Proceedings of the 8th Irish Shellfish Safety Workshop

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Edited by Terry McMahon, Bryan Deegan, Joe Silke and Micheál Ó Cinneide

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BIM Bord Iascaigh Mhara Irish Sea Fisheries Board

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INTRODUCTION AND OBJECTIVES OF THE 8th IRISH SHELLFISH SAFETY WORKSHOP

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

On behalf of the Marine Institute and our co-sponsors, BIM and the Food Safety Authority of Ireland (FSAI), I would like to welcome all the participants to Oranmore.

This is our 8th annual Shellfish Safety Workshop. We are glad to welcome colleagues from the regulatory agencies, universities, research labs and industry members from the Irish Shellfish Association, as well as visitors from Scotland, England and Northern Ireland.

Objectives

Our specific objectives for the 2007 Workshop are:

- **Communications:** Provide a forum for the partners in the Irish Shellfish Monitoring system to meet and take stock of developments and results for 2007
    - Build a shared understanding of risks/data on biotoxins and microbiology issues, in order to support risk management decisions
    - Provide a forum for debate and feedback.

- **Science**: This is an opportunity to present key findings (viruses in shellfish, biotoxins, gene probes and the toxicology of azaspiracids.) and to encourage scientific collaboration with agencies/researchers.

- **Benchmarking:** Review the evolution of shellfish safety systems in Ireland and in other EU countries such as the UK

- **Promotion:** Assist the Irish industry & State agencies (BIM) in the promotion of high Quality, safe Shellfish**.**

Key questions for research and rebate.

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

- Can we develop early warning tools and manage the risks of human viruses in shellfish?
- How far have we progressed on the Shellfish Water Directive?
- What were the seasonal trends in Irish toxicity in mussels and oysters in 2007?
- How can we use probes for rapid screening of phytoplankton?
- What lessons can we learn from the UK biotoxin system?
- What is future direction of the EU biotoxin and microbiology monitoring programmes?
- What are our future research priorities in shellfish safety?

Irish Shellfish Safety programme, a Snapshot of 2007

- The Shellfish Safety programme has evolved as a Partnership between MI + FSAI, SFPA, BIM and industry
- **Management Cell** was used for 35 decisions (year to date), which is down from 87 cases in the year 2006 (total of 276 in last 4 years). The Management Cell enables rapid decision making, according to protocols which were drafted by MSSC members
- Industry, SFPA, BIM & MI held 5 regional meetings in Autumn 2007 on the ***Good Practice Guide for Microbiological Monitoring***
- A key MI role is to manage an integrated programme of monitoring with 7,700 analyses of shellfish and phytoplankton
- MI has built up well developed **Quality** systems, with laboratory accreditation from INAB in the areas of phytoplankton, bioassay and chemical testing
- **95 %** of DSP/AZP results are reported by the Marine Institute within 3 days
- **Lower Toxicity** this year (**17%** of mussel samples tested positive in 2007. It was 29% in 2006!)
- MI has two research projects are ongoing – BIOTOX and Phytotest

Challenges for the Irish Molluscan Shellfish Programme from 2007

Our challenges for the coming year include:

- Department of Environment and DAFF (with the support of the SFPA, MI and BIM) will need to roll out a programme of Designations, under the Shellfish Waters Directive, 79/923
- MI will be working with FSAI and industry to build shared expertise in risk management of microbiology hazards, as we have done for biotoxins
- MI will work with industry and academic partners to implement new research programmes under *Sea Change* (for the period 2007 to 2013)
- Supporting the continued development of the Irish shellfish industry – no human illness, no product recalls.

Conclusion

The Irish shellfish industry has continued to show that is a sustainable marine sector with growth potential. The chart below (Figure 1) shows the steady growth in value of Irish molluscan shellfish from €7 million in 1990 to over **€63** million in 2006.

