7th Irish Shellfish Safety Workshop

Marine Institute, Galway
30th November 2006

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

Contents

INTRODUCTION AND OBJECTIVES OF THE 7TH IRISH SHELLFISH SAFETY WORKSHOP

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On behalf of the Marine Institute and our co-sponsors, BIM, the Food Safety Authority of Ireland (FSAI) and the Irish Shellfish Association, I would like to welcome all the participants to the new Marine Institute facilities in Oranmore. This is our 7th annual Shellfish Safety workshop. We are glad to welcome colleagues from the regulatory agencies, universities, research laboratories and industry members, as well as visitors from Scotland, England and France.

Objectives

Our specific objectives for the 2006 Workshop are to:

- Present the Irish shellfish monitoring system and the results gathered during 2006
- Assess key developments on the biotoxin side since our last Workshop in December 2005
- Summarise current Irish research work in Harmful Algal Blooms (HAB's), gene probes, isolation and toxicology of azaspiracids
- Strengthen our focus on shellfish microbiology, including viruses, water quality and risk management
- Take stock of events surrounding the oyster closures in France in 2006
- Provide a forum for debate and feedback.

Key questions for research and debate

Some of the key questions that have been investigated in Irish research that will be debated at this year's workshop include:

- What are the seasonal patterns and toxicity trends in mussels & oysters?
- Can we predict the start of PSP toxicity in Cork Harbour?
- How can we use gene probes for rapid screening of phytoplankton?
- How toxic are azaspiracids?
- Can we develop early warning systems for and manage the risks of, human viruses in shellfish?

Irish Shellfish Safety Programme, a snapshot of 2006

- The Shellfish Safety programme has evolved as a partnership between the MI, FSAI, DCMNR, BIM and the shellfish industry
- Within this, the role of MI is to manage an integrated programme of monitoring with 7,700 tests *per annum*
- All results for phytoplankton, bioassay and chemistry are posted on the MI and FSAI Websites
- There is a rapid turnaround of MI results (over 90% within 3 days).
- MI has a well developed quality system with laboratory accreditation in the areas of phytoplankton, bioassay and chemical testing
- Toxicity has been high in 2006, **(29%** of mussel samples tested by MI in 2006 were positive)
- The Molluscan Shellfish Safety Committee (MSSC) had 4 meetings, chaired by the FSAI; sub groups met to review microbiology issues and the Management Cell
- The Management Cell was used on 87 occasions to date in 2006**,** in order to enable rapid decision making, according to protocols which were drafted by MSSC members.

- Three major research projects have come to completion (BOHAB, ASTOX and REDRISK).
- Two research projects are ongoing – BIOTOX and PHYTOTEST

Context: Harmful algal events in Ireland and elsewhere have led to the setting up of World Class monitoring regimes.

- SPAIN – a major DSP/PSP outbreak in Galician mussels in 1996 led to a major investment in the phytoplankton and toxin programme, led by the *Centro para Control da Calidade do Medio Marino* in Pontevedra
- CANADA – the ASP outbreak in Price Edward Island mussels in 1987 led to a major investment in the phycotoxin research programme, led by the National Research Council laboratory in Halifax, Nova Scotia
- NEW ZEALAND – the NSP outbreak in 1994 led to a restructured, industry led toxin and plankton monitoring programme, with leadership from the Food safety Authority and collaboration with Cawthorn Institute, Nelson and other laboratories
- IRELAND – the discovery of AZP and long closures in 1999/2000 led to a fundamental review by the Molluscan Shellfish Safety Committee, with input from a number of agencies. After 5 years of investment and partnership, we believe that Ireland now has one of the best shellfish monitoring programmes in the northern hemisphere

Ensuring that Shellfish Safety remains a EU priority

According to the Surveillance Unit of DG SANCO (Health & Consumer Protection) in the European Commission, the EU food safety system faces an ongoing challenge with molluscan shellfish.

The data for Rapid Alerts and Information Events (Border recalls etc) is as follows:

Rapid Alerts and Information relating to Biotoxins in Molluscs

Year	2000	2001	2002	2003	2004	2005	2006	Total
No of Cases	14	14	14	5	9	11	21	88 cases

Countries of Origin:
Spain (20); Greece (15); UK (10); Belgium (9); France (8):
Italy (5); Tunisia (3), Ireland 2 (both events occurred in the year 2000)

Rapid Alerts and Information relating to Microbiological Contamination in Molluscs

Year	2000	2001	2002	2003	2004	2005	2006	Total
No of Cases	15	22	22	21	41	451	19	188 cases

Countries of Origin (within the EU):
Greece (20); Spain (18); France (9); Italy (4), Ireland 3 (All events occurred in the year 2002)

Countries of Origin (outside the EU):
Vietnam (20); India (17); Chile (15); Thailand (14); Mexico (80) Malaysia (8);
Tunisia (3).

Challenges for the Irish Molluscan Shellfish Programme from 2007

Our challenges for the coming year include:

- The Sea Fisheries Protection Authority (which comes into being on 1st January 2007). The Marine Institute, FSAI and industry need to implement the Good Practice Guide, which is based on the EU Hygiene Directive.

- DCMNR and other Government Departments will develop the programme of designations and monitoring under the Shellfish Waters Directive, 79/923.
- MI will be working with FSAI and industry to build up our shared expertise in the Risk Management of Microbiological hazards, as we have done in biotoxins.
- We must ensure that all shellfish species, production areas and exports are adequately tested; e.g. given the growth in bottom mussel exports, the level of testing needs to increase from the level of 178 samples in 2006.
- MI will be working to define new research priorities under the ***Sea Change*** programme for the period 2007 to 2013.

Future Evolution of the Irish Molluscan Shellfish Programme

We envisage that some of the main elements in the future evolution of the Irish shellfish safety regime will include:

- Continued development and international validation of the LC-MS method for multi toxin analysis; we support the policy as set out in the EC Regulation 2074/2005 which stated that "***provision should be made for the replacement of biological tests as soon as possible***"
- Ireland will work with partners in the EU Network of Reference Laboratories, with the European Food Safety Authority (EFSA) and with the European Commission (DG SANCO) towards the updating of legislation to allow for alternative shellfish test methods
- Reduced use of the mouse bioassay in low risk species and low risk periods
- Investment in research leading to the increased use of alternative test methods and risk management
- Development of HAB models and early warning systems for industry and coastal communities

Conclusion

The Irish shellfish industry has continued to show its resilience and potential in the past five years. The Price Waterhouse review of the Rope Mussel sector has made a set of valuable recommendations. The Marine Institute, BIM and other agencies will support the ongoing development of the shellfish sector to develop new markets, with no product recalls.

The ethos of the Marine Environment & Food Safety team is: **Service, Quality and Innovation.**

I would like to convey our thanks to our MSSC colleagues, the samplers, the laboratory staff and all the members of the shellfish safety team for your efforts in 2006.

SHELLFISH MICROBIOLOGY -IMPLEMENTATION OF THE HYGIENE REGULATIONS AND GOOD PRACTICE GUIDE

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Introduction

On 1st January 2006 a new package of Food Regulations were introduced throughout the EU. These regulations are commonly referred to as the "hygiene package". The package includes five pieces of regulation which provide a framework for food safety controls through all stages of food production. With regard to shellfish production, the new regulation replaces EU Directive 91/492/EEC laying down the health conditions for the production and the placing on the market of live bivalve molluscs, which was in place for nearly 15 years. In terms of specific microbiological controls of shellfisheries, there are few differences from the Directive. However, a significant difference is a requirement that where the Competent Authority decides in principle to classify a production or relay area, it must undertake a sanitary survey and that results for the sanitary survey must be used when establishing an ongoing sampling programme. Legally this requirement applies to new shellfish production areas only.

In addition to the introduction of new food hygiene regulations in 2006, a good practice guide for microbiological monitoring of shellfish harvesting areas in Europe was published in 2006. The guide was produced by a European working group commissioned by DG Sanco and with the European Community Reference Laboratory taking the scientific lead. The good practice guide promotes the use of best practice for microbiological monitoring of shellfisheries during the classification programme. In order to consider the route forward for implementing the good practice guide in Ireland a Working Group chaired by the FSAI has been formed. The working group consists of representatives from the MI, DCMNR, the Irish Shellfish Association (ISA), Sea Fisheries Protection Authority (SFPA) and BIM. The aim of the working group is to develop a Code of Practice for microbiological monitoring of shellfisheries for Ireland based on best practice.

This paper highlights the work of the group to date and the steps being taken towards producing the Code of Practice. It also details some of the most significant specific procedures which are proposed in the current draft of the Code of Practice.

Code of Practice for Microbiological Monitoring of Bivalve Mollusc Production Areas

Implementation and consultation

Ultimately the responsibility for classification of shellfish harvesting areas within Ireland lies with the DCMNR and up to this year this has meant the Sea Food Control Division in particular. However, this changes from the 1st of January 2007 with the establishment of the SFPA. It is the working group's understanding that the ultimate responsibility will continue to lie with DCMNR, but implementation will be carried out by the SFPA. Therefore, once an initial draft of the Code of Practice is developed it will be presented to both the DCMNR and the SFPA for formal agreement and sign off. At this stage the Code of Practice will be considered a draft and will enter into a round of consultation with stakeholders. It is planned to hold regional meetings to present the proposed Code of Practice to local stakeholders and gather feedback. A further draft incorporating feedback as appropriate will be produced with an ultimate aim of having a finalised Code of Practice ready for use in the next classification exercise planned for October. The proposed timescale for implementation is as follows;

- Completion of the final draft -March 2007
- Presentation and sign of draft by SFPA and DCMNR -May 2007
- Local consultation with industry and other relevant stakeholders - Summer 2007

- Final Code of Practice published – September 2007
- Next classification exercise carried out using new procedures –October 2007

Specific Proposed Procedures

Although much of the proposed content of the Code of Practice simply involves formalising existing procedures, there are number proposals which are a significant departure from the current arrangements for undertaking the classification exercise in Ireland. The most significant of these are highlighted below.

One of the most important of the changes is the procedures for data interpretation. It is proposed that classifications will be undertaken annually following a review of the last three years worth of data for the site, using a minimum of 24 results over the three-year review period. Currently, classifications are undertaken every six months and are primarily based on reviewing 6 months data from the same period in the previous year. However, variations in the concentration of faecal indicators in the polluting sources, together with variability in the way environmental factors, affect the microbiological quality of shellfisheries. This suggests a thorough assessment of the status of areas requires a relatively large number of samples spread over time. The new arrangements are believed to provide an improved assessment of the underlying background contamination levels in a harvesting area and a more accurate reflection of the classification status of the area. Seasonal classifications are still catered for, but only in areas where genuine differences in *E. coli* levels between seasons are demonstrated over the full data set.

A further change is the inclusion of proposals to take action when high *E. coli* results are observed during the routine monitoring programme. Two levels of action are proposed depending on the magnitude of the *E. coli* levels involved (Table 1.). Firstly where the *E. coli* levels exceed the alert state immediate investigations should be undertaken by the competent authorities to ascertain whether there are potential risks associated with the "event" and whether intervention management actions may be appropriate. It is proposed that a similar Management Cell approach could be taken as used for biotoxins. *E. coli* results above the classification limit for the area in question but below the alert state limit will also warrant further investigation to establish whether the result can be attributed to one off exceptional events and the result can thus be discarded during the classification exercise. It is proposed that this investigation should primarily be led by local industry with support as appropriate by state agencies. Protocols for both circumstances are developed within the Code of Practice.

Criteria for discarding results from the classification process

- Failure to comply with sampling or laboratory protocols.
- Failure of the sewage treatment system that has been rectified and where the authority responsible for controlling pollution identifies that such a failure is not expected to recur.
- Extreme rainfall event with a return period of 5 years or greater.
- Any other clearly identified one-off pollution event that is unlikely to recur.

Table 1: Proposed action levels for two tiered response for high E. coli levels

	E. coli Level MPN $100g^{-1}$	
Classification	Alert State	Above class. limit and below alert state
Cat. A	>1,000	>230 <1,000
Cat. B	>18,000	>4,600 <18,000
Cat. C	>46,000	>46,000

Within Hygiene Regulation EC No 854/2004, it is a requirement to undertake a sanitary survey for new shellfish harvesting areas designated after January 1st 2006. However, the European Good Practice Guide recognised that only undertaking sanitary surveys in new areas would create a two tier system and recognises that this is undesirable. It recommended that by January 1st 2009 Member States should introduce a programme of work to complete sanitary surveys for all harvesting areas classified at 21st December 2005 by 1st January 2011 at the latest. This theme will be picked up within the Code of Practice for Ireland and it is proposed that a programme of work should be adopted to undertake sanitary surveys in all shellfish harvesting areas in Ireland. Detailed procedures for conducting sanitary surveys are developed in the Code of Practice.

A sanitary survey may involve four stages

- A desk based study to identify pollution sources
- A shoreline survey to confirm initial findings of the desk based study
- A bacteriological survey
- Data assessment

However, it is clear that the introduction of sanitary surveys has resource implications and it is not possible to say at this stage, whether it will be possible to accept and implement this requirement.

A further important distinction proposed in the Code of Practice is that where sanitary surveys are conducted use of the information gathered should not just be restricted to developing the sampling programme, as stated in the legislation, but will also be used to develop risk management procedures and decisions in the areas concerned.

The Code of Practice recognises that work proposed under the classification monitoring programme has strong synergies with work required under the Shellfish Waters Directive. Both programmes of work represent significant and important monitoring programmes with clear overlaps and a number of key State organisations are involved in both. It recognises that in order to maximise the benefit from both State monitoring programmes it would be desirable to develop a shared data resource between the principal State bodies involved in shellfish monitoring in Ireland. In particular, a shared resource facilitating exchange of information between the monitoring programmes for the Shellfish Waters Directive and for classification purposes should be developed. This will principally require development and subsequent management by BIM, SFPA, MI and the FSAI (Figure 1.)

Summary

- On 1st January 2006 EC Directive 91/492 which had been the cornerstone of specific controls relating to placing bivalve shellfish on the market was replace by new hygiene regulations.
- In 2006 a good practice guide for microbiological monitoring of shellfish harvesting areas in Europe was published. The guide was produced by a European working group and outlines best practice for classification monitoring programmes.
- A Code of Practice on Microbiological Monitoring of Bivalve Mollusc Production Areas is being developed in Ireland through a working group chaired by the FSAI. The working group consists of representatives from the MI, ISA, BIM, DCMNR and SFPA.
- Following a period of consultation with local and national stakeholders the Code of Practice will be published in September and used as the basis for determining the next shellfish production area classifications in October.
- Currently the initial draft of the Code of Practice proposes significant changes to the way in which the current classification exercise is conducted.

- Currently the initial draft of the Code of Practice proposes that sanitary surveys should be undertaken in Irish Shellfish harvesting areas. This has significant resource implications.
- The working group recognises significant synergies between the work undertaken as part of the shellfish waters Directive and shellfish classifications and the Code of Practice makes proposals for information sharing between the two programmes
- Contributors to the working group are;
    - David Lyons (FSAI)
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    - Fergal Guilfoyle (Marine Institute)
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    - Dominick Gallagher (DCMNR/SFPA)
    - Joe McElwee (ISA)
    - Richie Flynn (ISA)
    - Terence O'Carrol (BIM)

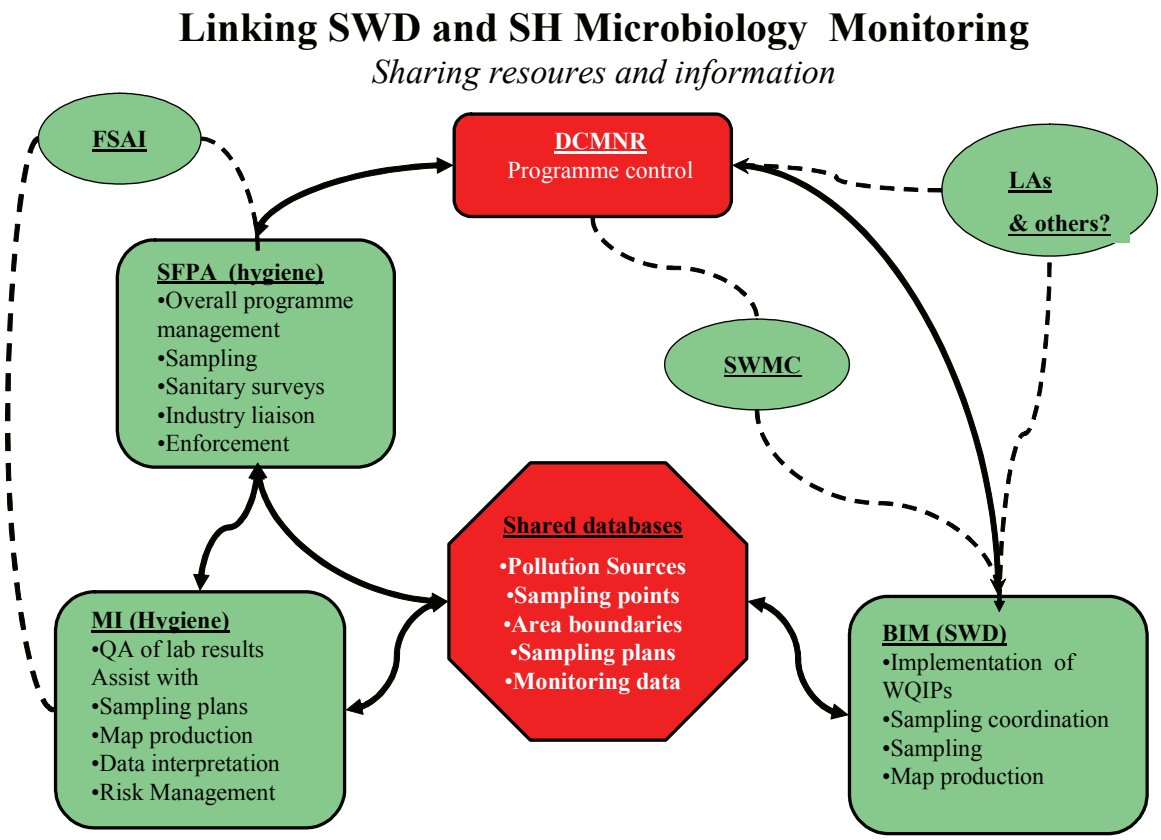

Figure 1: Proposals for sharing information between the monitoring programmes for the Shellfish Waters Directive and the Classification of Shellfish Productions Areas.
(LA = Local Authorities, SWMC = Shellfish Waters Management Committee, SWD = Shellfish Waters Directive)

IMPLEMENTATION OF THE SANITARY SURVEY REQUIREMENTS OF REGULATION 854/2004 IN SCOTLAND

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Introduction

Regulation (EC) No 854/2004 states that if the Competent Authority decides, in principle, to classify a production or relaying area, it must:

(a) make an inventory of the sources of pollution of human or animal origin likely to be a source of contamination for the production area
(b) examine the quantities of organic pollutants which are released during the different periods of the year, according to the seasonal variations of both human and animal populations in the catchment area, rainfall readings, waste-water treatment, etc.
(c) determine the characteristics of the circulation of pollutants by virtue of current patterns, bathymetry and the tidal cycle in the production area
(d) establish a sampling programme of bivalve molluscs in the production area which is based on the examination of established data and with a number of samples, a geographical distribution of the sampling points and a sampling frequency which must ensure that the results of the analysis are as representative as possible for the area considered.

(Items (a) to (c) constitute what is known as a sanitary survey.)

Sanitary surveys are a basic requirement in the US National Shellfish Sanitation Programme (NSSP) and thus have been undertaken in the US and countries exporting there, e.g. Canada, New Zealand, Mexico, Korea. The primary outcome of the sanitary surveys undertaken under the NSSP differs from that under the EU legislation and therefore the methodology is not directly applicable. However, much of the experience is relevant and therefore the EU Community Reference Laboratory (CRL) for monitoring bacteriological and viral contamination of bivalve molluscs has liaised closely with the relevant experts in the US Food and Drug Administration. This liaison is intended to continue and there are plans to hold a joint US:EU sanitary survey workshop during 2007.

The CRL has published a Good Practice Guide on the Microbiological Monitoring of Bivalve Mollusc Harvesting Areas. This contains recommendations as to how the requirements of the legislation may be met.

Classification of Shellfish Production Areas in Scotland

Scotland currently has 189 classified shellfish production areas, covering 246 individual harvesting sites. Six main species of bivalve mollusc are classified. The majority of the production areas are found in the Shetland Isles, Argyll and Bute, and across the Highland areas of Lochaber, Skye and Lochalsh, Ross and Cromarty, Sutherland and Inverness. Further classified production areas are found in Orkney, Western Isles. North Ayrshire, Edinburgh, Moray, Dumfries and Galloway and Fife. The latter areas tend to be home to wild shellfisheries rather than aquaculture sites and hold species such as clam species and common cockles. Within aquaculture areas common mussel is the main species with Pacific oyster also being a major species particularly in the Argyll and Bute area of Scotland. Figure 1 shows the location of main shellfish production areas.

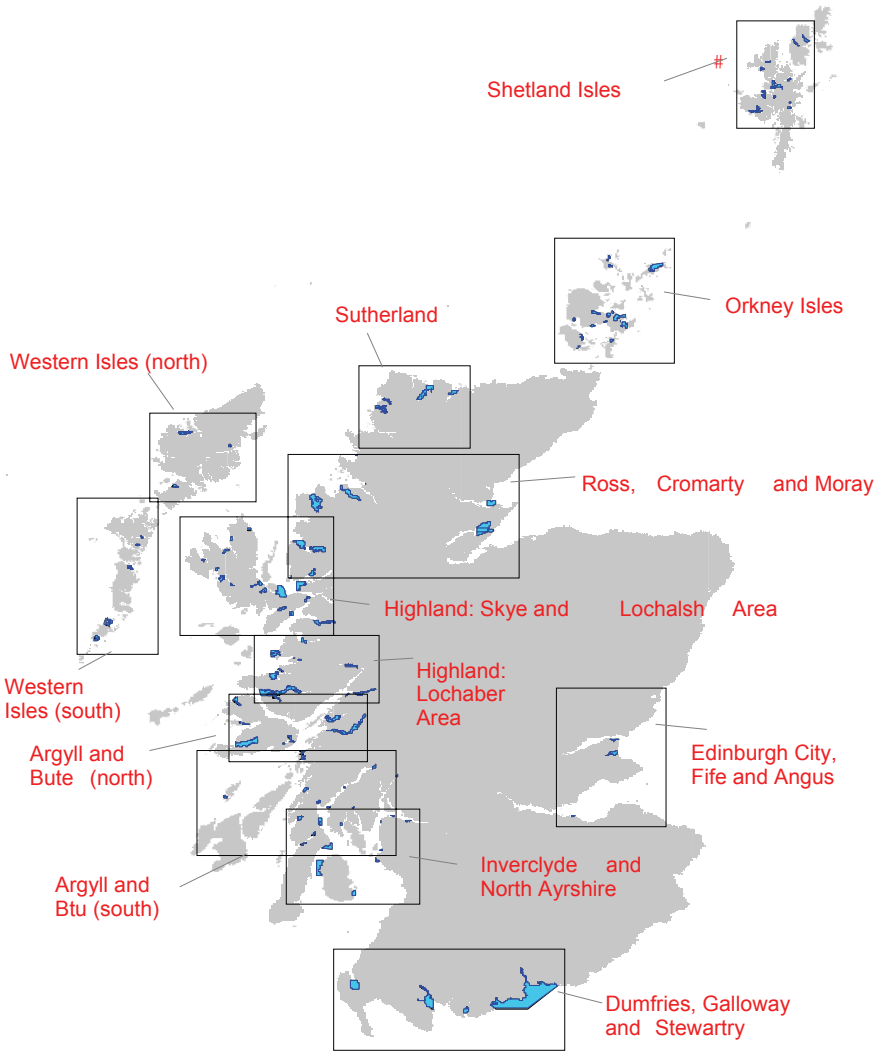

Figure 1. Location of the principal shellfish production areas in Scotland

In accordance with Annex II of the EU Hygiene Regulation 854/2004, the Food Standards Agency Scotland (FSAS) is required to establish the location and fix the boundaries of shellfish harvesting areas. The process involved in area classification is stringent and includes regular sampling of shellfish from representative monitoring points by Enforcement Officers from each Local Authority area, with the assistance of shellfish harvesters. Twelve local food authorities are involved in the programme. The regulations stipulate that the Competent Authority must monitor the levels of *E. coli* within the harvesting area and that according to the sample results, must classify the area as being one of three categories; A, B or C. An A classification allows for the product to be placed directly on the market, whereas a B or C classification requires the product to go through a process of depuration (purification), approved heat treatment or relaying before it can be placed on the market.

As indicated above, the regulations also require the Competent Authority to undertake sanitary surveys for all new areas and this requirement is detailed further below. It is additionally the intention to undertake such surveys on all of the existing classified production areas over a reasonable period of time. Currently FSAS carries out its annual review of classifications every December and awards provisional, seasonal or full classifications on the basis of three years historical *E. coli* monitoring data. A minimum of 6 samples is required, taken in separate months, between January and December to maintain

an area classification. However, if the shellfish harvesting area is a new site and is yet to be classified, or has a history of fluctuating results during specific months, then the minimum sampling frequency will be recommended to be more frequent. A separate fast track classification system also exists, which allows for the immediate harvesting of previously unclassified or a declassified area for a single season in that year. A provisional B classification is awarded for this harvesting period after a general desktop survey has taken place by FSAS Officers.

Definitions

The sanitary survey constitutes an inventory of pollution and how this impacts on the shellfishery. It consists of a number of elements. A significant amount of information can be gathered from existing sources in the form of a desk study. This is then supplemented by a shoreline survey and where necessary, a bacteriological survey. As part of the development of sanitary surveys in Scotland, it was identified that it was important that all interested parties understood key elements of the terminology. To this end, a number of definitions were established. These are as follows:

Sanitary survey: Identification of potentially contaminating sources in the vicinity of the production area and the way in which these may affect the microbiological status of the shellfishery

Desk top survey: Collation of all relevant existing information for conducting the survey, e.g. sewage discharge locations, loadings and treatment levels, land and farm use, population, rainfall, bathymetry and hydrodynamics.

Shoreline survey: A physical site audit verifying desktop information and identifying any additional sources of contamination.

Bacteriological survey: The collection of a small number of samples of flesh and/or water at several locations to clarify the outcome of the other elements of the sanitary survey, where necessary. Samples are to be tested for *E. coli* and also Norovirus in some circumstances.

Desk study components – recommendations from the EU Good Practice Guide

The EU Good Practice Guide (GPG; CRL (2006)) recommends that the desk study review information in the following areas:

- Fisheries
- Point sources (continuous, CSO, SO, EO)
- Land use
- Farm animals
- Wildlife
- Ships and boats
- Meteorology
- Bathymetry & hydrodynamics

For fisheries, the recommended information to be collated includes, the location and extent, the species involved and whether these are wild or aquaculture stocks, the seasonality of harvest and whether there are any closed seasons. This information is critical to the interpretation of the other data on potentially polluting sources. With regard to the latter, the principal information of concern relates to the location, size and treatment of continuous sewage discharges and the location, maximum flow rate and spill frequency of intermittent sewage discharges. Supplementary information on any tidal phasing, microbial or sanitary content and seasonal variation in content or flow is also relevant. Other potentially important sources of microbiological contamination are farm and wild animals; manure, sludge and

slurry storage and application sites; discharges from boats and ships. Whether, and how, potential sources of contamination may impact on the shellfishery may depend on rain and wind and the currents prevailing in an area under different conditions.

