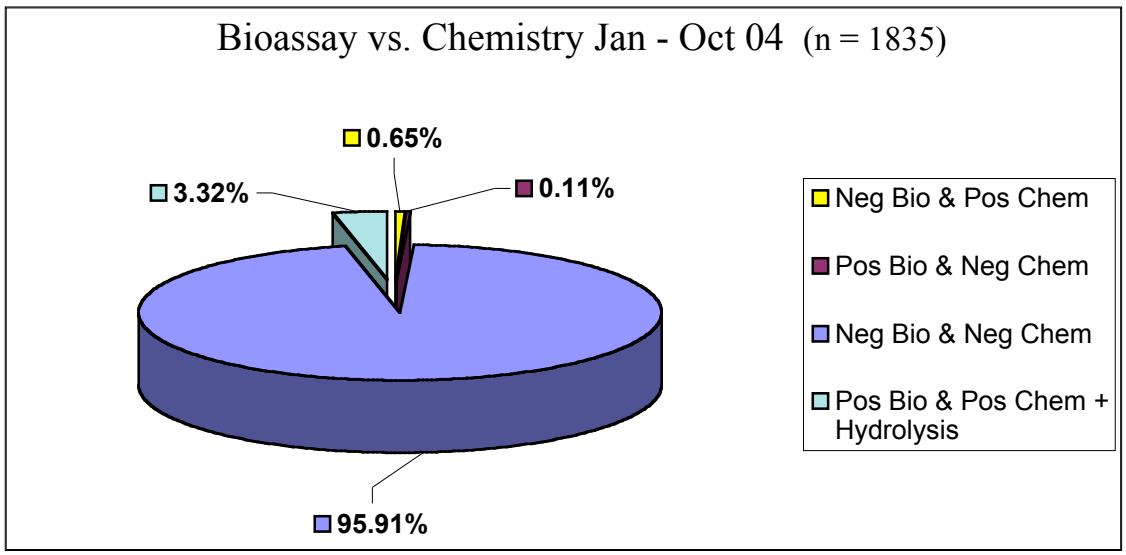

Figure 5. Comparison of Bioassay vs. Chemical Analysis (n=1835)

In 2003, the post hydrolysis step was conducted on a number of samples, primarily where discrepancies were observed in samples where positive bioassays and the chemistry for OA equivalents were below 0.16 µg/g.

This approach was adopted for 2004 for all but one sample where these discrepancies were observed, where the presence of Okadaic Acid esters analysed in the post hydrolysis step accounted for all of the discrepancies observed. This resulted in 0.1% discrepancy for samples with a positive bioassay and negative chemistry, compared to 1.26% discrepancy observed for 2003.

The number of post hydrolysis steps conducted on samples for 2004 has revealed a similar pattern in the presence of Okadaic Acid esters observed as for 2003. The average yield of Okadaic Esters was on average 52% of total DSP Toxicity in samples, with a range of 28% to 100% yield, illustrated in Figure 6. It was also

observed that in July the main acyl OA Esters were of Okadaic Acid, whereas in August the predominant acyl derivative was of DTX-2 in samples from the south-west.

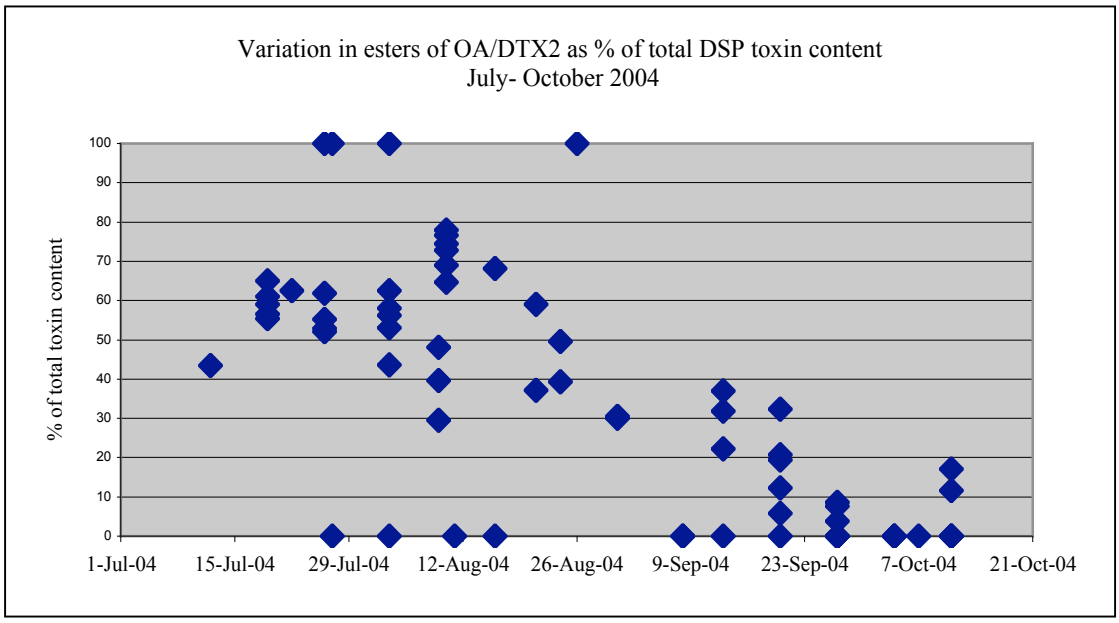

Figure 6. Variation in Esters of OA/DTX-2 of total DSP Toxin Content

The number of discrepancies where a negative bioassay with positive chemistry (> 0.16 µg/g whole flesh OA equivalents with post hydrolysis) was observed to increase for 2004, the majority of discrepancies occurring at or near the regulatory level, particularly in the month of October during the shoulder toxicity period as levels of DTX-2 were observed to be decreasing.

Paralytic Shellfish Poisoning (PSP)

A number of significant improvements in the National Programme for the detection of PSP was undertaken in 2004.

This included the full adoption and implementation of the AOAC Method for the PSP bioassay, which has been internally validated. This bioassay is quantifiable due to the use of Certified Reference Material, where results can be expressed as µg STX equiv/100g. Previously results could only be expressed as Positive/Negative for the presence of PSP Toxins

Due to the low occurrence of PSP toxins in Irish Shellfish and in a move to further reduce the number of bioassays conducted, a negative screening method has now been introduced and implemented in the National Monitoring Programme.

The Jellett Rapid Test Kit for PSP is an in-vitro immunoassay diagnostic test kit, which utilise polyclonal antibodies and work on the principal of lateral flow immuno-chromatography using a strip format. The polyclonal antibodies have been well characterised for their cross-reactivity and limit of detection. The critical reagent forming the basis of the test kit is the mixture of antibodies, which collectively are able to detect all common analogues of PSP toxins.

The Jellett Rapid Test Kit has been designed to be easy to use both in the laboratory and the field and to give a qualitative result within 20mins.

Each kit (Figure 7) consists of individually sealed strips that contain antibodies and coloured particles (which are re-suspended on the addition of running buffer and sample). Strips consist of an absorption pad, a membrane striped with a mixture of toxin analogues (T line on the right) and an antibody-detection reagent (C line on the left), a sample pad and a conjugate pad containing the antibodies. A visible T line indicates absence of toxin in the sample (below detection limit) and no line indicates presence of the toxin (Figure 8). The C line indicates that the sample fluid has sufficiently re-suspended and mobilised the anti-body complex.

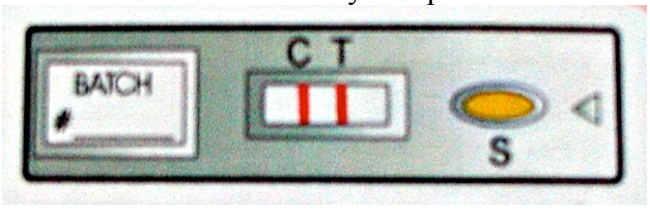

Figure 7. Jellett Rapid Test Kit for PSP

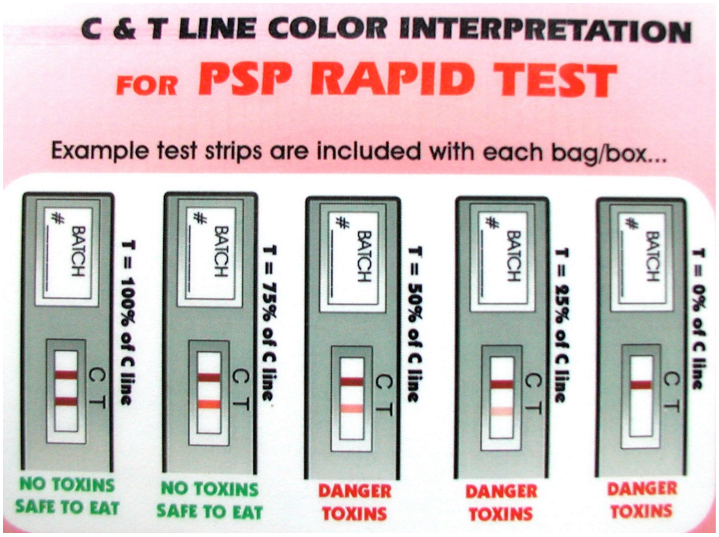

Figure 8. Jellett Rapid Test Kit – Colour Interpretation

The test kit has been internally validated and run in parallel with samples analysed by the PSP bioassay and has successfully determined the test kits suitability in determining the PSP toxicity profile in Ireland.

The test kit has now been fully introduced into the Monitoring Programme as a negative screening method, where if a positive result is observed, the confirmatory reference method the AOAC PSP bioassay is conducted to determine the level of PSP toxins in the sample (this approach has been adopted by the MSSC).

For 2004, two samples (of 130 analysed) of *M.edulis* were observed to contain PSP toxins above the detection limit from Cork Harbour in June at levels of 45 & 65µg STX equiv/100g. High concentrations of *Alexandrium sp.* (>75,000 cells/litre) were observed at this time in the area.

As the detection limit of the Jellett Test Kit is lower than that of the PSP bioassay, very low levels of PSP toxins have for the first time been detected in samples of shellfish from areas outside Cork Harbour, in Oysterhaven and Wexford, again

coinciding with the presence of Alexandrium sp. in these areas, Oysterhaven 23,280 cells/litre and in Wexford 8,360 cells/litre were observed. The low level sensitivity of the kit is also extremely valuable in detecting and indicating the onset of any potential increases in PSP toxicity in samples prior to detection via the PSP bioassay.

Azaspiracid Shellfish Poisoning (AZP)
In the 1835 samples analysed, all samples were below the regulatory limit of 0.16µg/g whole flesh, typically levels were less than the limit of detection/quantification.

Amnesic Shellfish Poisoning (ASP)
In 2004 (up to mid Oct 04), 523 analyses of scallops had been conducted for the presence of Domoic Acid (DA) & Epi-Domoic Acid.

Approximately 7.6% of gonad tissues analysed (compared to 8.8% for the same time period in 2003) had levels of DA greater than the regulatory limit (>20 µg/g), with the maximum level observed 103.7 µg/g.
For the adductor muscles analysed, no samples were observed to have levels of DA above the regulatory limit, with the maximum level observed 17.3 µg/g.
For the "Remainder" tissues, 69% analysed were observed to have levels above the regulatory limit (compared to 79% for the same time period in 2003), with the maximum level observed 1379.6 µg/g.
All *M.edulis*/*C.gigas* samples analysed for DA & Epi-DA presence up to Oct 04 were all below 20 µg/g Total Tissue,

Report Turnaround
Of the 1835 samples analysed (up to Mid October 04) for DSP/AZP & PSP, 85.2% of samples were reported within less than / equal to 3 working days (91.4% for 03) from initial lab receipt in bioassay laboratories (Figure 9).

A number of technical problems arose with the LC-MS in the Dublin facility in 2004, and resulted in downtimes and a reduction in the percentage report turnaround. This has led to a number of improvements implemented by MI, including increased equipment maintenance contracts, Uninterrupted Power Supply equipment and procedures to facilitate sample transfer in event of extended downtimes to the LC-MS to the Galway facility in the shortest possible time. This should reduce any further downtimes and lead to an improvement in percentage report turnaround for 2005.

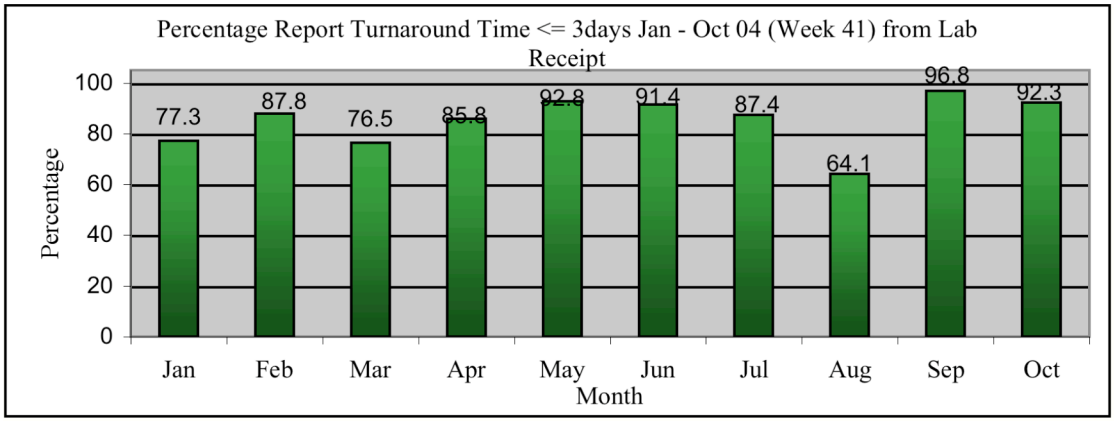

Figure 9. Percentage Total Report Turnaround for DSP/AZP/PSP for 2004

Figure 10 illustrates the report turnaround time for ASP analysis, with 100% of samples reported within 4 days. Since the transfer of ASP analysis via the new HPLC to the MI Galway facility in July 03, report turnaround has greatly improved, where 89% of samples were reported within 2 days of lab receipt (Average 1.6 Days)

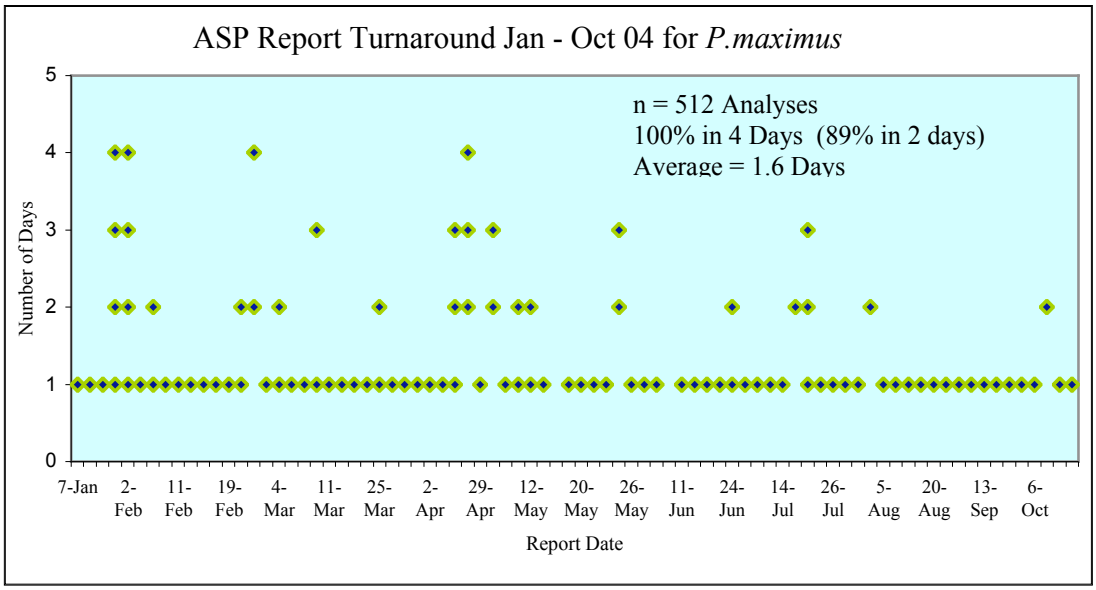

Figure 10. Percentage Total Report Turnaround for ASP analyses for 2004

Quality System

In 2004 the MI Biotoxin Chemistry Unit in Galway achieved accreditation for ASP analysis via HPLC from the Irish National Accreditation Board (INAB). Accreditation was also obtained for Okadaic Acid analysis via LC-MS in Dublin. In September 2004 application for accreditation was submitted to INAB for the analysis of DTX-1, DTX-2, OA Esters and Azaspiracids 1,2 & 3 via LC-MS in Dublin.
The Biotoxin Chemistry unit also participated successfully in Domoic Acid via HPLC proficiency testing (QUASIMEME).