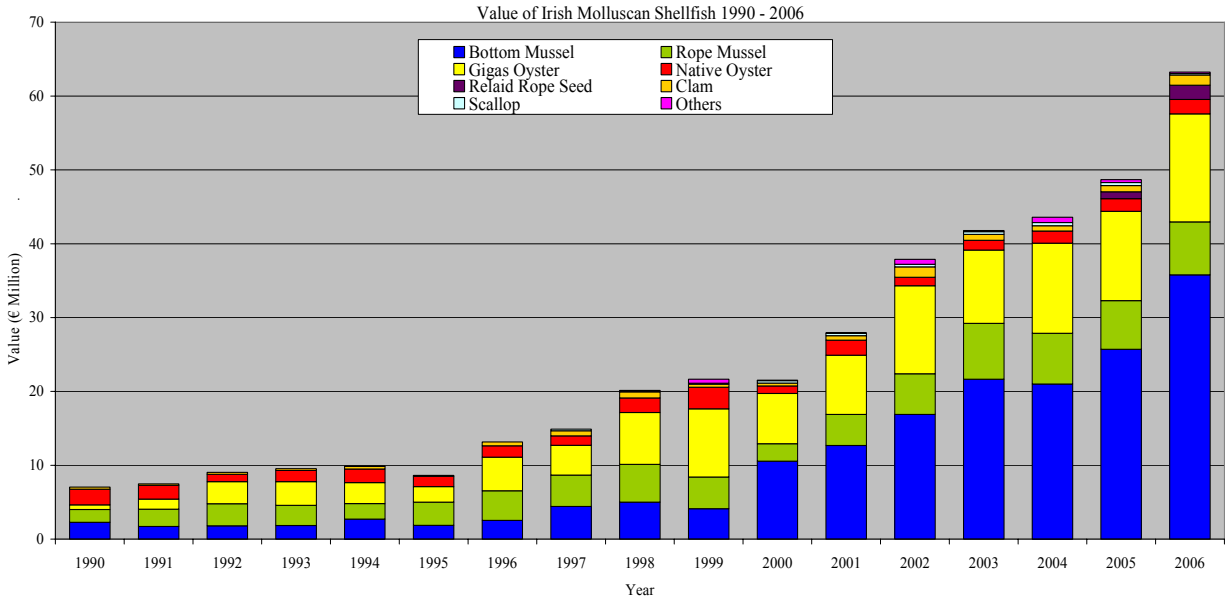


Figure 1. Value of Irish molluscan shellfish 1990-2006 (Source: Status of Aquaculture Report, 2006.)

The Marine Institute, BIM, FSAI and other agencies will work together to 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, sea fisheries officers**,** the laboratory staff and all the members of the shellfish safety team for your efforts in 2007. *Ár mbuiochas don bhFoireann uile !*

THE SEA-FISHERIES PROTECTION AUTHORITY AND THE MICROBIOLOGICAL MONITORING OF SHELLFISH PRODUCTION AREAS IN IRELAND.

Marie Henson, Sea Fisheries Protection Authority, Clonakilty, Co. Cork

The Sea Fisheries Protection Authority (SFPA) is an independent statutory agency established on 1st January 2007 under the provisions of the Sea Fisheries and Maritime Jurisdiction Act, 2006. The Authority is based in Clonakilty, Co. Cork, with offices in ports around the coast at Killybegs, Ros a Mhíl, An Daingean, Castletownbere, Dunmore East and Howth. The SFPA is the lead agency for the enforcement of sea-fisheries protection legislation and is an official agency for the enforcement of food safety law for the purposes of the Food Safety Authority of Ireland Act 1998.

The SFPA's mission is:

"To enforce Sea Fisheries Conservation legislation and Seafood Safety legislation fairly and consistently to ensure that the marine fish and shellfish resources from the waters around Ireland are exploited sustainably and may be consumed safely for the long term benefit of all"

The principal functions of the Authority are:

- To secure efficient and effective enforcement of sea-fisheries law and food safety law
- To promote compliance with, and deter contraventions of, sea-fisheries law and food safety law
- To detect contraventions of sea-fisheries law and food safety law
- To provide information to the sea-fisheries and seafood sectors on sea-fisheries law and food safety law and relevant matters within the remit of the Authority, through the Consultative Committee, or by any other means it considers appropriate
- To advise the Minister in relation to policy on effective implementation of sea-fisheries law and food safety law
- To provide assistance and information to the Minister in relation to the remit of the Authority
- To collect and report data in relation to sea-fisheries and food safety as required by the Minister and under Community law
- To represent or assist in the representation of the State at national, community and international fora as requested by the Minister
- To engage in any other activities relating to the functions of the Authority as may be approved of by the Minister

Consultative Committee

Section 48 of The Sea Fisheries and Maritime Jurisdiction Act 2006 provides for a Consultative Committee. This 'Sea-Fisheries Protection Consultative Committee' is an advisory body appointed by the Minister, and is made up of fourteen people drawn from the Fishing, Seafood Processing and Aquaculture Sectors, together with other marine experts. Their main functions include informing the SFPA of concerns and views of the sea-fisheries and seafood sectors regarding the functions of the authority to keep the sea-fisheries and seafood sectors generally informed of the applicable sea-fisheries law and food safety law, as well as standards, guidelines, practices and procedures operated by the Authority in relation to the enforcement of those laws. The Consultative Committee has no function in relation to detailed operational matters or individual cases or groups of cases with which the Authority is or may be concerned.

Complaints Officer

Independent of the SFPA under Article 49 of the Sea-Fisheries and Maritime Jurisdiction Act of 2006, the Authority will appoint a Complaints Officer from outside the organisation. Their role and responsibilities will include

- Consider and report on any complaint received.
- The SFPA must respond to the Complaint Officer's report and to the complainant.
- Issues subject to court proceedings/Ombudsman enquiries are excluded from their remit.
- Complaints must be made within 28 days of the alleged incident concerned.

Seafood Safety: Structure and Functions.

As the Competent Authority for fish and fishery products the SFPA is responsible for all aspects of seafood safety within the Irish fish and shellfish sectors and its overall structure is shown in Figure 1. In order to carry out its functions effectively, the SFPA have developed Service Level Agreements and Memoranda of Understanding with a number of agencies. Formal working relationships exist with:

- The Food Safety Authority of Ireland.
- The Irish Naval Service.
- The Irish Air Corps.
- The Marine Institute.
- An Garda Siochana.