Outcome of the Sanitary Survey

A formal report will be produced for each area, containing the outcome of the various elements together with an overall assessment of the effects on the shellfishery. The survey information will be reviewed on a regular basis and the local food authorities will contribute information to this review. The final product will be the sampling plan(s) for the area. The information contained in these plans will include:

- Production area
- Site Name and ID
- Geographical location (grid ref)
- Depth of sampling (if relevant)
- Frequency of sampling
- Responsible authority
- Authorised sampler(s)

Progress towards implementation

A Scottish sanitary survey working group was established in April 2005 to advise and contribute to the development of a sanitary survey programme. Four meetings have been undertaken so far. The working group undertook two pilot sanitary surveys, one of an aquaculture area and one of a wild harvest area. A risk matrix was developed for the determination of the monitoring required for wild harvest areas, taking into account the known major sources of contamination and estimates of microbiological risk, including classification status, results of other microbiological testing (including Norovirus) and any occurrence of shellfish-associated illness.

A risk matrix which includes the population in the vicinity of a shellfishery, the classification status over a 3-year period, the occurrence of unusual *E. coli* results and the occurrence of any shellfish-associated illness is being used to determine the priority of existing classified areas for sanitary surveys. A list of areas to be covered in first year will be drawn up accordingly. Data acquisition for the desktop surveys is being progressed. Local food authority sampling officers will assist in the shoreline surveys and these are to be recruited and provided with baseline training by March 2007.

Further data needs

The following additional items of data are not currently available in Scotland and are necessary to implement the recommendations of the EU Good Practice Guide and to enable proper assessment of the potential impact of specific pollution sources on shellfisheries:

- Actual microbiological content of sewage discharges, including seasonal and short-term variations
- Spill volume event recording for storm overflows
- More detailed farm census data
- Seasonal variation in manure, slurry and sludge applications (and microbiological content)
- Microbiological monitoring of freshwater inputs (rivers, streams)

Research needs

Simple water quality models have been developed for shellfish production areas where the movement of water is dominated by tidal effects (Tattersall, *et al*., 2003). There is a need to develop analogous models for areas with little tidal exchange, such as sea lochs with sills, where water movement is dominated by wind and density effects.

There is also a need to evaluate existing tools, such as catchment level models, to assist in the assessment of the impact of different sources of contamination, particularly in relation to low class B/class and A areas.

Finally, given the general recognition of the shortcomings of the use of faecal indicator bacteria to fully assess the risk of shellfish-associated microbial illness, it is necessary to look to the possibility of other monitoring requirements being introduced in the future and to consider how sanitary surveys would be undertaken with respect to pathogens, particularly viruses. Specifically, this will require an understanding of the occurrence and variation of the pathogens in potential sources of contamination, the variation of pathogens in harvesting areas (in terms of both space and time). Inevitably, this would result in at least some modifications to the ways that sanitary surveys are conducted and the results assessed.

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HUMAN VIRUSES IN SHELLFISH: VIRUS METHOD STANDARDISATION AND VIRUS SURVEY 2006/07

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Real-time PCR methods for the detection of norovirus in shellfish

In 2005 MI, as the National Reference Laboratory for Shellfish Microbiology, introduced up to date real-time PCR procedures for the detection of norovirus (NoV) in shellfish. Since the introduction of this method MI has applied them to a number of activities and has been actively involved in efforts to produce standard virus methods. To this end, the NRL has participated in a European Committee for Standardisation (CEN) working group for the standardisation of methods for the detection of viruses (norovirus and hepatitis A) in food.

At the 6th Irish Shellfish Safety Workshop an overview of the technical aspects associated with the detection of NoV in shellfish was described (Keaveney *et al.,* 2006). The technology used for the detection of NoV in shellfish involves the use of real-time PCR. This approach has proven to be a sensitive, robust and reproducible method for the detection of NoV in shellfish. Separate assays have been developed for genogroup I and II NoV (Jothikumar *et al.,* 2005). The use of NoV GI or GII specific probes allows for "in built" confirmation of a NoV positive result without the need to clone and sequence the PCR product. The NRL has applied the real-time procedure to research activities such as the EU research project REDRISK (Keaveney *et al*., 2006). This project highlighted the feasibility of applying procedures for NoV on a routine basis to provide comprehensive information about NoV contamination in shellfisheries. Although the technical feasibility of using NoV monitoring has been proven, doubts continue regarding interpretation of the results obtained from such testing. In order to obtain more information on the levels of NoVs commonly seen in shellfish and to help place results in context, a survey of NoV levels in shellfish harvesting areas in Ireland was commenced in 2006. Furthermore, the Irish NRL was, for the first time in 2006, in a position to respond to testing requests from the Food Safety Authority of Ireland (FSAI) and the Department of Communications, Marine and Natural Resources (DCMNR). The NRL's progress in the area of NoV testing in 2006 is reviewed in this paper

Progress in virus detection method standardisation

Provision for virus standard in EU legislation

There is currently no standard in EU legislation regarding virus, NoV and Hepatitis A (HAV), levels in shellfish. At the time of preparation of current regulations it was considered that the EU was not technically ready for the introduction of standards for viruses in shellfish because of lack of standardized methods. However during drafting of the current hygiene regulations it was recognised that virus standards in the area of shellfish hygiene was desirable and should be introduced as soon as technically feasible. Therefore provision was made in (EC) No. 854/2004 (Hygiene 3) in Article 18, Special decisions that stated that implementing measures may be laid down for a number of areas including:

> **"***13. Criteria for the classification of production and relaying areas for live bivalve molluscs in cooperation with the relevant Community Reference Laboratory, including:*
>
> *(b) Virus testing procedures and virological standards"*

The EU Community Reference Laboratory (CRL) for shellfish microbiology has been conducting ring trials for viruses (NoV and HAV) in shellfish for the past 5 years. Although it has been noted that there is a steady increase in the number of National Reference Laboratories (NRL) participating in the ring trials, the large number of different approaches to virus detection has also been highlighted. Therefore, with this regard, a need was also recognised for a consensus within Europe for virus detection in shellfish. Consequently the CEN (European Committee for standardisation) working group (TC 275/WG6/TAG4) was established in 2004 with a remit of developing a standard method for the detection of viruses (NoV and HAV) in food.

The overall aim of the TC 275/WG6/TAG4 (Detection of viruses in food) is to develop a standard horizontal method for the detection NoV and HAV in a range of foods including bottled water, soft fruits, salad vegetables, food surfaces and shellfish, which should eventually become an ISO standard method. Ireland (Marine Institute) joined the group in June 2006 with a special interest in shellfish matrix.

A number of key decisions have been made by the group with regard to the various stages of the detection of viruses in food, and the group is now at the stage of finalising proposed methods for a validation exercise under the CEN mandate. Figure 1 outlines the proposed method for the detection of NoV and HAV in shellfish. At this stage in the process, two elements of the procedure remain to be finalised, these are in relation to NoV; the reverse transcription stage and also the preparation of the NoV control material. These issues should be resolved over the coming months which will then allow for the commencement of the validation exercise of the proposed method for NoV. The proposed method for HAV has been agreed and finalised. This highlights one of the main differences between the two viruses in terms of their detection; the high strain variability within NoV makes it a more difficult detection target, especially for the preparation of the control material and hence one of the reasons why the NoV assay lags behind the HAV assay.

The internal control virus, Mengo virus, is used to measure the overall virus extraction efficiency of the procedure. A known concentration of the virus is spiked into each sample at stage 2 (Figure 1) of the procedure. The Mengo virus is therefore "co-extracted" with NoV/HAV (if present) in the sample, and is subsequently detected in a similar manner at the real-time PCR stage.

The validation exercise is due to commence in the first quarter of 2007, and the Irish NRL will participate on the shellfish matrix only.

Norovirus survey of harvesting areas 2006/07

To date, the application of real-time methods has generally been restricted to problematic harvesting areas that have been involved in outbreaks of illness or are highly polluted. To fully understand the distribution of NoV in shellfish harvesting areas, a need was identified to undertake a survey of a range of more representative sites using the new quantitative real-time PCR procedures. In 2006, the NoV detection procedures were employed to undertake a survey of representative shellfish harvesting sites from around Ireland. The intention of the ongoing survey is to provide additional information on the levels of NoV found in a number of shellfish harvesting areas. A further aim is to determine if the levels observed can be related to the predicted risk of viral contamination in those areas as assessed during the desk based study.

	HAV and NoV method	Controls
1.	**Sample size:** 6 oysters or 10 mussels Hepatopancreas (digestive gland) isolated from shellfish and chopped finely and weighed	
2.	2 g of hepatopancreas tested for each sample.	Sample spiked with **internal process control virus (Mengo virus)** at this point. This is used to measure overall virus extraction efficiency.
3.	**Virus extraction** with Proteinase K enzyme solution	↓
4.	**Viral RNA extraction** with Guanidine thiocyanate (GITC) and silica beads (Boom method)	
5.	**Reverse transcription of RNA to cDNA** Hepatitis A Norovirus GI and GII *Some technical work to be completed to finalise this aspect of the procedure*	
6.	**Real-time PCR detection** **Hepatitis A** *Primers and probes have been decided, as well as HAV control material for quantification* **Norovirus GI and GII** *Primers and probes have been decided, some work to be carried out on NoV GI and GII control materials*	**Mengo virus** *Primers and probes have been agreed*

Figure 1: Summary overview of proposed method for the detection of NoV and HAV in shellfish (oysters and mussels)

The survey is currently in progress and focuses on oysters harvested from 20 selected production areas (Figure 2) throughout Ireland. The survey is restricted to oysters as these are generally recognised to represent the major potential risk to public health. The survey will link to the existing monthly monitoring programme for classification purposes. The survey commenced in August 2006 and will continue for a 12 month period. Initially a desk-based study was undertaken to evaluate the risk of NoV occurrence in the 20 sites. This took into account risk factors such as population numbers, level of sewage outflow, level of sewage treatment, etc. present in the harvest area. The actual virus results obtained will be assessed based on this perceived risk in each area. Table 1 describes briefly the factors taken into account when developing the desk-based evaluation of the risk of NoV contamination and the number of sites ascribed to each of the predetermined risk-ranking.

Initial results (August to November 2006) appear to demonstrate a good correlation between the desk-based evaluation of the sites and the actual NoV levels found in the shellfish tested from the harvesting areas (Table 2). NoV was not detected in any of the samples collected in August. However, with the onset of winter, a steady increase in the frequency of NoV positive samples was observed in the sites that are ranked as a higher risk of NoV

contamination. The increase in virus level in shellfish at this time of year is consistent with an expected increase in levels of NoV found in the general community in line with increased infections. In addition, due to the use of real-time PCR, an increase in NoV levels was also detected with the onset of winter, particularly in the samples collected from the areas ascribed with higher risk of NoV contamination. In these areas, medium to high levels of NoV have been recorded.

Table 1: Desk based evaluation of NoV occurrence in each survey shellfish harvesting sites. Risk was ranked on a scale of 1-6 using the predetermined risk factors shown. Each was site was ascribed to one of the rankings based on evaluation of available data and information

Risk of occurrence	***Ranking***	***Number of sites***	***Risk factors***
HIGH ↓ LOW	1	3	Close proximity to large urban area; minimal or no sewage treatment in area
	2	4	Close proximity to smaller urban area; minimal or no sewage treatment in area
	3	3	Smaller population numbers; level of sewage treatment unknown
	4	3	Low population numbers; different zones in area could be exposed to specific risks
	5	4	Low population numbers; low risk of sewage contamination (septic tanks)
	6	3	Very low population numbers; no immediate risk of sewage contamination

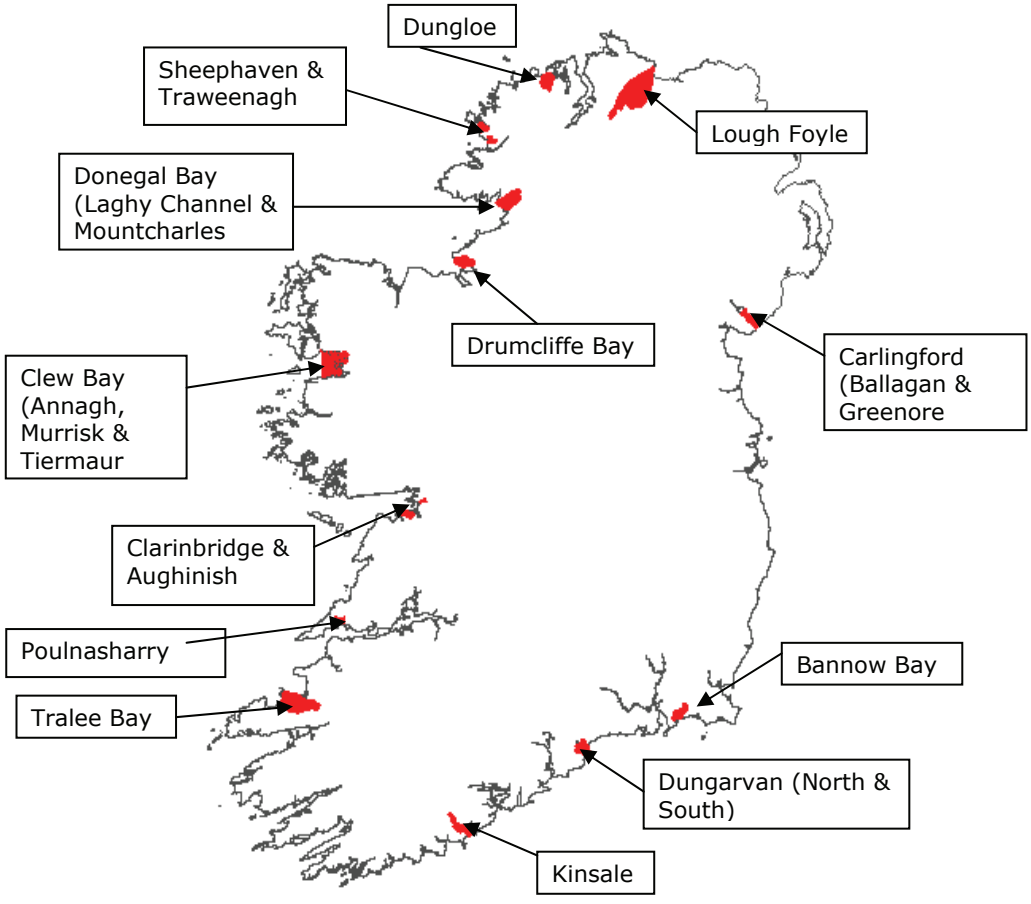

Figure 2. Location of shellfish harvesting sites for the NoV survey 2006/07

Table 2: Preliminary results from NoV survey (August to November 2006). The frequency of NoV positive results and relative levels are shown for each site in each risk category. Relative NoV levels are determined on the basis of Ct results shown in table 3.

Risk of occurrence	*Ranking*	*Number of sites*	*Aug*	*Sep*	*Oct*	*Nov*
HIGH ↓ LOW	1	3	Negative	Negative	2 Positive results (medium levels)	3 Positive results (medium – high levels)
	2	4	Negative	1 Positive result (very low level)	1 Positive result (very low level)	2 Positive results (very low level
	3	3	Negative	1 Positive result (very low level)	Negative	1 Positive result (very low level)
	4	3	Negative	Negative	Negative	Negative
	5	4	Negative	Negative	Negative	Negative
	6	3	Negative	Negative	Negative	Negative

Interpretation of Norovirus real-time PCR results

The interpretation of NoV results based on real-time PCR allow for an indication of the level of NoV present in a shellfish sample. The units of quantification in the real-time PCR are known as cycle threshold (Ct) values. The Ct value is the cycle number at which the fluorescence generated from the amplification of the target sequence crosses the threshold. **The lower the Ct value the more virus is present in the sample.** The quantity of target sequence i.e. virus copies, in the samples could be determined by extrapolation from a prepared standard curve. However, appropriate standards for the absolute quantification of NoV are not readily available and therefore absolute quantification is not currently possible; nonetheless close observation of the Ct values allows relative quantification of the virus level in a sample.

Although absolute quantification is not yet possible, an increase in the Ct value of 3 has been demonstrated to equate approximately to a 10-fold increase in virus levels (Keaveney *et al.,* 2006). The real-time PCR Ct values obtained for NoV levels in shellfish range from 32 (higher level of NoV) to 40 (low level of NoV). These values have been observed in shellfish samples tested in our laboratory and appear to correspond to levels found in other European laboratories undertaking real-time PCR analysis. It has been observed that Ct values returned for many shellfish samples are greater than 37 and in such samples NoV is often detected in only 1 or 2 of the 3 replicates tested in the assay. Such Ct values represent NoV levels at, or just above, the limit of detection of the NoV GI and GII assays, and indicate a low level of NoV contamination in a sample. Based on results observed to date and to assist in interpretation of norovirus results we propose a NoV reporting scheme of low, medium and high based on the corresponding Ct values (Table 3).

Table 3: Interpretation of real-time PCR results for NoV in shellfish samples

Ct values	*Level of NoV*
> 37*	Low
35 - 37	Medium
< 35	High

**Not always 3 positive replicates*

It is important to note that this low, medium and high designation relates only to the relative level of virus present and that how this translates to risk of infection when the shellfish is consumed is unknown. Currently, it remains difficult to interpret the risk of NoV infection from the consumption of shellfish contaminated with NoV. The ability of norovirus to cause infection will not only be influenced by the level present (infectious dose), but also the viability of the virus and the strain present. Sufficient uncertainty remains in relation to the health significance of norovirus real-time PCR results to prevent an assessment of the level of risk of infection associated different levels of NoV in shellfish to be made. It is recognised that this is an area that needs further investigation before the full benefit of the introduction of the real-time PCR procedures can be realised.

Summary points

(i) The Irish NRL has established real-time PCR methods for the detection of NoV GI and GII which are sensitive, robust and reproducible.

(ii) In 2006, the Irish NRL was able to respond for the first time to NoV testing requests from DCMNR and the FSAI with incidences associated with suspected illness outbreaks.

(iii) A concerted effort is being made in Europe to develop a standardised method for the detection of NoV and HAV in food (including shellfish) through the CEN working group. Ireland, through MI, participates on this group and is therefore keeping pace with the latest developments in virus detection methods in Europe.

(iv) The Marine Institute commenced a survey of twenty representative shellfish harvesting sites for NoV contamination (August 2006 – July 2007). A desk-based assessment of each site was undertaken to evaluate the risk of NoV occurrence in each site. The sites were subsequently ranked based on possible risk factors. Virus results obtained from sample analysis will be assessed based on the perceived risk in each area.

(v) The Marine Institute proposes that virus results will be reported based on the Ct value obtained (Table 3). Results will be reported as low, medium and high level of NoV contamination.

(vi) The level of NoV in a shellfish sample does not indicate the risk of NoV infection and as it stands this factor remains difficult to interpret. Further studies are required to establish the link between NoV levels observed in shellfish and the health risks for consumers.

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REDRISK: REDUCTION OF THE VIRUS RISK IN SHELLFISH HARVESTING AREAS.

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Introduction:

Filter feeding bivalve shellfish can accumulate human pathogenic bacteria and viruses if they are grown in sewage-contaminated waters. Current consumer protection legislation relies on classification of harvesting areas based on their sanitary quality, using *E coli* as an indicator of sewage contamination. Advances in viral monitoring have shown that *E coli* can underestimate the extent of the contamination.

The most common cause of gastroenteritis associated with shellfish is norovirus, commonly known as winter vomiting virus. The REDRISK project was undertaken to investigate the main environmental factors that cause viral contamination in shellfish. The REDRISK project is part of an EU research pillar with parallel research being undertaken in the UK, France and Spain. A recently developed technique to quantify norovirus in shellfish, real-time PCR, has been used in the REDRISK project.

Clew Bay, in Co. Mayo was chosen as the study area in Ireland. The Bay is generally considered to have good water quality but, in certain areas is subject to intermittent sewage contamination. The cooperation of local producers and organisations such as the Clew Bay Marine Forum and the Native Oyster Co-op greatly helped the project. The project was divided into a two-phased approach. Phase one involved the identification of contamination sources impacting the bay through a sanitary survey and selection of appropriate sites for further study. Results of the first phase of this study were presented previously at this forum (Keaveney, *et al* 2006) and the characteristics of the sites selected for study and locations within the bay are shown in Table 1 and Figure 1 respectively. The second phase of the project focused on monitoring environmental conditions and microbiological levels in shellfish to identify environmental conditions leading to viral contamination. This paper reports on the finding of this monitoring.

Material and methods

Samples were collected from the sites on 40 occasions between August 2005 and July 2006. On each occasion, 24 Pacific oysters (*Crassostrea gigas*) were collected from each site. Samples were then sent to the laboratory within 30 hours under chilled condition (<15°C) for *E coli*, FRNA bacteriophage and Norovirus analysis. Concurrent measurements of river flow, rainfall, outflow volumes from the wastewater treatment plant, as well as salinity on site, were also recorded. Upon receipt in the laboratory, oysters were cleaned and scrubbed under running potable water. A minimum of 10 oysters were shucked and homogenised for *E. coli* and FRNA bacteriophage analysis. Homogenates were analysed for *E. coli* using a standard ISO procedure (ISO/TS 16649-3). The same homogenate was centrifuged at 2000 x g and supernatant analysed for FRNA bacteriophage using a standard ISO method (ISO method 10705 – 1). Hepatopancreas was dissected from a further 6 oysters and analysed for norovirus using an established real-time PCR assay (Jothikumar *et al,* 2005).

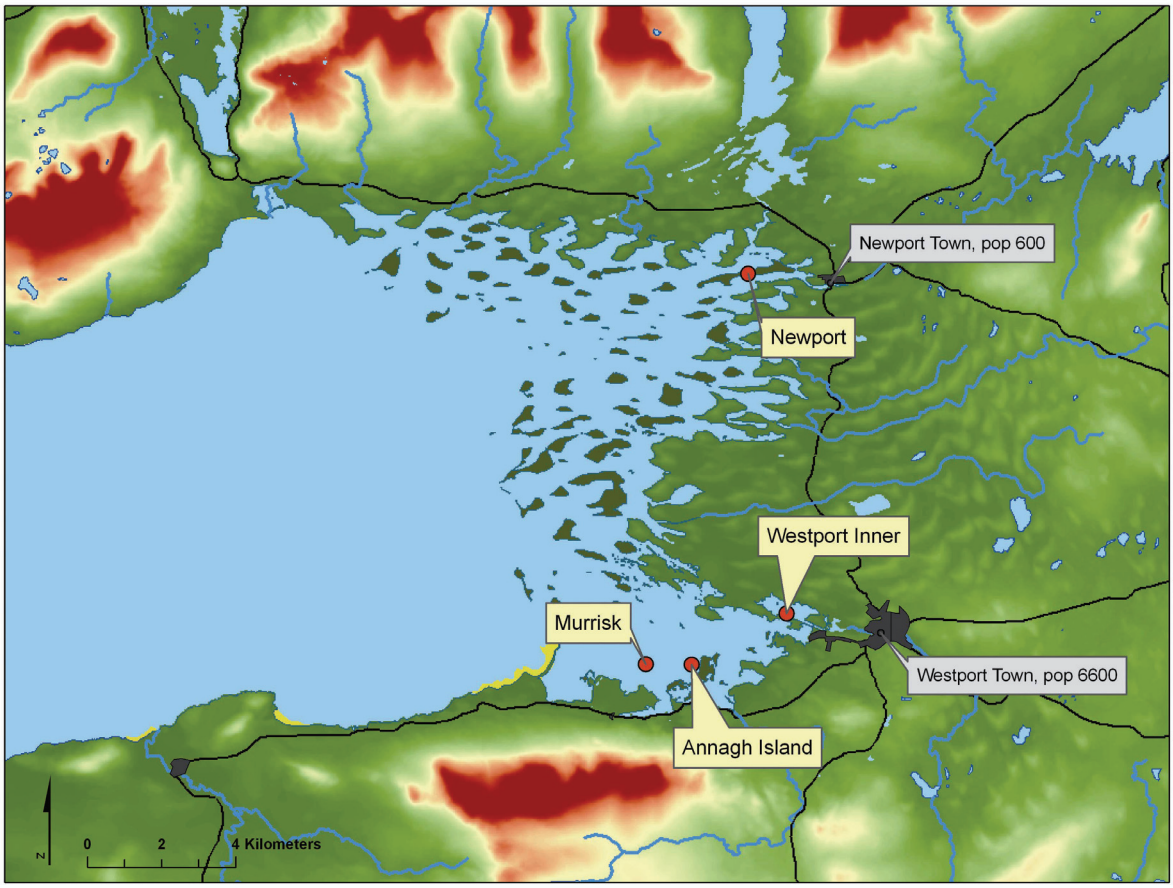

Figure 1: Indicating the 4 experimental sample sites chosen to monitor microbiological contamination. Also indicated are the 2 main towns in the bay and the main rivers.

Table 1: Key characteristics for each sampling site informing potential risk of viral contamination.

Site	**1**	**2**	**3**	**4**
Classification	Category B	On cat. A/B boundary	Category A	Category B
Previous *E. coli* results:	None available	24/26 Cat A results	178/193 Cat A results	7/13 Cat A results
Distance to nearest WWTP* outfall	300m	3500m	4500m	1500m
Local population	6600	Minimal	Low	600
Freshwater input	River A av. flow 0.96 m^3/s)	River B (av. flow 1.5 m^3/s)	Very little freshwater input	River C (av. flow 5.2 m^3/s)
Animal population	1300 sheep and 1200	Some local animals	Some local sheep farming	3200 sheep and 1900
Potential risk of virus contamination	**High**	**Medium**	**Low**	**High**

*WWTP-Waste Water Treatment Plant

Results

The microbiological results are presented for each of the individual sites in figures 2 to 7. A high frequency of norovirus positive results was observed at the Westport site (Figure 3). Although norovirus contamination was present for most of the year (Figure 3) levels showed a clear seasonal trend with peak PCR unit levels observed during the winter period and in particular January and February of 2006. Despite high norovirus levels at the site, *E. coli* levels were consistent with a category B classification throughout the year.

At Annagh Island both frequency of norovirus contamination and levels were significantly lower than at Westport. Norovirus was absent for much of the time (Figure 5). Norovirus occurrence in oysters at the site appeared to be linked to periods of peak WWTP influent flow causing overflows of untreated sewage. The initial occurrence of norovirus at the site coincided with a storm event in October of 2005 resulting in sewage overflowing at the WWTP site. A sewage overflow event in January 2006 also appeared to coincide with a prolonged spell of norovirus contamination at the site (Figure 5). This contamination appeared to last through until the middle of February. During this period, despite no further sewage overflows, levels of norovirus GII appeared to increase in oysters at the site. This may be a genuine increase in virus contamination at the site at this time, caused by further unidentified contamination. Alternatively it may be a feature of the low virus levels observed during the period. Apparent differences in norovirus levels in the shellfish sampled at this time may in fact be an artefact of the accuracy of the relative quantitative aspects of the assay at this level.

Norovirus contamination at the Murrisk site was observed only rarely throughout the study period (Figure 7). When norovirus contamination was observed, it was at very low levels which equate to the limit of detection of the assay. In this site norovirus contamination again appeared to coincide with sewage overflow events related to increased influent levels at the WWTP in October 2005 and January 2006.

Untreated sewage also overflowed from the WWTP on two further occasions during the study period, once in August 2005 and again in May 2006. No norovirus contamination in the Annagh Island and Murrisk sites were observed during these two events. This would coincide with the fact that norovirus associated illness in the population would be lower at this time of year, given the usual seasonal course of infection in community. Therefore, levels of norovirus in sewage effluent during this period would be considerably reduced compared with levels during the winter period.