In September 2004, the MI Biotoxin Bioassay Unit in Galway submitted applications for accreditation of the AOAC PSP Bioassay Test Method and the Jellett Rapid Test Kit for PSP immunoassay to INAB.

In October 2004 the MI Biotoxin Bioassay Unit in Galway participated in the EU-National Reference Laboratory Intercomparison exercise for PSP & DSP Bioassay analysis. In July 2004, the MI conducted two DSP Bioassay intercomparison exercises, with chemical confirmatory analysis. One exercise was held internally to assess individual staff performance, and the second exercise with the two regional Bioassay laboratories where satisfactory results were obtained. Also in 2004, internal PSP intercomparison exercises were conducted, again satisfactory results were obtained.

Also in October 2004, the EU Food & Veterinary Office conducted a conducted a detailed audit mission on the Biotoxin Monitoring Programmes in Ireland.

BIOTOXIN CHEMICAL AND TOXICOLOGICAL RESEARCH

Philipp Hess[1], Nils Rehmann[1], Jane Kilcoyne[1], Pearse McCarron[1], Katrin Bender[1] Gavin Ryan[2], Michael Ryan[2]

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Introduction

In 2003 the Marine Institute started a collaborative project funded by the NDP under the Marine RTDI programme called Azaspiracid Standards Isolation and Toxicology (ASTOX). Azaspiracids (AZAs) and Dinophysistoxins (DTXs) have become a major problem to the Irish shellfish industry over the last number of years. The toxins can accumulate in shellfish to levels that cause human illness. The project focuses on the isolation and purification of standards for AZAs 1 to 3, and DTX-2. It will also focus on the preparation of reference materials (shellfish matrix) for the above-mentioned four compounds. These standards and reference materials will then be used to further evaluate the acute and chronic effects of AZAs and DTX-2. International collaborators include the University College Dublin, scientists from the Health Biotechnology Research Development Centre, Okinawa and Chiba University, Japan, as well as toxicologists from the National Oceanographic and Atmospheric Administration (NOAA) in the U.S.
The work packages are divided among the collaborative groups and a summary of the progress after 18 months is presented.
The Marine Institute is also carrying out a survey on behalf of BIM to investigate the level of contamination of domoic acid, the biotoxin that causes amnesic shellfish poisoning, in scallops, to reassess the current situation of marketing scallops in Ireland.

For further information visit:
http://www.marine.ie/funding/marine+institute+funding/marine+rtdi+fund/Strategic+Marine+RTDI/ST_02_02.htm

ASTOX Work Package 1: Retrieval of contaminated material

The objectives of this work package are to obtain and store raw and processed contaminated shellfish materials.
In addition to the 2.4 tons of material contaminated with different levels of biotoxins obtained in 2003, a further two 500kg lots contaminated with OA and DTX-2 have been retrieved from the South West Coast of Ireland in August 2004.
Collaboration with the Norwegian School of Veterinary Science has enabled the project to gather 10kg of material from a recent toxic event involving azaspiracids in Norway. This will be used for method development.

ASTOX Work Package 2: Preparation of reference material 2

The objectives of this work package are:

(i) To produce at least four shellfish tissue laboratory reference materials (LRMs), contaminated with AZA –1, -2 and –3 and DTX-2
(ii) To demonstrate the homogeneity of those materials
(iii) To demonstrate the stability of those materials

The aim of this work package is the preparation of stable, homogeneous shellfish tissue materials naturally contaminated with both azaspiracids (AZA-1,-2,-3) and dinophysistoxins (OA and DTX-2).

The initial method developed for these preparations resulted in materials with unsatisfactory homogeneities (>10 %CV) with regard to the toxins of interest.

As method development continued further steps were added; these included the autoclaving of tissues for stabilisation (this also resulted in the tissues becoming less viscous), the addition of a solvent and use of a peristaltic pump for dispensing materials. The %CVs of the two toxin groups in material #8 prepared using the optimized method including these steps are shown in table 1.

Table 1. Conc. (µg/g), toxic equivalent and %CV of AZP/DSP LRM 03-2-R7 (#8)

	Conc (µg/g)	Toxic Equivalent (µg/g)	%CV (rep:18)
OA	0.16	0.16	6.590
DTX-2	0.76	0.76	5.080
Sum*		0.92	5.050
AZA-1	0.19	0.19	5.240
AZA-2	0.05	0.09	6.880
AZA-3	0.02	0.03	6.090
Sum*		0.31	4.970

* %CV of toxic equivalents calculated from sum of OA & DTX-2 or AZA-1,-2&-3

The outcome of this was a material with better homogeneity for the two toxin groups (ca. 5% for both). The addition of ethanol increased the homogeneity of the lipophillic toxin in what is a predominantly hydrophilic matrix. Autoclaving the tissues resulted in the material being easier to homogenise while the use of the peristaltic pump maintained the homogeneity of the material as a bulk while dispensing into aliquots.

During the method development stage materials were prepared in up to 1kg lots and the improved method produced good results for materials this size. However the next step was to see whether the methodology could be applied to a medium scale preparation (1-10kg). A medium scale material (AZP/DSP LRM 04-1-R) was prepared and the % CVs for both toxin groups were ca. 5%. This shows that it is possible to reproduce the results obtained for a pilot scale preparation on a medium scale.

Stability of the Materials

All dispensed aliquots of all reference materials were stored at –20°C.
AZP/DSP LRM 04-1-R was incorporated as an LRM in the routine monitoring programme of biotoxins in Ireland (which is carried out by the Marine Institute). These LRMs are used routinely in the chemical analysis of samples from the monitoring. Figure 1 is a control chart of the OA equivalents in the LRM over 75 batches demonstrating the stability of the material when stored at freezer temperature.

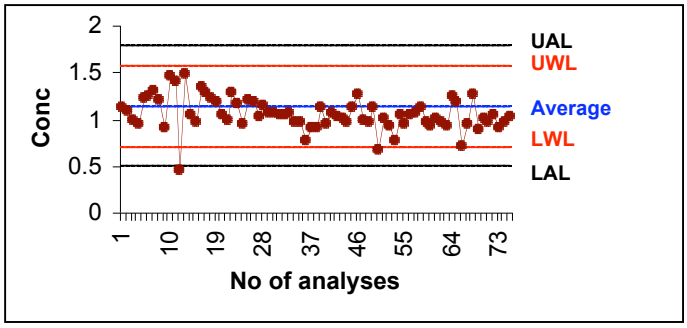

Figure 1. OA equivalent LRM control chart: AZP/DSP LRM 04-1-R

Use of Reference Materials in Method Validation

One of the prepared reference materials has been used as part of method validation exercises. As it had already demonstrated satisfactory homogeneity and stability, it was an ideal material for determination of the extractability of OA and DTX-2 using different solvents (Table 2).

This shows that 100% methanol as the extraction solvent produced the greatest recovery with an average of 0.064µg/g and 0.5µg/g for DTX-2 and OA respectively. The coefficients of variations using this extraction solvent are also satisfactory with the best result of 10.2% being obtained for OA and 3.8% for DTX-2.

Table 2. Extractability of OA and DTX-2 from a LRM using different concentrations of methanol in the extraction solvent.

	OA		DTX –2	
	Mean µg/	%CV	Mean µg/g	%CV
80% MeOH	0.056	11	0.41	5.1
90% MeOH	0.062	10.8	0.46	1.3
100% MeOH	0.064	10.2	0.5	3.8

Proficiency Testing Schemes

Numerous proficiency-testing schemes have now been set up or supplied by the Marine Institute as a result of the progress made in the production of LRMs by ASTOX.

Tissue reference materials have been produced for intercomparisons between Irish laboratories such as the Marine Institute (Galway/Dublin), the Public Analysts (PA) laboratory (Galway) and the Department of Agricultural and Rural Development (DARD) laboratory (Belfast).

In addition, test-materials have been prepared by the Marine Institute for use in an ASP QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring) proficiency-testing scheme. QUASIMEME is a global proficiency testing scheme provider. In late 2003 the first ever QUASIMEME organised ASP proficiency testing scheme was run using materials prepared by the Marine Institute. Results for the second round of the initial pilot study on Domoic acid (DA) and Epi-Domoic acid are shown (Figure 2).

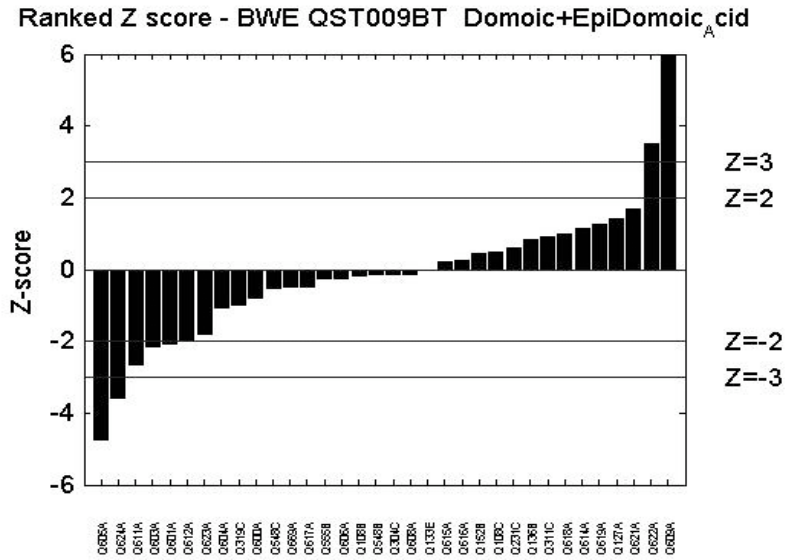

Figure 2. Z-score plot - whole scallop tissue QUASIMEME round 2.

For this ASP pilot study there were 34 participants of which 27 produced satisfactory results (Z Score between +2 and –2). The success of this has meant that the QUASIMEME ASP proficiency scheme is being provided routinely from now on.
Materials for the second year of the study have been prepared by the Marine Institute and sent to QUASIMEME for dispatch to the participating laboratories.
Proficiency testing schemes for other toxin groups are also being considered with the possibility that a pilot study for the Okadaic Acid (OA) group will be run in late 2005.

ASTOX Work Package 3: Isolation of pure toxin material
The objectives of WP3 are the isolation of AZA-1 and it's analogues (AZA-2 and AZA-3) as well as DTX-2 for toxicological studies and standard reference material for instrument calibration. The isolation scheme, shown below, is based upon a procedure developed by the Japanese collaborators and is also being optimised under this work package.

1. Triple blending extraction. 3L Acetone, 2x 3L MeOH
2. Partitioning I (EtOAc/H_2O)
3. Partitioning II (n-Hexane / 80/20 vv methanol/water)
4. Silica gel open column chromatography
5. SEC on HW-40S
6. Develosil column*
7. DEAE anion exchanger
8. CM650 cation-exchanger **
9. HPLC final separation on ODP-50 (Showa Denko)

* Another gel permeation step (Sephadex LH-20) was introduced before this step.
** This step has been taken out of the isolation scheme

Some changes were made to the existing isolation scheme. A further gel permeation material was introduced (Sephadex LH-20). The cation exchange step was taken out of the procedure due to poor performance and low recovery of the toxins.

To date 1.8mg of AZA-1 has been isolated from shellfish material using existing and optimised techniques. The purities achieved ranged between 20-80%. Although 80% purity can be sufficient for standard material, it is not sufficient for toxicological studies. NMR analysis of the purified toxins indicated that the remaining impurities were due to residues in the solvents being used throughout the purification process. All toxins isolated to date will be re-chromatographed using high purity solvent, yielded through distillation. This may result in a loss of small amounts of AZA-1, however it is acceptable if higher purities can be achieved. The overall recovery throughout the batches is ca.18%.

Along with AZA-1, 800μg of DTX-2 have been isolated, and its high purity has been determined using NMR by our Japanese collaborators. More isolation batches are required to determine the overall recovery for DTX-2.

From the isolated AZAs and DTX-2 a stock solution containing a combination of all toxins was prepared. This stock solution was used to carry out spiking experiments to determine the matrix effects of different shellfish tissues in the LC-MS test method. These experiments were used for validation studies and helped to obtain the INAB accreditation. Isolated AZA-1 and DTX-2 will be used in future toxicology studies.

ASTOX Work Package 4. Toxicological Studies: Conway Institute, UCD

The objectives of this Work Package are:

(i) to develop an *in vitro* test as an alternative to the *in vivo* mouse bioassay
(ii) to establish the mode of action of AZA to provide an understanding of the basis of the toxicity and means to prevent it
(iii) to establish comparative toxicity of AZA and okadaic acid (OA)

As the main symptoms of AZA toxicity in humans are gastrointestinal disturbances, human intestinal cells were chosen to develop an *in vitro* assay relevant to the *in vivo* effects in humans. This also served to eliminate any species-to-species variations that could occur with animal based models. The human colon carcinoma cell line Caco-2 was selected for these studies due to their ability to form tight junctions and generate a transepithelial electrical resistance (TER). Transepithelial electrical resistance is a useful index of the function of these cells in maintaining the transport of solutes and water. The effect of AZA-1 on cellular viability was initially assessed using the Alamar Blue assay. Reduction of Alamar Blue from an oxidised (non-fluorescent, blue) form to a reduced (fluorescent, red) form was used as a measure of metabolic activity. Caco-2 cells were exposed continuously to AZA-1 (0.01 – 100 nM) for periods of 24, 48 and 72 hours. This assay did not detect any loss in cellular viability (data not shown).

Transepithelial electrical resistance studies were carried out using the WPI REMS Autosampler, an automated electrical resistance measurement device. After a stable TER reading was achieved cells were exposed continuously to AZA-1 (0.1 - 100 nM) for periods of 24, 48 and 72 hours. No significant change in TER was observed at any time-point up to a concentration of 2.5 nM. With 5 nM AZA-1 or higher concentrations a significant decrease were observed at 24, 48 and 72 hours (Figure 3). A complete loss of TER was observed at 48 hours with concentrations of 5 nM or higher. This decrease in TER correlates with an increase in paracellular permeability. The ability of the marine toxin okadaic acid (10 – 500 nM) to alter TER in our model was then assessed. With 100 nM OA a significant decrease in TER was observed at 24, 48 and 72 hours (Figure 4). This indicates that our model is significantly more sensitive in the detection of AZA-1 than OA.

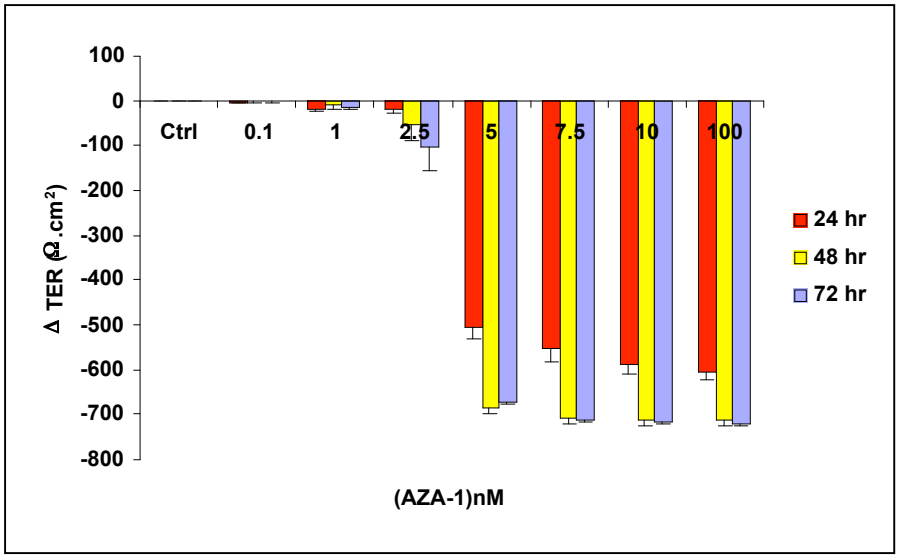

Figure 3. Effect of azaspiracid-1 (AZA-1) on the transepithelial electrical resistance across Caco-2 cell monolayers using the REMS autosampler.