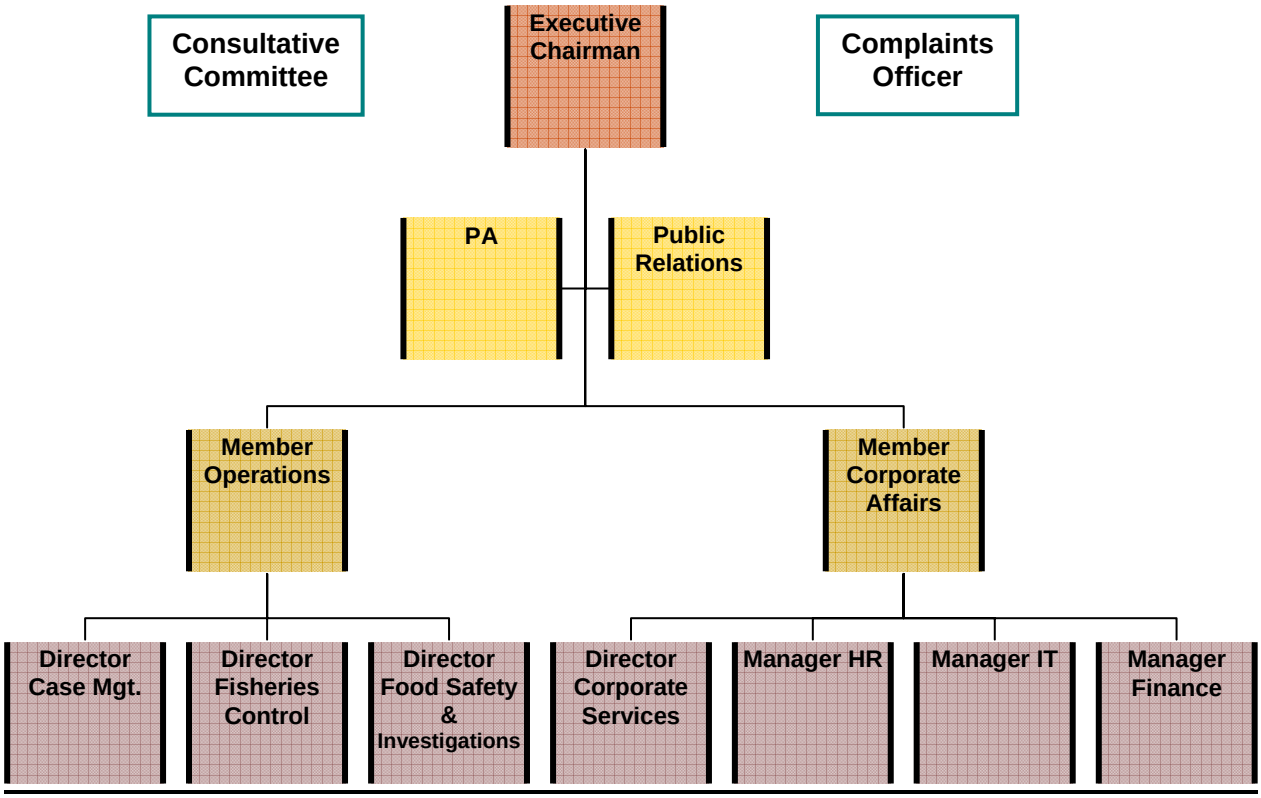
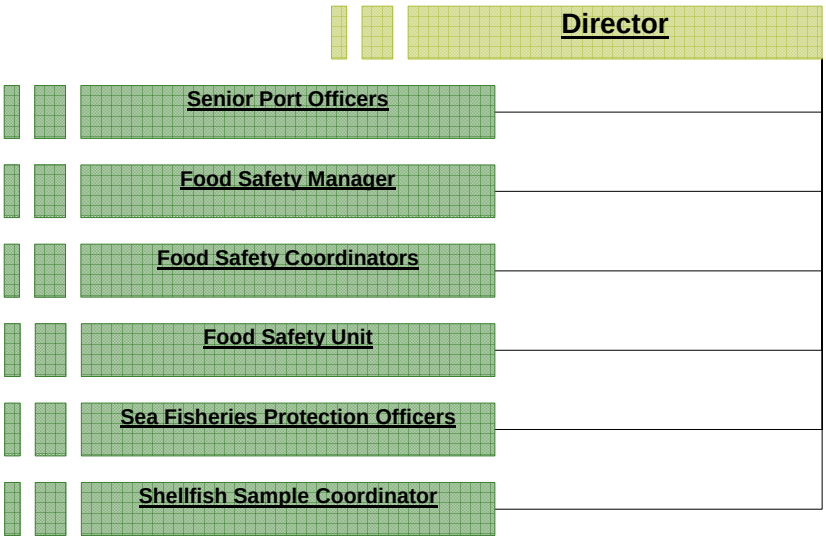


Figure 1. Sea Fisheries Protection Authority Structure

Within SFPA, seafood safety functions are managed nationally by the Director of Food Safety and Investigations, with input from Sea Fisheries Protection Officers, Senior Port Officers and other SFPA Personnel as described below.



The food safety activities undertaken by the SFPA include:

- The approval and/or registration of premises and vessels.
- The development of Codes of Practice and Standard Operating Procedures.
- The development of a Quality Management System.
- The application of import and export controls.
- The management of food incidents/outbreaks.
- Auditing of premises and vessels.
- Participation in the Shellfish Waters Management Committee.
- Participation in the Molluscan Shellfish Safety Committee.
- The classification of shellfish production areas, based on data from the microbiological monitoring programme.