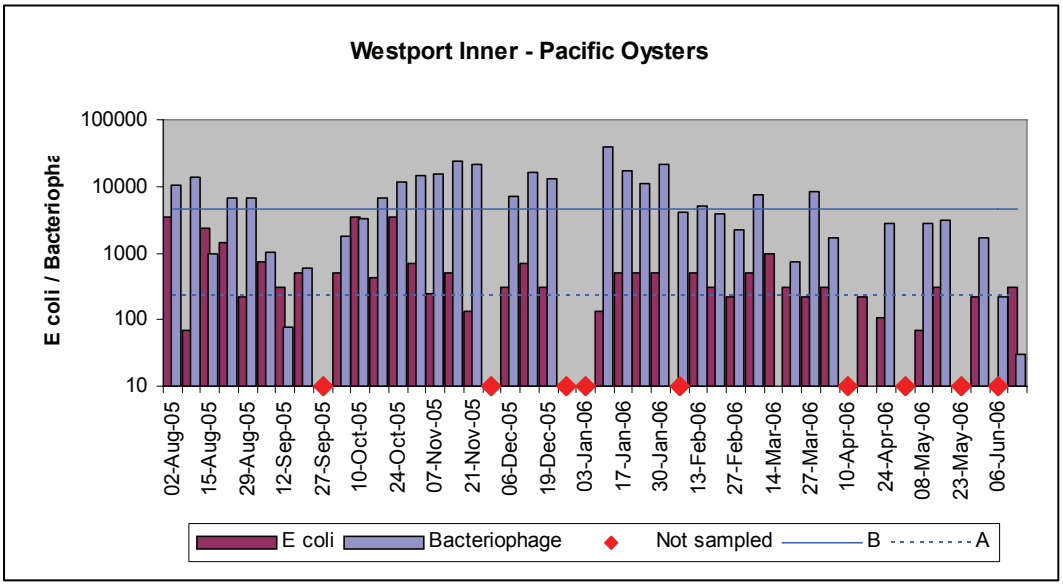

Figure 2. E. coli (MPN $100g^{-1}$) and bacteriophage (pfu $100g^{-1}$) levels in pacific oysters from Westport Inner. The category A and B classification limits are indicated. The weeks not sampled are indicated.

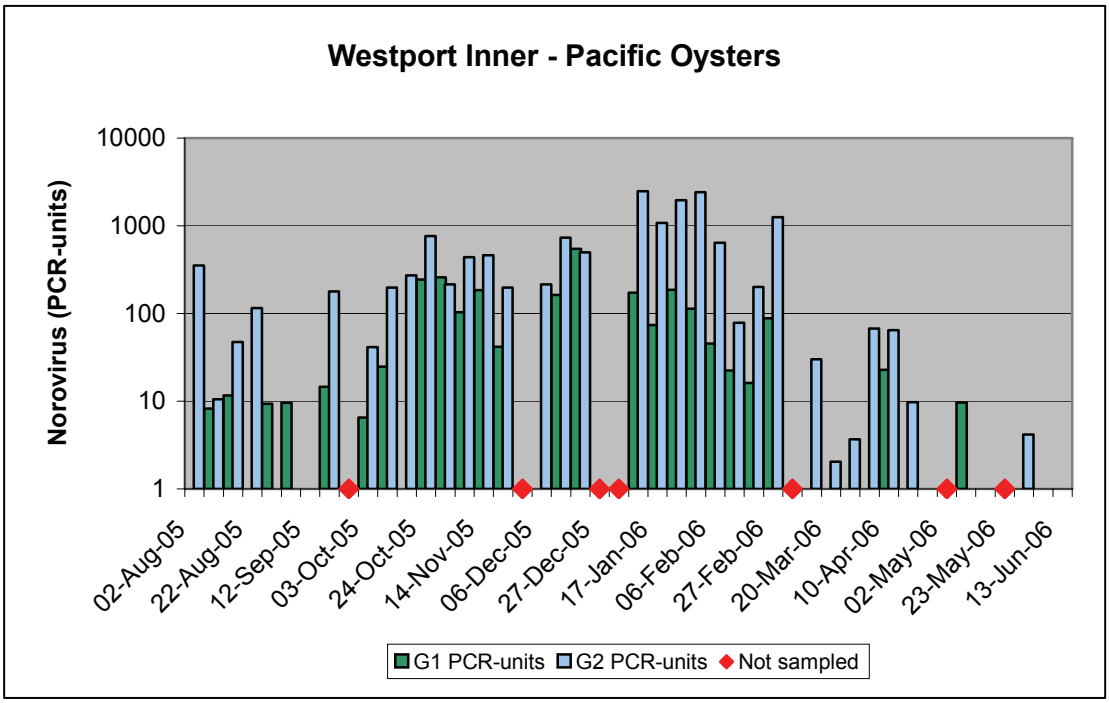

Figure 3. Norovirus GI and GII levels (PCR units) in Pacific oysters at Westport Inner. The weeks not sampled are indicated.

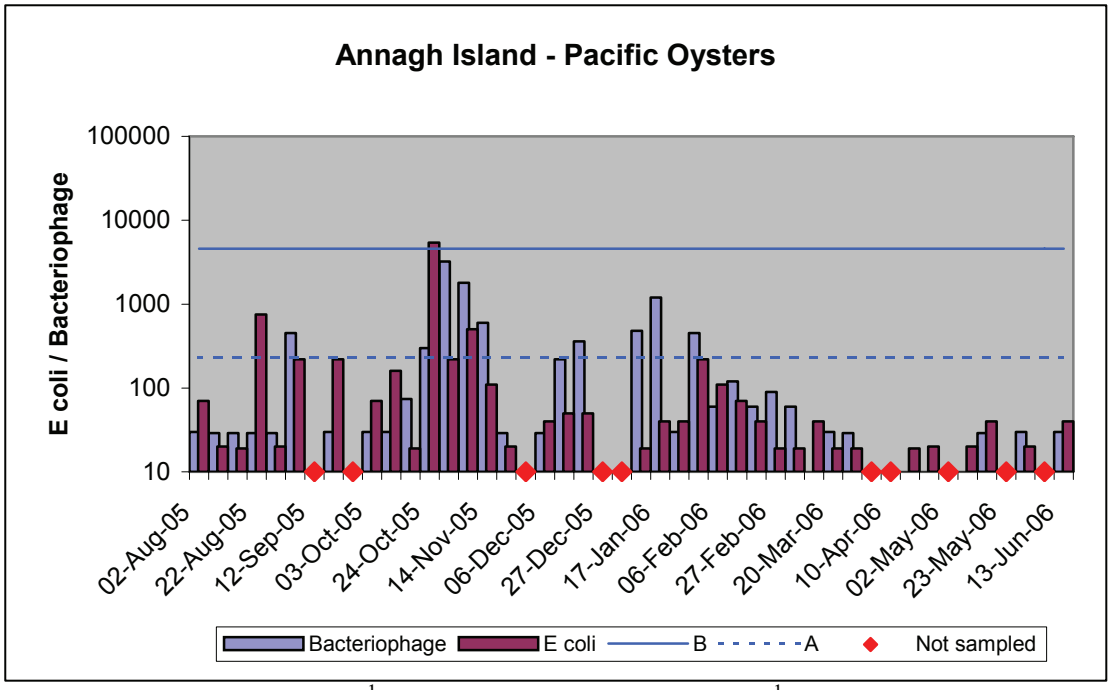

Figure 4. E. coli (MPN $100g^{-1}$) and bacteriophage (pfu $100g^{-1}$) levels in pacific oysters from Annagh Island. The category A and B classification limits are indicated. The weeks not sampled are indicated.

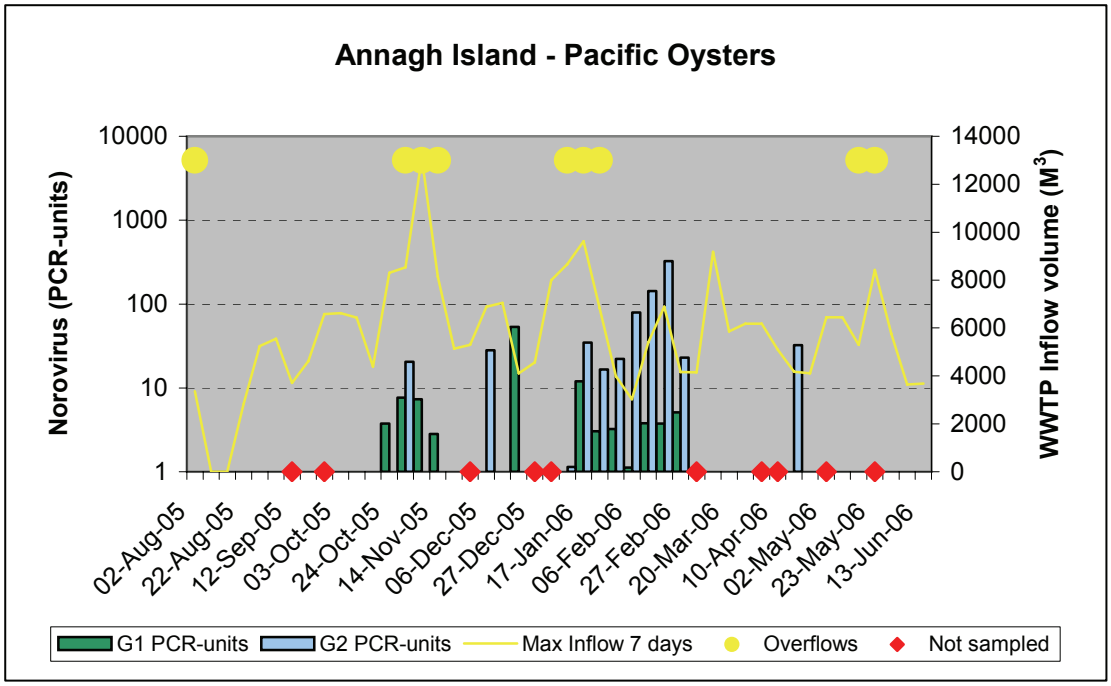

Figure 5. Norovirus GI and GII levels (PCR units) in Pacific oysters from Annagh Island. The weeks not sampled are indicated. The inflow volume to the WWTP and the periods of overflow are indicated.

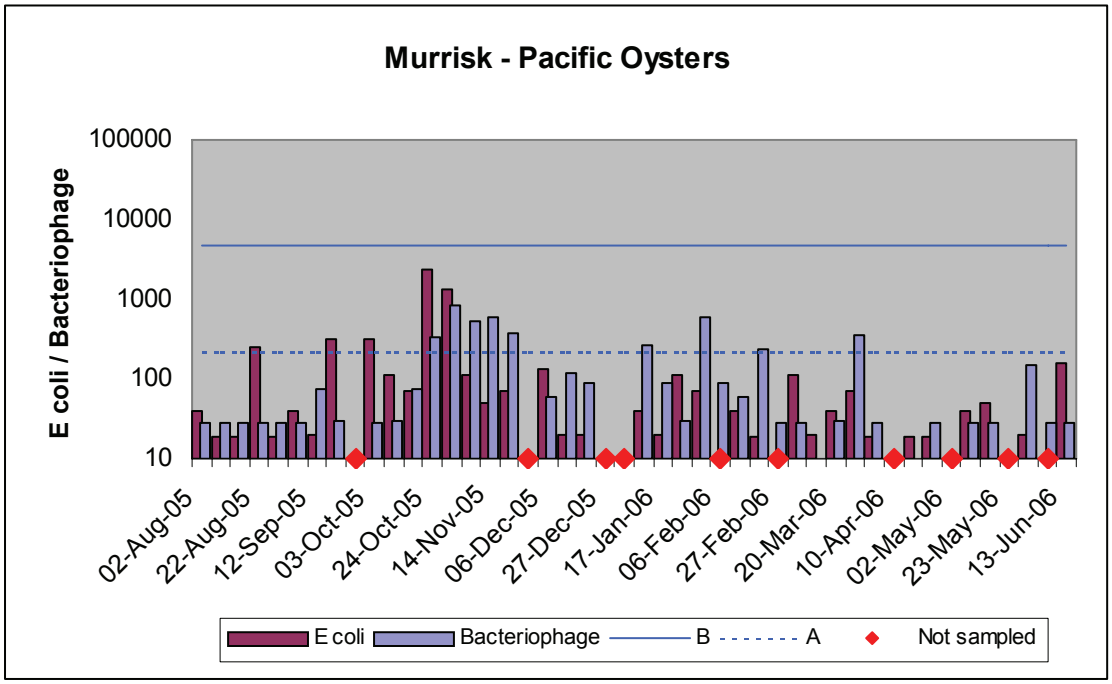

Figure 6. E. coli (MPN $100g^{-1}$) and bacteriophage (pfu $100g^{-1}$) levels in pacific oysters from Murrisk. The category A and B classification limits are indicated. The weeks not sampled are indicated.

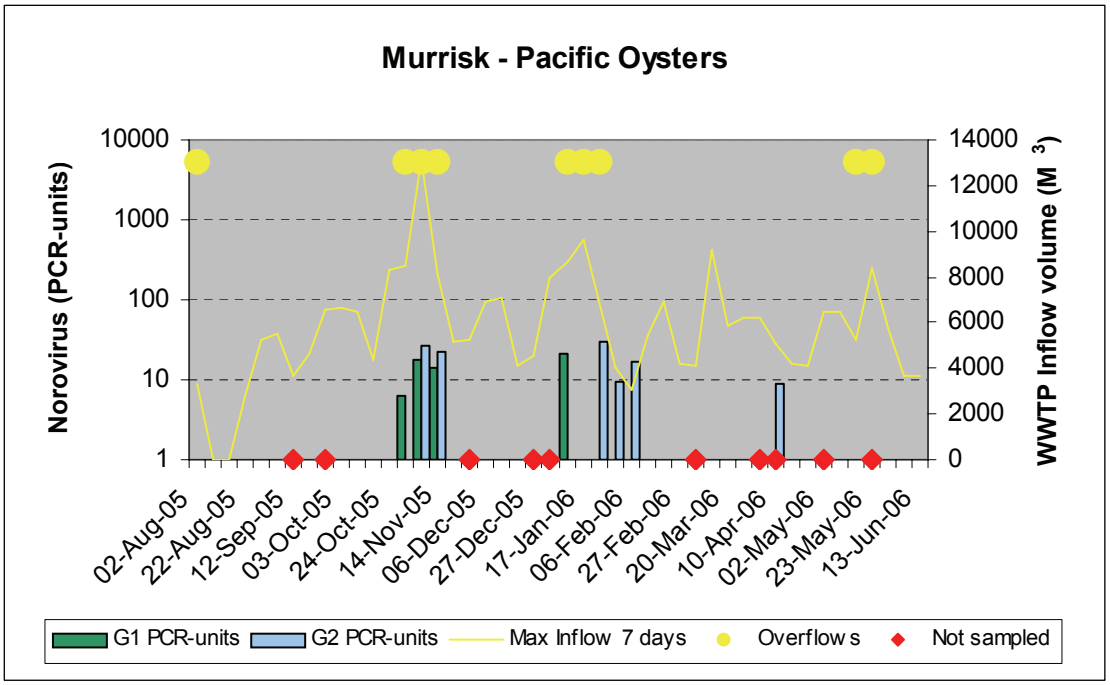

Figure 7. Norovirus for GI and GII levels (PCR units) in Pacific oysters from Murrisk. The weeks not sampled are indicated. The inflow volume to the WWTP and the periods of overflow are indicated.

Discussion

Although, long-term, the most effective way forward to control the health risks associated with shellfish consumption is reduction of levels of sewage initially impacting shellfisheries at source (Pommepuy *et al*, 2004), there is an immediate need to implement active risk management procedures. In moving towards developing active risk management procedures this study demonstrates that the identification of factors leading to norovirus contamination in shellfish is possible using new real-time PCR methods. This information can be used to determine when intervention measures should be introduced to limit the exposure of consumers to contaminated shellfish. However, as demonstrated at the Westport site in this study, the almost continuous incidence of norovirus contamination at some sites (despite compliance with the existing *E. coli* standard) preclude the suitable introduction of intervention controls. Therefore a pre-requisite for the use of active risk management procedures is that shellfish harvesting areas should be relatively free from sewage pollution and subject to only intermittent norovirus contamination. Therefore a first step in developing site-specific risk management procedures is to extensively characterise the shellfishery in question. In this study the sanitary survey successfully identified sites at less risk of norovirus contamination. Sanitary surveys in other area could also be used to determine areas likely to be impacted by intermittent contamination.

Where intermittent norovirus contamination was observed, it was closely linked to discharge of untreated sewage as a result of storm events. Procedures for rapid identification of these events and communication to relevant shellfish producers and risk managers is a key step in identifying high risk periods requiring intervention to manage the risk. Developing these links represents a major challenge requiring resource and commitment from all parties. The adoption of appropriate management options in each area will depend on local circumstances and the level of viral contamination.

The introduction of real-time PCR procedures allows the effectiveness of the control measures in preventing significant norovirus levels reaching consumers to be monitored, as well as providing information on how long the extra procedures should be in place. However, further work is required to relate the risk of viral illness to virus levels found in shellfish to determine whether complete removal of virus is required to provide a safe product, or whether there is an acceptable virus level which can be considered to present a level of risk.

Conclusions

1. The sanitary survey accurately predicted the relative risk of norovirus contamination in oysters at each site within the study area.
2. The three major factors influencing norovirus contamination were proximity to sewage input, season, with winter representing a higher risk, and the influence of untreated sewage inputs as the result of overflows from the WWTP.
3. The introduction of active risk management procedures is only appropriate in areas subjected to intermittent contamination. Sanitary surveys can provide an initial assessment of the likely risk of norovirus contamination and determine the suitability of a shellfish production area for the application of active risk management procedures.
4. One site was shown to be almost continuously contaminated with norovirus through the study period. This was despite complying with European hygiene regulations for shellfish harvesting as judged by *E. coli* data.
5. Intermittent norovirus contamination in two sites appeared to be associated with untreated sewage from overflows. These events could be used to trigger management

action at those sites. Close links between WWTP managers, shellfish producers and risk managers should be developed.

6. The highest incidence and levels of norovirus contamination in shellfish occurred during the winter months. Closer links between health professionals and shellfish risk managers should be developed to more accurately determine high-risk periods from the surveillance of outbreak data.
7. Further studies are urgently required to establish the link between norovirus levels observed in shellfish and health risk in consumers. Such studies will indicate the level of management and treatment required to provide an acceptable risk in shellfish.

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Acknowledgements:

The Redrisk project could not have been undertaken without the help of the following people, we at the NRL would like to extend our gratitude to:

Terence O'Carroll and laboratory personnel - BIM
Niall O'Boyle, Sean O'Grady, Mike Struth – Clew Bay Marine Forum
Jimmy Carney - DCMNR
Alan Stoney – Clew Bay Native Oyster Co-op
Hugh McGinley - EPA
Walter Hughes – Westport Urban District Council

THE SHELLFISH WATERS MANAGEMENT COMMITTEE

Kieran Burns
Department of Communications, Marine and Natural Resources

The Directive

The EC Shellfish Waters Directive (79/923/EEC), adopted on 30th October 1979, aims to protect or improve shellfish waters in order to support shellfish life and growth, therefore contributing to the high quality of shellfish products directly edible by man.

The Directive sets physical, chemical and microbiological water quality requirements that designated shellfish waters must either comply with ('mandatory' standards) or endeavour to meet ('guideline' standards).

The Shellfish Waters Directive is designed to protect the aquatic habitat of bivalve and gastropod molluscs, including oysters, mussels, cockles, scallops and clams. The Directive does not cover shellfish crustaceans such as crabs, crayfish and lobsters.

Under the Directive, Member States must designate specific waters as shellfish growing waters. Once designated, an action plan must be put in place for each water, in order to monitor water quality for the parameters listed in the Directive, to ensure that there is no deterioration and to work towards improving those waters where necessary.

The Directive was transposed into Irish law by Statutory Instrument 200 of 1994. At that time 14 Shellfish waters were designated as needing protection and or improvement. These regulations were amended by S.I. No. 459 of 2001 to establish action programmes for the 14 designated waters.

European Court of Justice

A complaint was made to the European Commission that Ireland had not implemented meaningful action programmes for the 14 designated shellfish waters. The matter was referred to the European Court of Justice (case C-67/02). On 11th September 2003 the court gave a ruling in favour of the commission. Ireland complied with the ruling in 2006 by implementing meaningful action programmes run by BIM and set out in regulations SI 268 of 2006. The EU dropped any further action in this case.

A second case against Ireland was also taken by the Commission (case C-148/05) on 1st April 2005. The basis of the commissions claim is that Ireland has breached Council Directive 79/923/EEC on the quality required of shellfish waters by failing to designate all shellfish waters and by failing to implement action programs for these additional waters.

Shellfish Waters Management Committee

It is recognised that the Department of Communications, Marine and Natural Resources does not have control over financial and operational measures needed to protect and improve shellfish waters, therefore, a decision was sought from Government to convene and inter-departmental/agency committee to progress matters.

This committee is comprised of those bodies that have a function/control in the protection of shellfish waters, and the focus is on cross body co-ordination and co-operation.

The following bodies are represented.

Marine Institute
Bord Iaschaigh Mhara
Environmental Protection Agency
Department of Environment Heritage & Local Government
Department of Communications Marine & Natural Resources
Department of Finance
Department of Agriculture & Food
Sea Fisheries protection Authority

In addition, a representative of the relevant local authority, the regional authority, the Central Fisheries Board, the relevant Regional Fisheries Board and others may be requested to attend individual meetings as deemed appropriate.

The terms of reference of the committee are:

- reviewing progress in relation to actions identified under existing programmes for designated shellfish waters;

- advising the Minister for CMNR in relation to shellfish waters where additional actions may require to be initiated, the authority responsible for any such actions and proposed timeframes for such actions;

- reviewing the ongoing sampling and analysis programme in order to identify any particular trends in relation to the quality of waters in any individual shellfish waters; and

- the development and overseeing of action programmes in respect of any additional areas to be designated as shellfish waters for the purposes of the Regulations.

The committee has met 5 times since June 2006. The main topics dealt with by the committee are updates on the status of existing designated waters, working towards the designation of all appropriate shellfish waters, the production of guidelines for Local Authorities , harmonisation of Shellfish Waters testing with Hygiene testing and the estimation of the capital costs of designating all shellfish waters.

The Committee has undertaken to produce a report for government in Quarter 1 2007.

REVIEW OF PHYTOPLANKTON MONITORING 2006

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Introduction

In recent years the national phytoplankton monitoring programme has become more and more important with its integration into Management Cell decision operations as part of the biotoxin monitoring programme. This programme fulfils national obligations to monitor toxic phytoplankton under Regulation (EC) No 854/2004 which enforces the following in relation to the monitoring of harmful algal species (extract):

> *"(a) Periodic sampling to detect changes in the composition of plankton containing toxins and their geographical distribution. Results suggesting an accumulation of toxins in mollusc flesh must be followed by intensive sampling;"*
> *"(b) Periodic toxicity tests using those molluscs from the affected area most susceptible to contamination."*

These sampling plans, as provided, must in particular take account of possible variations in production at relaying areas in the presence of plankton containing marine biotoxins. The sampling plans must be organised to detect changes in the composition of the plankton containing toxins and the geographical distribution thereof. Information leading to a suspicion of accumulation of toxins in mollusc flesh must be followed by intensive sampling.

The Irish monitoring programme provides robust and thorough toxic phytoplankton data that fulfils the following requirements:

- Provides early warning of potential shellfish toxicity
- Focuses attention on potential toxins for analysis that might otherwise not be detected
- Provides information on which algae are responsible if a new biotoxin event occurs
- Guides management decisions on protecting consumer safety

Recently the phytoplankton monitoring has taken an extra dimension with its incorporation into the Water Framework Directive as a required biological classification element. In developing an index of water quality in Ireland and Europe the existing Irish monitoring programme has been extremely valuable for the purpose of determining threshold values and providing intercomparison information. The programme also regularly provides important public health information to County Councils, Environmental Health Officer's and the public during times of bloom events.

Overview

The following paper provides an overview of phytoplankton sampling, analysis and reporting in 2006. The occurrence of potentially toxic and harmful phytoplankton found in Irish coastal and shelf waters in 2006 is compared with the previous year. The succession of phytoplankton blooms in Bantry is described and environmental data that may explain the onset of toxic species is described.

Methodology

Sampling Sites

Phytoplankton sampling sites are located around the Irish coast, usually within shellfish production areas or adjacent to finfish sites. Generally, samples submitted from south-west to south-east coastal sites are analysed in the MI laboratory based in Bantry, Co. Cork, while all remaining samples submitted are analysed in the MI laboratory based in Galway.

Throughout 2006, 2034 samples were submitted to the phytoplankton laboratories. Of these, almost 86% were processed as part of the National Monitoring Programme, from 46 shellfish sites and 42 finfish sites around the coast. The remaining were analysed as part of various research projects, surveys and quality control checks.

Sampling Protocol

The Lund tube sampling method accounted for the majority of samples collected in 2006. A smaller proportion was collected by surface sampling or discrete depths where the Lund tube was not suitable. A proportion of samples are still not adequately labelled with over 11% of samples received by the laboratories giving no information on sampling method.

In total, 82 samples or (5%) were rejected in 2006, broadly similar to the previous year and down from 9.9% in 2004 and 12.6% in 2003. This drop is due to a combination of improvements made to both procedures and sampling strategies.

Sampling Analysis & Reporting

All samples analysed for the presence of toxin producing/ problematic phytoplankton were examined using the Utermöhl method (Trondsen, 1995) following INAB accredited procedures. The method has a sensitivity of 40 cells.l-¹. By the end of 2006, the results of a total of over 1532 samples were reported back to the industry and related bodies, in 290 individual phytoplankton reports, issued on a daily basis. The overall turnaround time from laboratory receipt to reporting is ~ 80% within one working day, and 95% in two working days (Figure 1), well exceeding the 80% within two working days requirement as stated in the service agreement between the MI, FSAI and DCMNR.

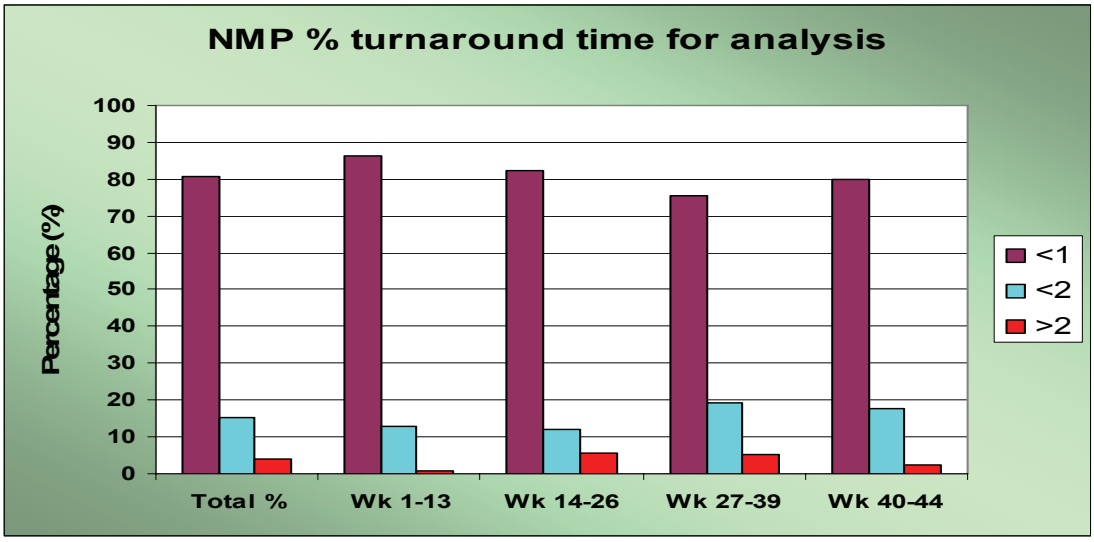

Figure 1. Turnaround of phytoplankton samples from receipt to reporting (Overall actual turnaround time 2006: 95% <2 working days)

Toxic phytoplankton in Irish waters in 2006

At present there are four main toxic algal groups that occur in Irish waters. These are the phytoplankton species that produce the toxins that cause

- Diarrhetic Shellfish Poisoning (DSP)
- Paralytic Shellfish Poisoning (PSP)
- Amnesic Shellfish Poisoning (ASP)
- Azaspiracid Poisoning (AZP)

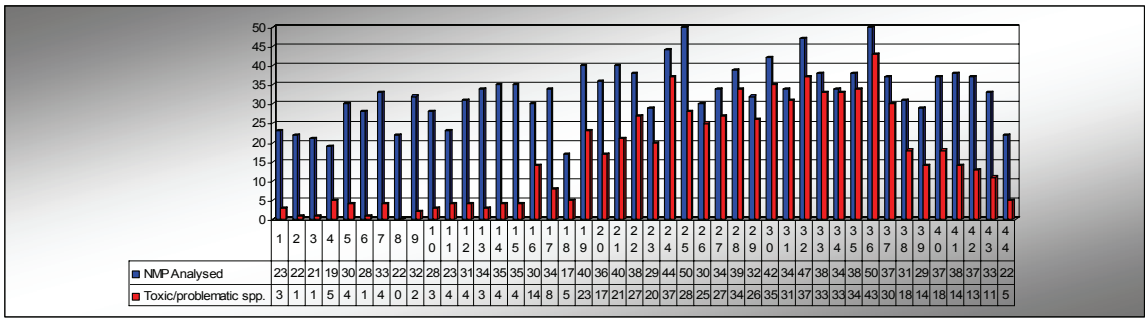

Figure 2. Graph showing the total number of National Monitoring Programme (NMP) samples analysed on a weekly basis (blue columns) and the number of samples containing one or more toxic species (red columns)

Similar to in 2005, prolonged closures also occurred in 2006 due DSP and AZP events. In addition there were localised closures in Cork Harbour due to PSP events.