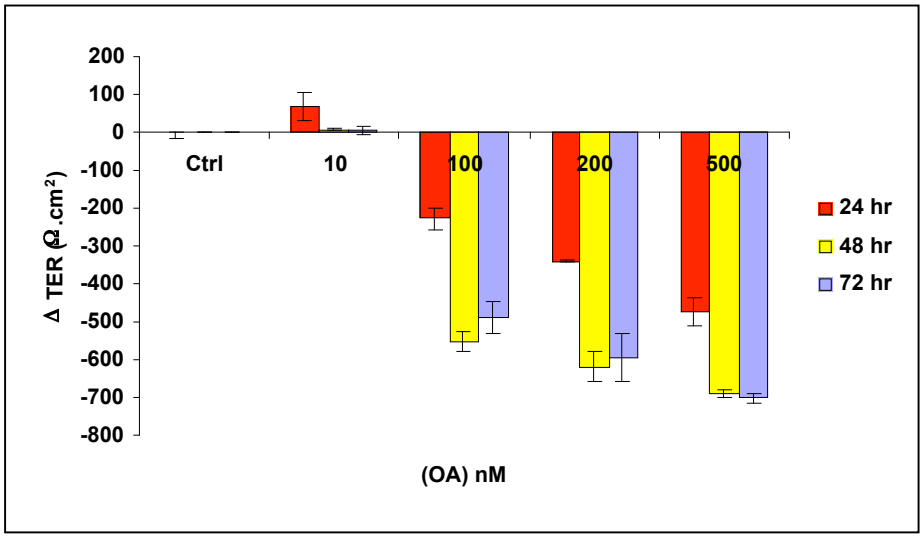

Figure 4. Effect of okadaic acid on the transepithelial electrical resistance across Caco-2 cell monolayers using the REMS autosampler.

In nature, AZA-1 and OA have often been found to co-occur. We examined the effect of combinations of AZA-1 and OA in our model system. No additional decrease in TER was seen when both toxins were added together compared to when either toxin was added alone (Figure 5).

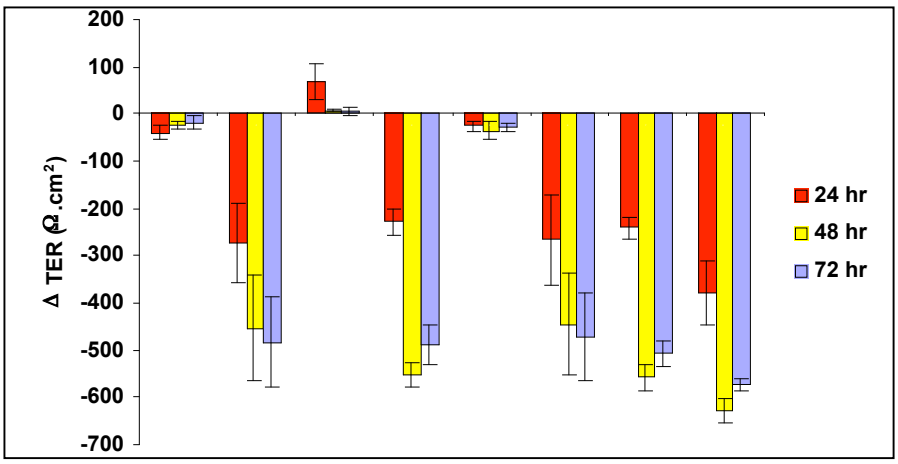

Figure 5. Effect of AZA-1 in combination with OA on the transepithelial electrical resistance across Caco-2 cell monolayers using the REMS autosampler.

The ability of AZA-1 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. The integrity of the barrier is maintained by intercellular junctions, the most apical of which is the tight junction. Disruption of tight junction components can lead to abnormalities in barrier function. The ability of AZA-1 to alter these components e.g. occludin, claudin and the signalling mechanisms involved in these alterations are currently being examined in our model.

The current regulatory limit is 0.16 μg of azaspiracid/g edible mollusc part, which equates to approximately 190 nM. The ability of this assay to detect levels of 5 nM (0.0042 μg/g) is potentially very useful. While the sensitivity of this model in detecting pure AZA-1 induced alterations in epithelial function are clear, it must be stressed that in real life *in vivo* human situations, dilution factors and possible breakdown in the gastrointestinal tract would have to be taken into account in determining a 'No Observed Adverse Effect Level' (NOAEL).

BIM Scallop Survey

In 2003 BIM (Bord Iascaigh Mhara) approached the Marine institute with the request to carry out a contamination study for ASP in Scallops (*Pecten maximus*) from Southeast Irish coastal waters around the Wexford grounds. The scallops were fished at 100 different dredge stations varying in depth and substrate.

BIM is seeking a market for fresh Irish scallops distributed in the shell as well as fresh processed scallops. Since Domoic Acid (DA) levels within the entire shellfish are much more likely to exceed the set limits than in adductor muscle and gonad only, there is an increasing demand for efficient quality control. BIM are also considering the benefits of performing analysis for DA in scallops autonomously in the field. Therefore, alternative methods to HPLC have to be found and tested for their feasibility and cost effectiveness.

A competitive direct Enzyme-Linked Immuno Sorbent Assay (ELISA) for DA, which is presently marketed by Biosense AS, Norway was used in this study. The ELISA was compared to the existing HPLC method, considering performance, results and investment (Figure 6). The precision and robustness were also investigated.

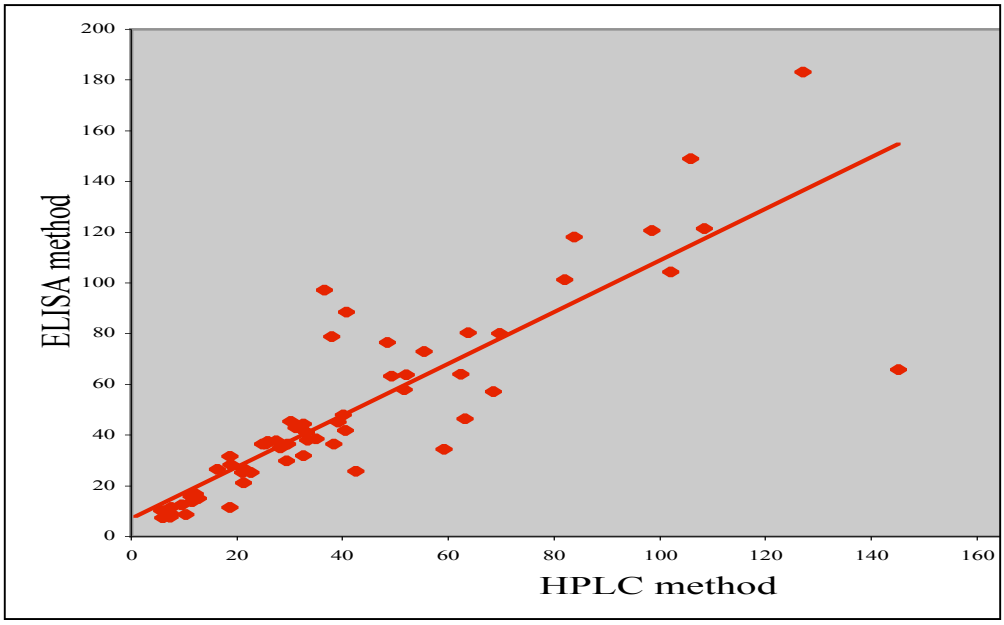

Figure 6. Bias Plot HPLC-DAD vs. ELISA (Entire Tissue results)

The experiments carried out by the Marine Institute indicated that the ELISA test correlated quite well with the HPLC method currently used as the routine monitoring method for DA by the Marine Institute. A bias plot comparing the two methods is shown in figure 5 above.

Acknowledgments
The ASTOX project (Grant AID agreement No. ST/02/02) is carried out with the support of the Marine Institute, University College Dublin and the Marine RTDI Measure, Productive Sector Operational Programme, National Development Plan 2000 – 2006.

THE BIOLOGICAL OCEANOGRAPHY OF HARMFUL ALGAL BLOOMS (BOHAB) PROGRAMME, 2004.

Caroline Cusack[1], Joe Silke[2], Georgina McDermott[1], Glenn Nolan[2], Maeve Gilmartin[2] & Robin Raine[1].

[1]Martin Ryan Institute, National University of Ireland, Galway
[2]Marine Institute, Galway Technology Park, Galway, Ireland

In 2004, the BOHAB project focused on two activities. The first of these involved processing and analysing data from field surveys carried out in 2003. Secondly a multi-national survey studying thin layers of phytoplankton within Killary Harbour was carried out in July 2004.

The data analysed covered a very broad range of measurements and included spatial and temporal distributions of harmful phytoplankton species, the physical characteristics of the water during surveys, moored current meter and temperature sensor measurements, meteorological conditions (wind and rainfall), and shellfish gut content analysis.

Analysis of water column structure along the western shelf and outside Killary Harbour showed the existence of bottom density fronts in the summer months. Baroclinic jet-like flows generated by these fronts would allow a persistent northward transport of phytoplankton populations past the mouth of Killary Harbour. These flows, which are maximum at a depth of about 20 – 40 m, result from competition between tidal mixing and seasonal heating of the upper water column in the summer. The fronts play an important role in forcing the Irish Coastal Current northwards along the west coast of Ireland from May to September. Deployment of current meters by Amergen International Ltd. at the location of the bottom front off Killary confirmed the jet-like flow during 2003. Deployment of a second mooring nearer the mouth of Killary pointed towards the presence of a gyre that may act as a retention zone for phytoplankton populations, including HAB species, in the area (Fig. 1). The water circulation was slack and tides were relatively weak with limited horizontal advection. Growth of resident phytoplankton would be encouraged within the gyre, including slow growing, long lived organisms such as *Karenia mikimotoi*. Given the right conditions these blooms would subsequently be advected inshore. The sudden appearance and disappearance of harmful phytoplankton in Killary Harbour during the summer months indicates that the HAB populations are being physically transported into and out of the fjord.

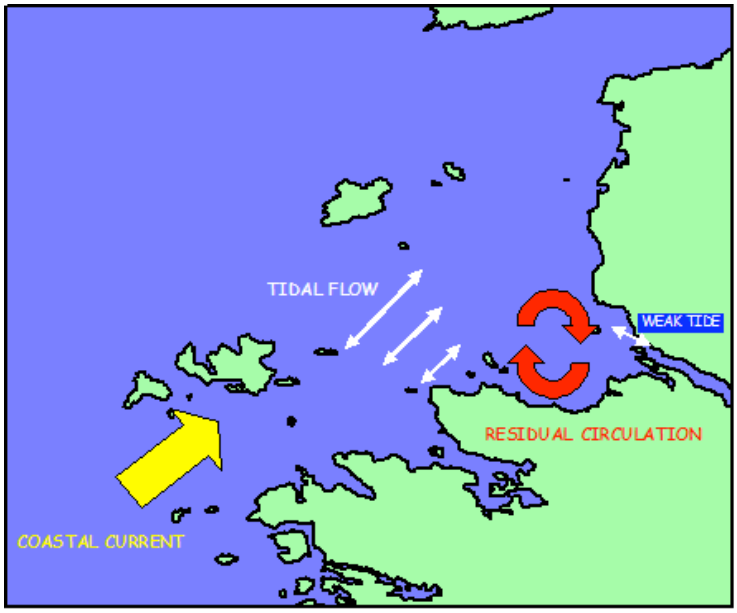

Figure 1. Illustration showing the offshore currents and possible gyre at the mouth of Killary Harbour derived from current meter data.

Fieldwork in 2004 concentrated on the investigation into the existence of phytoplankton in thin layers. A multidisciplinary team of scientists from France (CREMA-IFREMER), Spain (Instituto Español de Oceanografía, Centro Oceanográfico de Vigo), the USA (Graduate School of Oceanography, University of Rhode Island) and Ireland (NUIG, MI) took part in the survey. This was performed by deploying unique high-resolution instruments from France and the US which measured physical and biological properties of the water column at a very fine scale (Fig. 2). Hydrographical data (temperature, salinity, nutrients, chlorophyll and quantitative phytoplankton distribution) was also collected using conventional CTD casts and tows of Scanfish PS19 (Fig. 2). In all, a total of 151 stations were sampled within and outside the fjord (Fig. 3).

Data analysed to date has shown that the HAB species *Karenia mikimotoi* was present in two thin layers in the survey area. This organism was recorded both inside and outside the fjord, and was the predominant phytoplankton species with highest densities evident at the mouth of the Harbour. This co-incides with the gyre, or retention zone, referred to earlier. The layers were located at depths of 5.2 m (*Karenia mikimotoi* 220,000 cells per litre) and 13.7 m (47,000 cells/L). *Ceratium lineatum* was the second-most dominant species in the layers examined. Several *Protoperidinium* spp. (*P. brevipes*, *P. divergens*, *P. leonis*, *P. pallidum*, *P. steinii, P. oblongum*, *P. pyriformes* and *P. steinii*) were present in the samples examined, although not in large quantities. The diversity of *Protoperidinium* spp. decreased towards the inner part of Killary Harbour where only *P. brevipes* and *P. steinii* were present. The *Protoperidinium* species were seen to be feeding on other phytoplankton species, in particular *C. lineatum.*

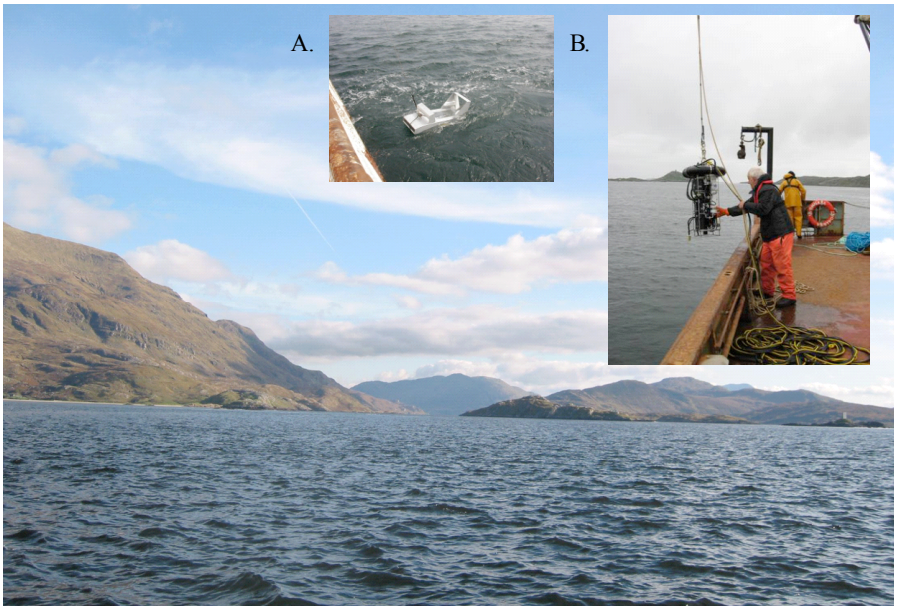

Figure 2. Pictures showing; A. The Scanfish PS19 (IFREMER) towed vehicle which gives a picture of the hydrodynamic structure of a water body and B. High Resolution Profiler (URI) which gives the high spectral resolution needed to identify species groups. Photos courtesy of Michel Lunven (IFREMER), Georgina McDermott (NUIG) and Pauline O'Donohoe (MI).