Microbiological Monitoring of Shellfish Production Areas.

The Microbiological Monitoring Programme is devised and implemented by SFPA with scientific advice and support from the Marine Institute and input from the Molluscan Shellfish Safety Committee. It is designed to comply with the requirements of Irish and European Legislation, including:

1. Regulation (EC) 852/2004 on the hygiene of foodstuffs.
2. Regulation (EC) No 853/2004 laying down specific hygiene rules for food of animal origin.
3. Regulation (EC) 854/2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption.
4. Regulation (EC) 2073/2005 on microbiological criteria for foodstuffs.
5. The European Communities (Hygiene of Fishery product and Fish Feed) Regulations 2006 (SI NO 335 of 2006) giving effect to EU regulations in so far as they relate to fishery products in Ireland.

The annual sampling plan provides for monthly sampling of all active production areas, the Shellfish Sample Co-ordinator manages all data associated with the programme and drafts the annual classification order each year, based on the sample results received and the parameters specified in the legislation:

Parameters for the classification of bivalve mollusc harvesting areas.

Classification	Standard per 100g of LBM[4] flesh and intra-valvular fluid	Treatment required
A	<230 *E. coli* per100g of flesh and intra-valvular liquid[1]	None
B	LBMs must not exceed the limits of a five-tube, three dilution Most Probable Number (MPN) test of 4,600 *E. coli* per 100 g of flesh and intra-valvular liquid[2]	Purification, relaying in class A area or cooking by an approved method
C	LBMs must not exceed the limits of a five-tube, three dilution MPN test of 46,000 *E. coli* per 100 g of flesh and intra-valvular liquid.	Relaying for a long period or cooking by an approved method
Prohibited	>46,000 *E. coli* per 100g of flesh and intra-valvular fluid[3]	Harvesting not permitted

Notes: [1]By cross-reference from Regulation (EC) No 854/2004, via Regulation (EC) No 853/2004, to Regulation (EC) 2073/2005.
[2] By way of derogation from Regulation (EC) No 854/2004, the competent authority may continue to classify as being of Class B areas for which the relevant limits of 4,600 E. coli per 100g are not exceeded in 90% of samples.
[3]This level is not specifically given in the Regulation but does not comply with classes A, B or C.
[4]LBM: Live Bivalve Molluscs.

Scientific support for this programme is provided by the Marine Institute who, as Irelands National Reference Laboratory for microbiological and virological contamination of live bivalve molluscs, organise comparative testing between the various laboratories, audit all laboratories and monitor and advise on results.

Code of Practice for the Microbiological Monitoring of Bivalve Mollusc Production Areas.

This document has been devised by a Working Group with membership from the SFPA, The Food Safety Authority of Ireland, The Marine Institute, The Irish Shellfish Association and Bord Iascaigh Mhara. This Code of Practice draws on best practice in Europe and statutory requirements and outlines procedures for:

- Producing sampling plans and conducting sanitary surveys
- Requirements for sample collection and testing
- Procedures for making classifications, including data interpretation
- Communication
- Additional risk management procedures including reacting to high *E. coli* results

A series of regional meetings with industry have taken place to outline the provisions of the control system and provide a forum for discussion, it is intended that the final document will be published by SFPA in 2008. The current draft version of the document is available on www.sfpa.ie. It is envisaged that the 2008 Classification Order will be produced in line with the provisions of the Code of Practice. The dataset used to assign classifications will be larger, covering a time period of up to three years, and will allow for seasonal classification of production areas based on clear local trends supported by the available data. In addition to ongoing participation in the Molluscan Shellfish Safety Committee and the Shellfish Waters Management Committee, it is envisaged that SFPA will develop, and make available on the internet, maps of classified areas showing a range of detail including sampling points and production areas.

UPDATE ON NOROVIRUS SURVEY OF IRISH SHELLFISH HARVESTING AREAS

Sinéad Keaveney, John Flannery and Bill Doré
Shellfish Microbiology National Reference Laboratory (NRL), Marine Institute

Background and rationale for NoV survey in Ireland

There is currently no standard in EU legislation regarding the contamination of bivalve molluscs with human pathogenic viruses, namely norovirus (NoV) and Hepatitis A virus (HAV). However, the introduction of specific virus controls once standardised methods are available is viewed as a high priority. In recognition of this a CEN working group (TC 275/WG6/TAG 4 – Detection of viruses in food) in Europe is currently developing a standardised method based on quantitative real-time PCR for the detection of NoV and HAV in food, including bivalve molluscs. The work of this group represents a concerted effort towards the technical ability required for the inclusion of a virus standard in future EU legislation. However, uncertainty still remains about what such a virus standard should be and the impact on both public health and the shellfish industry of its introduction.