In contrast to the previous year however, there was a significant reduction in the presence, intensity and distribution of toxic species in 2006. The highest counts for both 2005 and 2006 for the main toxic species are shown in Table 1. In 2006 *Alexandrium* peaked at 18% of the 2005 high count. Similarly, *Dinophysis acuminata* showed only 2.4% and *Dinophysis acuta* 10.4% of 2005 levels. *Pseudo-nitzschia* spp. was also notable by its reduction to 14.7% of the previous year's intensity.

Table 1: The highest counts of toxic phytoplankton detected in 2006, and corresponding high counts in 2005

	2005		**2006**	
	Cells/l	Location	Cells/l	Location
Alexandrium spp.	49,680	Banc Fluich, Castlemaine Hbr	9280	Oysterhaven, Cork
Dinophysis acuminata	82,547	Sheephaven, Donegal	2000	Drumcliff Bay, Sligo
Dinophysis acuta	2680	Kealincha-Inishfarnard, Kenmare Bay	280	Dunmanus Inner, Cork
Pseudo-nitzschia seriata group	2,304,272	Hawks Nest, Mannin Bay	339,592	Rosroe, Killary Hbr
Protoperidinium crassipes/curtipes	80	9 sites	120	Cuigeal, Galway

Dinophysis spp.

The phytoplankton responsible for Diarrhetic Shellfish Poisoning (DSP) toxins (okadiac acid and DTX's) are mainly produced by the dinoflagellates of the *Dinophysis* genus in Irish waters. Historically, the majority of closures in Irish production areas have occurred as a result of this toxin. Toxicity in shellfish can be recorded at very low cell counts (>200cells/l). In 2006, the occurrence of two main species (*D. acuminata* and *D. acuta*) is shown in figure 3.

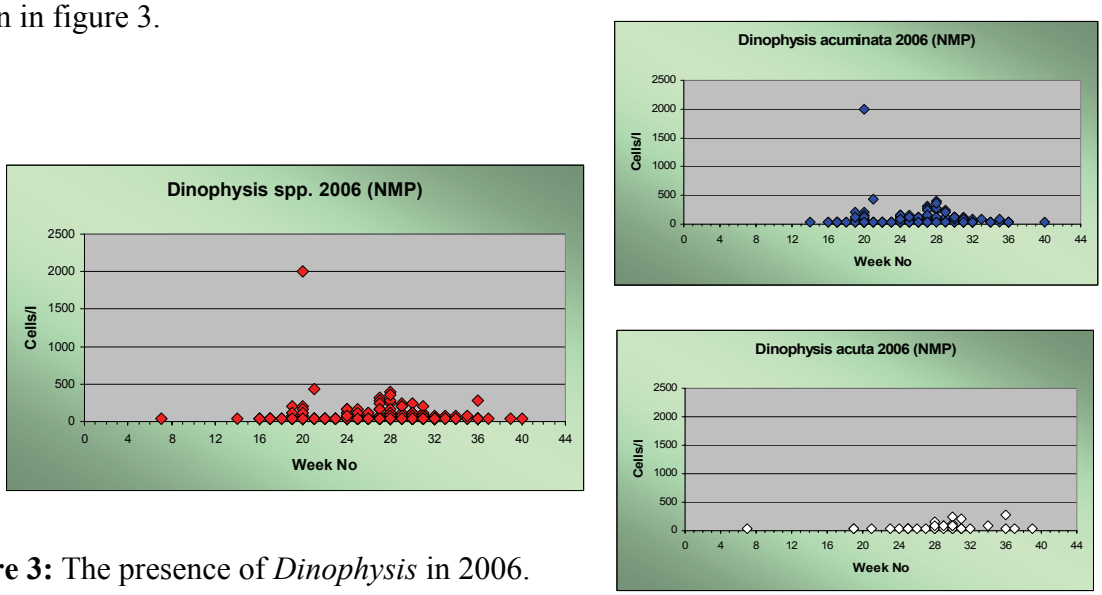

Figure 3: The presence of *Dinophysis* in 2006.

The onset of *Dinophysis* was detected in early summer with low levels in May and June. These levels were observed to increase in a small number of locations in the south west, but typically remained at very low levels in comparison to previous years. While the presence of these did result in DSP toxicity in shellfish, the toxin levels were not particularly high and cleared out of most areas by late September.

Pseudo-nitzschia spp

As the phytoplankton group responsible for Amnesic Shellfish Poisoning (ASP) toxicity, *Pseudo-nitzschia* spp are carefully monitored in Irish waters. Where high counts and in particular monospecific blooms are detected, extra shellfish are sampled and analysed for domoic acid (the toxin responsible for ASP). ASP is usually only found at high levels in the digestive system of scallops, but in 2005 mussels and a lower number of oyster closures occurred in the south west and to a lesser extent in the north west due to this toxin (Clarke *et al*., 2006).

In 2006, *Pseudo-nitzschia* spp did not reach particularly high levels (Figure 4) and apart from one minor detection at borderline levels in mussels from Ardgroom (June), only the usual scallop toxicity was detected above regulatory levels. A relatively minor bloom of the species occurred in autumn with no toxicity in shellfish occurring.

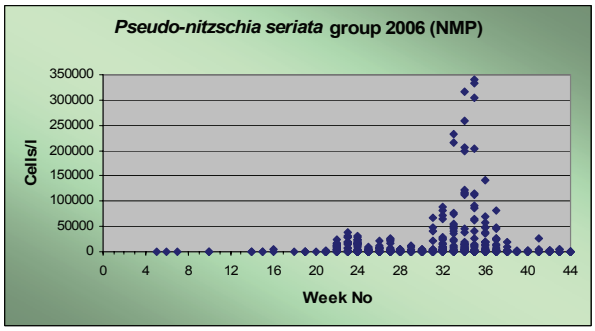

Figure 4. The presence of *Pseudo-nitzschia* spp in 2006

Alexandrium Spp.

One of the most potentially dangerous toxin producers in Irish waters are the Paralytic Shellfish Toxin (PSP) producing *Alexandrium* spp. Due to the potential severity of this neurotoxin, the presence of this species in water samples triggers increased testing of shellfish samples for PSP toxins. To date the main production area that has experienced closures due to PSP toxins is North Channel in Cork Harbour. Levels of *Alexandrium* spp. were generally observed at low levels in 2006 (Figure 5) with the highest levels observed as usual in the summer in North Channel and Oysterhaven, County Cork. Toxicity levels just over the regulatory threshold of 81.4µg/100g were observed in mussels from North Channel, on 20th June (Week 25). Following this, the levels and distribution of *Alexandrium* spp. decreased over the summer months and no other unusual events were observed.

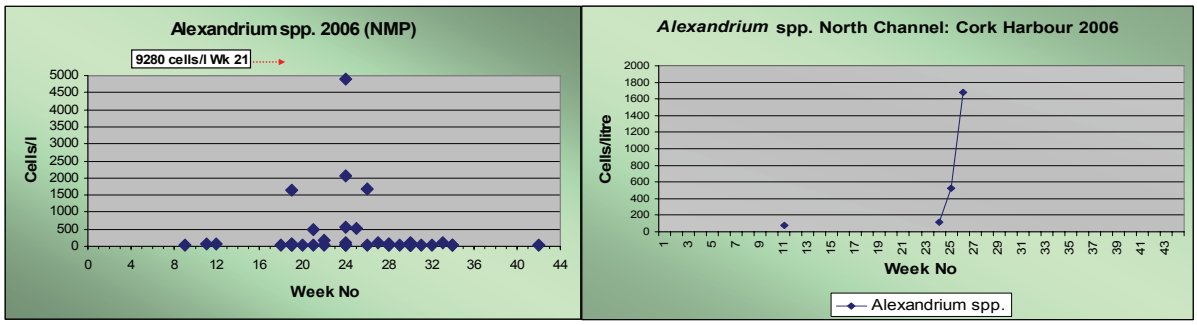

Figure 5. The presence of *Alexandrium* spp in 2006.The bloom observed in North Channel around week 25 corresponded with low level toxicity in shellfish

Protoperidinium spp

This genus of dinoflagellates have been associated with the presence of Azaspiracid shellfish toxins (AZA), however the conclusive proof of this is still outstanding. The correlation between the presence of *Protoperidinium* and Azaspiracid intoxication in shellfish has never been clearly shown. Nonetheless, *Protoperidinium* spp are monitored and their presence in 2006 showed little correspondence to the Azaspiracid event that occurred in the late summer through to winter period. The *Protoperidinium* spp distribution is shown in figure 6. Highest levels observed were in Drumcliff Bay on the 15th May with 74,880 cells/l and a later presence of *P. brevipes* in Greenore on 4th Sep, at a level of 2,000 cells/l.

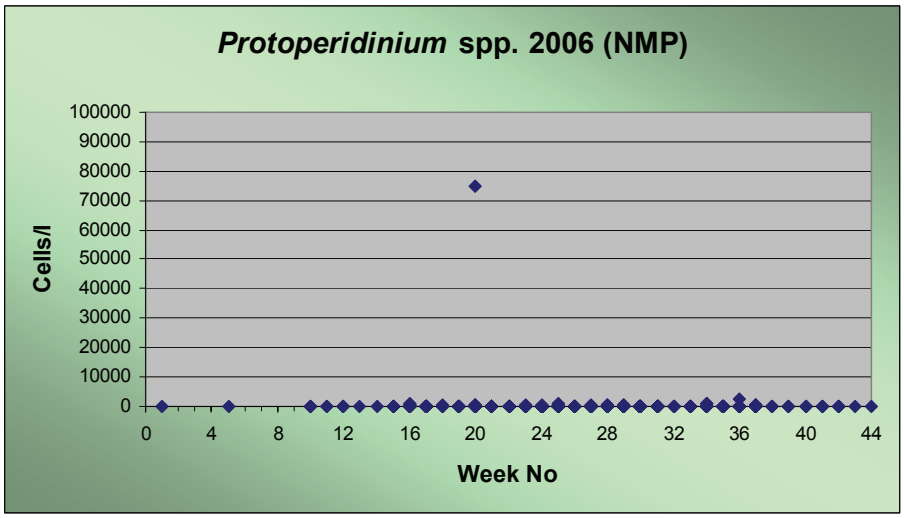

Figure 6. The presence of *Protoperidinium* spp in 2006.

Conclusion:
The extent and intensity of toxic phytoplankton in Irish waters was much reduced than in previous recent years. This resulted in lower levels of toxins observed in fewer areas. The most probable reason for this was the difference in wind climate between 2005 and 2006.

It has been suggested that in the main rope mussel growing region of Ireland (The south western bays) that *Dinophysis* and possibly other toxic species are delivered into these bays by relaxation in upwelling caused by wind direction. (Raine and McMahon 1998, Mc Dermott *et al.* 2004 and Cusack *et al.* 2006). This relaxation results in upper warm water exiting the bay which is then replaced with colder deeper water. This cold water is most likely to be the most significant delivery mechanism for *Dinophysis* species into these south west bays of Ireland.

The Marine Institute maintains a network of temperature probes around the country. These temperature probes (loggers) record hourly temperature at each site at various depths providing a comprehensive time series of temperature around Ireland. This information is invaluable in understanding oceanographic events using temperature as a marker.

The data shown in Figure 7 shows the temperature obtained at one of the temperature logging stations in Bantry in 2005 and 2006. The red arrows indicate cold water pulses in the summer period, which suggest intrusions of water from outside the bay in response to wind stress. The contrast between these events in 2005 and 2006 are obvious with much less colder deeper water pulses observed in 2006. This may explain the reduced observations of *Dinophysis* and consequent toxicity in 2006.

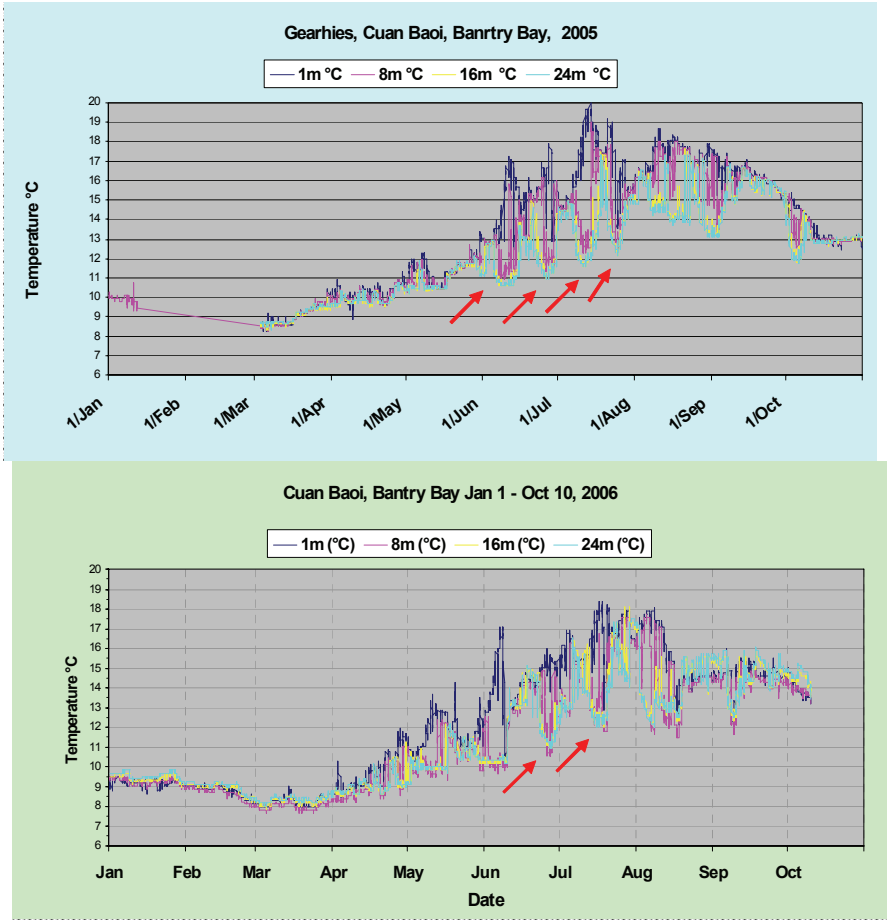

Figure 7. *Sea temperatures measured at 1m, 8m, 16m and 24m in Gerahies Bantry Bay for 2005 and 2006.*

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Cusack C., Silke J., Mc Dermott G., Noklegaard T., Nolan G., Gilmartin M., and Raine R. (2006) The Biological Oceanography of Harmful Algal Blooms (BOHAB) Programme: Special emphasis on the Dinoflagellate Genus Dinophysis. In: Marine Environment and Health Series No. 23: Proceedings of the 6th Irish Shellfish Safety Workshop, Marine Institute Galway

A REVIEW OF SHELLFISH TOXICITY MONITORING IN IRELAND FOR 2006

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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 Galway, under contract to DCMNR and FSAI.

Samples of shellfish species are routinely analysed for the presence of marine biotoxins by bioassay and chemical methods in accordance with Commission Regulation (EC) No. 1664/2006, Regulation (EC) No. 853/2004 and Regulation (EC) No. 2074/2005.

The Marine Institute as NRL for marine biotoxins are required as part of their NRL duties under Council Decision 93/383/EEC, *of 14 June 2003 on reference laboratories for the monitoring of marine biotoxins,* to coordinate the activities of the National Laboratories in respect of biotoxin analysis under the National Biotoxin Monitoring Programme which includes the organisation of intercomparison exercises and the regular auditing of the National Laboratories, En-Force Laboratories and Charles River Biological Laboratories.
The MI also participates in a number of proficiency testing schemes and intercomparison exercises including Quasimeme, BEQUALM, with the Community Reference Laboratory and also a number of individual laboratories and organisations.

During 2006 Azaspiracid (AZA) toxicity persisted for a prolonged period in a number. of sites as a carry over of the toxic event which began in 2005. This was followed by DSP toxicity during the summer months, followed by another AZA toxic event during the last quarter of the year. This resulted in prolonged closures in many sites, in particular the south west, where some sites were closed for several months, which have led to economic losses for producers and processors.

Tables 1 and 2 illustrate the number and locations of closures due to ASP, DSP and AZA toxins for 2005 and 2006. For 2006 (up to Oct) there was 22 site closures (all *M.edulis*), predominantly in the south west. Both the DSP and ASP events observed this year were present on a smaller scale and distribution when compared to 2005.

ASP	
DSP	
AZP	
DSP + AZP	

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

Production Area	Table 1. Site Closures 2005 – 38 Site Closures
SOUTH WEST	
Adrigole	
Ardgroom	
Banc Fluich	
Beare Island	
Castletownbere	
Cleandra	
Cromane East	
Dunmanus Bay	
Gearhies	
Glengarriff	
Gouladoo	
Kilmakilloge	
Newtown	
North Chapel	
Roaringwater	
Snave	
South Chapel	
Tahilla	
Templenoe	
Whiddy Point	
WEST	
Curraun	
Poulnaclough	
Aughinish	
Inislaughil	
Island eddy	
Killala	
Killary Inner	
Killary Middle	
Killary Outer	
Killeenaran	
Kinvarra	
Mannin	
NORTH WEST	
McSwynes Bay	
Mountcharles	
Gweebarra	
Tra Eanach	
Laghy Channel	
Mulroy Bay	

ASP	
DSP	
AZP	
PSP	
DSP +	

Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec

Production Area	Table 2. Site Closures 2006 – 22 Site Closures (up to Oct)
SOUTH WEST	
Adrigole	
Ardgroom	
Beare Island	
Castletownbere	
Cleandra	
Coosmore	
Dunmanus Bay	
Gearhies	
Glengarriff	
Gouladoo	
Kilmakilloge	
Newtown	
North Chapel	
Snave	
South Chapel	
Tahilla	
Whiddy Point	
SOUTH	
Cork Harbour	
WEST	
Inverin	
Mweeloon	
NORTH WEST	
Mountcharles	
McSwynes	

Amnesic Shellfish Poisoning

Report turnaround time of ASP analysis in shellfish samples from Jan – Oct 06 was 89% within 4 days of sample receipt. During Jan-Aug, 418 analyses for ASP were conducted on scallop tissues (*P. maximus*), typically gonad and adductor muscle tissues, where the levels observed on adductor muscle tissues (182 analyses) were all below the regulatory limit (highest level observed 9.2 µg/g in Feb in Kenmare River). One of 182 Gonad tissues analysed were observed to be > 20 µg/g, where the highest level observed was 24.4µg/g in February from Kenmare River.

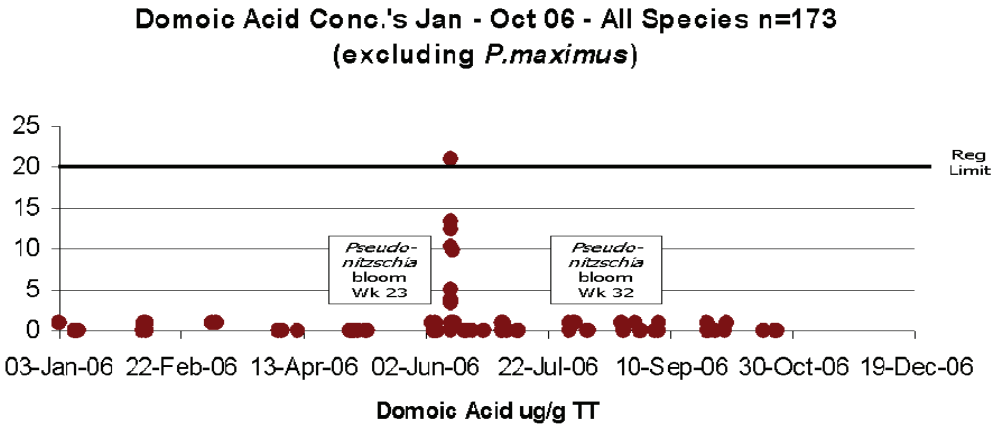


Figure 1. Domoic Acid conc.'s in Shellfish samples (except scallops) for 2006

Additionally during this time period, a total of 173 samples of *M. edulis, C. gigas, C. edule, E. siliqua* and *T. philippinarium*, were analysed for the presence of domoic & epi-domoic acid (DA). A small ASP event was observed in June in the south west, where one sample of *M. edulis* was observed to be slightly above the regulatory limit (Figures 1 and 2). The quantifiable levels of ASP observed in samples of *M. edulis* during this time period coincided with a bloom of *Pseudo-nitzschia spp.* observed in the area the week prior to ASP levels being detected in *M. edulis* samples. A second bloom of *Pseudo-nitzschia spp.* was observed during August in the south west. However no ASP was observed in any shellfish samples. Nationally, typical levels were observed to be **< Limit of Detection** throughout the year.

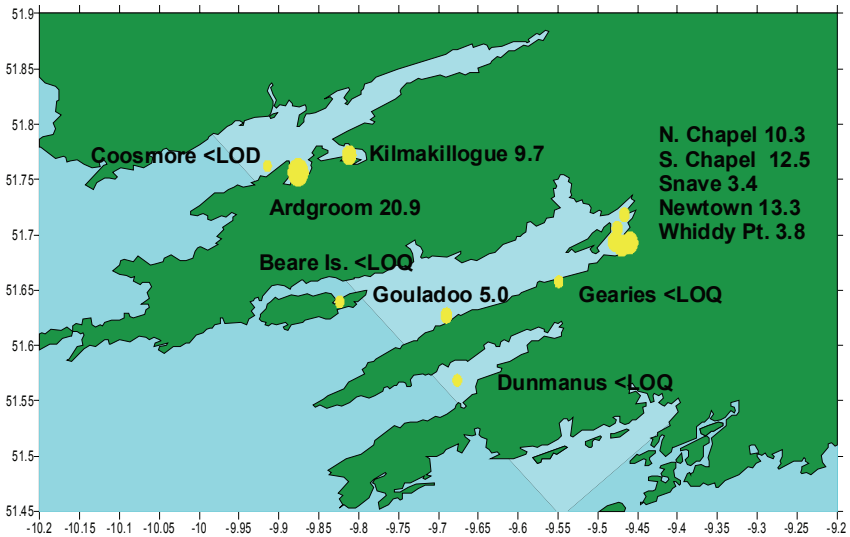


Figure 2. • Domoic Acid µg/g TT south west June 2006

Diarrhetic Shellfish Poisoning (DSP)

For 2006 (to end of October 2006) 2096 samples (2400 samples projected year end 2006 compared to 2546 samples submitted for 2005) were submitted for bioassay and chemical analysis for the presence of DSP (Okadaic Acid equivalents (OA, DTX-1, & DTX-2)) and AZA's (Azaspiracids 1, 2, 3).

Figure 3 illustrates the number of samples submitted from 1988 – 2006 for bioassay analysis. Following agreement at the Molluscan Shellfish Safety Committee (MSSC) meeting in March 2006 in addressing issues associated with the carryover of AZA toxicity from 2005 in samples into 2006 and some discrepancies observed between biological and chemical analysis, the frequency of testing of samples by bioassay was reduced to monthly from the end of February to the beginning of May. This was based on a poor correlation observed between the bioassay and chemical methods for the determination of AZA toxicity in samples. The results showed that during periods of AZA toxicity, greater emphasis should be placed on the chemical analysis in producing reliable and consistent results and in determining the production status of areas. This allowed for a number of sites to be re-opened as levels detoxified to under the regulatory level from January to May. From November 2006, this agreement was applied again following another AZA intoxification event observed in samples of *M. edulis*, predominantly in the south west from October.

The MSSC also agreed during 2006 that, where DSP toxicity was present, no hydrolysis would be conducted on samples for the analysis of Okadaic acid esters. In cases where there were discrepancies between the two methods, bioassay results would be used to determine the production area status.

Mussel (*Mytilus edulis*) samples were submitted on a weekly basis throughout the year. All other species were submitted on a monthly basis until May, fortnightly during June to November, reverting back to monthly frequency from December.

The Report Turnaround Time for samples submitted for DSP/AZP analysis was **91.2%** within 3 days from laboratory receipt.

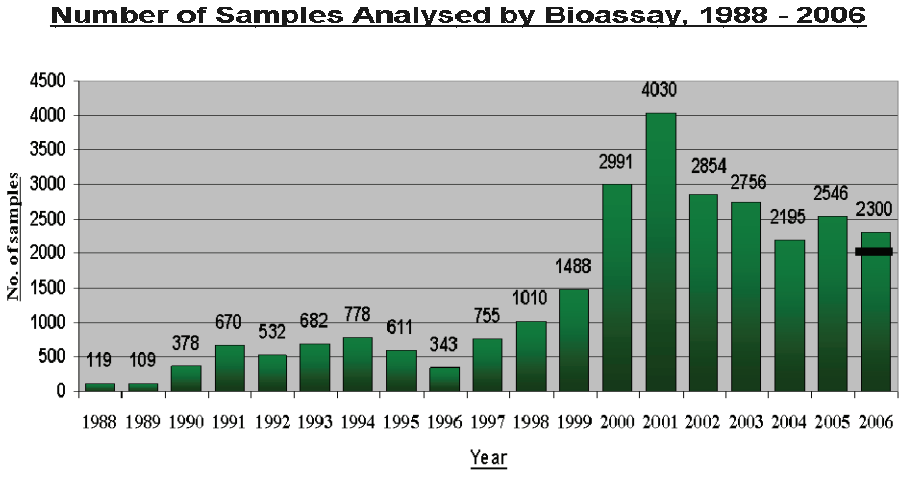


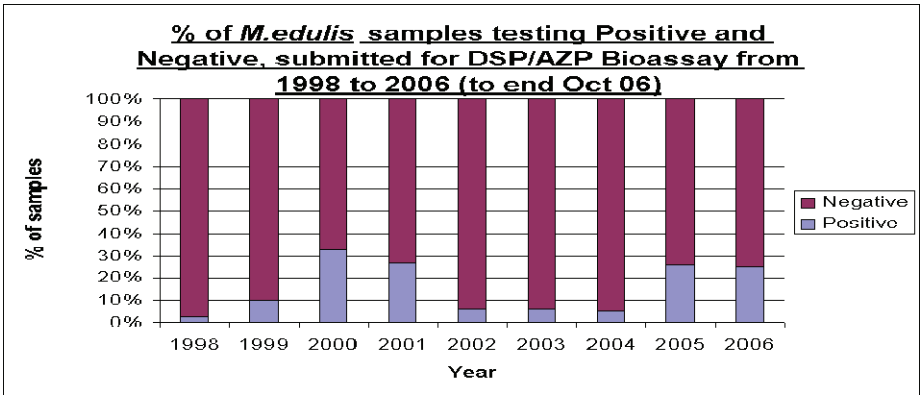
Figure 3. No. of all samples submitted for DSP/AZP analysis for 2006

Overall for 2006, (to October 06) 16% of all samples tested positive (all *M. edulis*) by mouse bioassay compared to 15% over the same time period for 2005. A breakdown of percentage positives by species for *M. edulis* reveals 25% of samples tested positive (of 1183 samples) compared to 2005,

where 23.9% samples tested positive (of 1564 samples). Figure 4 illustrates the percentage of mussel samples testing positive/negative via DSP Bioassay from 1998 to 2006.