Other work in the project involved the investigation of the use of fuzzy logic to give a probability of a short range (ca. 5 day) prediction of a toxic or harmful event occurring during the summer in Bantry Bay. The model is based on certain knowledge rules which include the time of year, presence of a harmful phytoplankton population and water exchanges in Bantry Bay. The likelihood of water exchange can be predicted using a 5 day weather forecast of wind speed and direction. In order to achieve a high probability of a harmful event, the model requires three days of easterly winds followed by two days of southwesterly winds blowing along the axis of the bay. This sequence of events allows phytoplankton populations to be transported around to the mouth of the bay and subsequent advection inshore. The output of the model gives a wind index based on wind speed and direction, which, combined with the possible presence of a harmful population (derived from time of year) gives a high, medium or low risk of a harmful event. The robustness of this model will be tested during 2005.

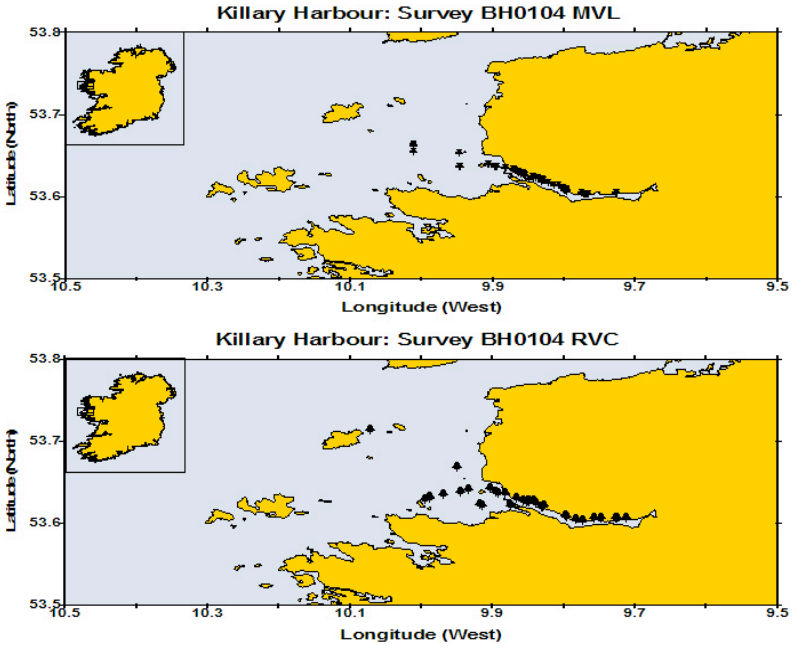

Figure 3. Map of Killary Harbour showing the 151 stations sampled aboard the MV Lughnasa and RV Conamara (bottom) (top), 19-30 July 2004.

Acknowledgements:
Representatives from the Aquaculture Industry including Mike Salmon (Cuan Baoi Salmon Farm), Deirdre Collins (Bantry Bay Seafoods), Danny McNulty, Simon Kennedy, Kevin Lydon, Abden Ryan, Anthony Murphy (Killary CLAMS group), Niall O'Boyle (Clew Bay Marine Forum), William Murphy, Dan O'Shea (Bantry Bay)
Micheál O'Cinneide, Dave Clarke, Siobhan Moran, Philipp Hess, Tara Chamberlain, Rafael Salas, Josephine Lyons, Jeeny Ronan, John Flannery, Bursars, Jane Kilcoyne, Myra Keogh, Billy Gibbons, Niamh Mulcahy, Maria McCarron (MEFS, MI), Guy Westbrook, Kieran Lyons, Sheena Fennell (OSS, MI).
Paul Connery and other SFOs (Dept. of Marine), Robert Boyne (OPW consultant, Eowen Associates), Shane O'Boyle (EPA), Tomas Burke (BIM), Martin White, Christian Mohn, Linda Ahern (NUIG), Yvonne Bogan, John Slatter (LYIT), Tracy Griffin (NUIC), Kristin Gribble (WHOI), Willemien VanHoeve (Met Éireann).

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THE DYNAMICS OF *ALEXANDRIUM* BLOOMS

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Introduction

Alexandrium is a genus of gonyaulacoid dinoflagellates. Species within this genus are known to produce a suite of saxitoxins, the naturally occurring neurotoxins responsible for paralytic shellfish poisoning (PSP) in consumers of contaminated shellfish. Two species are relevant to the present study: *Alexandrium tamarense* and *Alexandrium minutum*. In this particular case *A. tamarense* is non-toxic and *A. minutum* produces the toxins GTX2 and GTX3 (Touzet *et al*., 2003). The life-cycle contains two distinct phases: the resting cyst (Figure 1) and the vegetative cell (Figure 2). The resting cyst allows the species to over-winter in benthic sediments, while the vegetative cells live in the water column as part of the phytoplankton community and grow by repeated cell division. Under optimal conditions, vegetative cells can occur in very high densities which is known as a bloom.

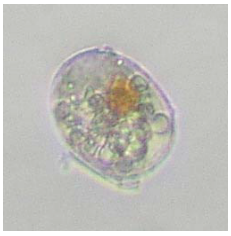


Figure 1a

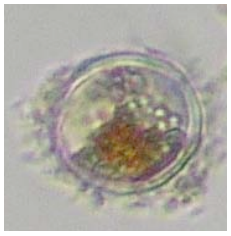


Figure 1b

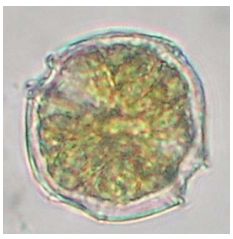


Figure 2a

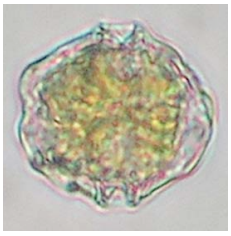


Figure 2b

Figure 1a: Resting cyst of *A. tamarense*
Figure 1b: Resting cyst of *A. minutum*
Figure 2a: Vegetative cell of *A. tamarense*
Figure 2b: Vegetative cell of *A. minutum*

Background

Vegetative cells of *Alexandrium* spp. have been identified from a large number of sites around the Irish coast (Touzet *et al.*, 2004). However, just one area has a history of harvest closures due to positive PSP bioassays on shellfish. This is Cork Harbour, or more specifically the North Channel of the harbour. Marine Institute records of *Alexandrium* counts in Cork Harbour over the past four years show that three distinct blooms have occurred (Figure 3). The first of these was in June 2000 and the following two were in September 2003 and June 2004. On each of these occasions a positive PSP bioassay was detected and closures in the area ensued. Two smaller blooms are also evident, one in June 2003 and the second in September 2004, although neither of these blooms led to positive PSP bioassays. There does, however, seem to be a temporal pattern in the history of *Alexandrium* in the region, with blooms forming in early (June) and late (September) summer.

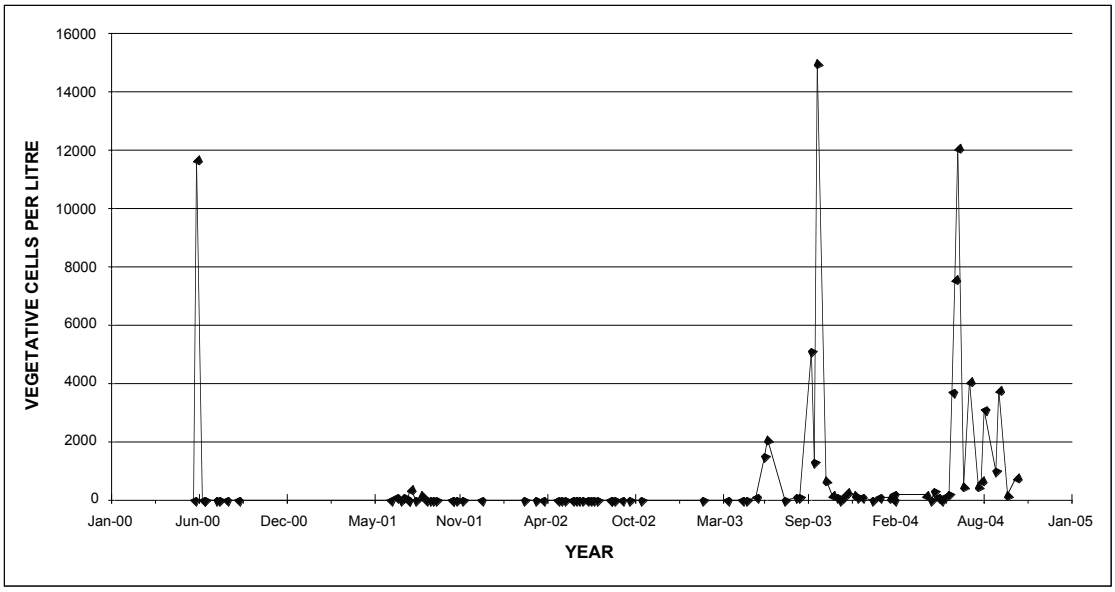


Figure 3: Results of the Marine Institute Phytoplankton Monitoring Program for the North Channel, Cork Harbour between 2000 and 2004.

There are several stages within the life-cycle of *Alexandrium*, all of which are affected by environmental parameters such as light and temperature (Figure 4). The first stage is excystment where a vegetative cell hatches from the cyst and begins life in the water column where it grows and divides. The growth rate is dependant on both light and temperature and so varies depending on the time of year and the relative stability of the water column. Towards the end of the life-cycle, for reasons which are still not clear, the vegetative cells encyst to form a resting cyst once again. This resting cyst then requires a certain amount of time in the sediment to mature before it can again excyst. The length of time in question depends on temperature .

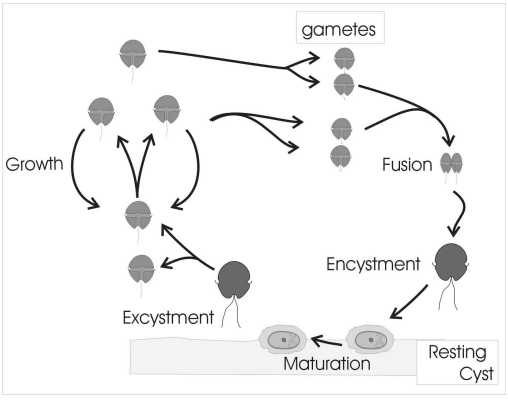


Figure 4: Life-cycle of *Alexandrium* spp.

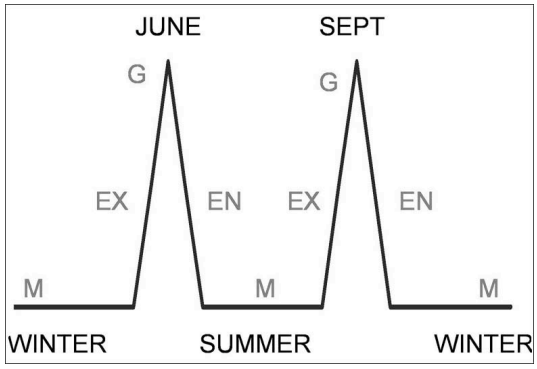


Figure 5: Theoretical annual bloom pattern
M=Maturation G=Growth
EX=Excystment EN=Encystment

Given the stages of the life-cycle, their apparent dependence on environmental conditions and the climatic conditions prevalent in Ireland, one might expect the following annual bloom pattern for the species (Figure 5): The cyst matures and/or remains dormant, during the winter months in the sediment. With the onset of brighter and warmer days, the cyst excysts into a vegetative cell, which, under optimal conditions, will grow and divide in the water column. The bloom then declines and the vegetative cells encyst. These new resting cysts require time to mature before they

can excyst once more. At this stage, in the summer, temperatures are at their warmest in Irish coastal waters. The maturation time for the cysts would be at its shortest, thus potentially allowing a second bloom within a year. This sequence matches the historical pattern of peaks that have occurred in Cork Harbour to date, most often in June and September.

Methods

During the summer of 2004, intensive field sampling was carried out to investigate the dynamics of *Alexandrium* blooms. The work included sampling the phytoplankton as well as measurements of the temperature and light levels in the area. Nutrient, chlorophyll and salinity samples were also taken and brought back to the laboratory for analysis. Running concurrently with the field work were laboratory experiments designed to examine the factors that affect each stage of the life-cycle. These included the effects of light and temperature on excystment and growth, and the effect of temperature on maturation rates. Water from the entire harbour area was sampled but efforts were concentrated in the North Channel (Figure 6)

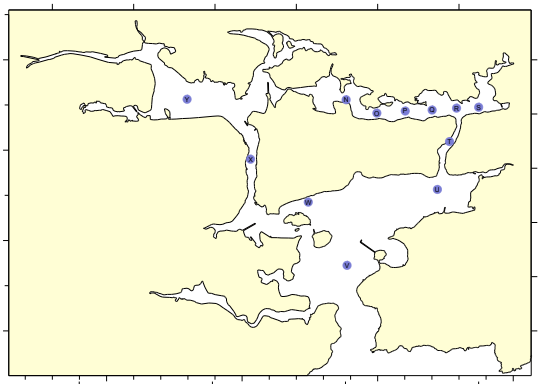
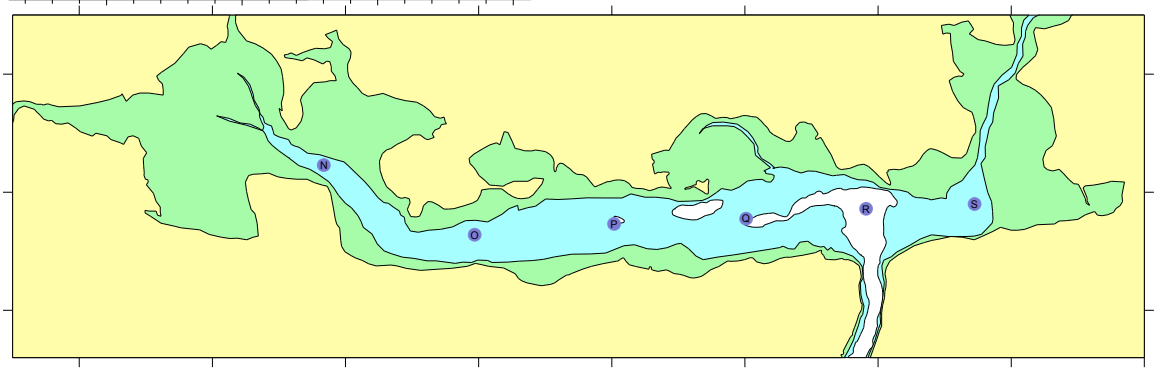


Fig.6a [left]: Map of water sampling stations in Cork Harbour.

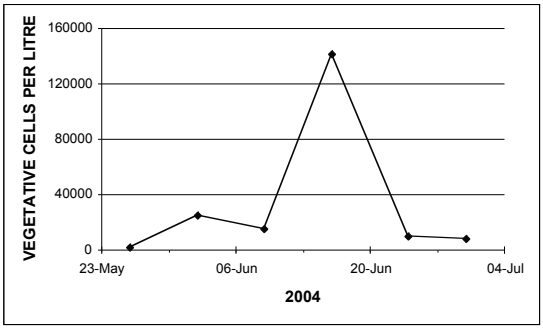
Fig.6b [below]: Map of water sampling stations in the North Channel of Cork Harbour.

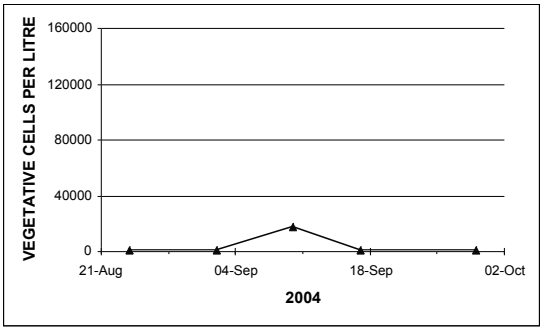


A six week intensive survey was carried out in June 2004. During this period, an intensive bloom that peaked on June 16th was observed. A positive PSP bioassay was recorded by the Marine Institute Biotoxin Monitoring Program on June 21st, which led to a closure of shellfish harvesting in the area. The intensive survey was repeated in September. A second *Alexandrium* bloom was observed that appeared to last for about two weeks with a peak on September 10th. There were no positive PSP bioassays recorded this time and thus no closures.