Previous surveys of oyster production areas in Ireland and elsewhere in Europe have generally been restricted to problematic harvesting areas that have been involved in outbreaks of illness or that are known to be highly polluted. Consequently, high levels of virus positive results have been recorded in these studies. In a study undertaken in Ireland over the winter of 2002/03, shellfish were tested for NoVs from 8 suspected problematic sites. NoV was detected by reverse transcription (RT)-PCR in 59 % of all samples tested during the study period (unpublished data). In a study in the UK NoV was found in 56 % of all samples analysed by RT-PCR in from one category B classified site (Henshilwood *et al.,* 1998). Similar results have been found in other European countries (Le Guyader *et al.,* 2000; Myrmel *et al.,* 2004; Croci *et al.,* 2007). These surveys shared a fundamental characteristic in that analysis was biased towards sites impacted by sewage pollution or associated with outbreaks of illness. Therefore, in this study a survey of oyster production areas in Ireland was conducted to provide information on NoV contamination in oysters from a range of representative sites using a real-time PCR method allowing relative quantitation of NoV levels. An additional feature of this survey was to consider the relative risk of virus contamination in shellfish harvesting areas by taking into account the occurrence and impacts of potential NoV contamination in an area in a desk based study.

The two main objectives from this survey were: (i) to gather information on the relative levels of NoV found in oysters in Irish shellfish harvesting areas using semi-quantitative real-time PCR, and (ii) could a simple risk matrix approach using existing data in a desk based study be used to determine the relative risk of NoV contamination?

Selection of sites and risk categorisation

A desk-based sanitary survey of each of the production areas was undertaken using available local information and data previously collected as part of the implementation of the Shellfish Waters Directive. Prior to the survey commencing, each site was ranked according to the expected extent of virus contamination based on factors such as population density, proximity to waste water treatment plants and level of sewage treatment. Subsequently each site was categorised into three levels depending on the risk of NoV contamination, namely low, medium and high (Table 1). Included in the 18 sites were two non-commercial control sites from the REDRISK study, Newport and Westport (Clew Bay), that were highly impacted by secondary and primary treated waste-water, respectively. The *E. coli* results, from the

REDRISK study, for Newport and Westport complied with category C and B classifications respectively under EU regulations.

Table 1. Desk based evaluation of NoV occurrence in each of the survey oyster production areas. Each site was ranked in order of the likely risk of NoV contamination determined by the desk based sanitary survey. Sites were subsequently categorised into high, medium or low risk of NoV contamination. The *E. coli* classification status of each area is given in brackets beside each site. Factors characterising the areas in each category are also given. Sites 1 (Newport) and 2 (Westport) were non-commercial high risk control sites. Independent *E. coli* analysis indicated a C and B classification respectively.

Site	Risk factors
High risk of NoV contamination	
1 (C)	• Close proximity to urban areas • Impacted by large population numbers • No/Minimal sewage treatment in area
2 (B)	
3 (B)	
4 (B)	
5 (B)	
Medium risk of NoV contamination	
6 (B)	• Smaller population numbers • Intermediate distance to pollution sources • Minimal sewage treatment in area • Level of sewage treatment unknown in area
7 (B)	
8 (B)	
9 (B)	
10 (B)	
Low risk of NoV contamination	
11 (B)	• Low population numbers in area • No immediate population in area • Low risk of sewage contamination (septic tanks) • No immediate risk of sewage contamination • Distant from human pollution sources
12 (A)	
13 (A)	
14 (A)	
15 (B)	
16 (B)	
17 (A)	
18 (B)	

Methodology

Sampling of the 18 oyster sites commenced in August of 2006. Alongside monthly samples of oysters collected routinely for the *E. coli* classification programme an additional 24 oysters were collected by Sea Fishery Protection Officers. Samples were transported to the laboratory by courier and were received within 48 hours under chilled conditions (< 15ºC). The hepatopancreas from 6 oysters were analysed for NoV using previously published real-time PCR procedures (Jothikumar *et al.,* 2005). Three replicates were tested for each sample in the real-time PCR assays for NoV GI and GII.

The real-time PCR assays for NoV provide semi quantitative data which can be used to compare NoV levels between survey sites. 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 can be determined by extrapolation from the standard curve. Appropriate standards for the absolute quantification of NV are not readily

available. However, close observation of the Ct values allows for relative quantification of the virus copy number. For the purposes of presenting the results from the first year of the virus survey the resulting Ct values were converted to PCR units. This makes use of the weighting provided by the number of positive replicates to yield a more usable value. A PCR unit is converted from the Ct value by assigning a value of 100 PCR units to a Ct value of 37. An increase in Ct value of 1 is considered to correspond to a dilution of 1 in 2 and therefore a Ct value of 38 would be assigned a PCR unit of 50, etc. The three replicates are assigned values in this manner and an average was taken, this was the final PCR unit assigned to that result. In this way a sample with Ct values of 37, 0, 0 is given a PCR unit value of 33.3 whereas a sample with CT values of 37, 37, 37 would be assigned a PCR unit value of 100, thereby weighting the fact the sample tested with three positive Ct values. *It must be noted that the values reported here as PCR units do not represent actual virus numbers. The current real-time PCR assay does not provide absolute quantitation, but provides semi-quantitative data indicating the relative levels of NoV in the survey samples.*

To assist presentation of the results NoV levels were categorised as negative (<25 PCR units), low level (25-99 PCR units), moderate level (100-399 PCR units) and high level (>400 PCR Units) on the basis of previous data from using the real-time PCR assay for NoV. However, it should be noted that *this is an indication of the relative level of NoV in the sample and is not intended as an assessment of the risk to public health.* The public health significance of NoV levels in shellfish remains unclear.