Quantifiable levels of DSP were first observed in May below the regulatory limit in 2 sites in Bantry. Levels of DSP above the regulatory limit were first observed towards the end of May in Inverin. Throughout June – September, quantifiable DSP levels were observed predominantly in the south west in Kenmare, Bantry and Dunmanus Bays. During this time period there was a lower correlation between the bioassay and chemical results, which resulted in discrepancies between positive bioassays and chemical DSP concentrations below the regulatory levels. From October onwards the quantifiable DSP levels in the south west were observed to decrease further. Figures 5 and 6 illustrate the levels of DSP toxins and their locations during 2006.

In all except one sample, Okadaic Acid was the predominant toxin of the DSP group observed in samples during 2006. Levels of DTX-2 were extremely low during 2006, and were usually <LoD or <LoQ except one sample from Dunmanus which was observed to have DTX-2 levels above the regulatory limit. For 2006, in the cases where samples were observed to contain the presence of DTX-2, all DTX-2 results were calculated and reported using a toxicity conversion factor of 0.6



Year	1998	1999	2000	2001	2002	2003	2004	2005	2006
% Positive	2.5%	11.2%	32.7%	26.7%	6.03%	5.89%	5.06%	25.8%	24.9%
Highest OA µg/g Total Tissue	NA	NA	NA	NA	3.81	0.57	3.83	4.00	0.29
Highest AZA µg/g Total Tissue	NA	NA	NA	NA	0.23	0.19	0.02	>ULQ (8)	1.63

Figure 4. Percentage of Mussel Samples Positive for 2006 (end Oct 06)

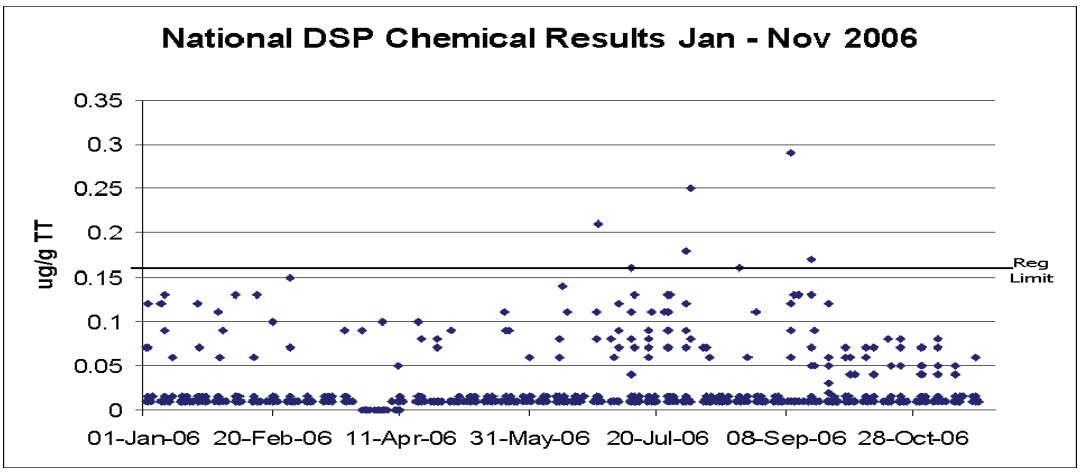


Figure 5. Concentrations of OA equivalents in samples submitted

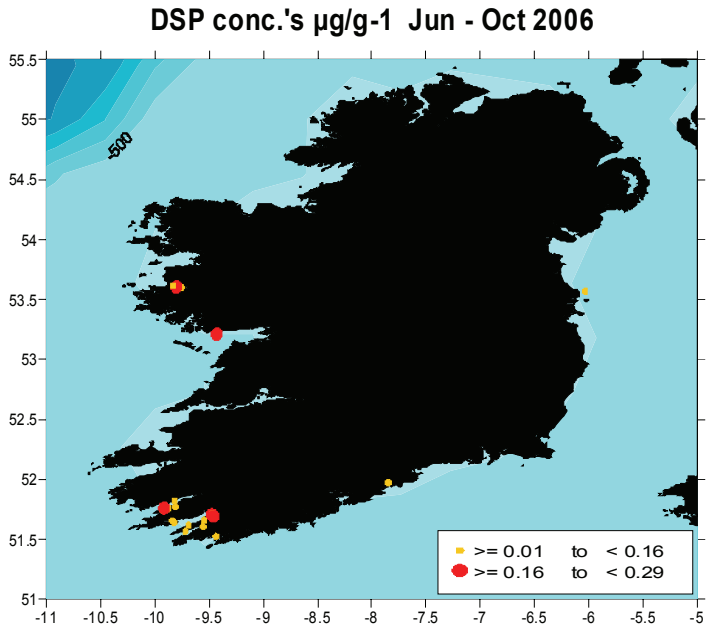


Figure 6. Concentration of OA equivalents in samples submitted June to October 2006

Azaspiracid Shellfish Poisoning (AZP)

Figure 7 illustrates the trends observed in concentrations observed in samples for OA, DTX-2 & AZA's 1,2, & 3 for 2006. Figures 8 and 9 illustrate the distribution and concentrations of Azaspiracids in samples observed from January - November 2006.

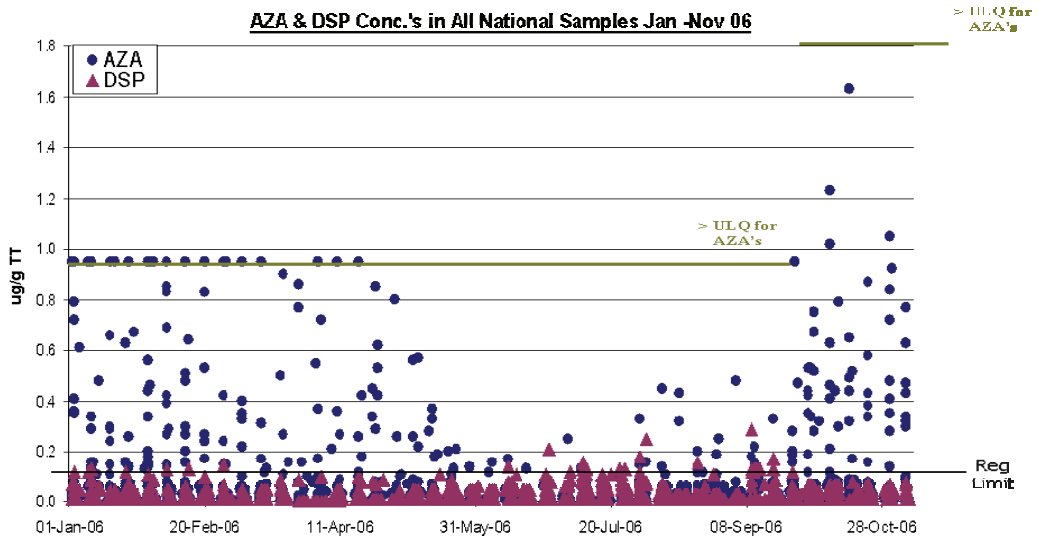


Figure 7. Concentrations of OA, DTX-2 & AZA's 1, 2 & 3 in samples submitted

There was a significant amount of AZA carryover in samples from 2005 into 2006 in samples predominantly from the south west and also from 2 sites in the north west. These AZA levels were observed to decrease during February and March resulting in openings of some sites, no significant decreases were observed during April, with further decreases observed through May however in some sites AZA toxicity remained above regulatory levels until May.

AZA toxicity was observed to be below the regulatory limit in all samples throughout June and through the majority of July, except two samples in July observed to be above the regulatory level, one sample from the north west and one from Kenmare. During this time quantifiable levels below the regulatory limit were observed in several sites in the south west.

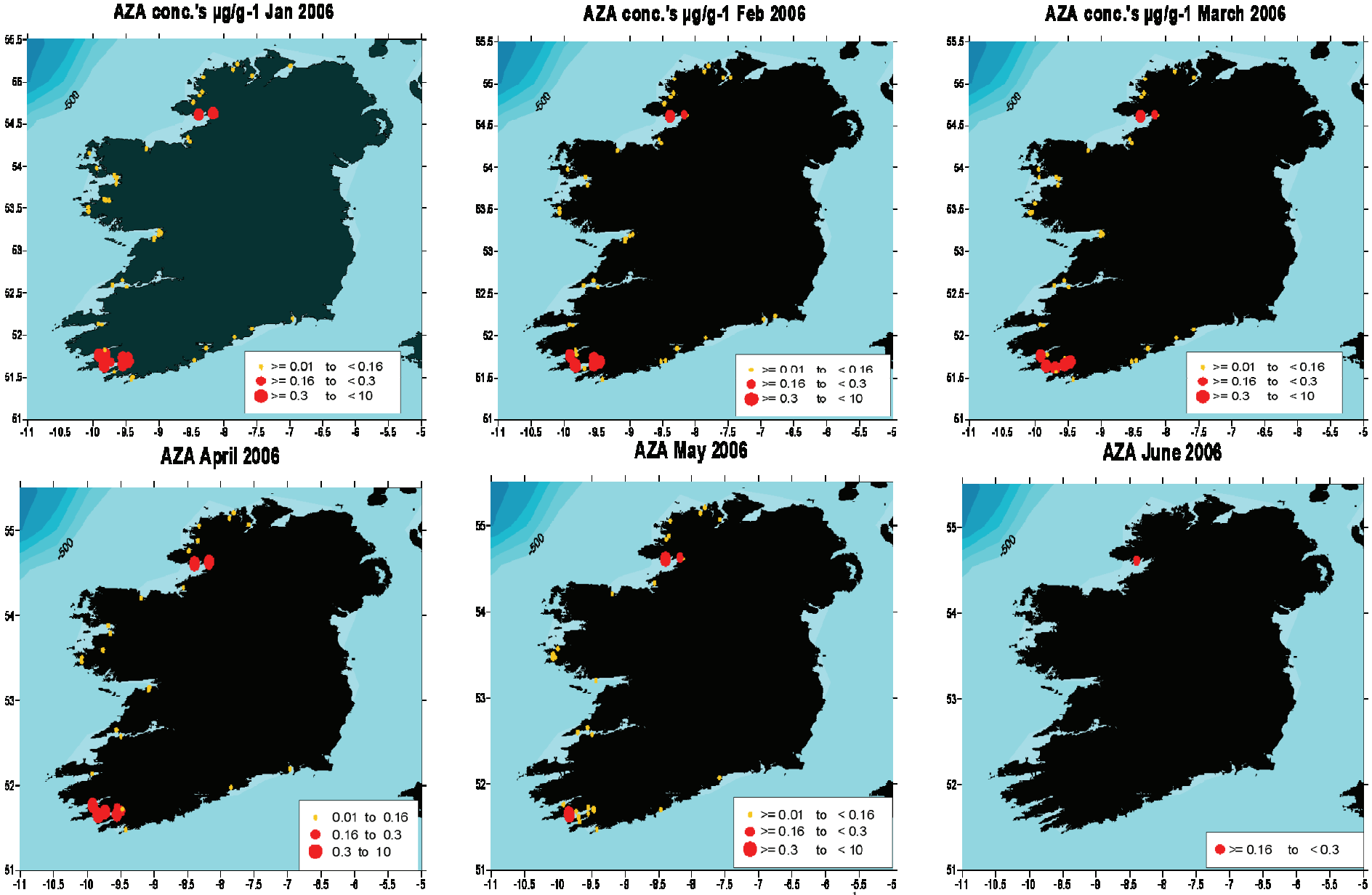


Figure 8. AZA Results January to June 2006. Total Azaspiracids AZA's 1, 2 & 3 in µg/g TT^{-1}

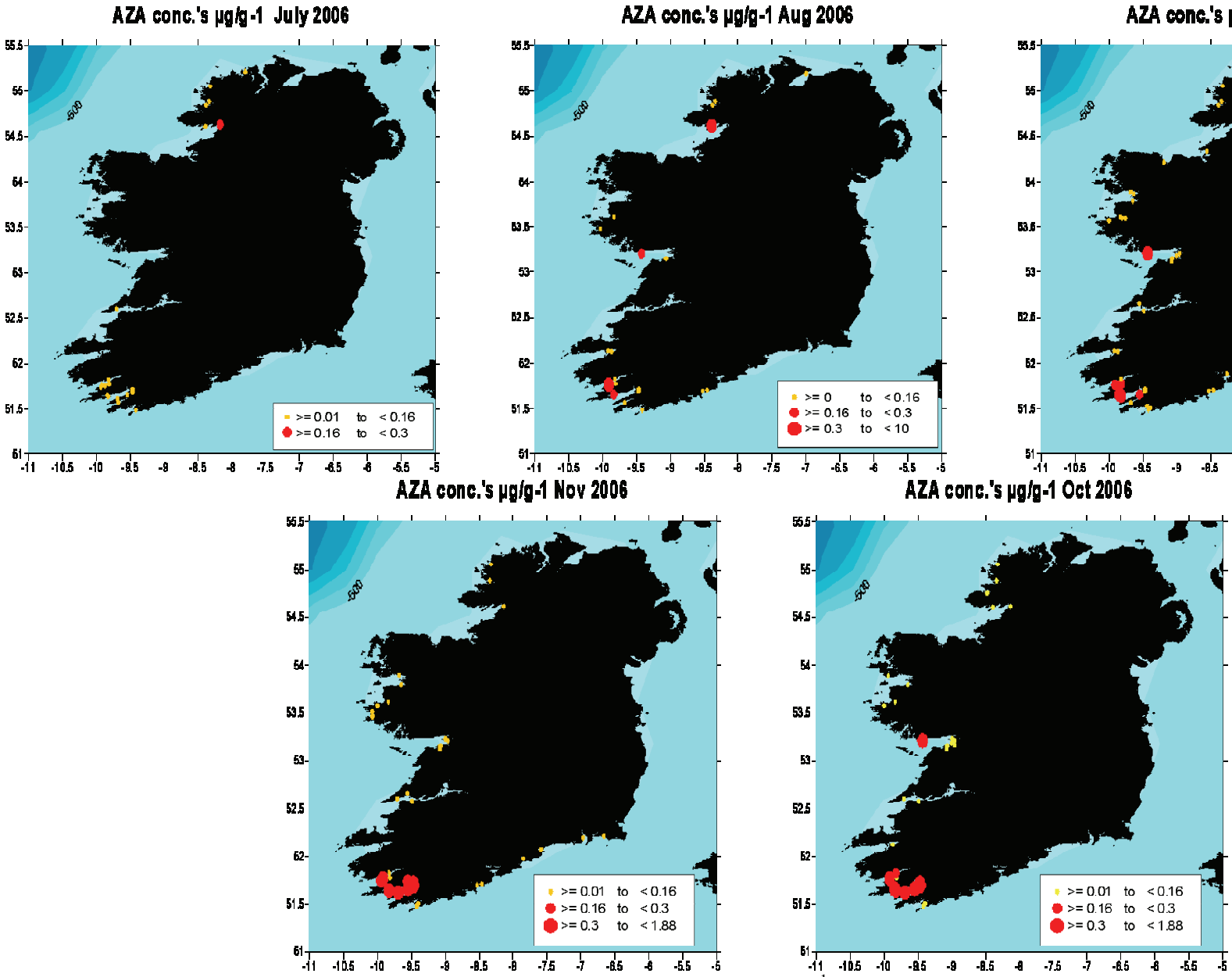


Figure 9. AZA Results Jul –Nov 06 Total Azaspiracids AZA's 1, 2 & 3 in µg/g TT^{-1}

In August, further sites in the north west, west and south west were observed to be above the regulatory level, with a number of sites in the north west to have quantifiable AZA levels, below the regulatory level. Throughout September and October AZA levels increased sharply, predominantly in the south west. However, in November decreases were observed in these sites. When the 2006 AZA data is compared to the September 2005 AZA intoxification event, it can be seen that overall the AZA levels during 2006 autumn/winter intoxification period were lower than those observed at the end of 2005.

Comparison: Bioassay & Chemical Analysis

The bioassay results were compared to the chemical results for OA equivalents & AZA's (n= 1883 samples January to November 2006) and are illustrated in table 3. Overall, an 89.5% correlation was obtained between the two methods for 2006. This is a significant decrease on those levels observed in previous years. When bioassay and chemical results are compared for AZA, there was a poor correlation between the two methods, where it appears that bioassay analysis is less specific and sensitive for the determination of AZA's. It was also observed during the summer months, that there was a larger discrepancy between positive bioassays and negative chemical results for the determination of DSP toxins than observed in previous years. However, the hydrolysis step for the chemical determination of okadaic esters, which the bioassay detects, was not conducted on samples where there was a discrepancy. Previous years have seen the percentage discrepancy reduced between positive bioassay and negative chemistry when the hydrolysis step is conducted.

Table 3. Comparison of bioassay vs. chemical analysis from 2002 - 2006

Year	Positive MBA & Positive Chemistry	Negative MBA & Negative Chemistry	Total Correlation
2002	2.5	96.3	98.8
2003	1.3	96.2	97.5
2004	3.3	95.9	99.2
2005	11.3	83	94.3
2006 (Nov 05)	8.7	80.8	89.5

Paralytic Shellfish Poisoning (PSP)

During January to November, 143 samples were submitted for PSP analysis. All samples were negative via AOAC PSP Bioassay and or Jellett Rapid Test Kit (used until June 2006), apart from two *M. edulis* samples observed at the end of June from Cork Harbour. The maximum level observed was **81.4** µg STXdiHCl $100g^{-1}$. Typically all other samples were <LoQ. MI have been working on the recently AOAC validated method for the determination of PSP toxins via HPLC (*Lawrence et al.* method) to use this method as a future alternative to the bioassay.

Quality System

Following the relocation of laboratories from Dublin and Galway into the new MI premises in March 2006, all accredited test methods for the analysis of phytoplankton and shellfish toxins through bioassay, immunoassay and chemical analysis were temporarily suspended from March. All methods were quickly reinstated in the new facilities with no significant downtime in the sample report turnaround time observed. All methods were fully re-accredited in June 2006.

THE MANAGEMENT CELL

Richie Flynn
Executive Secretary, Irish Shellfish Association

The "Management Cell" (MC) is both a physical group of people and a process that has been applied to the Irish shellfish biotoxin management regime since 2004. The cell structure and objectives are described in Section 3.3 of the Code of Practice published at http://www.fsai.ie/sfma/cop/ chapter3.asp#c33. These have been refined and adapted since the setting up of the cell, which was an initiative sought by the ISA as an essential guidance tool to ensure that decisions on opening and closing bays took all available evidence into account and balanced consumer safety with commercial sustainability.

The Management Cell applies risk management principles to real-life situations as marginal or disputed results are thrown up by the bioassay, chemical and plankton monitoring systems. Decisions of the MC are based on all available evidence, carried out by representatives of all the relevant stakeholders (competent legal authority, scientific analysis lab, producers and food safety body). It is the aim of the MC to reach all decisions by consensus where possible, with the final casting vote being given to the FSAI as the ultimate consumer protection stakeholder.

In 2006, there were 91 decisions up to week 40 – the most up to date information available for this workshop. At the outset it is important to thank all involved in the work of the MC – particularly those MI staff and the other stakeholders, especially the voluntary ISA members, who are often dealing with an MC decision late on a Friday evening when everyone else is relaxing!

This year the issue causing such a large number of MC decisions was the prolonged presence of AZA at relatively low levels. A decision had been taken by the Molluscan Shellfish Safety Committee in late 2005 to regulate AZA by means of chemistry results alone. Many decisions had to be take as regards opening or closing individual harvesting areas based on one or two micrograms above or below the very low regulatory limit of 16 micrograms per gram of AZA. The other significant development in 2006 was the request on behalf of the mussel processors request to be recognised as a stakeholder and have a formal input into MC decisions. The ISA agreed with this point as long as the processors agreed that the contact put forward would be chosen democratically and represent all processor interests equally.

The primary issue raised in 2006 as a result of MC work was the decreasing confidence among producers and processors that the 16-microgram limit was a sufficiently robust scientific level upon which to risk the future of the entire rope mussel industry. The ISA has repeatedly raised fears that the original intoxication by AZA based on the Arranmore incident in 1997 was not properly documented or sufficiently detailed or swift in its back-up research to justify the fixing of such a low figure. Industry is very anxious that all available Irish research capacity in this area is focused on ascertaining the true toxicity of AZA.

The other point raised by growers and processors is the need to improve the value ratio to industry i.e. to build upon the "protection" aspect of the monitoring regime and develop a "promotional" aspect to the work of so many dedicated professionals and volunteers. Monitoring must be used to improve the image of industry not undermine it.

The most important lesson learned from the experience of 2006 is first and foremost that the MC works! It has consistently been shown that MC decisions have been fair and balanced and crucially have not resulted in a single intoxification incident since the system was set up. In addition, any fears raised in 2004 that industry may have tried to use the MC to push for unwarranted harvesting from potentially toxic areas have been shown to be completely unfounded and ISA's representatives have acted completely in the interests of the industry's customers – the public.

It has been shown that the MC can't deal with all situations e.g. AZA toxicity. The MC is not a scientific research body and can only rely on the latest interpretations and regulations. The MSSC must push for more research into those areas that continue to cause concern.

The final question is whether the same risk management model could be applied to microbiology/virus situation? The industry is prepared to trial this and is heartened by the risk management features slowly coming into practice in the area of viruses through the recently published "Good Practice Guide for the Microbiological Monitoring of Shellfish" by DG SANCO which will hopefully be interpreted by the MSSC for the Irish industry in 2007.

ASTOX PROJECT
(Isolation and purification of azaspiracids from naturally contaminated materials, and evaluation of their toxicological effects (ST/02/02))

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Background
Since 1995, when several people became ill following consumption of shellfish from Ireland, azaspiracids have been known as shellfish toxins, causing symptoms associated with gastro-intestinal disorders, including diarrhoea, vomiting, headaches and other symptoms. The aims of the ASTOX-project were to provide control tools for the analysis of azaspiracids in shellfish, i.e. calibration standards and tissue reference materials, and to clarify the toxicity of azaspiracids in qualitative and quantitative terms, i.e. to understand the mode of action of AZAs and to derive a No-Observed-Adverse-Effect-Level (NOAEL) for safe consumption of shellfish.

Project design
Activities relating to the retrieval of contaminated shellfish, the preparation of homogeneous and stable reference materials (RMs) and the isolation and purification of azaspiracids and dinophysistoxin-2 were mainly carried out at the MI, with advice and quality control being provided by Tohoku University, (TU; Japan), the Japan Food Research Laboratory, (JFRL), and the Tropical Research Centre, (TTC; Okinawa, Japan). Further collaboration also included external partners such as the Norwegian Veterinary Institute, (NVI; Oslo, Norway) and the Institute for Marine Biosciences, National Research Council, (NRCC; Halifax, Canada). Activities relating to the evaluation of the toxic effects of azaspiracids were mainly carried out at the Conway Institute, University College Dublin, (UCD; Ireland), Chiba University, (CU; Japan) and at the Centre for Coastal Environmental Health and Biomolecular Research, National Oceanic and Atmospheric Adminstration, (NOAA: Charleston, US). Additional collaborations included the Norwegian School of Veterinary Science, Oslo (Norway), the Centre for Bio/molecular Science and Engineering, Naval Research Laboratory (US) and the University of Modena (Italy).

Retrieval of shellfish tissues and preparation of RMs

These were secured from natural toxin occurrences in 2000, 2001, 2004 and 2005 were contaminated up to 5 mg/kg AZA1 and up to 1.5 mg/kg with DTX2, and were successfully used to prepare 17 different shellfish tissue reference materials (100 to 3,700 portions of 1 to 8 g). Factors affecting homogeneity were mostly related to water content and dispensing procedures. Optimised procedures were published. Factors affecting stability were temperature, water content and container sealing; methods investigated to improve stability included the addition of antibiotics, antioxidants, γ-irradiation, heat treatments and freeze-drying. Some of the materials resulting from these studies have been and continue to be employed, in the QC of the Irish national statutory monitoring for marine biotoxins. Others have been used for comparison of the detection method used at MI, with up to 16 other laboratories worldwide, through proficiency testing and method validation exercises. The collaboration with NRCC allowed for the preparation of a candidate certified mussel tissue reference material (CRM) for AZAs, which will be made available globally once certification is complete (ca. 3,700 portions of 8 g), (Figure 1).

Figure 1. Candidate certified tissue material for azaspiracids produced during collaborative study between the Marine Institute and the Institute for Marine Biosciences, National Research Council Canada.

Isolation and purification studies

Isolation and purification studies included a total of 14 isolation batches and led to purified AZA1 (ca. 8 mg). The initial isolation procedure developed by the Japanese collaborators was implemented and tested; a detailed standardised protocol for the purification of AZAs has been established. The amount of toxin obtained was not sufficient to conduct formal long-term oral exposure studies in mice since this would require more than 250 mg. However, aliquots of the purified standard have been provided for *in-vitro* toxicology studies as well as method validation studies. The collaboration with NRCC allowed for the preparation of a candidate CRM for pure AZA1, which will be made available globally once certification is complete (ca. 3,500 ampoules of 0.5 mL at ca. 1.5 μM AZA1). The collaboration with NRCC is continuing and 4 reference standards are under development (AZA1, 2, 3 and DTX2). Small amounts of AZA1 standard and shellfish tissues contaminated with AZAs have already been made available to the EU Community Reference Laboratory (CRL), individual National Reference Laboratories (NRLs) and the Public Analyst Laboratory in Galway, another Irish official control laboratory.

Toxicology studies

A summary of toxicological observations in humans and animals was prepared for a risk assessment on AZAs, and cellular and molecular studies were designed to investigate the mode of action underlying the toxicity of AZAs. Studies conducted on 7 different mammalian cell lines showed that AZAs strongly affect most cell types, an important observation in light of the initial report of multiple organ damage in mice following exposure to AZAs. In addition, a functional assay was developed for the specific detection of AZAs using morphological changes of pseudopodia in lymphocyte-T cells (Jurkat). These cytoskeletal effects provided some of the first insights as to the molecular target of AZAs. In parallel, cytoskeletal effects have also been observed in human colon cells (Caco-2), used in a separate functional assay developed to mimic the effects of AZAs in the human digestive tract. Caco-2 cells form a tight layer, across which an electrical resistance can be measured in the assay. When the cell-layer is exposed to AZAs, it is disrupted and the electrical resistance decreases. These assays reflect the *in vivo* effects of gastrointestinal upset in humans. Figure 2 shows how human symptoms can be related to *in vivo* and *in vitro* studies and mechanistic studies.