Results and Discussion

The *Alexandrium* bloom that occurred in June 2004 contained a significantly higher density of cells than the one in September 2004 (Figure 7a). With both blooms plotted on the same time axis (Figure 7b), a reflection of the theoretical life-cycle timescale (Figure 5) can be seen, albeit with a significantly smaller second bloom.





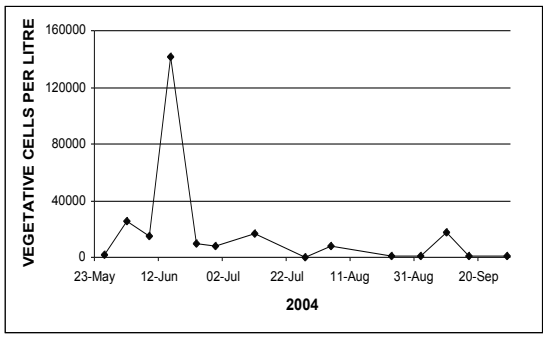


Figure 7a(i)[above left]:
Results of June bloom.

Figure 7a(ii)[above right]:
Results of September bloom.

Figure 7b[left]:
Results of summer 2004 survey.

The first four weeks of the June survey were made up of glorious, warm sunny days and flat calm seas. As expected there were three neap tides over the six week period. Neap tides equal a smaller flushing volume than spring tides, and thus a more stable and calm water column with warmer temperatures and greater light penetration. There was one neap tide just days before the peak of the bloom that, along with the prevailing weather conditions provided the optimal levels of light and temperature for bloom development. Tidal flushing of the North Channel is much smaller in neap tides than with spring tides, therefore phytoplankton can grow more successfully. Two neap tides occurred during the development of the *Alexandrium* bloom in June 2004. This bloom also occurred during a period of very warm, sunny and calm weather resulting in optimal growth conditions for *Alexandrium*.
When the weather conditions deteriorated however, so did the bloom. The bad weather, coupled with a spring tide just days after the peak of the bloom, coincided with the demise of the bloom.

In September the sequence of events was similar. Ireland experienced a so-called 'Indian summer', which gave the warm calm conditions that lead to blooms of *Alexandrium*. Again, neap tides occurred shortly before the peak of the bloom. The bloom subsequently terminated when the weather broke during a spring tide. If the weather had stayed warm and calm through to the following neap tide, then one can speculate that the cell counts would have increased culminating in an intense bloom similar to that observed in the same time period in 2003.

Conclusions

The observations presented here strongly suggest that the limits of *Alexandrium* blooms in Cork Harbour are inextricably linked to the weather, with tides also playing a key role. Bloom development hinges on the establishment of a stable water column with minimum turbulence and tidal flushing. In general terms, dinoflagellates do not survive well in turbulent environments, which would be produced by strong winds blowing over a shallow water column such as exists in the North Channel. Weather patterns definitely play an integral role in both creating and destroying the calm turbulent-free water column. Further analysis of the effects of the environmental parameters measured on the blooms in question has yet to be done.

Acknowledgements

This Project (PHD/01/005) is carried out with the support of the Marine Institute and the Marine RTDI Measure, Productive Sector Operational Programme, National Development Plan 2000 – 2006. We greatly appreciate the assistance of our skippers Brian Byrne and Donie Geary and the advice of the local oyster farm Atlantic Shellfish Ltd.

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AMNESIC SHELLFISH POISONING RESEARCH AT LETTERKENNY INSTITUTE OF TECHNOLOGY

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Introduction

Amnesic Shellfish Poisoning (ASP) caused widespread closures of the scallop fishery off the west coast of Scotland in the summer of 1999. Given the location of Letterkenny Institute of Technology (LYIT) on the north coast of Ireland and the involvement of some of the staff in scallop research, an undergraduate student research project on domoic acid (DA), the toxin responsible for ASP, commenced in October 1999. Analytical equipment suitable for the analysis of DA was available within the Department of Science. Following confirmation of the toxin in Irish waters in December 1999, this project was continued with funding from the Marine Institute for the summer of 2000. Three further undergraduate research projects followed with funding provided internally by the Department of Science. Two postgraduate research projects funded by the Higher Education Authority Technological Research Programme: Strand 1 commenced in 2002. This presentation features a collation of some of the results from these research projects.

Council Decision 2002/226/EC refers to the 20µg/g limit for the toxin in a whole scallop homogenate, the 4.6µg/g limit for the toxin in the edible parts following the processing of scallops from fisheries exhibiting 20-250µg/g in the whole scallop, the use of HPLC (High Performance Liquid Chromatography) techniques for measurement, a sample size of 10 scallops and the need for weekly sampling in harvesting areas. There are however no references in the Directive to several practical aspects of any monitoring programme such as:

- What are the precise details of the measurement procedure ?
- Whether scallops should be sampled from the seabed or from hanging culture in locations where both exist, such as Mulroy Bay in Co. Donegal ?
- Is toxin concentration related to scallop size ?
- Is toxin concentration related to the water depth ?

These questions have formed the basis of the research programmes undertaken at LYIT to date.

ASP is caused by the consumption of shellfish contaminated with the toxin, domoic acid, produced by diatoms of the genus *Pseudo-nitzschia*. In Ireland ASP has been reported predominantly in the king scallop, *Pecten maximus*. The toxin accumulates mainly in the hepatopancreas of this species (Figure 1).

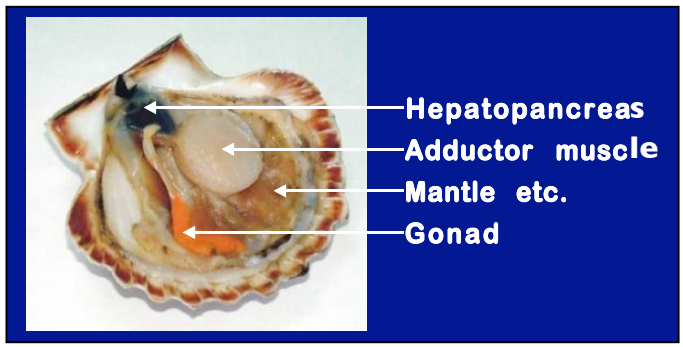


Figure 1. Internal organs of *Pecten maximus*

Measurement Procedure

Hardstaff *et al.* (1990) used an aqueous methanol extraction procedure in their initial work on the identification of domoic acid. Both 50 and 75% aqueous methanol exhibited good recovery for exhaustive extraction, the recommended technique for the highest level of accuracy. A single, dispersive extraction was recommended for use in regulatory monitoring programmes, under pressure to provide results. The single extraction procedure using 50% aqueous methanol produced the best extraction efficiency and is nowadays widely used by regulatory laboratories.

Following extraction and measurement of the domoic acid concentration in the extract by HPLC (µg/ml), the concentration of domoic acid (µg/g) in tissue samples is calculated according to the formula below based on the work of Quilliam *et al* 1995.

$$\text{Tissue DA Conc. (µg/g)} = \frac{\text{HPLC DA Conc (µg/ml) x 5 x 20}}{\text{Wt. of tissue sample (g)}}$$

This equation assumes that in the extraction procedure, the domoic acid is equally dispersed between the tissue pellet and the extraction solvent, hence the factor of 20 (16ml + 4g) used in the formula. Alternatively, if the domoic acid were completely extracted into the solvent, a factor of 16 (16ml of solvent) should be used in the formula (Figure 2). According to the literature, this is a 95% partial extraction with the reduced extraction yield causing an underestimate of the tissue concentration. However, the 4g of tissue is composed of approximately 75% water resulting in a supernatant volume of 19ml, rather than 20ml, leading in turn to a 5% overestimate of tissue concentration. Conveniently the overestimate and underestimate cancel each other from the equation (Quilliam *et al* 1995).

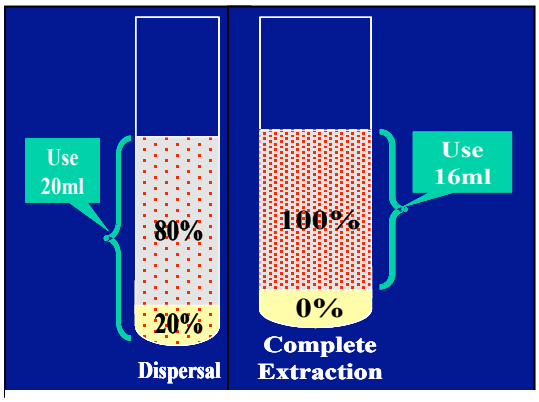


Figure 2. Effect of different extraction efficiencies

Research at LYIT compared the extraction efficiency of the single extraction used in routine testing with the exhaustive extraction of the same sample. Eight different tissue types were examined, including scallop hepatopancreas, adductor muscle, gonad and remainder, whole mussel, whole cockle, whole variegated scallop, whole Pacific oyster and whole native clam. Each tissue type was examined at up to five different DA concentrations to investigate the effect of toxin concentration on extraction efficiency. Six replicates of each of the tissue types at each of the concentrations were examined. Each replicate of each tissue type at each concentration was extracted exhaustively to determine the total domoic acid concentration and this value compared to the domoic acid concentration extracted by a single extraction.

Results demonstrated that:

- The efficiency of the single extraction varied between different shellfish matrices.
- The efficiency of the single extraction varied with toxin concentration.
- Implementation of a double extraction procedure, each of 1-minute duration, combination of the extracts and dilution to a fixed volume resulted in approximately 95% extraction efficiency in all of the tissues and at all concentrations examined.

Seabed or hanging culture as the origin of scallops for regulatory sampling

Since 1999, several reasons have arisen for concern over the origin of scallops provided for regulatory monitoring. Initially interest in the existence of a difference between scallops sampled from hanging culture nets, or from the seabed, was focussed on the occurrence of both farming systems in Mulroy Bay, County Donegal and also on the convenience of sampling for regulatory monitoring. The requirement of one sample for regulatory monitoring and the possible existence of variations in toxin concentration between scallops from two farming systems had the potential to close one or other farm unfairly.

Following confirmation of ASP toxin in Irish scallops and the reported slow rates of toxin depuration in Scotland, consideration was given to the possibility that scallops with domoic acid concentrations exceeding the EU regulatory limit could be suspended in richer phytoplankton waters where the rate of toxin depuration might be increased.

Latterly scientific concerns have been expressed that in addition to species of the diatom *Pseudo-nitzschia*, some benthic diatoms or bacteria may also be capable of producing the ASP toxin, domoic acid.

Ten samples comprising 12 large scallops (100-115mm) from the seabed and 12 large scallops from a lantern net suspended in mid-water were provided from the same site in Clew Bay, Co. Mayo over a one-year period. Individual analysis of the hepatopancreas and analysis of a pooled composite of gonad and adductor from each size group was performed.

Analysis consisted of domoic acid extraction from approximately 4g of tissue homogenate with 16mls of 50:50 extraction solvent (methanol:water) for 4 minutes. The extract was centrifuged at 4000rpm for 30 minutes. The supernatant was filtered using a methanol-compatible 0.45µm syringe filter. The concentration of domoic acid in the filtered extract was determined by HPLC/DAD. In the earlier part of the project the concentration of domoic acid was determined using HPLC/UV. Quality assurance of the data was provided using Laboratory Reference Material's provided by the Marine Institute. Statistical analysis was performed using SPSS.

Domoic acid concentration in the hepatopancreas of scallops from the two different farming systems has exhibited no significant difference (Figure 3). Individual analysis of scallops demonstrated the high variability in toxin concentration that occurs in scallops. The results demonstrated that suspension of scallops from the seabed in hanging culture nets has no potential as a technique for increasing the rate of toxin depuration. Domoic acid concentration in the gonad of scallops from the two different farming systems exhibited variable results. These variations were more difficult to understand because of the lower toxin concentrations involved in this tissue and the influence of the scallop reproductive cycle on toxin concentration in the gonad.

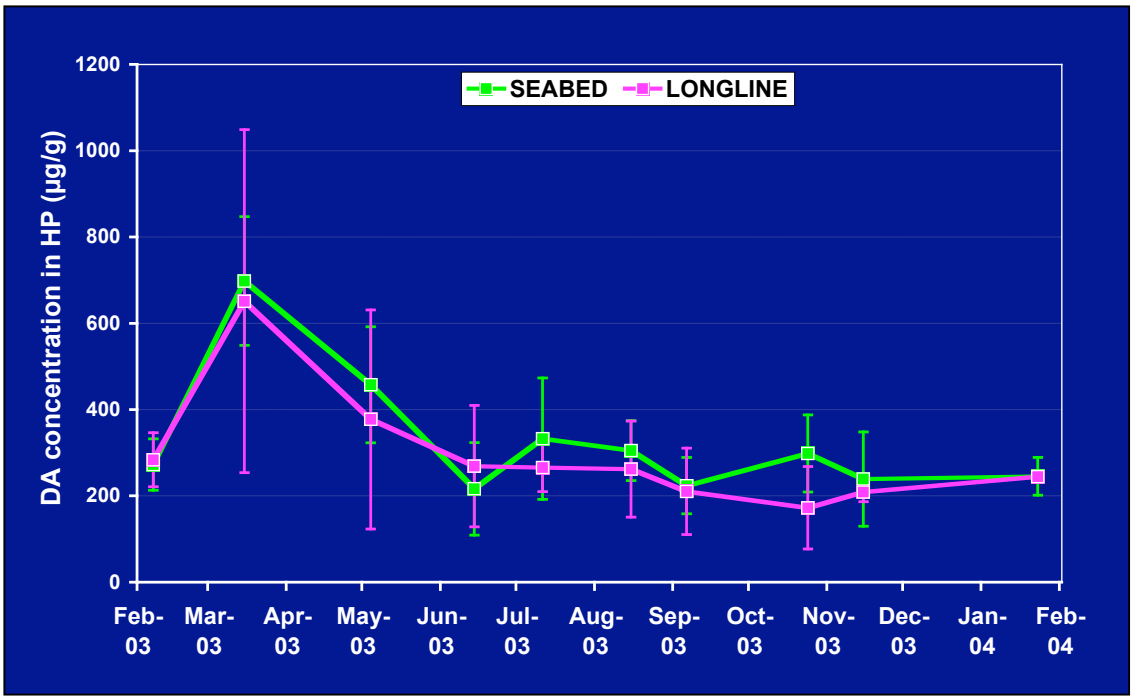


Figure 3. Domoic acid concentration in the hepatopancreas of scallops from both seabed culture and hanging culture in Clew Bay, Co. Mayo from Feb 03 to Feb 04.

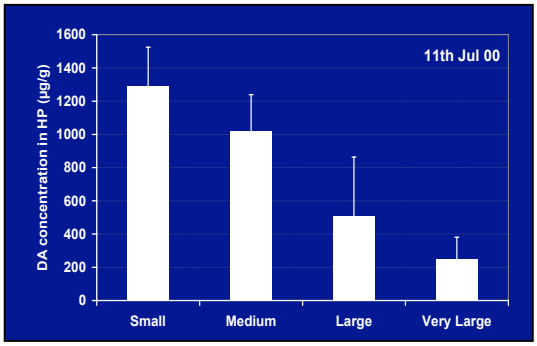
Variation in toxin concentration with scallop size

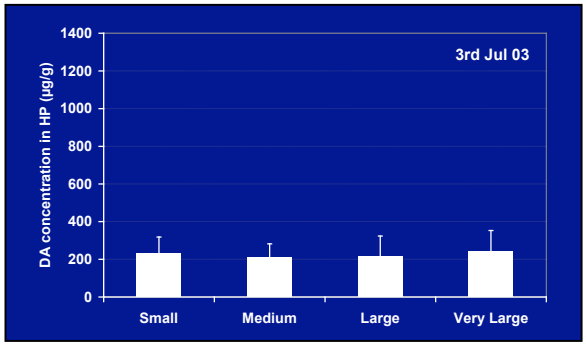
Samples comprising 12 scallops, from each of four different size groups, small (70-85mm), medium (85-100mm), large (100-115mm) and very large (>115mm) have been obtained from Casheen Bay, Co. Galway; Valentia Harbour, Co. Kerry; Strangford Lough, Co. Down and from the Wexford fishery. Regular monthly samples over a one-year period were provided from Clew Bay, Co. Mayo. Individual analysis of the hepatopancreas and analysis of a pooled composite of gonad and adductor from each size group has been performed to provide data on variability in toxin concentration.