To put the results from the survey sites into context, control sites from the REDRISK study over August 2005 to June 2006 were included in the analysis of NoV data. Newport and Westport were used as positive control sites. It is important to note that the data used from these control sites was collected in the preceding twelve months to this survey (August 2005 to June 2006).

Results virus survey – August 2006 to July 2007

NoV results (PCR units) from samples collected during twelve month period of the survey (August 2006 – July 2007) are presented (Table 2.). The frequency of NoV contamination and the relative levels of NoV GII in each site as judged by the PCR units demonstrate a clear correlation with the risk category ascribed. In the high risk site category NoV levels were significantly higher than in the medium and low risk sites, with the highest levels recorded in samples taken during the winter months (November 2006 – January 2007). A similar seasonal trend was also observed in the medium risk site category where higher levels of NoV GII were recorded in January 2007 compared with other periods of the year.

Significantly, a high proportion of samples over the study period were negative for NoV. Of particular note is the fact that in oysters sampled in the low risk site category, all areas, apart from two, were free from NoV contamination. On the whole, the results from this study demonstrate that just over 40 % of commercial sites tested were free from NoV contamination throughout the study, including during the higher risk winter period.

In addition it is worth noting that NoV levels in the majority of samples from the commercial harvest areas were low. Only four results from these sites demonstrated levels above those designated as low (<99 PCR units). These results indicate a lower level of contamination than found in previously studies in Ireland and elsewhere in Europe. This is may be due to previous studies concentrating on high-risk problematic harvesting areas, but probably also reflects the relatively pollution free sites used for oyster production in Ireland compared with other parts of Europe.

Table 2. Results from NoV survey (August 2006 to July 2007). The frequency of NoV positive results and relative levels (PCR units) are shown for monthly samples from each site in each risk category.

Site	Aug 06	Sep 06	Oct 06	Nov 06	Dec 06	Jan 07	Feb 07	Mar 07	Apr 07	May 07	Jun 07	Jul 07
HIGH RISK												
1 (C)*	1125			133	1866	2753	497	71			47	
2 (B)*	353		41	214	215	2470	643					
3 (B)				53	25	89						
4 (B)			81	1070								
5 (B)						96						
MEDIUM RISK												
6 (B)						180	42		54	46		
7 (B)						42	30					
8 (B)			28			29						
9 (B)												
10 (B)						63	167	175				
6 (B)		29										
LOW RISK												
11 (B)												
12 (A)												
13 (A)												
14 (A)							79		32			
15 (B)												
16 (B)									40		89	
17 (A)												
18 (B)												

	No sample
	< 25 PCR units
	25 – 99 PCR units
	100 – 399 PCR units
	> 400 PCR units

* Sites 1 and 2 are non commercial high-risk control sites. NoV data for these sites was collected during August 2005 to June 2006 and are used to place results from the current survey sites in context.

The seasonal occurrence of NoV found in shellfish was demonstrated during the twelve month study period coinciding with the increased levels of NoV found in the general population during the winter months (November – March). Figure 1 demonstrates this seasonal peak of NoV found in the survey samples, where in February 2007 approximately 50 % of the samples tested from all 18 sites were positive for NoV.

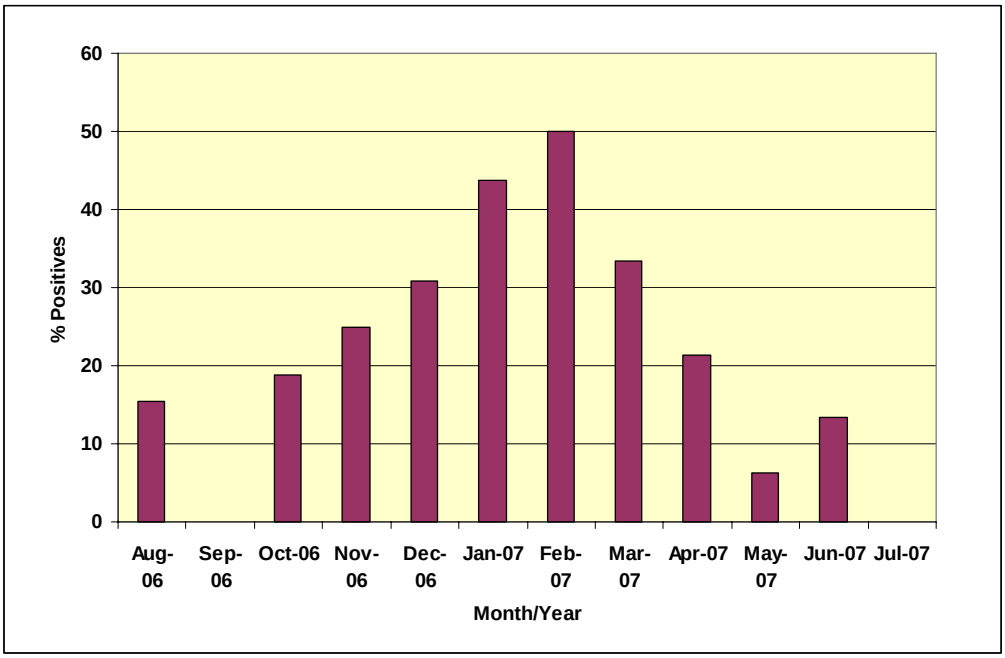


Figure 1. Seasonal occurrence of NoV found in samples collected from all virus survey sites over twelve month period of August 2006 to July 2007

Preliminary NoV data for year 2 of virus survey (August 2007 – October 2007)

Preliminary NoV data collected from the first three months of year 2 of the survey indicate a slight increase in the occurrence of NoV in samples collected from the medium risk category sites compared to the same time in 2006. NoV was not detected in samples from the low risk sites, which is also consistent with the time of year (summer, early autumn) and the reduced level of NoV found in the general community.