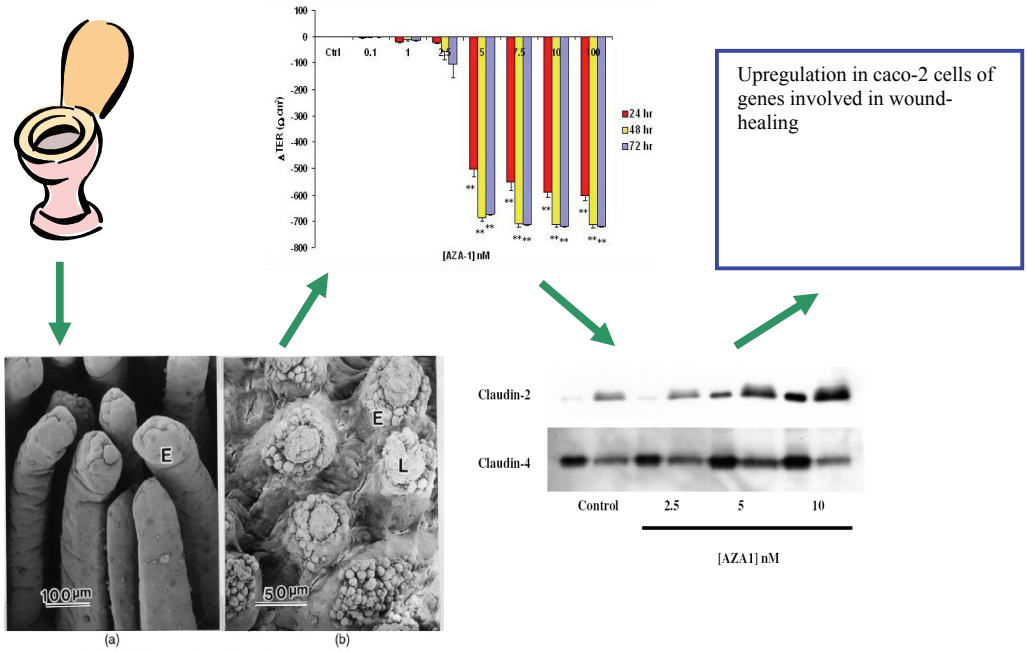

Figure 2. Toxicological studies from human epidemiology (symptom diarrhoea), over observations in mice intestines, reduction of TEER in Caco2-cells, changes in protein expression of Claudins to changes in gene expression due to insults by azaspiracid.

Mechanistic studies

At molecular level, the cytoskeletal effects in Caco-2 cells were shown to be related to the proteins actin and claudin-2 and claudin -4. These studies demonstrate that AZAs strongly interferes with the intestinal barrier, consistent with the human symptom of diarrhoea. Parallel studies on enzymes affected by okadaic acid (OA), namely protein phosphatase-1 and –2a, showed that AZAs do not bind to these enzymes, thereby, distinguishing their toxic mechanisms clearly from that of OA and DTX2. Using intraperitoneal injection into mice, the toxicity of DTX2 was compared to that of OA and DTX2 was found to have only ca. 60% of the potency of OA (Figure 3); a similar reduced potency of DTX2 compared to OA was also noted in the protein-phosphatase inhibition assay. Azaspiracids also showed toxic effects on neuronal cells in spinal cord networks and in the expression of E-cadherin in MCF-7 and Caco-2 cells. Structure-activity relationship of AZAs was further clarified to be stereo-specific, since a C1-20 epi-AZA1 showed significantly reduced toxicity in mice at up to 4 times higher concentrations as AZA1. The studies in this project did not unequivocally demonstrate the primary target(s) of AZA. However, alterations of gene expression in lymphocyte-T and Caco-2 cells following exposure to AZA1 were investigated and point toward possible pathways of molecular interaction.

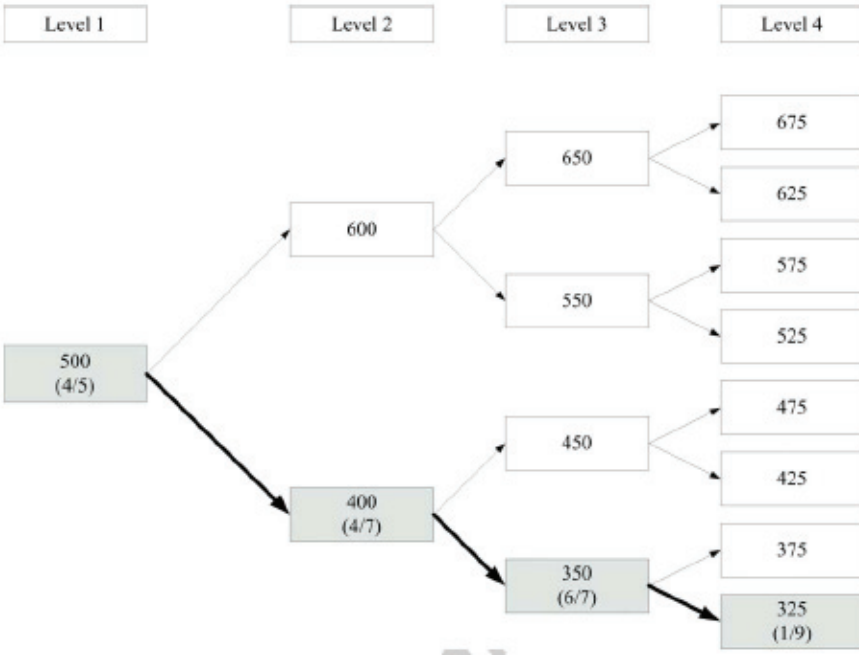

Figure 3. Exposure of mice to Dinophysistoxin-2, an analogue of Okadaic Acid, regularly occurring in shellfish from Ireland. The relative toxicity of DTX-2 was established to be 0.6 compared to Okadaic Acid (Aune *et al*., 2006).

Risk assessment

The project was initially designed to carry out long-term animal exposures to derive a lowest observed effect level and subsequently a no observed effect level for a safe level of AZAs in shellfish. However, following international guidance, the risk assessment to which scientists of this project contributed, was based on data from human illness. The Food Safety Authority of Ireland's risk assessment from 2001 was reviewed in light of the experience gained in Ireland since then. Evidence from routine monitoring on profiles of analogues, studies on the heat-stability of AZAs, as well as the distribution of AZA throughout different mussel tissues allowed the revision of the initial intake for people involved in one of the first poisoning incidents in Ireland. Those data, in combination with the experience from risk management during 2001 and 2005, allowed the establishment of a safe level of AZA in shellfish at ca. 0.16 mg/kg shellfish flesh. This risk assessment was adopted by the FSAI in August 2006, and has been made available to international bodies, e.g. the European Food Safety Authority and *Codex alimentarius*.

Scientific publication output

The scientific aspects of this work have led to 22 presentations at international conferences and 11 peer-reviewed papers (5 in press) and these are listed below. At least 8 further peer-reviewed publications are in preparation.

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UPDATE ON BIOTOX RESEARCH – SPATT TECHNOLOGY AND LC-MS METHOD DEVELOPMENTS

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General Introduction

BIOTOX is a three-year EU funded project, involving 12 partners from 6 countries. The project focuses on the development and validation of cost effective assays for the detection of lipophilic marine toxins. The different work packages (WPs) and objectives of the project were presented in detail at the 6th Irish shellfish workshop (Bire *et al*., 2006). The Marine Institute is involved in several WPs including, the development of a liquid chromatography mass spectrometry (LC-MS) based reference method (WP2) and its validation (WP5), a feasibility study on the production of a candidate certified reference material (WP6) and the development of an early warning system to forecast accumulation of lipophilic toxins in bivalves (WP7). This paper describes the progress in WP2 and WP7. It was agreed that WP7 would be carried out using two independent approaches. The first approach consists of the development of gene probes and is carried out in The Netherlands Institute for Fisheries Research (IMARES). The second approach, carried out at the Marine Institute, is based on the use of passive sampling technique referred to as Solid Phase Adsorption Toxin Tracking (SPATT).

A- Development of an early warning system (WP7)

Introduction

The use of passive sampling has shown major advantages for the monitoring of organic pollutants in the aqueous environment (Stuer-Lauridsen, 2005). The ability to obtain a long term integration of an analyte, including episodic contaminations, without the need of energy and avoiding bio-transformation, make passive sampling an attractive tool to obtain temporally and spatially integrated levels of contaminants. In 2004, Lincoln MacKenzie developed Solid Phase Adsorption Toxin Tracking (SPATT) bags, which were passive samplers designed for the detection of lipophilic marine toxins (MacKenzie *et al.,* 2004). The study emphasised that SPATT bags accumulate toxins prior to their accumulation in shellfish. Therefore, it was concluded that the SPATT technology could be a way of forecasting harmful algae blooms and hence providing an early warning system to the industry. This technology was evaluated in Irish waters during the summer 2005.

Experimental

Sampling sites

Three locations on the west coast of Ireland were selected according to their history of contamination in recent years. Field sampling during the summer 2005 was set up in three sites in Killary Harbour (Killary inner [53 36’0.0001”N, 9°45’19.9008”W]; Killary middle [53 36’14.0039”N, 9°48’10.0080”W]; Killary outer [53°36’52.9919”N; 9°49’59.0160”W]), one site in Bantry [51°41’35.1600” N, 9°28’41.9880”W] and one site in Bruckless [54°36’47.9879”N, 8°23’31.9920”W].

Sampling system

A 15 m polypropylene rope was used to suspend the SPATT discs in the water column. Three SPATT were attached to PVC tubes going through the rope at three different depths: surface, 5 m and 10 m. Nets containing 300 g of uncontaminated mussels (*M. edulis*) were placed at the same depths, as the SPATT discs and are referred to hereafter as transplanted mussels. The rope was ballasted with a weight and attached either on a mussel rope, fish cages or buoy, depending on the location. The SPATTs and the uncontaminated mussels were replaced on a weekly basis.

SPATT preparation

HP20 DIAION resin was weighed (3.00 ± 0.05 g) and activated in 100 ml of methanol by a 40 min shaking step in a multitube vortexer prior to a filtration on 95 µm mesh (≈ 21 x 12mm). The resin was wrapped with the mesh and clipped in an embroidery frame (diameter 8.8 cm) allowing exposition on both sides of the frame. Methanol residues were removed by a 10 min sonication step in 500 ml water. The SPATT discs were stored in MilliQ water at 6 °C until deployment.

SPATT extraction

Method development

The extraction efficiencies were assessed using naturally contaminated resins. Following several attempts to improve MacKenzie's method (MacKenzie *et al.,* 2004), it was found that a slow elution gave the best recovery. This was assessed by packing the naturally contaminated resin in a preparative glass column, which was connected to a pump set at 1 ml/min flow with methanol as mobile phase. The elution was carried out over 60 minutes and 5 ml fractions were taken and analysed by LC-MS.

Extraction method

SPATTs were rinsed twice in 500 ml of MilliQ water and vigorously shaken to remove salts and the excess of water. The contaminated resins were removed from the mesh and inserted into empty solid phase extraction (SPE) glass cartridges placed on a manifold. Vacuum was applied in order to remove the remaining water. The Killary-middle results presented here were the first set of SPATT discs that were analysed and in order to ensure that the limit of detection (LOD) was achieved all extracts were concentrated by a factor 5 after elution with 25 ml of methanol at ca 1 ml/min. It was found that the concentration step could be avoided, as the toxins of interest were quantifiable in a 25 ml solution. Therefore, the following procedure was applied for the remaining samples. A 23 ml methanol portion was used to elute the resin at ca 1 ml/min flow rate. The extracts were transferred into 25 ml volumetric flasks and an additional 2 ml was used for rinsing and to complete up to the mark. Aliquots were taken from the volumetric flask, filtered on 0.2 µm disc filters, and inserted into LC vials for LC-MS analysis.

Mussel extraction

Upon receipt, the transplanted mussels were steam cooked and stored frozen until extraction while the indigenous mussels from the same location were analysed fresh as part of the national shellfish safety monitoring program. The extraction procedure consisted of a double methanolic extraction which was previously described by Hess *et al.* (2005).

Instrumentation
Two systems were used for quantitative LC–MS analysis, using a binary mobile phase with A (100% aqueous) and B (95% acetonitrile) both containing 2 mM ammonium formate and 50 mM formic acid. The indigenous mussel samples were analysed using 2695 Waters HPLC coupled to a Micromass Quattro Ultima (triple quadrupole) equipped with a z-spray ESI source. The Quattro Ultima was operated in multiple reaction-monitoring (MRM) mode, analysing two fragment ions per compound. Monitored transitions were reported elsewhere (Hess *et al.,* 2005). A C_8 BDS Hypersil (50 x 2 mm, 3 µm particle size, guard column, 10x2 mm, 3 µm) was used with a gradient elution, starting with 30% B at time zero linearly rising to 90% B at 8 min. Then, 90% B was held for 0.5 min, decreased to 30% B over 0.5 min which was held again for 3 min until the next run.

The SPATT discs and the transplanted mussels were analysed using a 2795 Waters HPLC equipped with a C_{18} ACE (30 mm x 2.1 mm) column coupled to a Micromass quadrupole-time-of-flight hybrid (Q-TOF Ultima) also equipped with a z-spray ESI source. The Q-TOF was used in TOF-MS-MS mode, where the molecular ion is isolated in the quadrupole and after fragmentation in the collision cell, the whole fragmentation spectrum is obtained in the TOF. The parameters of the Q-TOF are reported in Table 1. AZAs and PTX2 were analysed in positive ionisation mode with an isocratic run of 60% B for 7 min and OA, DTX2 and YTX were analysed in negative ionisation mode with an isocratic run of 55% B for 6.5 min.

Table 1: LC-MS Q-TOF parameters

	ESI +	**ESI -**
LC System	2795 Waters HPLC	2795 Waters HPLC
MS System	Micromass Q-TOF Ultima z-spray ion source	Micromass Q-TOF Ultima z-spray ion source
Column	C8-ACE 30 x 2mm, 3 µm particle size, 0.2 µm particle filter	C8-ACE 30 x 2mm, 3 µm particle size, 0.2 µm particle filter
Column Temperature	30 ºC	30 ºC
Mobile Phase Composition	60 % mobile phase B	55 % mobile phase B
Run Length	7 min	6.5 min
Flow Rate	0.25 ml/min	0.20 ml/min
Injection Volume	5 µl in partially filled 50 µl loop	5 µl in partially filled 50 µl loop
Capillary Potential	2.8 kV	2.8 kV
Desolvation Temperature	350 ºC	350 ºC
Source Temperature	140 ºC	140 ºC
Desolvation Gas Flow	Nitrogen at 450 l/h	Nitrogen at 450 l/h
Cone Gas Flow	Nitrogen at 50 l/h	Nitrogen at 50 l/h
Cone Voltage	35 V	35 V
Scan Time	0.5 s	0.5 s
Collision Energy	50 V	10 V

Results and discussion

Water samples were taken on a weekly basis in Killary-middle during the study period using a long tube. Known toxin-producing dinoflagellates were only found at one occasion, on the 22nd of August 2005 where *D. acuta* reached 160 cells/l. This explains the increase in OA and DTX2 obtained in both SPATT discs and transplanted mussels on the 24th of August as shown in Figure 1.

During the three weeks prior to the toxic event, the SPATT discs indicated a constant level of OA and no DTX2. The presence of *D. acuta* led to an increase of OA and to the appearance of DTX2 confirming previous study (James *et al.*, 2006). The amounts of toxins that accumulated on SPATT discs were significantly different along the water column and usually accumulated several micrograms of toxins in all sampling stations (data not shown). The transplanted mussels accumulated quantifiable levels of OA and DTX2 only once during the study despite the presence of toxins in the water. This suggests that the mussels were only able to accumulate the toxins when they were feeding on toxic plankton. The indigenous mussels did not accumulate toxins to a great extent even after the toxic event.

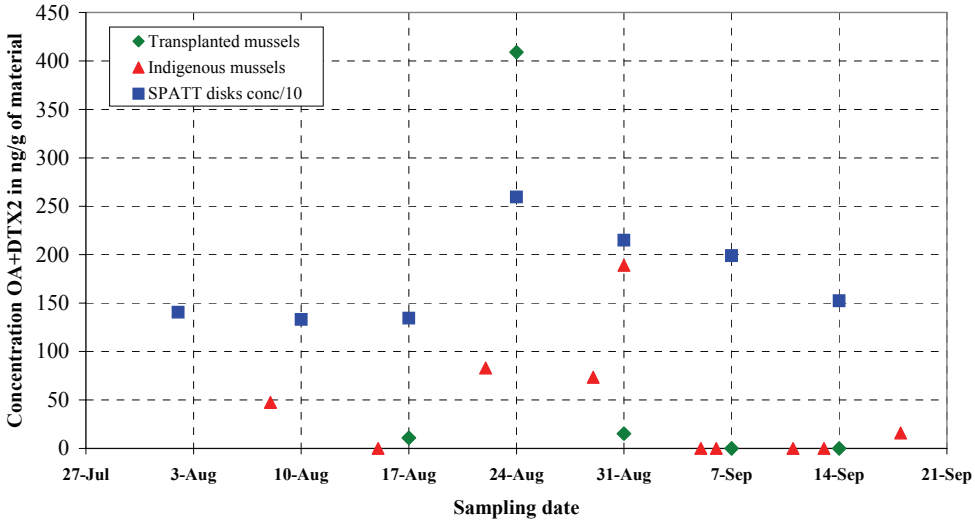

Figure 1. Concentration of OA and DTX2 found in SPATT, transplanted and indigenous mussels in Killary middle from the 27th of July until the 21st of September.

There could be two explanations for the large difference observed between the amount of toxins accumulated in the transplanted mussels and in the indigenous mussels: i) since the transplanted mussels were cooked when returned in the lab, the loss of water resulted in a concentration step as we are dealing with lipophilic toxins (Hess *et al.,* 2005). ii) the transplanted mussels were purchased after storage in clean water tanks that did not contain food and spent one to two days in the post, which resulted in a starvation period. Therefore, once back in the water, the bivalves fed intensively which would result in more accumulation. Early warning at one week was not observed in our conditions. This could be due to the oceanographic conditions leading to the toxic event. During the summer it is common in this area to observe distinct bodies of water separated by a thermocline.

Conclusions

The extraction procedure presented here shows a significant improvement compared to MacKenzie's method in terms of solvent and time consumption. In our oceanographic conditions and when using a SPATT retrieval frequency of one week, no early warning was obtained as the concentration of toxins in the SPATT increased at the same time as in the mussels. It was found that OA was present at relatively high levels in the water despite the absence of toxic plankton. However, shellfish did not accumulate toxins directly from the water.

B- Development of analytical reference method (WP2)

Introduction

The reference method described in the EC regulation for lipophilic toxins is the mouse bioassay (MBA) but there is growing acceptance of the need to develop and implement non-animal based methods of toxin detection. Hess *et al.* (2006) described the possibility of acceptance and validation of alternative methods. Recent advances in analytical instrumentation have enabled the development of alternative methods such as LC-MS. This is becoming the method of choice for the detection and quantification of several marine toxins and has been used by several research and monitoring laboratories following in-house validation (Hess *et al.,* 2005, Aasen *et al.*, 2005, McNabb *et al.,* 2005, MacKenzie *et al.,* 2005, James *et al.,* 2003, Lehane *et al.,* 2002, Draisci *et al.,* 2000, Puente *et al.,* 2004, Stobo *et al.,* 2005 and Suzuki *et al.,* 2003).

The ACQUITY ultra-performance liquid chromatograph (UPLC) is a novel chromatographic system employing 1.7 μm stationary phase particles in an elevated pressure system. This allows faster analysis times at the same flow rates, and the improved separation and peak shape affords higher sensitivity and selectivity at the detector (Schwartz, 2005). Multiple reaction monitoring (MRM) experiments using a tandem quadrupole mass spectrometer are generally considered the best method for optimal quantitative and confirmatory analytical performance. However, the fast switching required between transitions in a multi-analyte method, with the fast run times afforded by UPLC, exceeds the capability of traditional instruments. The latest generation of mass spectrometers utilise a travelling wave device which clears the collision cell very rapidly between transitions, thus allowing fast switching and shorter dwell times, meaning more data points can be acquired across the narrow peaks. The travelling wave device described here is similar to that described by Kirchner (1993). Improved electronics also allow for fast polarity switching, allowing for analysis of positive and negative ions at the same retention time (Giles *et al.,* 2004).

Material and methods

Reagents

HPLC grade methanol and water were purchased from Fischer Scientific, Loughborough, UK. Chromasolv HPLC Acetonitrile (ACN) and ammonium formate (97 %) were purchased from Sigma-Aldrich, Steinheim, Germany and formic acid (98 %) from BDH laboratory, Poole, UK. OA, PTX2, YTX, gymnodimine (GYM), spirolide 13-desMe-C (SPX 13-desMe-C) certified calibration solutions and a mussel tissue reference material (CRM) CRM-DSP-Mus-b were obtained from the National Research Council (NRC), Halifax, Canada. AZA1

was isolated in the Marine Institute from naturally contaminated mussels from the South West of Ireland (Rehmann *et al.,* 2006).

UPLC-MS/MS analysis

An Acquity UPLC system coupled to a Quattro premier XE mass spectrometer (Waters-Micromass, Manchester, UK) equipped with a Z-Spray ESI source was used. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (1.7 µm 2.1 x 100 mm) equipped with an in-line 0.2 µm Acquity filter. Mobile phase A was 100 % aqueous and mobile phase B 95 % aqueous ACN, both containing 2 mM ammonium formate and 50 mM formic acid. A gradient from 30 % B rising to 90 % B was run over 3 min and then held for 1.5 min. At 4.5 min the gradient was set back to the initial composition and equilibrated for 2 min. The flow rate was set at 0.4 ml/min and 10 µl of each sample (maintained at 5ºC) was injected onto the column at 30°C. The electrospray source was operated simultaneously in both positive and negative mode by rapid switching. The capillary potential was set at 2.5 kV, desolvation temperature 350°C, source temperature 120°C, desolvation gas flow 850 l N_2/h, cone gas flow 50 l N_2/h. Analyses were performed in MRM mode with a collision gas flow of 0.32 l/h.

Where toxins were available in sufficient amount, cone voltages and collision energies were optimised on two fragment ions by infusion (quantification and confirmatory transitions). Where toxins were not available in sufficient amount for infusion, one MRM transition was set based on theoretical calculation or on those reported in the literature (Aasen *et al.* (2005), MacKenzie *et al.* (2005) and James *et al.* (2003). Cone voltages and collisions energies were set to those used for compounds with similar structures. Transitions, cone voltages and collision energies used in the method are reported in Table 2.

Calibration curves were obtained for the following toxins: OA, YTX, PTX2, AZA1, GYM and SPX 13-desMe-C. In order to calculate concentrations of compounds for which no calibration curves were generated, it was assumed that related analogues would give a similar response to that of the parent toxins.

Shellfish samples for selectivity assessment

Shellfish samples with different toxin profiles were used, including one extract from Norwegian mussel hepatopancreas, one from Japanese scallop (*Patinopecten yessoensis*) hepatopancreas as well as whole flesh mussel extracts from Ireland (*M. edulis*) and Italy (*M. galloprovincialis*). All extracts were made in 100 % methanol.

Extraction procedure of the certified reference material DSP-Mus-b

Triple methanolic extraction was performed by weighing 2 g of sample in a 50 ml plastic centrifuge tube to which 6 ml of methanol was added. The extracts were vortex mixed for 1 min at the highest speed and centrifuged for 15 min at 6,000 rpm. The supernatant was transferred to a 20 ml volumetric flask and the pellet was re-extracted in the same way. The third extraction was carried out by adding an additional 6 ml of methanol and blending the extract (ultraturrax) at 11,000 rpm for 1 min. The extract was centrifuged and the supernatant transferred to the volumetric flask with the two previous extracts. The final volume was made up to the mark using 100% methanol.

Table 2. MRM, cone voltages and collision energies of toxins incorporated in method

Compound	MRM	Mode	Cone voltage / V	Collision Energy / eV
GYM	508.3 > 392.4	positive	50	35
	508.3 > 490.4			24
SPX-13-desMeC	692.5 > 164.2	positive	50	60
	692.5 > 444.4			55
YTX	1141.5 > 1061.5	negative	40	55
	1141.5 > 925			55
45-OH-YTX	1157.5 > 1077.5	negative	40	55
Carboxy-YTX	1173.5 > 1094.5	negative	40	55
Homo-YTX	1155.5 > 1075.5	negative	40	55
Carboxyhomo-YTX	1187.5 > 1107.5	negative	40	55
45-OH-homo-YTX	1171.5 > 1091.5	negative	40	55
Carboxy-hyroxy-YTX	1189.5 > 1109.5	negative	45	40
OA & DTX2	803.5 > 255.2	negative	70	50
	803.5 > 113			65
DTX1	817.5 > 255.5	negative	70	65
	817.5 > 113			90
AZA1 & AZA1b	842.5 > 654.5	positive	50	55
	842.5 > 362			55
AZA2	856.5 > 672.5	positive	30	55
	856.5 > 654.5			45
AZA3	828.5 > 362	positive	50	55
	828.5 > 640.5			55
PTX2	876.5 > 823.5	positive	40	40
	876.5 > 212.5			50
PTX1	892.5 > 839.5	positive	40	25
PTX6	906.5 > 853.5	positive	40	25
PTX2sa & 7-epi-PTX2sa	894.5 > 805.2	positive	40	40

Results

Selectivity

Figure 2 shows the combination of chromatograms for all compounds monitored obtained from the analysis of several shellfish extracts using their quantification trace. All 21 toxins monitored eluted within 3.8 min with a total run time of 6.6 min. The first group of toxins to elute was the cyclic imine group with GYM and SPX 13-desMeC eluting at 1.18 and 1.52 min respectively. This was followed by the YTX group with 45-hydroxy-carboxy-YTX eluting at 1.95 min, 45-hydroxy-YTX and 45-hydroxy-homo-YTX both eluting at 2.29 min, and carboxy-YTX, carboxy-homo-YTX, 1-homo-YTX and YTX eluting at 2.32, 2.38, 2.92 and 3.07 min respectively. The chromatographic conditions did not allow for complete resolution of carboxy-YTX, carboxy-homo-YTX, 45-hydroxy-YTX and 45-hydroxy-homo-YTX similarly to other reported LC-MS method using the Hypersil C8 column, where carboxy-YTX and 45-hydroxy-YTX peaks overlapped (Ciminiello *et al.,* 2003). OA, DTX1 and DTX2 eluted at 2.86, 3.43 and 3.03 min respectively. The PTX group eluted between 2.31 and 3.09 min starting with PTX1 at 2.31 min, PTX6 at 2.46 min, PTX2sa at 2.79 min,

7-epi-PTX2sa at 2.98 min and PTX2 at 3.09 min. The last group to elute was the AZAs with AZA3 at 3.22 min, AZA1 and AZA1b at 3.44 and 3.35 min and AZA2 at 3.56 min.

In order to examine the efficiency of the separation of the UPLC method, the resolution of OA and DTX2 peaks was calculated and compared with the resolution obtained by the HPLC method in place at the Marine Institute (Hess *et al.,* 2005). The resolution obtained for OA and DTX2 by UPLC was 3.81 against 2.96 obtained by HPLC. The tailing factor for a typical OA peak was 1.05 for UPLC against 0.66 by HPLC. These results show that the chromatographic separation and peak shapes obtained on the UPLC system were better than those obtained by HPLC, even though the run time was decreased by a factor of two.

Accuracy

Accuracy of the UPLC-MS determination was checked by triple methanolic extraction, as described in section 2.4, of two separate portions of the CRM-DSP-Mus-b that contained 10.1 ± 0.8 µg/g of OA. Both extracts were subsequently diluted by a factor 20 and injected in duplicate. The concentrations obtained were 9.55 and 9.48 µg/g indicating an average of 93.7 % accuracy.