Analysis consisted of domoic acid extraction from approximately 4g of tissue homogenate with 16mls of 50:50 extraction solvent (methanol:water) for 4 minutes. The extract was centrifuged at 4000rpm for 30 minutes. The supernatant was filtered using a methanol-compatible 0.45µm syringe filter. The concentration of domoic acid in the filtered extract was determined by HPLC/DAD. In the earlier projects extracts were cleaned-up using SAX SPE cartridges and analysed using HPLC/UV. Quality assurance of the data was provided using Laboratory Reference Material's provided by the Marine Institute. Statistical analysis was performed using SPSS.

Individual analysis of scallops demonstrated the high variability in toxin concentration that occurs between scallops from a single size group, from the same site sampled on the same date. Relative standard deviations (RSD) within a size group in this study ranged between 11-82%. The relationship between scallop size and domoic acid concentration has exhibited a negative correlation in some samples, i.e. small scallops had a higher concentration of domoic acid (Figure 4.); a positive correlation in others, i.e. small scallops had a lower concentration of domoic acid (Figure 6.); and most commonly no relationship, i.e. different sizes of scallops exhibited a similar toxin concentration (Figure 5).

Current thinking to explain the variations observed in the relationship between different sizes of scallop and toxin concentration are based on the hypothesis that during the development of a toxic *Pseudo-nitzschia* bloom, small scallops achieve a maximum concentration before larger scallops, resulting in a negative correlation between toxin concentration and scallop size. There follows a period where toxin concentration and scallop size are unrelated. Following termination of the bloom, depuration of the toxin from small scallops is achieved before larger scallops resulting in a positive correlation between toxin concentration and scallop size. Such variations, observed in the field samples to date, may result from differential rates of toxin uptake and depuration in different sizes of scallop or alternatively may be due to differences in the size of the hepatopancreas and relative amounts of tissue for toxin dilution in the different sizes of scallop.





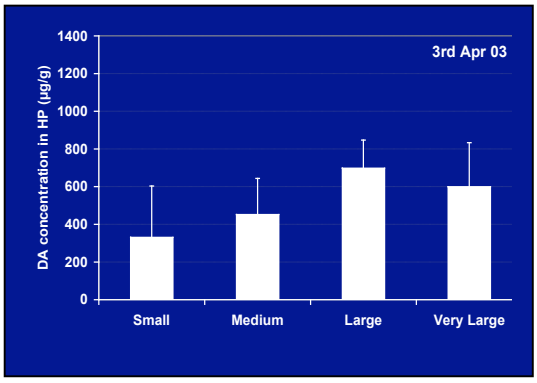


Figure 4. Variation in the domoic acid concentration of the hepatopancreas of different sizes of scallop from Casheen Bay, Co. Galway on 11th July 2000. (top left)

Figure 5. Variation in the domoic acid concentration of the hepatopancreas of different sizes of scallop from Clew Bay, Co. Mayo on 3rd July 2003. (top right)

Figure 6. Variation in the domoic acid concentration of the hepatopancreas of different sizes of scallop from Clew Bay, Co. Mayo on 3rd April 2003. (bottom left)

Variation in toxin concentration with water depth

Several investigations have been performed over the last five years and these are outlined separately below:

Analysis consisted of domoic acid extraction from approximately 4g of tissue homogenate with 16mls of 50:50 extraction solvent (methanol:water) for 4 minutes. The extract was centrifuged at 4000rpm for 30 minutes. The supernatant was filtered using a methanol-compatible 0.45µm syringe filter. The concentration of domoic acid in the filtered extract was determined by HPLC/DAD. In earlier projects extracts were cleaned-up using SAX SPE cartridges and analysed using HPLC/UV. Quality assurance of the data was provided using CRM and LRM provided by the Marine Institute. Statistical analysis was performed using SPSS.

Beirtrabui Bay project

Samples of 12 king scallops from three different depths – shallow site (11.1-12.3m), a middle site (16.5-16.7m) and a deep site (25m) were collected in a standard scallop dredge from Beirtrabui Bay, Co. Galway in February 2002 as part of a Bord Iascaigh Mhara fishery survey. Mean shell lengths of the scallop from the three depths were 128.4mm, 129.8mm and 132.8mm respectively. Individual analysis of the hepatopancreas and analysis of a pooled composite of gonad and adductor from each depth group were performed to provide data on variability. The results demonstrated that mean domoic acid concentration increased with water depth. Statistical analysis using one-way ANOVA demonstrated that the mean concentration of domoic acid in the scallop hepatopancreas was statistically different at the three depths.

Isle of Man projects

Irish boats from the Wexford fleet dredge on the scallop grounds around the Isle of Man (IOM) in the spring of each year. Samples of 12 king scallops were provided from six scallop beds in October 2003 and from eight scallop beds in July 2004 during a stock assessment survey performed by the Port Erin Marine Laboratory on behalf of the Manx Government. Mean shell lengths of the scallop ranged between 97.0mm to 116.7mm and between 104.6mm to 107.8mm on the two dates respectively. Individual analysis of the hepatopancreas of each scallop from all the scallop beds was performed to provide data on variability. Samples demonstrated that mean domoic acid concentration exhibited no relationship with water depth on either of the sampling dates. Domoic acid concentrations were significantly higher on the west coast of the IOM than on the east coast of the IOM.

Waterford project

ASP has caused restrictions on the sale of fresh scallops (in-shell), which achieve a higher market price than the frozen processed product. Scallop beds off the Wexford and Waterford coast and in the south Irish Sea are larger than in other locations around the Irish coast and extensive offshore grounds exist in the Celtic Sea and English Channel (Tully et al 2002). The fleet is based mainly in Kilmore Quay and Duncannon, Co. Wexford and currently accounts for 90% of Ireland's scallop landings. Attempts to re-establish the sale of whole, in-shell scallops whilst satisfying the 20µg/g legal limit for domoic acid concentration prompted this study on the spatial variation of domoic acid concentrations within one fishing area. The principal objectives of the study were to provide data on the extent of spatial variation within a single fishing ground and to investigate any relationships with water depth and scallop size within the same fishing ground.

Mean domoic acid concentration in whole tissue of the scallop from 69 stations was 40.6 ± 30.8µg/g within a range from 6.5 to 154.3µg/g (Figure 7.). The stations were grouped into four concentration groups based on the domoic acid concentration in the whole scallop: 0-20µg/g, 20-50µg/g, 50-80µg/g and >80µg/g. Scallops from the four concentration groups had similar mean physical characteristics, viz. length, height and age; however mean total tissue weight increased with mean domoic acid concentration. The increased total tissue weight was attributed to large increases in gonad weight with a smaller increase in adductor muscle weight. The domoic acid concentration in the whole scallop and water depth at all 69 stations exhibited a significant negative correlation (r= -0.71), i.e. domoic acid concentration decreased with water depth.

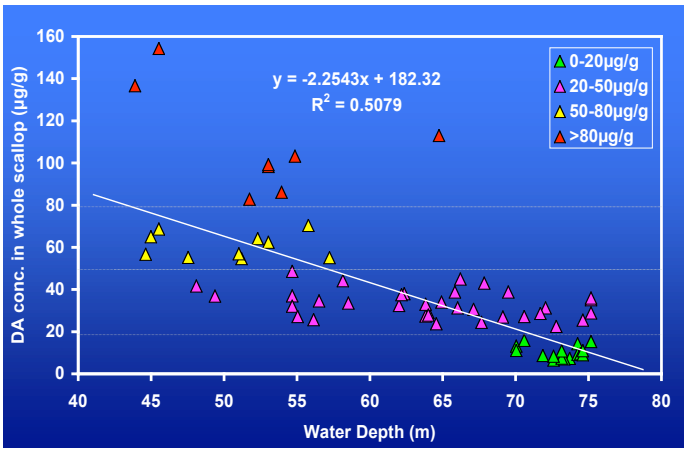


Figure 7. Variations in the concentration of domoic acid in whole scallop (µg/g) vs. water depth (m).

Conclusions

Measurement procedure

A double extraction procedure, combination of the supernatant and dilution to volume has been shown to extract approximately 95% of the domoic acid over a range of concentrations and in a range of different shellfish matrices.

Seabed or hanging culture origin of scallop samples

No significant difference was observed between domoic acid concentrations in the hepatopancreas of scallops sampled from the seabed and those sampled from hanging culture over a 12-month period.

Scallop size

Domoic acid concentration can exhibit variations with scallop size.

Water depth

Domoic acid concentration can exhibit variations with water depth.

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MICROBIOLOGICAL AND VIRAL CONTAMINATION OF SHELLFISH: WORK OF THE IRISH NATIONAL REFERENCE LABORATORY AND CURRENT PROGRESS IN VIRUS DETECTION METHODS

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Marine Institute, Galway Technology Park, Parkmore, Galway.

Introduction

The Marine Institute (MI) was designated as the National Reference Laboratory (NRL) for monitoring microbiological and virological contamination of bivalve molluscan shellfish for Ireland, in accordance with European Council Decision 1999/313/EC. The first staff were recruited to this area in Autumn 2004. This paper details the proposed work plan for the NRL over the coming year. It also outlines the current state of progress towards developing improved virus detection methods for Noroviruses in shellfish in Europe.

Molluscan bivalve shellfish that are grown in sewage-contaminated waters can accumulate pathogenic bacteria and viruses. Such shellfish can present a significant health risk when consumed raw or lightly cooked. The potential for shellfish to act as a vector for disease is illustrated by largest recorded outbreak of food-borne illness, which occured in Shanghai in 1988, and where almost 300,000 people contracted hepatitis A following the consumption of contaminated clams (Halliday, 1991). To control these risks in Europe, shellfish harvesting areas are classified depending on the extent of sewage contamination that they receive based on levels of faecal indicator bacteria present. The classification determines the degree of treatment required before placing on the market (Table 1.).

Table 1. Criteria for Microbiological Classification of Shellfish Harvesting Areas

Category	Microbiological Standard	Treatment required
A*	<230 *E.coli* or 300 faecal coliforms	May go direct for human consumption
B	<4,600 *E.coli* and 600 faecal coliforms (90% compliance)	Must be depurated, heat treated or relayed to meet class A requirements
C	<60,000 faecal coliforms	Relay for 2 months to meet class A or B requirements-may also be heat treated
D	>60,000 faecal coliforms	Harvesting prohibited

*Shellfish going directly for consumption must also be free from *Salmonella* spp.

Such control measures have been successful in virtually eliminating bacterial illness associated with shellfish throughout Europe. However outbreaks of viral illness continue to occur (Ang, 1998; Christensen *et al*, 1998). Most noticeably, such illness is associated with Noroviruses (NVs) causing gastroenteritis and hepatitis A virus (HAV) causing infectious hepatitis.

HAV infections associated with shellfish tend to be more closely associated with southern countries in Europe. For example, it has been suggested that shellfish may be responsible for as many as 70% of all hepatitis A cases in Italy where specific endemic populations may be found (Salamina and D'Argenio, 1998). Norovirus associated gastroenteritis is more widely distributed and has been associated with

shellfish consumed throughout Europe (Lees, 2000). To date the implementation of successful viral control measures has been handicapped by the lack of suitable virus detection methods for shellfish.

National Reference Laboratory Activities

The implementation of current control measures relies heavily on the work of the network of National Reference Laboratories (NRLs) in European member states, which are coordinated by the Community Reference Laboratory (CRL) in CEFAS, UK.

The duties of the NRL are defined by Article 2 of European Council Decision 1999/313/EC. These are;
(a) co-ordinating the activities of national laboratories.
(b) assisting the competent authority in the member state to organise a system for monitoring viral and bacteriological contamination of bivalve molluscs
(c) organising comparative tests between the various national laboratories
(d) disseminating the information provided by the CRL to the competent authorities and national laboratories

In response to the designation as the NRL, the MI has begun the process of recruiting a team dedicated to discharging these responsibilities. A Team Leader was appointed in October 2004. A further scientist has been recruited and will be starting in January 2005. A laboratory technician/fellowship will be completed shortly.

Introduction of test methods into the NRL

The first and most critical task facing the NRL in the coming year is the introduction of appropriate methods into the MI. For bacterial parameters this will include;

- The CRL recommended five-tube three-dilution most probable number (MPN) procedure for *Escherichia coli* (Donovan *et al*., 1998)
- the CRL recommended method for *Salmonella* spp. based on the ISO Standard method (Anon, 2002).

The NRL aims to have these methods accredited by the Irish National Accreditation Board (INAB).

The NRL will also develop a virus testing facility for shellfish in Ireland. Initially, this will focus on introducing a method to detect NVs using a quantitative real-time PCR procedure. Priority will be given to introducing procedures for NVs as this group of viruses represents the most clearly defined risk in Irish shellfish.

Organising national laboratories

The NRL is responsible for organising national testing laboratories so that they provide an effective facility for testing shellfish in Ireland. This includes both laboratories carrying out testing for classification purposes and laboratories carrying out end-product checks for producers.

The MI and Food Safety Authority of Ireland carried out an audit of official laboratories performing tests for classification purposes in April/May 2004. Corrective Action Plans were drawn up and implemented. In the early part of 2005, the MI will place the national programme of *E .coli* testing of shellfish for

classification purposes for tender. Contracts will be awarded to laboratories that can demonstrate compliance with rigorous methodological and quality criteria. Audits of laboratories undertaking own check tests for *Salmonella* spp. and *E. coli* are also planned for 2005. To ensure continued delivery of an effective national microbiological testing facility the NRL will introduce a programme of annual audits for national laboratories for subsequent years.

Dissemination of Information.
A significant role for the NRL is to disseminate information from the CRL to the national testing laboratories. This includes information on technical developments in methods, advice on sampling and sample storage, and information on quality assurance schemes. As well as *ad hoc* contact between testing laboratories and the NRL, the MI will fulfil this function by two formal mechanisms.

Firstly, by creating a microbiology and virology information centre on the MI shellfish safety website. This will contain background information, regular updates on the work of the NRL and a mechanism for publishing the latest information from the CRL. It is also envisaged that it will provide a facility for information exchange between laboratories.

Secondly, the NRL will hold annual meetings of national testing laboratories. This will provide a technical forum that will not only allow dissemination of information to the laboratories but will also provide an opportunity for laboratories to feed information back to the CRL through the NRL. This will provide a mechanism to allow Irish testing laboratories to influence technical issues at a European level. The NRL will complete the formal link for the flow of information between the testing laboratories and CRL by attending the annual meeting of the network of NRLs and representing the agreed views of Irish testing laboratories.

Assistance in organising the national monitoring programme
A further key role for the NRL will be to assist the competent authority (DCMNR) to organise a system for monitoring viral and bacteriological contamination of bivalve molluscs. This will involve the provision of scientific advice on selecting appropriate sampling points, sample storage and transport analysis and interpretation of monitoring data. Scientific input with regard to data interpretation is likely to occur both during the biannual classification process and on the receipt of high results during routine monitoring. Of particular significance here is the role that direct monitoring for viral pathogens should play in the national monitoring programme. The role of new molecular detection methods in assessing the risk associated with shellfish is far from clear and the NRL proposes to produce a position paper on the use of virus testing in Ireland. This paper will be presented to the Molluscan Shellfish Safety Committee for discussion.

Noroviruses in Shellfish: Progress Towards Improved Detection Methods in Europe

Despite widespread and determined efforts to develop a culture-based detection system for NVs no such system is available. Detection systems using immune electron microscopy and Enzyme Linked Immunosorbent Assays have been developed and are used in the clinical setting. However these systems have been shown to lack the sensitivity required to detect low levels of virus often found in the environmental setting. Therefore detection methods for NVs and other pathogenic viruses in shellfish have centred on the use of molecular techniques. In particular the Polymerase Chain Reaction (PCR) has been used to amplify target DNA to a level whereby it can be visualised on an electrophoresis gel. However detection of NVs in shellfish using this procedure present a particular problem.