Conclusions from virus survey in Ireland – Year 1

i. The real-time PCR method applied to this study for NoV detection proved to be a robust and reliable procedure. The method provided semi-quantitative data allowing comparison of relative levels of NoV in oysters at the survey sites. This survey represents the first systematic application of real-time PCR to investigate relative NoV levels in a range of broadly representative harvest areas.

ii. Application of a simple desk-based risk assessment using existing, readily available data sources provided a relatively accurate indication of the relative risk of virus contamination in shellfisheries.

iii. The frequency of NoV positive samples in this survey were significantly lower than studies reported previously in Ireland and elsewhere in Europe. This probably reflects the fact that previous studies were targeted at problematic harvest areas and that harvest areas used for oyster production in Ireland may be impacted less by human sewage than more densely populated areas used for oyster production in many parts of Europe.

iv. In general levels of NoVs found in samples were low, often approaching the limit of sensitivity of the assay. The public health significance of low-level real-time PCR results remains unclear.

Acknowledgements
The authors would like to acknowledge part funding from the Sea Fisheries Protection Authority (SFPA), Ireland for this survey and the assistance from the Sea Fisheries Protection Officers (SFPOs) with sample collection.

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OPPORTUNITIES FOR RISK VIRUS MANAGEMENT IN SHELLFISHERIES

Bill Doré
Shellfish Microbiology National Reference Laboratory (NRL), Marine Institute

Background

Sewage contaminated bivalve shellfish can present a risk to consumers when consumed raw or lightly cooked (Lees, 2000). This risk is well documented. In Europe and elsewhere in the world there are a range of regulatory controls to reduce or prevent illness associated with shellfish consumption. The critical control points during the production of live bivalve shellfish which currently receive attention in European regulations are seen in Table 1.

Table 1. Controls points currently regulated during the production of live bivalve shellfish.

Control point	Control
Sewage contamination of shellfisheries	**Shellfish Waters Directive 79/923/EEC** –Limits sewage contamination of designated shellfisheries
Sanitary quality of shellfisheries	**Regulation (EC) No 854/2004** -Harvesting areas classified on the basis of the *E. coli* levels in shellfish and determines the level of post harvest treatment required for each category of classification.
Post harvest treatment standards	**Regulation (EC) No 853/2004** –Standards for treatment levels applied post-harvesting e.g. depuration relaying and heat treatment
End product quality control	**Regulation 2073/2005** –bivalve shellfish must meet end product standards of <230 *E. coli* 100^{-1} g, absence of Salmonella in 25g

Currently, the major control point during the production of shellfish uses *E. coli* monitoring to determine the sanitary quality of shellfish. In particular, shellfish production areas are classified on the basis of *E. coli* levels in shellfish. This determines the level of treatment required prior to sale. Despite these controls, illness following the consumption of raw shellfish can occur. Of particular concern are outbreaks of viral gastroenteritis caused by Norovirus associated with the consumption of oysters (Le Guyader *et al.*, 2006; Lees, 2000). There is, therefore, recognition that improved public health controls to prevent NoV illness associated with oysters are required. Ultimately the most effective control would be to prevent the initial contamination of shellfisheries. However, it has to be recognised that progress in providing protection for shellfish from sewage contamination will take significant time and expenditure. This therefore constitutes a longer-term approach to managing the virus risk. Until pollution control procedures are fully developed in the future, improved public health controls are required, in the shorter term. The most appropriate health controls are likely to develop risk management protocols to reduce the viral risk associated with such shellfish.

Until recently, the task of developing risk management procedures has been made difficult because of a lack of quantitative methods for detecting NoVs in shellfish. The recent development of molecular PCR methods for the detection of NoVs in shellfish has facilitated research into the mechanisms of environmental contamination of shellfish. In a previous research study, the Marine Institute has demonstrated that environmental conditions leading to intermittent viral contamination of shellfisheries can be identified (Keaveney *et al.*, 2006). Identification of the conditions leading to viral contamination in shellfisheries is a first step towards developing risk management procedures. It is proposed that where conditions leading

to contamination are identified and can be predicted, this should trigger additional control procedures to reduce the risk. The previous research project also highlighted that a risk management approach is only likely to be suitable for shellfish areas which are intermittently contaminated. It is likely that in areas where shellfish are more heavily or even continuously contaminated, additional control measures are likely to be required to be put in place on a permanent basis.

This paper outlines a proposed approach to the development of a risk management procedure in oyster fisheries and introduces a research project to trial such an approach.

Approaches to developing risk management procedures for NoVs

A three stage approach to the implementation of virus risk management in shellfisheries is proposed.