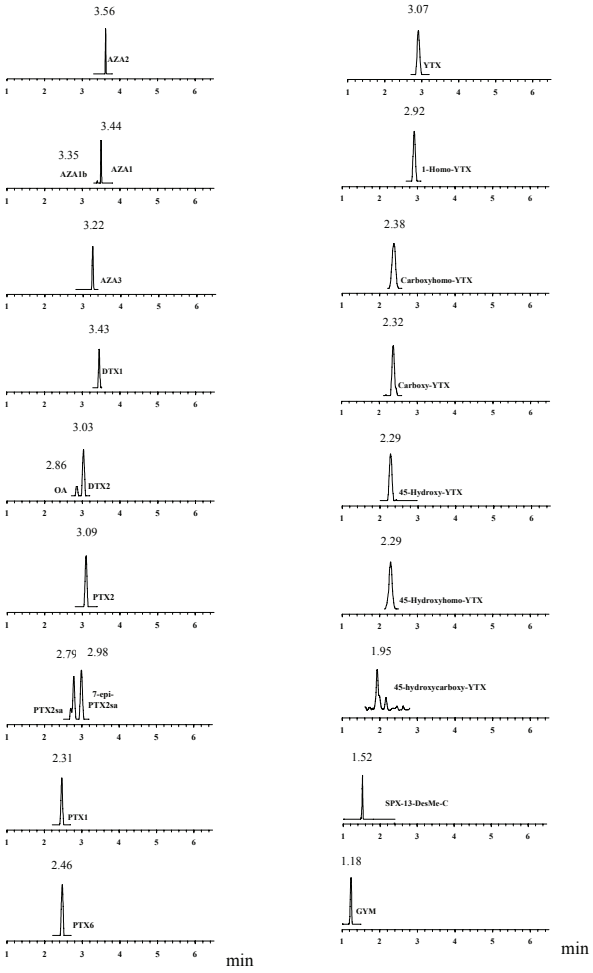

Figure 2. Combined UPLC-MS/MS chromatograms using the multi toxins method for lipophilic marine toxins

Calibrations

Calibration curves were generated for OA, YTX, PTX2, AZA1, GYM, and SPX 13-desMe-C. The equations, correlation coefficients, coefficients of variation and limits of detection obtained from standard injections are shown in Table 3. These results were obtained from triplicate injections. All correlation coefficients were ≥0.997 using a set of 10 standards for OA, YTX, AZA1 and PTX2 and a set of 6 standards for GYM and SPX-13-desMeC. The LODs ranged from 22 (SPX 13-desMeC) to 483 pg/ml (OA). Compounds analysed in positive mode showed LODs 10 times lower than those analysed in negative modes (based on lowest standards). The retention times were reproducible to ± 0.01 min over a 30 h run sequence comprising shellfish samples and standards (data not shown).

Table 3. Equations obtained for the calibration of OA, PTX2, AZA1 using 10 standards, and YTX, SPX 13-desMe-C and GYM using 6 standards. Values were calculated from triplicate 10 µl injections.

Toxin	**Slope** ± *SD*	**Intercept** ± *SD*	**Correlation coefficient** ± *SD*	**LOD** [a] **(pg/ml)**	**LOQ** [a] **(ng/ml)**	**Range of standards (ng/ml)**
OA	80.7 ± *1.2*	270.5 ± *39*	0.9970 ± *0.0012*	483.1	1.61	1.5 -232
YTX	31.9 ± *0.2*	-22.8 ± *7.5*	0.9969 ± *0.0009*	336.3	1.12	2.8 - 56
AZA1	877.0 ± *10.7*	-139.3 ± *188.1*	0.9996 ± *0.0004*	32.8	0.11	0.4 - 77
PTX2	769.9 ± *7.0*	188.5 ± *0.1*	0.9993 ± *0.0007*	47.7	0.16	0.5 - 96
GYM	1548.6 ± *1.18*	2478.2 ± *512.4*	0.9974 ± *0.0014*	60	0.2	1.5 - 111
SPX 13-desMe-C	1563.3 ± *59.5*	-318.85 ± *1086.3*	0.9966 ± *0.0037*	22	0.07	1 - 80

[a] LOD and LOQ were calculated from the lowest standard at S/N = 3 and S/N = 10 respectively

Discussion

The UPLC-MS/MS method described in this work allows the detection of 21 lipophilic toxins in a single run, which is the most comprehensive method reported to our knowledge. All 21 toxins monitored eluted within 3.8 min, with a total run time of 6.6 min demonstrating the speed of the method. Despite the fact that all toxins were not chromatographically resolved, the recent advances in instrumentation (last generation triple quadrupole MS) allows the monitoring of several transitions at the same time without loss in sensitivity. The method presented here covers the analysis of all the lipophilic toxins currently required under EU legislation apart from the OA and DTXs ester derivatives (DTX3), which are quantified by difference in the concentration of the parent compounds before and after hydrolysis. The Commission Decision 2074/2005 specifies that the MBA is the reference test for determination of lipophilic marine biotoxins in official control of food stuffs. However, for ethical reasons, laboratories are encouraged to use non-animal test methods, such as the LC-MS method proposed here. Some of the non-regulated, bioactive

compounds described here (SPX, GYM) may lead to false positive results by the MBA, and therefore to discrepancies with physicochemical methods, focusing on the determination of regulated toxins from the OA, AZA, YTX and PTX groups. Thus, the simultaneous determination of these bioactive compounds, in our method, may be useful in the interpretation of positive MBA results, where the regulated toxins are absent or present in low amounts.

The rapidity of the method is a strong advantage for monitoring laboratories, as it would allow a faster turnaround time of sample results compared to the current reference method and an increase in the number of samples that could be analysed on a routine basis. The selectivity (expressed as elution order) obtained on the BEH C18 column (1.7 µm 2.1 x 100 mm) was qualitatively identical to the C8 BDS Hypersil (2.1 x 50 mm, 3 µm) (Hess *et al.,* 2005 and Quilliam *et al.,* 2001) or the C18 ACE columns (2.1 x 30 mm, 3 µm) previously used in the author's laboratory (unpublished data – MI).

The method was found to be accurate, since the concentration found for the CRM-DSP-Mus-b standard extract was within the uncertainty of the certified concentration. However, it must be noted that matrix effects were minimised for this analysis since the high concentration of the material required a 1/20 dilution prior to injection. Linear ranges and LODs were established for six compounds, OA, AZA1, YTX, SPX 13-desMeC, PTX2 and GYM. The instrument was found to be more sensitive in positive mode than in negative mode and the LOQs for OA and DTXs, PTX, YTX and AZA were found to be well below the EU regulatory levels. OA had the highest LOQ of all the tested toxins, but the method was still sensitive enough to quantify 1/10 of the maximum regulatory limit (160 µg/kg).

Conclusions

A UPLC-MS method was successfully developed for the detection of 21 lipophilic marine biotoxins and proved to be a suitable and reliable method for rapid turnaround of analyses. All 21 toxins monitored eluted within 3.8 min with a total run time of 6.6 min demonstrating the speed of the method. Linear ranges and LOQs were established for OA, PTX2, YTX, AZA1, SPX 13-desMe-C and GYM.

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PREDICTING *ALEXANDRIUM* BLOOMS IN CORK HARBOUR

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The genus *Alexandrium* comprises 28 different species, some of which produce potent neurotoxins which if ingested by humans, give rise to symptoms referred to as Paralytic Shellfish Poisoning (PSP). Both toxin producing and non-toxic forms of one of these species, *Alexandrium minutum,* have been found in Irish coastal waters (Touzet *et al*., 2007). The distribution of the toxic form, which produces the toxins known as GTX2 and GTX3, appears restricted to Cork Harbour, where there has been a history of shellfish harvest closures due to positive PSP bioassays on shellfish.

The archive of the National Phytoplankton Monitoring Programme indicates that *Alexandrium* in Cork Harbour tends to proliferate, or bloom, in June. Smaller blooms have occurred between July and September in some years. Field results have shown that *Alexandrium* cell numbers do not increase before the end of May and achieve maximum cell densities in June. The blooms arise in the North Channel of Cork Harbour (Figure 1). This article shows how the onset of these blooms can be predicted from the simple premise that the cells grow at a rate governed by the amount of available light, so that they can photosynthesise and grow like other plants, and the water temperature. The overall observed increase in cell numbers represents the balance between growth and dilution by the tide.

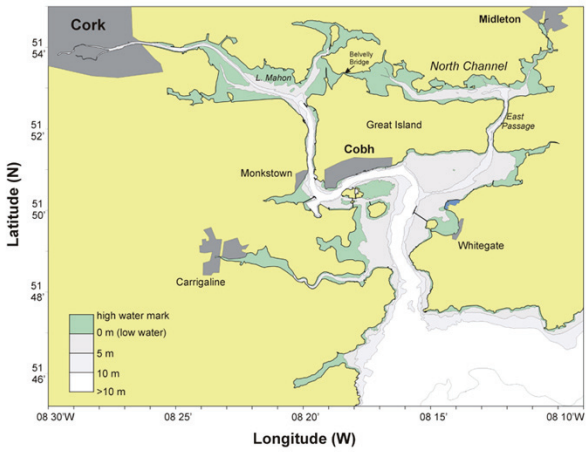
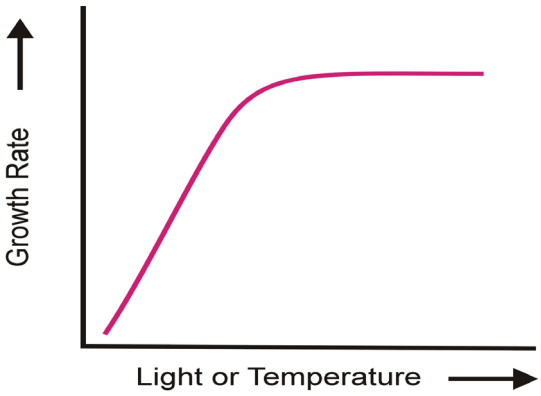


Figure 1. Map of Cork Harbour showing location of the North Channel

Alexandrium growth

Like other phytoplankton, *Alexandrium* grows through a process of cell division. The division of one cell producing two 'daughter' cells, and the subsequent division of each of the daughter cells, and so on, can produce very high cell densities in a relatively short time. Under optimum conditions it takes about 14 days to generate a bloom. Laboratory experiments on *Alexandrium* isolated from Cork Harbour have shown that the relationship between growth rate and both temperature and light is broadly similar (Figure 2). There is an initial increase in growth rate with increasing temperature and light, and there comes a point when any further increase in temperature (or light) does not increase the growth rate any more. We have experimentally determined that the values of temperature and irradiance where this happens are 15°C and 100 µM/ sq m/ sec. It takes about 30 hours for cells to divide when growing under optimum conditions of light and temperature.



The annual cycles of light and temperature in Cork Harbour are shown in Figures 3 and 4. It can be seen that the underwater irradiance is consistently at sub-optimal levels (less than 100 µM/ sq m/ sec) up until mid-March. Water temperatures attain 15 °C towards the end of May. Prior to this, water temperature is affecting growth rates. It is only once the water temperature has increased above 15 °C that maximum growth rates can be achieved.

Figure 2. The general relationship between growth of *Alexandrium* with light and temperature. Note that in both cases there comes a point where any further increase in light or temperature does not increase the growth rate further.

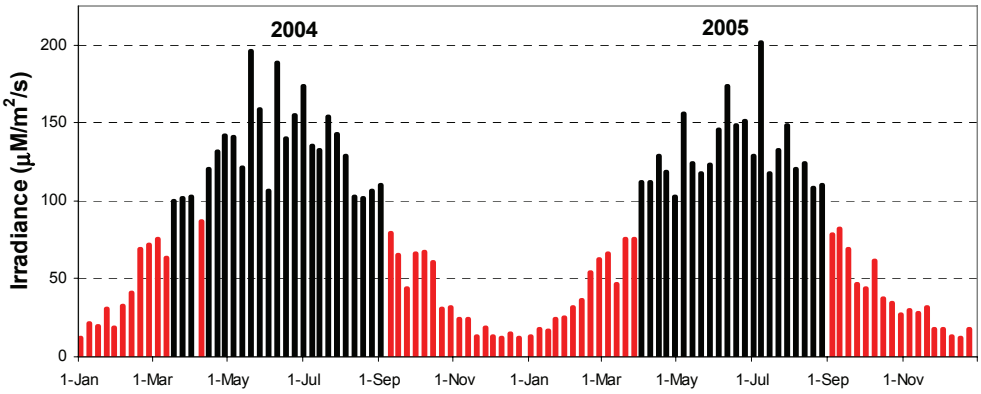


Figure 3. The annual cycle in the amount of underwater light, expressed as irradiance, available for *Alexandrium* to grow in the North Channel of Cork Harbour through 2004 and 2005. The data have been averaged into weekly mean values. Periods when light is not limiting growth are shown in black.

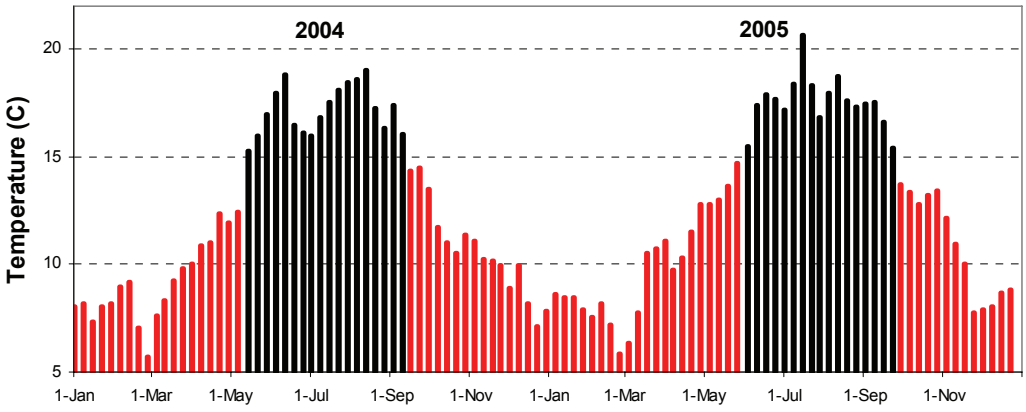


Figure 4. The annual cycle in sea water temperature in the North Channel of Cork Harbour through 2004 and 2005. The data have been averaged into weekly mean values. Periods when temperature is not limiting *Alexandrium* growth are shown in black.

Tidal Dilution.
The tidal range is the difference in height between a high tide and the subsequent low tide. The annual pattern in tidal range for Cork Harbour is shown in Figure 5. The spring-neap tidal cycle is immediately evident. There is also an additional pattern with the biggest spring tides occurring at the equinoxes in March and September and the smallest spring tides at the solstices in June and December. The tide flushes out, or dilutes, the North Channel at a rate which is directly related to tidal range.

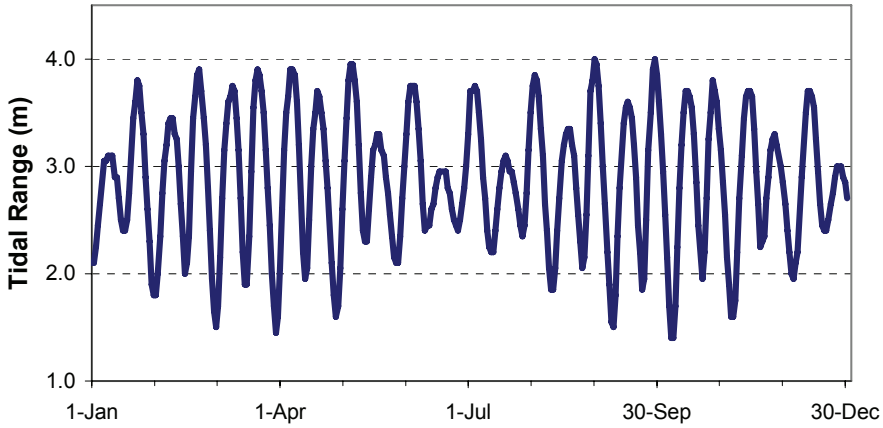


Figure 5. The annual variation in tidal range in Cork Harbour. The spring-neap cycle is clearly evident. It appears to be the small spring tides that occur in June which are important for the promotion of *Alexandrium* blooms in the North Channel. Tidal data are taken at Cobh.

At the equinoctial spring tides, *Alexandrium* cells are being flushed out of Cork Harbour at a rate quicker than their growth (or division) rate, even if the cells were dividing at their maximum division rate. Given that under optimum conditions it takes 2 weeks for cells to become sufficiently numerous to form a bloom and contaminate shellfish, then during most of the year spring tides are always diluting out the cells before a bloom can form. The biggest potential for a bloom is therefore when the spring tides are at their weakest, near the summer solstice.

Observations
Cell densities of *Alexandrium* in the North Channel of Cork Harbour observed in summer of 2004, 2005 and 2006 are shown in Figure 6. Blooms of cells occurred in June each year, with highest numbers observed in 2004 and lowest numbers in 2005. In each case water temperatures were above 15 ^{o}C when the bloom occurred. The tidal dilution rates are also shown in Figure 6 where it can be seen that the blooms achieve maximum cell densities following the first weak spring tide near the summer solstice. In any year this tide is nearly always the first spring tide in June.

It now appears that tidal dilution bloom control of *Alexandrium* is observed in other estuaries. For example, the Penzé estuary is a small estuary off the Bay of Morlaix in Brittany, France. Annual blooms occur here which also seem to coincide with the timing of the weak spring tides in June (Le Denn & Chapelle, 2006). Characteristics of the estuary are similar to the North Channel in Cork Harbour, i.e. a large intertidal region and generally shallow depth, with a pronounced spring-neap cycle in tidal range.

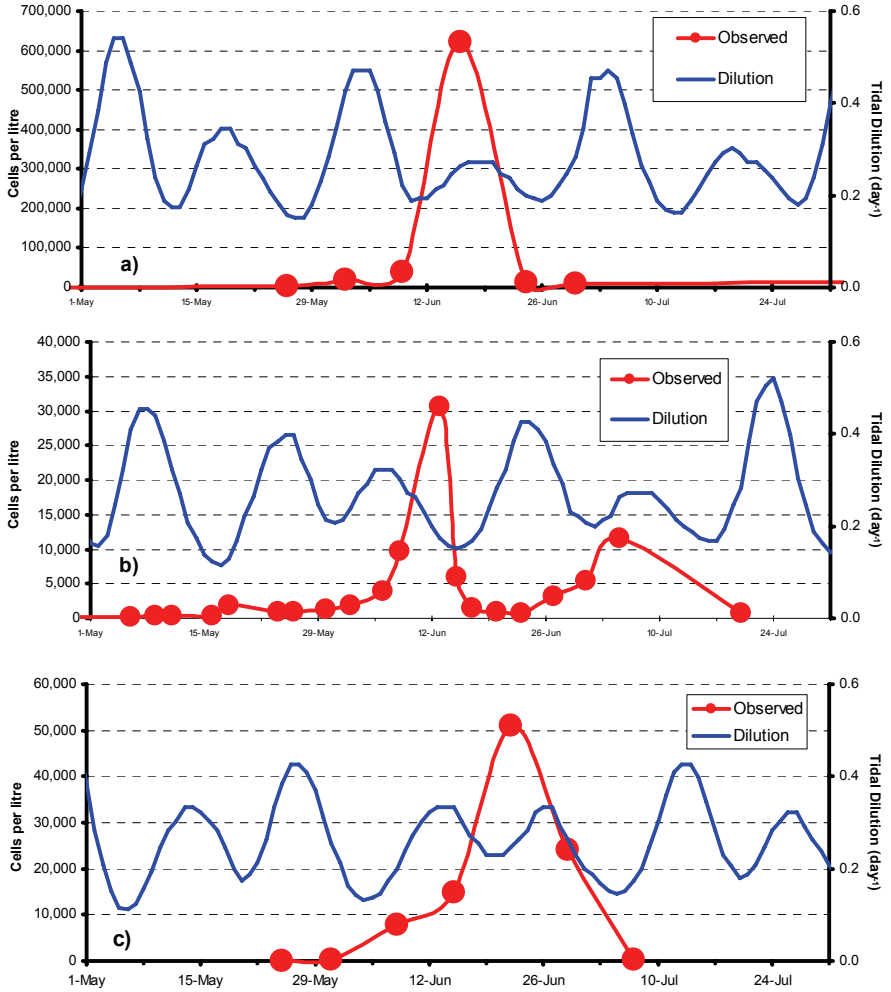


Figure 6. *Alexandrium* spp. cell densities and tidal dilution in samples taken from the North Channel in Cork Harbour in a) 2004, b) 2005, c) 2006. Although cell densities vary from one year to the next, the bloom peak occurs after the first (small) spring tide in June when tidal dilution is low over the spring tide.

Conclusions.

There is a strong link between the initiation of blooms of *Alexandrium* in Cork Harbour and tidal dilution. These blooms can therefore be predicted with confidence with knowledge of water temperature and tidal range. Given the regularity in the annual cycle of water temperatures in the area (Figure 4), a set of tide tables appears to be the main prerequisite for the successful prediction of *Alexandrium* blooms and PSP toxicity in Cork Harbour as blooms appear following the first spring tide in June.

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NUCLEIC ACID TESTS FOR TOXIC PHYTOPLANKTON IN IRISH WATERS-*PHYTOTEST*

Marine Strategic RTDI project AT/04/02/02-Research Update

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Rationale for the project:
The Phytotest project is a 3 year collaborative project funded through the Marine Strategic Programme in Advanced Technologies as part of the National Development plan 2000-2006. The project partners include the National Diagnostics Centre at NUI Galway and MI. The overall objective of the project is the development of nucleic acid tests (molecular methods) for the identification of key toxic phytoplankton species in Irish waters. In the final year of the programme the aim is to transfer the molecular methods developed in the project into MI to support their monitoring service. Currently, the monitoring for phytoplankton species in Irish waters is performed by light microscopy which can easily identify some plankton species based on distinctive morphological traits. Other species in particular, *Pseudo-nitzschia* spp. and *Alexandrium* spp. cannot be identified to species level by light microscopy. Identification of these species requires more sophisticated microscopic techniques such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM). These techniques cannot easily be integrated into a routine testing environment. Molecular methods utilise unique information contained within an organism's genome in order to identify it. This genetic information can be exploited in a range of molecular test platforms enabling microorganisms to be identified to species level. Additionally, there has been a major drive towards the development of highly automated platforms to support molecular tests for high-throughput testing in routine laboratory settings.

Summary of Project Tasks
The Phytotest project is organised as a number of discrete tasks which include:

1. Sourcing, isolation and culturing of *Dinophysis* and *Pseudo-nitzschia* species. The MI phytoplankton monitoring team are responsible for this task and are proactive in sourcing strains and wild samples for the project.
2. Generation of DNA sequence information for selected genomic targets in *Dinophysis* spp. and *Pseudo-nitzschia* spp. For *Dinophysis* species, *D. acuta* and *D. acuminata*, key DSP producers in Irish waters, the genomic targets selected for DNA sequencing included the small and large rDNA ribosomal sub-units (SSU and LSU). For *Pseudo-nitzschia* spp., the LSU and internally transcribed spacer regions (ITS1+2) were sequenced.
3. Design of the building blocks for molecular tests, DNA probes and PCR primers from the sequence information. To enable the design of PCR primers and DNA probes, sequence information generated in the project is compared with sequence information available in public databases (GenBank). Using selected bioinformatics tools, DNA

sequence regions suitable for the design of PCR primers and probes for the target organism are identified. Usually several candidate PCR primers and DNA probes are designed for each species of interest. They are tested for their ability to detect the species of interest and distinguish it from closely related species (specificity). The performance of the PCR primers and DNA probes for detecting different cellular concentrations of the species of interest is also performed (sensitivity).

4. Compilation of the *Dinophysis* spp. and *Pseudo-nitzschia* spp. tests onto specific test platforms. In Phytotest, real-time PCR (RT-PCR) and fluorescent *in situ* hybridisation (FISH) are the platforms being employed.
5. Performance testing is carried out using well characterised and wild samples.
6. The final task of the project due to commence in mid-2007 is the transfer of the molecular tests to the MI.

Overview of test methods:

FISH is a DNA probe based technique that has been applied to identify plankton cells (Adachi *et al.,* 1996; Scholin *et al.,* 1999; Touzet and Raine, 2006). The plankton cells are chemically fixed on a solid support and treated to make the cell wall permeable. A species-specific DNA probe labelled with a fluorescent reporter is added in a specific buffer (hybridisation buffer) to the cells. The probe binds or hybridises to a specific genomic target usually rRNA at a pre-determined temperature. Unbound probe is removed by a series of washing steps and the results of the DNA probe binding can be viewed by microscopy and the selected species identified.

Real-time PCR is a rapid and integrated *in-vitro* amplification and detection technology. The incorporation of specialised fluorescent dyes or labelled probes into the PCR reaction enables PCR product formation to be monitored in real-time. This provides the added advantage of eliminating the need for post-PCR processing while adding specificity to the PCR by including probes and minimizing the risk of contamination by performing PCR amplification and detection in a single closed tube system (Bustin, 2000). In this project, the LightCycler® and hybridisation probe technologies are being employed. Hybridisation probes also referred to as FRET probes or HybProbes comprise two specially designed sequence-specific oligonucleotide probes labelled with fluorescent dyes. The sequences of the two probes are designed to hybridise to the amplified DNA fragment in a head to tail arrangement. When the probes hybridise in this orientation, the two fluorescent dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited by the LightCycler™ LED (Light Emitting Diode) filtered light source, and emits green fluorescence at a slightly longer wavelength. This emitted energy excites the second dye (LC Red 640) attached to the second hybridisation probe which emits red fluorescence at an even longer wavelength. This energy transfer, referred to as FRET (Fluorescence Resonance Energy Transfer) is proportional to the increasing amount of PCR product generated. By combining hybridisation probe technology with an analysis function of the LightCycler™ software, melt peak analysis, it is possible to generate temperature specific melt peaks which can distinguish PCR products generated from the target species from PCR products generated from non-target species. For Phytotest, we have designed the species real-time PCR tests to have a common thermocycling regime providing the potential to identify a range of different *Dinophysis* spp. and *Pseudo-nitzschia* spp. in the same analytical run.

In addition to RT-PCR and FISH techniques, other molecular platforms including DNA probe sandwich hybridisation, molecular biosensors and microarray technology have been employed as diagnostic platforms for the identification of toxic phytoplankton species (Simon *et al.,* 2000; Metfies *et al.,* 2005; Medlin *et al.,* 2006). Another important consideration in the application of molecular tests for phytoplankton species identification is the optimisation of suitable sample preparation and nucleic acid extraction procedures. In Phytotest, the samples for testing are filtered onto a membrane and nucleic acids are extracted from phytoplankton cells using a commercially available kit, Qiagen Plant kit or an in-house method.

Results

Nucleic acid tests for Dinophysis spp.-D. acuta and D. acuminata:

The LSU D1-D2 region was PCR amplified from single *Dinophysis* species cells and the resulting PCR products were sequenced. Sequence information was generated for eighteen LSU D1-D2 regions and eight partial SSU regions of *Dinophysis acuta* and *Dinophysis acuminata* isolated from preserved wild samples from around the Irish coast. Sampling sites included Sheephaven Bay, Drumcliff Bay, Killary Harbour and Bantry Bay. Sequence alignments were performed with *Dinophysis* species sequences available from GenBank. Phylogenetic analysis revealed that Irish *D. acuta* clustered with *D. acuta* from the Scottish coastline. Irish *D. acuminata* clustered with *D. acuminata* from the Scottish coastline and isolates from USA, Sweden and Australia.

An internal D1-D2 region primer set was designed based on *Dinophysis* species LSU sequence alignments. DNA HybProbes were designed based on greatest sequence variation between species within this region, following analysis of the sequence alignments. A real-time PCR test was designed, incorporating the HybProbes, for the LightCycler™ for the specific detection and discrimination of *D. acuta* and *D. acuminata*.