- NVs are RNA viruses and cannot be amplified directly in the PCR. A reverse transcription (RT) step to produce cDNA for amplification is required.
- NVs are genetically diverse consisting of 2 major genogroups which infect humans containg over 20 genetically distinct virus genotypes.
- Virus levels in shellfish are extremely low. Nested PCR procedures have been commonly employed to increase amplification to the point that a DNA band can be visualised on an agarose gel following elcetrophoresis.
- Shellfish tissue contains high levels of substances that are inhibitory to the PCR.

Despite this, a number of PCR based methods to detect viruses in shellfish have been developed (reviewed in Lees, 2000). These methods, although major breakthroughs in their own right, have a number of drawbacks that make them difficult to use on a routine standardised basis.

Over the last few years attention amongst workers in this area has turned to the potential use of an alternative PCR procedure, namely Quantitative Real-Time PCR and in particular the TaqMan® assay. Quantitative real-time TaqMan® PCR assays exploit the 5' nuclease activity of *taq polymerase* to detect and quantitate the accumulation of PCR product amplification in 'real-time' using fluorogenic probes. The fluorogenic probes employed are oligonucleotide specific to the target of interest and contain both a reporter dye and also quencher which prevents fluorescence of the reporter dye. Cleavage of the probe by *taq polymerase* during exponential amplification separates the reporter dye and quencher thus increasing fluorescence. Therefore, the greater the initial level of target template in the sample the more rapidly exponential amplification is detected.

Real-Time PCR procedures have a number of advantages over conventional PCR. (Table 2). The procedure provides for the first time the real prospect of developing standardised methods for the detection of NV in shellfish because of it's potential for automation, and absence of a need for post PCR analysis. In addition the increased sensitivity of the fluorescent detection system means there is no need for two-stage nested PCR procedure thus reducing the need for technician manipulation and potential for sample cross-contamination.

Table 2. Comparison of conventional PCR and Real-Time TaqMan PCR

Criteria	RT-nested PCR	Real-Time PCR
Detection	Gel-electrophoresis requires post PCR manipulation	Fluorescent probe chemistry provides a single step detection method.
Sensitivity	Requires a 2 step (nested) PCR to achieve sufficient amplification for detection on agarose gel.	Fluorescence detection system requires less amplified DNA for detection removing need for two-step nested procedure
Confirmation	Requires secondary confirmation. (gene sequencing)	Specific probes provide in built confirmation step.
Quantitation	Not possible for procedures using a final amplified product detection system.	Log phase detection of fluorescence allows quantitation

Despite these advantages a major barrier to the use of Real-Time PCR for the detection of the genetically diverse NVs has been the lack of broadly reactive primer and probe sets for this virus. However, broadly reactive primer probe sets have recently been developed for genogroup I & II viruses by the Community Reference Laboratory in conjunction with the University of North Carolina. Full (CRL technical report, 2003) details of this work can be found on the CRL website. Therefore it is now possible to use real-time PCR procedures to investigate the presence of NVs in shellfish. The use of this technique has demonstrated that NV detection is prevalent in shellfish samples from category B harvesting areas although shellfish appear to contain very low concentrations of virus (CRL technical report, 2003). A recent survey of category B harvesting area in Ireland showed NVs were present in samples taken during the high risk winter period, as often as 100% of the time in some areas. Clearly the next challenge is to determine the significance of such results and whether it is possible to use this new technology, in particular the quantitative aspects of the assay, to identify shellfish that may make people ill.

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MICROBIAL DISPERSION IN BANNOW BAY, CO. WEXFORD.

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Background

Filter-feeding bivalve mollusks, such as oysters, concentrate microorganisms from the overlying water. Where the water is subject to microbial pollution from sewage or animal sources, there is the risk that human pathogens, such as *Salmonella*, *Campylobacter*, Norwalk-like and Hepatitis A viruses, may contaminate the shellfish, and on entry into the human food chain, endanger public health. Transitional waters used for shellfish farming are classified under Council Directive 91/492/EEC (EEC 1991) on the basis of the levels of faecal indicator organisms found in the shellfish flesh. The levels found are taken to index the degree of sewage pollution of the shellfish and therefore their potential for the transmission of human disease. Gastroenteritis is the most common form of illness associated with the consumption of sewage-contaminated shellfish grown in temperate waters (Rippey 1994). The classification categories are as follows.

‘A’ grade: live bivalve molluscs can be collected for direct human consumption provided they contain less than 300 faecal coliforms or less than 230 *E. coli* per 100g of flesh and intravalvular liquid, and do not contain salmonella in 25g of mollusc flesh.

‘B’ grade: live bivalve molluscs can be collected from these areas but must not contain more than 6,000 faecal coliforms per 100g of flesh or 4,600 *E. coli* per 100g of flesh in 90 % of samples. Only after purification treatment that achieves the A grade microbial standards, can they be placed on the market for human consumption.

‘C’ grade: live bivalve molluscs can be collected from these areas but must not contain more than 60,000 faecal coliforms per 100g of flesh. Only after prolonged or intensive purification treatment that achieves the A grade microbial standards, can they be placed on the market for human consumption.

Prohibited: Where faecal coliforms are at a higher concentration than for Class C, shellfish harvesting is not permitted.

The earlier Council Directive 79/923/EEC (EEC 1979) regulating the quality of shellfish waters does not specify a microbial water quality standard as such, but only a Guide value (that Member States should endeavour to observe) of 300 faecal coliforms per 100 ml for the shellfish flesh and intervalvular liquid (a volume of flesh rather than a weight is prescribed). While currently there is no microbial water-quality standard for the waters in which shellfish are farmed, it is likely that future actions under the EU Water Framework Directive 2000/60/EC (EU 2000) will lead to satisfactory control of the shellfish waters when registered as Protected Areas.

The Water Framework Directive (WFD) takes a holistic "joined up" approach to pollution regulation on a river catchment basis. The catchments, referred to as 'river basins' in the Directive, are grouped as Districts; cross-border catchments are assigned by agreement to 'International' River Basin Districts. There is no certainty that water quality improvements obtained under the WFD, that is focused on chemical and ecological quality, will effect parallel improvements in microbial water quality. Nevertheless, the management plans developed for catchments that drain to translational waters serving as shellfish harvesting areas should seek to deliver microbial water quality that ensures Grade A classification. There is obvious concern about the water quality management costs in achieving this, in particular the high costs involved in ameliorating the impact of high rainfall events that might involve substantial improvements to sewerage infrastructure and changes in agricultural practices. Understanding the complex relationship between the microbial content of the shellfish and microbial concentration of the water in which they are grown will be essential for effective management, although the scientific work needed to underpin this is at an early stage. The citations that follow give a flavour of this work.

Clearly the control of polluting discharges is important; Younger and Kershaw, S. (2004) reported that upgrades of sewage treatment plants discharging to the Solent (UK) achieved marked improvement in shellfish hygiene in nearby harvesting areas. Many factors operate; season, high/low tidal cycle and rainfall were found to be the principle ones affecting shellfish contamination (Lee and Morgan 2003). These complexities induce the need for advanced modelling techniques; Lee and Glover (1998) assessed the use of geographical information system (GIS) techniques to predicting the effect of change in sewage treatment type on the extent of contamination of nearby shellfisheries, and recently the site-specific relationships between indicators and pathogens for shellfisheries have been modelled successfully using artificial neural networks (Brion *et al*., 2004). Interestingly, the importance of sediment-associated microbial contamination is becoming evident. Over a decade ago, Valiela *et al.* (1991) found that the levels of sediment and shellfish faecal coliforms were significantly related, and they concluded that monitoring faecal coliforms in sediments provided a better assessment of shellfish contamination than sampling the water. More recently, the temporal and spatial distribution of enterococcus in sediment, in shellfish tissue, and in the overlying water were found to correlate well (De Luca-Abbott and Creese 2000).

It is relevant to point out that greater attention has been paid to the microbial pollution of bathing waters, protected under the EU Bathing Water Directive 76/160/EEC (EEC 1976). A number of catchment studies, focused on clarifying how catchment-derived microbial pollution affects impacted bathing areas, have been published within the past ten years. A Welsh-Irish (INTERREG-II funded) study of the Dargle catchment in Ireland (Bruen *et al*., 2001) and the Afon Rheidol and Afon Ystwyth catchments in Wales (Crowther *et al*., 2003) showed that the microbial water quality of bathing areas was vulnerable to rainfall-related runoff from adjacent rivers in a manner that related to the patterns of catchment land use. The pioneering catchment studies in the United Kingdom in the Island of Jersey (Wyer *et al*., 1995) had first evidenced this, as have subsequent studies in the Staithes Beck catchment (Wyer *et al*., 1996, 1998b), the Derbyshire Peak District (Tranter *et al*.,1996; Hunter et al., 1999), the Afon Nyfer catchment (Wyer et al., 1997), the Afon Ogwr catchment (Wyer *et al*., 1998a), the Holland Brook (Clacton) catchment (Wyer *et al*., 1999a), the River Irvine and Water

of Girvan catchments (Wyer *et al*., 1999b), the Ribble catchment (Crowther *et al*., 1999b; Crowther *et al*. 2001) and the Windermere and Morecambe Bay catchments (Crowther *et al*., 1999a). This experience suggests useful approaches to the study of shellfish microbial pollution.

Against the above background, a study commenced in the summer of 2003 on the influence of catchment run-off on the microbial quality of the transitional waters of Bannow Bay, Co. Wexford that are used for oyster farming. This communication summarises the progress made in the first eighteen months; the work will be concluded by summer 2005. The work is funded as an INTERREG-IIIa (Ireland - Wales) project, entitled *'Sustainable management of near shore water quality for aquaculture, recreation and tourism (SMART)'*. Other Irish work for the project is progressing in the Liffey catchment as an extension of the Three Rivers Project (Earle 2003), and is concerned with the recreational water quality of Dublin Bay.

The Bannow Bay study is focused on questions such as the following. How is microbial pollution, both point discharge and diffuse, dispersed through Bannow Bay; is there any correlation with the microbial quality of the shellfish produced there? How vulnerable is the Bay to microbial pollution; what are the likely catchment sources, and what proportions of the microbal pollution do they account for; what approaches might be taken to manage the catchment impact to abate the pollution to an acceptable level? Of especial interest is the influence of rainfall on the extent and impact pattern of the microbial pollution. The overall aim is to work out how the management of microbial pollution at the catchment level should cater for shellfish hygiene.

Planning

Construct a catchment model that relates land use and indicator *Escherichia coli* and intestinal enterococci budgets for point and diffuse upstream sources and for the bay. (Such models have been generated by previous studies in Ireland and in the United Kingdom.)

Construct a hydrodynamic model of microbial dispersion in the bay.

On the basis of these models, relate microbial water quality to shellfish flesh microbial test results reported by the approved laboratories.

Outline remediation strategies for the control of point sources and of diffuse sources, referencing good land-use practices.

Organisation and staffing

A multidisciplinary, trans-institutional team was formed to bring to bear the expertise necessary to tackle the work of the project. Details of the personnel involved and a brief outline of achievement to date (October 2004) and deadlines follows.

Field surveys and classify land-use classification

(M. Thorp, G. Mills, S. Meehan and P. Byrne, Department of Geography, University College Dublin.)

The land use surveys are completed (Figure 1), and statistical analysis is in progress. This will be completed in the second quarter of 2005.

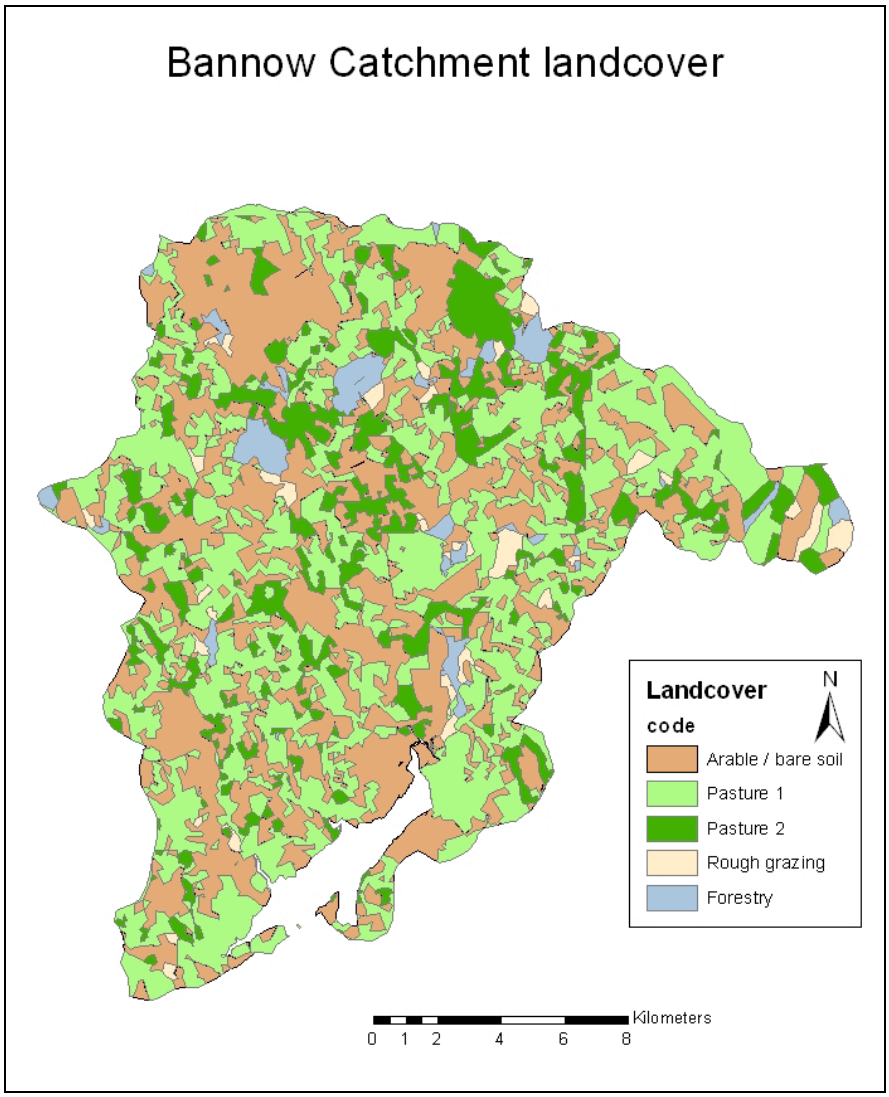

Figure 1. Land-use classification of the Bannow Bay catchment.

Estuarine hydrology modelling of microbial dispersal.
(M. Hartnett, Department of Civil Engineering, University College Galway, and A. Berry, Marcon Ltd..)
A working microbial dispersal model has been developed (Figure 2). Calibration, refinement and testing of the model will be completed in the second quarter of 2005.

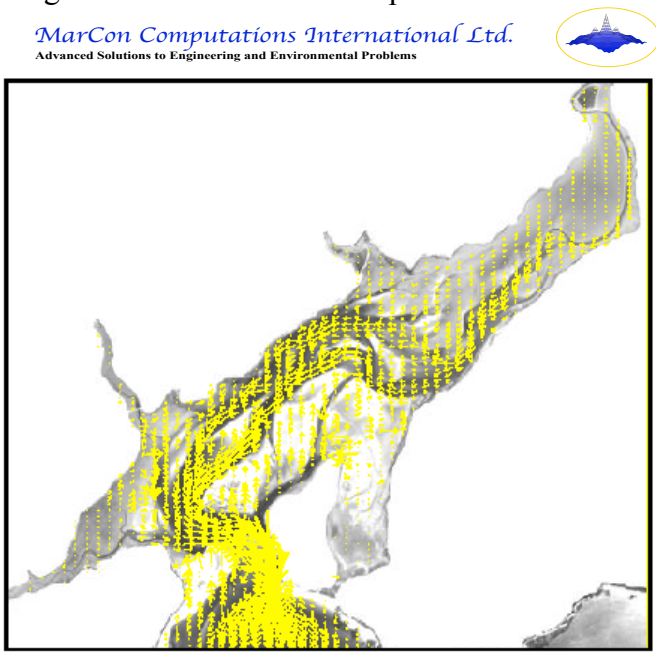

Figure 2. Hydrodynamic regime at mid-ebb tide.

Riverine hydrology to quantitate catchment run-off.
(M. Thorp, Department of Geography, University College Dublin, M. McCartaigh, Environmental Protection Agency, E. Hoare, Y. Mullooly and T. Quirk, Wexford County Council.)
Upgrading of hydrological stations at three sites of the catchment river system is progressing, and will be completed by the end of 2004 (Figure 3, 4). These installations are to provide both for the work required for the Water Framework Directive and for SMART (this project). Some additional enhancements of the installations are planned for early 2005.

Figure 3. Station at Foulksmills.

Figure 4. Station at Goff's Bridge.

Enumeration indicator bacteria in the catchment and Bay
(B. Masterson—Project Leader, K. Real, R. Chawla, and S. Fraser, Department of Biochemistry, University College Dublin.)
Six broad-ranging sampling excursions were conducted in Autumn 2003 and in Summer 2004, covering both high-flow and low-flow conditions, to establish the microbial sampling strategy required for the project (Figure 5,6). Synchronised riverine and estuarine sampling will commence as soon as the hydrological stations are operational. This work will be completed within the present oyster-harvesting season.

Useful synergy has been possible with another INTERREG-III project (iCREW) within which microbial source-tracking techniques are being developed. These techniques should enable indicator organisms from different animal sources (e.g. human and ruminant) to be distinguished. Some of the preliminary work has been done using samples collected during the SMART excursions; so far successful detections of ruminant indicators has been achieved. The iCREW work programme will conclude in September 2005, and it is expected that the results will add value to the SMART project too.


Figure 5. Sampling at Foulksmills.


Figure 6. Sampling at Taylor's Bridge.

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The Conway Institute, University College Dublin

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MICROBIOLOGICAL VULNERABILITY
A new approach for shellfish quality assessment and management

Sébastien Chantereau (Comité National de la Conchylicultre, France)
Richie Flynn (Irish Shellfish Association)

The European Shellfish Industry is moving rapidly to address the growing concerns in relation to the level of potentially harmful bacteria and viruses that could contaminate shellfish in inshore coastal areas and which originate from the human population adjacent to those areas or upstream of the local catchment system. Monitoring and control methods set down in EU regulations have focused to date on defining the risk of contamination of production areas by simple analysis of *E. coli* or faecal coliforms on a regular basis and then setting limits on the potential outlets for shellfish for those areas. This system has its flaws, mainly in the:

- Inability of the methods used to detect or respond to rapidly appearing and disappearing microbiological events.
- Lack of cohesion in the application of the regulations throughout the EU
- Absence of incentives within the regulations to deal with the causes of contamination

There have been two different responses to these recognised drawbacks in the system:

(a) From the Regulators (European Commission and Community Reference Laboratories) – a focus on increasing both the stringency of the limits set on allowable contamination in shellfish and the sensitivity of methods used to detect potential pathogens.
(b) From the industry (European Mollusc Producers Association, CNC) – a focus on limiting the potential causes of distribution of pathogens into the shellfish production environment and the establishment of a risk management based approach to preventing shellfish with potentially harmful contaminants from reaching the marketplace.

This paper sets out the industry's position on the ways and means to establish an effective risk management strategy for microbiological sensitivity around the coast of Europe, using work carried out mainly in France and sponsored by the CNC.

The French shellfish industry is made up of 3,750 companies, of which 78 % are owned by individuals or family. There are 21,700 people involved in the sector, including 10,400 full time employees. Intertidal and bottom-grown shellfish areas take up 16,000 ha with 1,700 km of bouchot poles. In total the sector produces 205,000 t with a sales turnover of €630 million.

Shellfish quality management in France is based on the same regulations that apply throughout the EU. Directive 79/923/EC, on the Protection of Shellfish Waters and Directive 91/492, which outline monitoring, control and classification of shellfish production areas, define purification and despatch centre controls, etc.

In France the responsibilities for various elements of the control of shellfish areas are handled by different agencies and groups:

- regulation at the prefecture level by the Ministry of Agriculture and Fisheries
- monitoring programme by IFREMER
- market sanitary control by the Veterinary Department of the Ministry
- self checking by the industry

In France, during 2004, the monitoring programme of shellfish areas classified 460 production zones in 314 separate geographical areas around the coast under Directive 91/492. Of these 32% were classed as "A", 49% as "B" and 12% as "C".

In France and in other countries, it is clear that this system does not take into account many important factors that have a clear bearing on the potential risk of consuming products. It is also evident that the current system of monitoring is not failsafe nor does it hold the confidence of producers. There is a lack of epidemiological evidence that shellfish cause many of the outbreaks of which it is accused. This is particularly important when one considers the many ways in which viruses and bacteria are spread between humans, animals, the environment and foodstuffs. There are difficulties in controlling the sampling of shellfish where *E. coli* could be artificially increased due to poor storage, etc. The test for *E. coli* outlined in the Directive is a very blunt instrument with many critics within the industry. Crucially, however, there are no data available for each production area on the sources of microbiological contamination that could be used to limit or prevent the initial problem, before penalising the shellfish producer.

In response to greater awareness and public concern about food safety issues in general, the European Commission has developed and evolved new food safety legislation. From simple vertical legislation which dealt with each foodstuff separately, the EC has produced a Hygiene Package which deals with controls and monitoring in a horizontal fashion and which puts emphasis on good management practices and the use of HACCP controls to protect consumer health. In addition, in the area of shellfish, this hygiene package became the subject of much debate during its various revisions in 2003-2004 as the inclusion of new monitoring tools were proposed, using bacteriophage as an indicator of viral presence. These new tools were seen as unworkable by national food safety agencies, regulators and the industry. Over 11 revisions of the draft legislation saw the bacteriophage proposal eventually dropped and a return to *E. coli* monitoring. This overall approach of dealing with the symptom rather than the cause of contamination is characteristic of the approach taken since the rather positive 1979 Protection of Shellfish Waters Directive. Producers regarded the historically palliative approach of DG SANCO of the Commission - where the effects rather than the sources of contamination are the key control point - as being fundamentally wrong and not a guaranteed protector of human health.

Producers' Approach

From the point of view of those on the front line of contamination of the inshore waters from the land, shellfish producers see the need for a complete change in the way the contamination is monitored and dealt with in order to protect consumer safety.

The basic principle upon which the producers' plan is built can be summarised thus: Quality Shellfish = Quality Coastal Waters. This concept puts consumers and the environment first, by laying emphasis on the parallel objectives of safe food and clean waters instead of simply accepting that there will inevitably be pollution and the only acceptable reaction is to place restrictions on the producers rather than dealing with the problem.

Building upon this concept, the common objectives of producers and the responsible agencies for health and environmental protection should be: A preventative approach to contamination of shellfish using real-time management which should be complementary to existing shellfish quality monitoring programmes (e.g. biotoxins, requirements under the Water Framework Directive) using agreed standard assessment methods, tools and indicators throughout the EU (Figure 1).

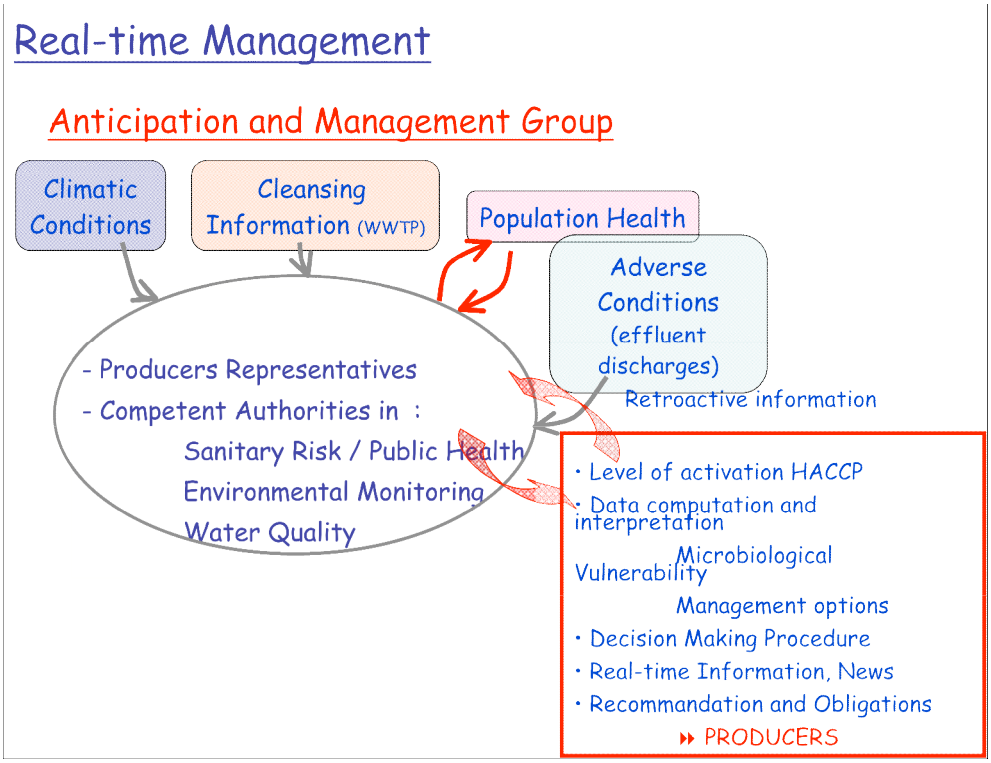

Figure 1. Real Time Management.

Achieving the preventative management approach required two major pieces of work, which have been pioneered and sponsored by the French industry and scientific community:

The Microbiological Vulnerability Concept – establishing the microbiological input risk of any given wateshed/catchment area.
Surveillance Mollusques (SUMO) – establishing the framework for a real time health management system.

The Microbiological Vulnerability Concept.
This begins with developing a model of Microbiological Sources and Processes for the basic understanding of the key coastal ecosystem components that potentially can affect the food safety status of shellfish grown in the adjacent inshore waters. A model of the various potential components in a typical area can be seen in Figure 2. It is important to understand that much of this data already exists. In the case of France, the industry found that for 15 international coastal areas extensively studied under various research projects (CORINE land cover study of the EU, AO study on Coastal Erosion, SIGMAZAL study in Spain, etc), 94 separate data sets were available on the various components outlined in the vulnerability model.

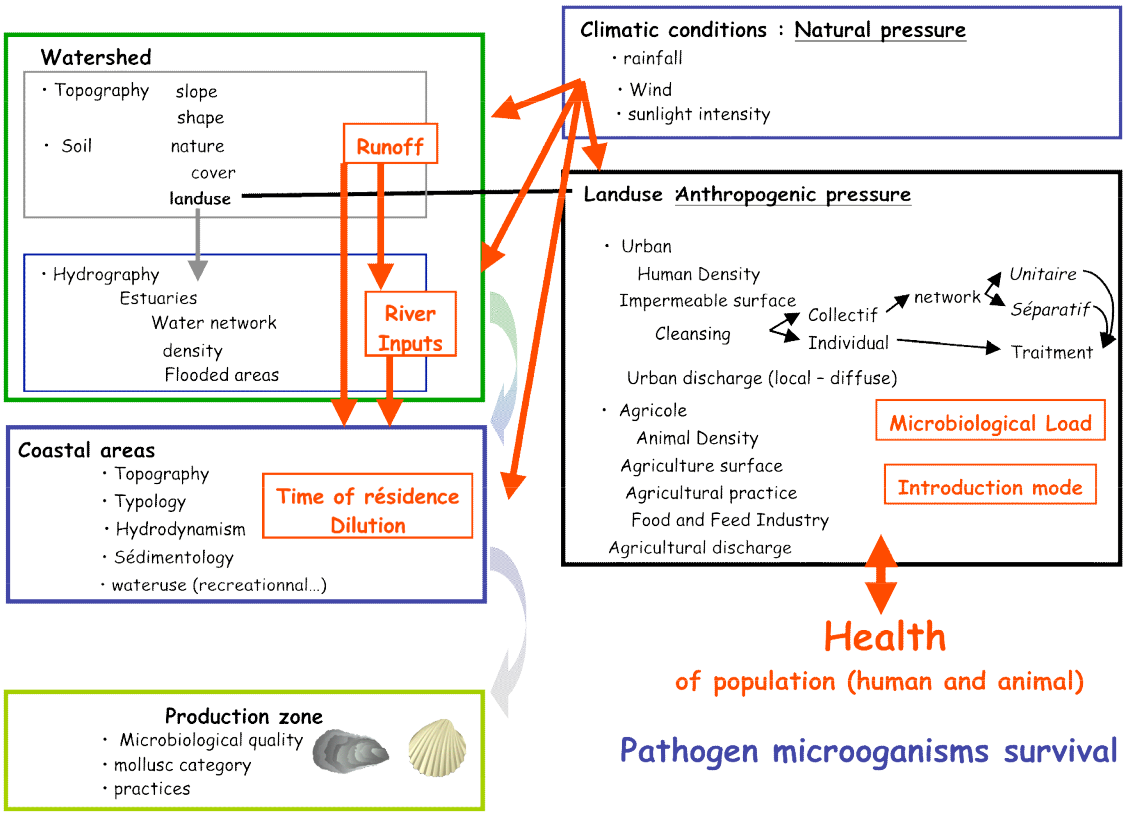

Figure 2. Factors affecting Microbiological Vulnerability of an area.

The development of a Microbiological Vulnerability model for any areas begins with the establishment of a Geographical Information System (GIS) to act as a reference foundation for future analysis and recommendations. The GIS will contain all the relevant information for the catchment area on human activities, the characteristic of the watershed, hydrodynamics of the river systems and inshore waters and climate. The product of this GIS will be to establish a reference model to compare actual conditions within the catchment/coastal area by way of ongoing monitoring of potential contamination sources, water quality and contamination within the shellfish themselves. As this builds with time, it will develop into a fully-fledged

microbiological quality assessment tool that will be feed into and be a key component of the overall management strategy for Shellfish Quality. The Management Strategy will be to quantify the impacts of events, assess options and review the effectiveness of corrective actions (which may range from short term voluntary harvest suspension by growers to longer term investment in improving local waste water treatment plants)

Given that consumer safety is the number one priority, side-by-side with longer-term investment in improvements to prevent pollution entering the catchment/inshore area, the production sector recognises the necessity to have real time management of shellfish production areas to ensure that potential contaminants do not find their way into the food chain. The development of an effective real time management strategy first depends greatly on detailed knowledge of the shellfish production area itself, including any data on previous incidents of noted contamination of shellfish or problems caused to human health from shellfish from the area at any time. The early management of risk also depends on identifying the Critical Control Points, such as drainage outlets and storm overflow pipes from towns and villages, individual septic tanks, potential agricultural discharges upstream. It is also necessary to ensure an early warning system as regards the state of health of the human population locally is known and that health professionals caring for the local population play a part in reporting potential risks.

Real Time Management can only be effective with the co-operation and active participation of producer representatives and the Competent Authorities in public health, environmental monitoring and water quality management working with accurate data on real time issues such as weather, the operation of local treatment plants, the local population health and any individual incidents of pollution. This group of people will take this information into account and combine it with the level of knowledge available on control of HACCP within the industry, the Microbiological Vulnerability Management Options taken from the GIS exercise which will all be underpinned by an agreed formal decision making protocol. The decisions of this group feed into the Shellfish Quality Management Strategy and producers act on the advice as regards harvesting control, depuration and recall of product where necessary.

In summary, the three main tasks to be undertaken in order to provide a much improved and better focused management strategy for shellfish quality are:

1. To collect standard data on each watershed to identify the potential sources and distribution processes of micro-organism inputs which result in shellfish contamination and input into a GIS (Microbiological sources and Processes)
2. Use the same tools to create a multi-criteria assessment method to allow comparison and classification of contamination conditions with respect to variables in each of the main contributing factors such as weather, local population health, etc. (Microbiological Vulnerability)
3. Improve data collection and distribution among a team of responsible actors within the production/health/environment sectors locally. Put in place management options and decision making plans according to real time conditions and microbiological vulnerability. (Surveillance Mollusques – SUMO)

The final objective should now be reached i.e. the firm establishment within the minds of consumers, Government and producers that:

Consumer Protection = Shellfish Quality = Water Quality

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