The real-time PCR test yielded species-specific melt peaks at 48 ^{0}C for *D. acuminata* and at 61 ^{0}C for *D. acuta* (Figure 1). The specificity of the test for detection and discrimination of *D. acuta* and *D. acuminata* was confirmed using *D. acuta* and *D. acuminata* single cells and also using clones containing target LSU regions from *D. acuta* and *D. acuminata* (Figure 2). The specificity of the test for *D. acuta* and *D. acuminata* was also tested using a panel of phytoplankton species found in Irish waters (Table 1). The test only detected *D. acuta* and *D. acuminata* and did not cross-react with other species tested to date. Twenty-five preserved wild samples from the phytoplankton monitoring program were tested and there was good correlation between *Dinophysis* species cells detected by light microscopy and by the HybProbes (Table 2). The real-time PCR test detected *D. acuta* in all samples identified to contain *D. acuta* and in 90% of samples determined to contain *D. acuminata* by light microscopy.

The detection limit of this test was determined using serial dilutions (10^{8} to 10^{1} copies) of the *D. acuta* and *D. acuminata* clones as templates in the real-time PCR test (Figure 3). Detection limits of 1-10 copies were consistently achieved for both targets indicating that the test can detect 1 cell of these species.

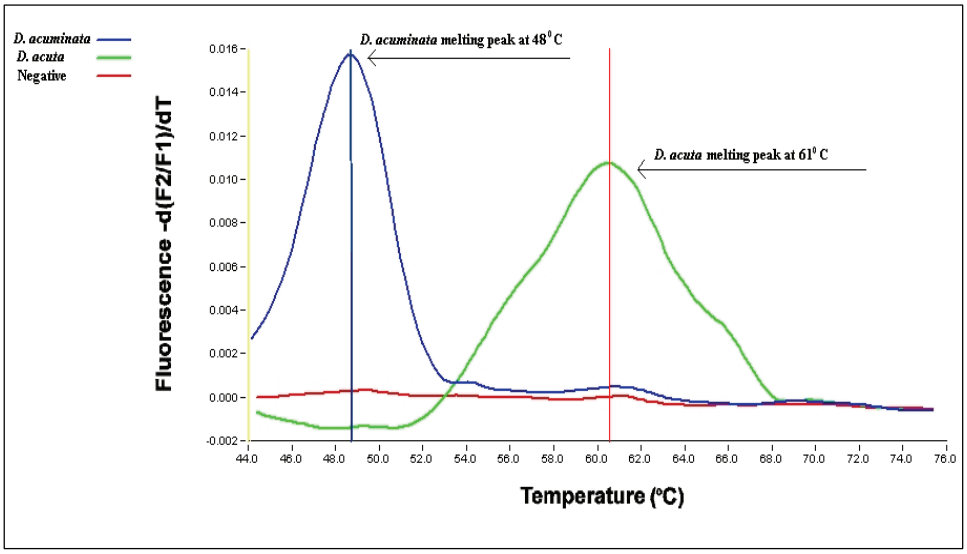

Figure 1. Real-time PCR (LSU) test for the detection of *D. acuta* and *D. acuminata* using HybProbes (FRET probes). *D. acuta* and *D. acuminata* are simultaneously identified in one test. The test target is the LSU D1-D2 region.

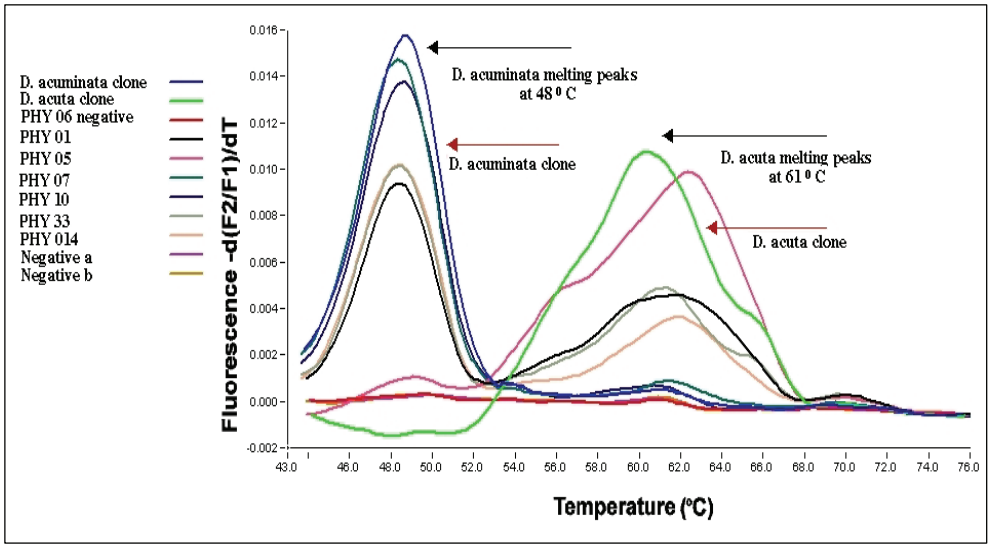

Figure 2: Real-time PCR (LSU) test designed for the detection of *D. acuta* and *D. acuminata* using HybProbes (FRET probes). Melt peak analysis of the HybProbes yielded melt peaks at 48 ^{0}C for *D. acuminata* cells and 61 ^{0}C for *D. acuta* cells. Two positive clones and samples obtained from the phytoplankton monitoring program were included in this run. Field samples tested were: PHY 01: 2 *D. acuminata* cells, 1 *D. acuta* cell, PHY 05: 2 *D. acuta* cells, PHY 07: 1 *D. acuminata* cell, PHY 10: 2 *D. acuminata* cells, PHY 33: 2 *D. acuminata* cells, PHY 014: 2 *D. acuminata* cells, 1 *D. acuta* cell, PHY 06: Negative - No *Dinophysis* species.

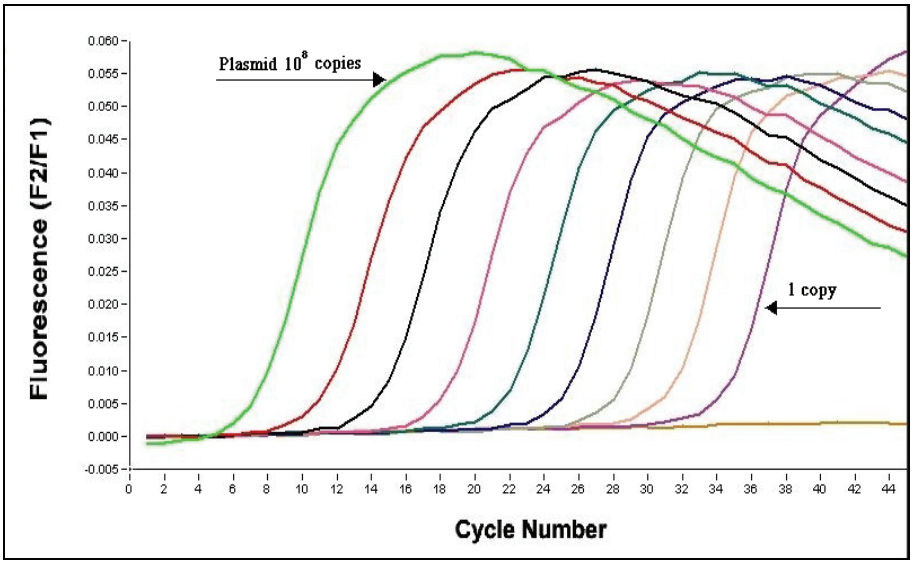

Figure 3. Quantification curves generated from serial dilutions of the *D. acuta* positive control clone in the *D. acuta/D. acuminata* real-time PCR test.

Table 1: Panel of species tested in the *D. acuta/D. acuminata* real-time PCR test.

Phytoplankton species tested with HybProbe probes	Melting peaks observed
Dinophysis acuta	61 ^{0}C
Dinophysis acuminata	48 ^{0}C
Pseudo-nitzschia australis	No melting peak observed
Pseudo-nitzschia fraudulenta	No melting peak observed
Pseudo-nitzschia delicatissima	No melting peak observed
Alexandrium minumum	No melting peak observed
Alexandrium tamarense	No melting peak observed
Alexandrium fundyense	No melting peak observed
Alexandrium catenella	No melting peak observed
Prorocentrum dentatum	No melting peak observed
Chaetoceros debilis	No melting peak observed
Asterionellopsis glacialis	No melting peak observed
Gymnodinium cf species	No melting peak observed

Table 2: Wild Samples tested in the *D. acuta/D. acuminata* real-time PCR test.

Sample #	Cell number/type observed in Phytoplankton Monitoring Program	Melt Peak 48 ^{0}C	Melt Peak 61 ^{0}C
PHY0(62800)1	2 *D. acuminata*, 2 *D. acuta,* 1 *D. dens*	+	+
PHY0(62800)5	2 *D. acuta*	-	+
PHY0(62700)6	4 *D. acuminata*	+	+
PHY0(62700)7	1 *D. acuminata*	+	-
PHY0(62701)0	2 *D. acuminata*, 1 *D. acuta*	+	+
PHY0(62003)3	2 *D. acuminata*, 1 *D. acuta*, 1 *Phalachroma rotundatum*	+	+
PHY0(62701)1	8 *D. acuminata*, 1 *D. dens*, 1 *Phalachroma rotundatum*	+	-
PHY0(62700)8	1 *D. acuminata*, 1 *Phalachroma rotundatum*	+	-
PHY0(62401)4	2 *D. acuminata*, 1 *D. acuta*	+	+
PHY0(62701)3	7 *D. acuminata*	+	-
PHY0(62402)8	1 *D. acuminata*	+	+
PHY0(61903)3	5 *D. acuminata*	+	-
PHY0(62700)2	1 *D. acuminata*	+	-
PHY0(62501)1	2 *D. acuminata*, 1 *Phalachroma rotundatum*	+	+
PHY0(61801)4	1 *D. acuminata*	+	-
PHY0(62001)6	1 *D. acuminata*, 1 *Phalachroma rotundatum*	+	-
PHY0(62402)7	1 *D. acuminata*	+	-
PHY0(62503)3	2 *D. acuminata*	+	-
PHY0(62004)5	3 *D. acuminata*	+	-
PHY0(62600)8	4 *D. acuminata*	+	-
PHY0(62001)7	5 *D. acuminata*	+	+
PHY0(62703)3	6 *D. acuminata*	+	-
PHY0(62404)6	1 *D. acuminata*	-	+
PHY0(62405)7	1 *D. acuminata*	-	+
PHY0(62405)8	1 *D. acuminata*	+	+

Nucleic acid tests for Pseudo-nitzschia spp.:

Cultures of indigenous *Pseudo-nitzschia* spp. and species donated by Dunstaffnage Marine Lab, Oban, Scotland were set-up by the Phytoplankton Monitoring Laboratory at MI. The LSU D1-D2 region was PCR amplified from a DNA extract generated from the indigenous *Pseudo-nitzschia* culture and the resulting PCR products were sequenced. Sequence analysis revealed that the culture organism was *P. fraudulenta*. Sequence alignments were performed using ITS1-ITS2 region sequences for a range of *Pseudo-nitzschia* species downloaded from GenBank and PCR primers were designed to amplify the ITS1 and ITS2 rDNA regions. ITS regions 1 and 2 were PCR amplified from DNA extracts generated from all of the *Pseudo-nitzschia* spp. cultures and the resulting PCR products were sent for sequencing. The ITS

region 1 primers were used to generate PCR products for sequencing from preserved samples taken from the MI phytoplankton monitoring program. *P. delicatissima* was identified in preserved samples from Selax, Invern, Hawks Nest and *P. australis* was identified in a preserved sample from Red Flag. Phylogenetic analysis of the sequence information revealed that Irish *P. fraudulenta* and Scottish *P. australis*, and *P. delicatissima* species sequences formed clusters with corresponding *Pseudo-nitzchia* species from the USA and Denmark.

Sequence alignments were performed using ITS1 and ITS2 sequences and ITS1-2 sequence information for other *Pseudo-nitzschia* species available from GenBank. DNA Hybprobes were designed for *P. australis*, *P. fraudulenta* and *P. delicatissima* based on analysis of sequence information for the ITS region 1. Three independent real-time PCR tests were designed for the identification of *P. australis*, *P. fraudulenta* and *P. delicatissima*. *P. australis* produces a melt peak at 57 ^{o}C with *P. australis* specific probes. *P. fraudulenta* produces a melt peak at 55 ^{o}C, with *P. fraudulenta* specific probes and *P. delicatissima* produces a melt peak at 60 ^{o}C with *P. delicatissima* specific probes. The specificity of the tests for detection and discrimination of individual *Pseudo-nitzschia* species was confirmed using DNA from all available *Pseudo-nitzschia* species. The *P. australis* real-time PCR test detected the presence of *P. australis* in a DNA extract of 173 cells isolated from a preserved seawater sample (Figure 4). This seawater sample was taken at Castlemaine Harbour as part of the routine phytoplankton monitoring program and was associated with a toxic event in April 2005. The identity of the organism as *P. australis* was further confirmed by sequencing of the LSU D1-D2 region and Transmission Electron Microscopy (TEM). Published FISH probes (Miller and Scholin 1996) for the identification of *P. fraudulenta, P. australis and P. delicatissima* are currently being investigated using the whole-cell hybridisation method.

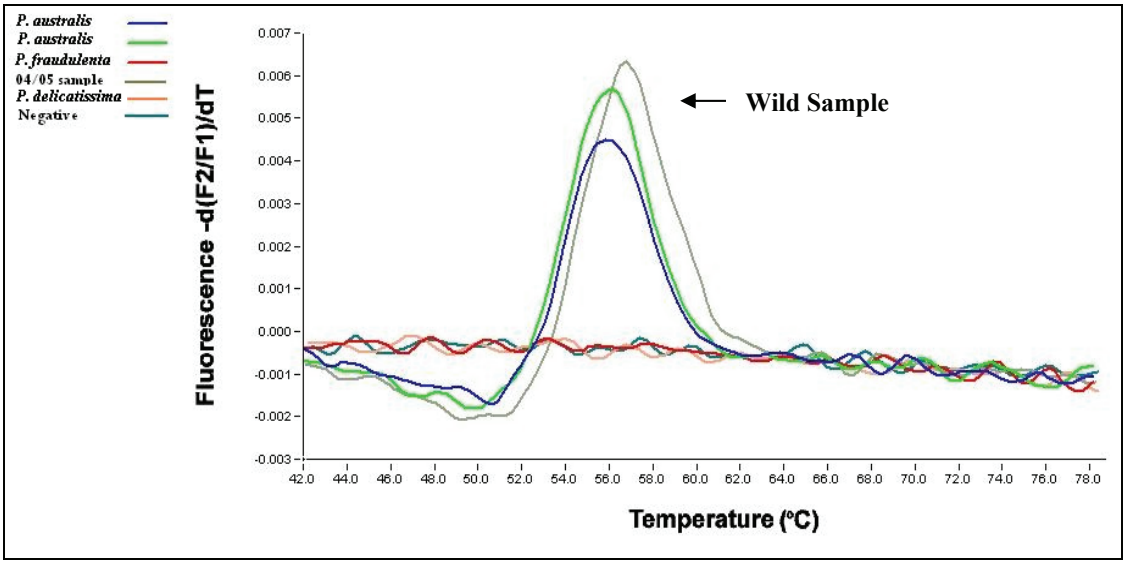

Figure 4. Real-time PCR (ITS-1) test designed for the detection of *P. australis* using FRET probes. Melt peak analysis of the FRET probes yielded a melt-peak at 57 ^{0}C for *P. australis*.

Conclusion and Future Work:

In the Phytotest project to date, real-time PCR tests on the LightCycler incorporating hybridisation probes (HybProbes) have been designed for the identification of *D. acuta*, *D. acuminata*, *P. fraudulenta, P. australis* and *P. delicatissima*. The application of these nucleic acid tests for the identification of these species in wild samples has been demonstrated. Evaluation of the real-time PCR tests is continuing. In parallel, the evaluation of FISH probes for the identification of *Pseudo-nitzschia* spp. is ongoing. The aim for 2007-2008 will be to optimise these real-time species assays as quantitative real-time PCR tests and to transfer the tests to MI.

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Risk Management & Communications in Shellfish – The Arcachon Case

Olivier Laban & Sylvie Latrille
Section Régionale de Conchyliculture (SRC), Arcachon

Introduction (by Chairman, Nicolas Ranninger, BIM Paris)
I would like to take the opportunity to look into biotoxin issues from a business and in particular, a market angle. Mollusc farming is expected by the FAO, to reach 10 million tons by 2010. The market outlook for bivalves farming looks promising. However, in the last few decades, bivalve farmers have been regularly exposed to harmful algal blooms, which affect their :

*Production level***,** caused by anoxia of inshore waters
A good example of this occurred in Ireland during the summer 2005, caused by *Karenia mikimotoi.*

Marketing level caused by biotoxins
The presence of biotoxins leads to the closures of farming sites. This directly effects the farmers business (turnover, cash flow, etc.), but also effects the market (product image and consumer confidence)

It is the damage in product image and consumer confidence which is the most difficult to measure, but has the most long-term devastating results. Due to modern media networks, the impact of a closure due to biotoxins can spread rapidly, far beyond the farming site, affecting the national and sometimes the international markets.

The possible presence of natural toxins in fish and shellfish has been known for a long time. Most of these toxins are produced by species of naturally occurring marine algae (phytoplankton). There are over 4,000 species of marine algae, but only 70-80 species (~2%) are known to produce toxins, (Scoging, 1998).

Outbreaks of foodborne disease attract media attention and raise consumer concern. However, cases of foodborne illness occur daily in all countries, from the most to the least developed. As most of these cases are not reported, the true dimension of the problem is unknown. On a worldwide basis, marine algal toxins are estimated to be responsible for more than 60,000 intoxication incidents per year, with an overall mortality rate of 1.5% (Source F.M. Van Dolah NOAA). However this must be tempered by the overall food intoxication, for example in the United States alone, there are some 76 million cases of foodborne illness, resulting in 325 000 hospitalisations and 5000 deaths (source WHO).

The question for the bivalves farming sector is how to find the correct balance between food safety, (consumer satisfaction) and farming viability, (minimum closing period and most importantly minimum market impact). In addition, how do you manage and control food & media crises in a period were western consumer sensitivity to risk has never been as high?

I sincerely thank Olivier Laban and Sylvie Latrille (vice presidents of the SRC, the French producer organisation of the Bassin d'Arcachon and respectively oyster farmers and packers) for having accepted our invitation to this workshop, to share their experience and thoughts about the crises they sector went through this summer.

Summary of Presentation by Olivier Laban & Sylvie Latrille

The Arcachon basin, in the south west corner of France, covers an area of 40 km2 at low tide and 156 km2 at high tide. The area contains 365 oyster farms, producing 12,000 tons of *C. gigas* oysters per year at a farm value of € 35 million per year. The basin also produces 600 tons of wild mussels and 600 tons of clams

Figure 1. The Arachon Basin

In 2006, during the risk period, the DSP monitoring of Arcachon was done weekly, when no DSP phytoplankton was identified in the water samples and twice a week if the presence of DSP plankton was identified, or if the Arcachon area was closed the previous week. The closures are specific to oyster or to mussels. Mussel bioassays are taken from one single area the Arguin Bank, whereas the oyster samples are taken from 3 different production areas namely: Arguin, Tés & Grand Banc. The basin is totally closed if one tested area is positive for the bioassay The bay can only be re-opened for harvesting, when bioassays are negative from all three zones twice consecutively with 48 hours gap. Water samples for phytoplankton analysis are taken weekly.

The French monitoring system moved to the 24 hour mouse bioassay in 2002. The French profession were always against this 24 hour test, as we believed that the 5 hour test had proven successful for 20 years.

During the period 2005 to 2006, the Arcachon basin experienced two periods of crisis. These were two years of serious pressure on the industry. The two periods of crises caused by repeated closures were:

April – August 2005
April – November 2006.

Due to the perverse effect of retroactive closures, test results which issue on a Wednesday/Thursday can lead to the destruction of product that has been packed and sold on Monday or Tuesday. During the busy period from September to December 2006, we faced a loss of turnover and a crisis in customer confidence

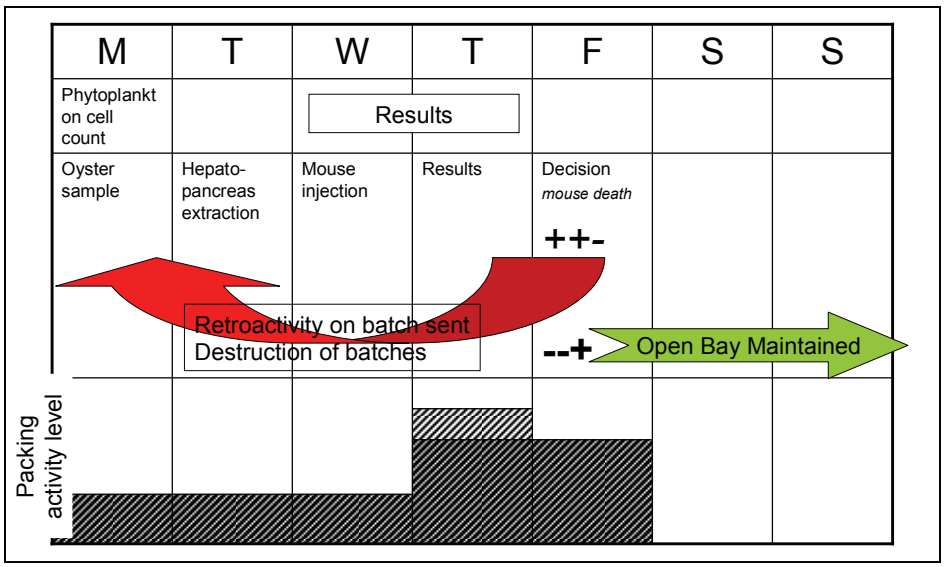

Figure 2. Weekly monitoring and sales activity.

On 6 September 2006, the SRC arranged a protest march in Arcachon to demonstrate against the criteria of the 24 hour mouse test. Photographs were taken of marchers carrying a "mock coffin" of an industry killed by the mouse (Figure 3).
People were in a hot tempered mood. Media photographs showed a fire burning outside the gate of the local Prefecture and riot police confronting the march.

Figure 3. Demonstrations against the criteria of the 24 hour mouse test (Source:Culture Marines)

However, on the same day, the French Ministry of Health issued a statement that two people had died in Arcachon, after eating oysters. There was a media explosion. While there was no immediate explanation for the deaths, there were suggestions of new toxic algal species. On the 7th September, there was a positive test result from the shellfish. However, no toxin or causative organism had been officially found in the shellfish by the laboratory.

Figure 4. Fire at the gate of the Prefecture of Arcachon. (Source:Culture Marines)

On 8th September, officials said that there was no link with one of the deaths and the consumption of oysters. On 14 September, the Arcachon basin was were re-opened after two consecutive negative bioassay results. Then, on 11th November the Prefect declared that none of the two deaths had been caused by the consumption of oysters.

In conclusion, 2 months of research and court cases were required to totally lift the suspicion of oysters in the deaths. The Arcachon story is not finished. We are seeking compensation from the State for the loss of sales. It is clear that industry's relations with the authorities had broken down. The communications were not well managed. We know that shellfish farming requires a lot of passion and that sometime this sector reacts strongly. However, there is a need for balance and trust between science and the industry side.

We recognise the need for monitoring the environment and for public health. However, we need to have a more transparent system of decision-making. It is the view of the French industry and of the European Mollusc Producers Association (EMPA) that closures should be based on a combination of test results, including phytoplankton, a 5 hours bioassay and chemical analyses.

Reference

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Appendix 1: List of Attendees

Surname	Forename	Organisation
Allison	James	DCMNR
Behan	Patrice	DIT
Bire	Ronel	Marine Institute
Brennan	Claire	NUIG
Connellan	Iarfhlaith	
Connery	Paul	DCMNR
Cooper	Lisa	Bantry Bay Seafoods
Coyne	Anthony	
Coyne	Patrica	
Cusack	Caroline	Marine Institute
Dabrowski	Tomasz	MarCon Computations International Ltd
Davidson	Keith	Scottish Association for Marine Science
DeBurca	Stiofan	DCMNR
Deegan	Bryan	Altermar
Devaney	Mike	Marine Institute
Devilly	Leon	Marine Institute
Donlon	Pete	BIM
Falvey	John	DCMNR
Farrell	Hazel	
Flannagan	Andrew	Public Analyst Laboratory
Flannery	John	Marine Institute
Foley	Barry	DIT
Gallagher	Edward	Irish Premium Oysters
Geary	Michelle	Marine Institute
Gilmartin	Maeve	Marine Institute
Harrington	John	Kush Seafarms
Hensey	Mary	Glan Uisce Teo
Henshilwood	Kathy	FSAI
Henson	Marie	DCMNR
Johnston	Maureen	Marine Institute
Keady	Evelyn	NUIG
Kennedy	Simon	
Kenny	Dermot	South East Shellfish Co-op Ltd
Kilcoyne	Jane	Marine Institute
Lardner	Caroline	Public Analyst Laboratory
Lawlor	Keelan	NUIG
Leitch	Jack	Charles River Laboratories
Lynch	Grainne	DCMNR
Lyons	Josephine	Marine Institute
Lyons	Vicky	BIM
Marie	McCarron	Marine Institute
McCarron	Maria	Marine Institute

McCarron	Pearse	Marine Institute
McElwee	Joe	IFA
McGrane	Paula	NUIG
McKeever	Dick	DCMNR
Mckeown	Martin	Clare Fish Ltd.
Milligan	Stephen	CEFAS
Minihane	Denis	Bantry Bay Seafoods
Moffatt	Rebecca	Marine Institute
Molloy	Pat	Connemara Seafoods
Mulligan	Steve	CEFAS
Murphy	Daniel	
Murphy	Patrick	
Murran	Sean	DCMNR
Nicholson	Gearoid	Marine Institute
Noklegaard	Tone	Marine Institute
Nolan	Glenn	Marine Institute
O'Boyle	Niall	
O'Byrne-Ring	Nuala	DIT
O'Carroll	Terrence	BIM
O'Connell	Michael	Blackshell Farms
O'Flynn	Sylvia	Public Analyst Laboratory
O'Loan	Brian	BIM
O'Malley-Quinn	Olivia	Connemara Seafoods
O'Shea	Dennis	Tralee Oyster Ltd
Pearse	McCarron	Marine Institute
Petersen	Andrew	En-Force
Pybus	Colin	GMIT
Rode	Daniela	Marine Institute
Rourke	Barry	Marine Institute
Salas	Rafael	Marine Institute
Scotter	Michael	CSL, UK
Shannon	Shane	Marine Institute
Smith	Breda	BIM
Swan	Sarah	Scottish Association for Marine Science
Tully	Oliver	BIM
Voitoux	Elodie	Marine Institute

Speakers

Burns	Kieran	Coastal Zone Administration Division, DCMNR
Clarke	Dave	Marine Institute
Doré	Bill	Marine Institute
Flynn	Richie	ISA
Fux	Elie	Marine Institute
Guilfoyle	Fergal	Marine Institute
Heffernan	Peter	Marine Institute
Hess	Phillip	Marine Institute
Kavanagh	Siobhan	NUIG
Keaveney	Sinead	Marine Institute
Laban	Oliver	Section Régionale de Conchyliculture, Arcachon
Latrile	Sylvie	Section Régionale de Conchyliculture, Arcachon
Lee	Ron	CEFAS
Lyons	David	FSAI
Maher	Majella	NUIG
McMahon	Terry	Marine Institute
Moran	Siobhan	Marine Institute
Murray	Lorna	FSA, Scotland
Ní Rathaille	Aoife	NUIG
O'Cinneide	Micheál	Marine Institute
Raine	Robin	NUIG
Ranninger	Nicolas	BIM
Silke	Joe	Marine Institute
Whelan	Peter	FSAI