Hazard Identification → Risk Analysis → Risk management

Hazard identification.

This involves identifying sources of human sewage that may impact directly on a shellfishery. For NoVs, this means identifying human sources of contamination. However, the identification of additional non-human contamination may also be important as this influences the *E. coli* content of shellfish and thus the classification status. In turn, this will determine the level of treatment already received by the shellfish. Hazard identification can be undertaken by conducting sanitary surveys in the vicinity of the shellfish harvest area. Sanitary surveys should identify all known sources of pollution in the area. Initially, existing information on pollution sources, which may be held by a variety of bodies including local authorities and the Environment Protection Agency, should be gathered through a desk-based study. Critically, data gathering should also be extended to using local knowledge of non-documented sources of pollution (e.g. Sea Fisheries Protection Officers and local shellfish producers) to ensure all hazards are identified. As a final step, a physical survey of the shoreline in the vicinity of the shellfishery may be required to complete the data gathering exercise.

Risk Analysis.

Data from the sanitary surveys will be analysed in conjunction with risk factors known to be responsible for NoV contamination to identify the risk of NoV contamination at the site. Sites will be categorised as low, medium or high risk on the basis of the extent of expected NoV contamination. Following risk categorisation of a site, environmental conditions that are likely to cause viral contamination in the harvesting areas will be identified. Conditions known to increase the risk of NoV contamination in a site include the occurrence sewage overflows due to high rainfall events, outbreaks of gastroenteritis in the general community as well as the season, with risk increasing during the winter months (Table 2). Site specific rainfall triggers will be identified to give an early warning of storm events that will result in overflow of untreated sewage into the harvest area. Current community outbreak data will also be reviewed to identify periods of peak NoV discharge in sewage which will also act as a trigger to implement additional control procedures to reduce the virus risk in shellfish at these times.

Table 2. Identification of high risk periods of NoV contamination shellfisheries using a risk matrix approach

	Site Risk Categorisation		
Risk Factors	**Low**	**Medium**	**High**
Summer (April-Sept.)			
Winter (Oct.-March)			
Community Outbreaks of NoVs			
Overflows			
Community outbreak of NoVs and overflow			

	No risk		Low risk		Med. risk		High risk

Oyster growing areas are categorised as low, medium or high risk sites based on the identified sewage inputs into the area. A risk profile is then determined for each site taking into account environmental conditions such as season, identification of outbreaks and occurrence of sewage overflows. Trigger levels should be set for each environmental condition which initiates a move to the next level of risk.

Risk Management

Following the risk analysis, site-specific management plans should be developed. The plan will establish procedures to control virus contamination during identified high-risk periods. Management options available for the various risk levels identified are outlined in Table 3 and include;

- Cessation of harvesting
- Increased treatment (e.g. longer depuration times, increased depuration temperature, heat treatment)
- Relaying in clean seawater

During periods of identified high risk, virus monitoring will also be undertaken to provide a real-time indication of the NoV risk and inform ongoing risk management decisions. In particular, NoV monitoring will provide an indication of when it will be suitable to return to the normal control measures.

Table 3. Possible actions to be taken identified risk periods

Virus Monitoring	**Level of Risk**	**Management option**
Yes	High	Suspend harvesting
Yes	Medium	Increased treatment
Maybe	Low	Maybe increased treatment
No	None	No action

Depending on the level of risk present in a harvesting area, at any particular time, different management options may be required. Ultimately suspension of harvesting may be required.

Future Risk Management Project

The Marine Institute and Sea Fisheries Protection Authority will undertake a joint research project to develop and trial risk management procedures to control the pathogenic human virus risk associated with commercial oyster fisheries in Ireland. The project will last two years and aims to implement and trial procedures based on the approach described above. We aim to identify three commercial oyster production sites which would be considered suitable for the study. Only sites which are believed to be intermittently contaminated with NoVs will be deemed suitable for investigation.

In conjunction with the oyster producers comprehensive sanitary surveys will be conducted in the harvest sites to identify sewage contamination sources in the vicinity of the shellfisheries (**hazard identification**). The impact of these sources of pollution on the shellfishery will be assessed in relation to their size (population equivalents), treatment levels and distance from the shellfish **(risk analysis)**. Historical data on the frequency of all sewage overflows will be reviewed where available. This will be related to rainfall data in order to establish trigger values for early warning of storm overflows.

For each area site-specific risk management procedures will be developed **(risk management).** Critically, management options selected will depend on the local circumstances at the oyster production area and will be developed with the local producer. Interventions will need to be sustainable and realistic. Options that will be considered include cessation of harvesting, extended depuration times or relaying oysters in clean seawater. Alternatively in larger production sites, harvesting may be switched to oyster beds which are not under threat of increased NoV contamination.

Once environmental triggers and management plans have been developed for each area, the project will enter a period of environmental and microbiological monitoring. Samples will be collected from each site on a weekly basis and analysed for NoVs using quantitative real-time PCR procedures (Jothikumar *et al.*, 2005)**,** FRNA bacteriophage, which have been shown to be a good model of virus behaviour previously (Doré *et al.*, 2000), and *E. coli,* which is the indicator on which sanitary quality is based. This will establish the true level of viral contamination in the shellfishery during the study period. Environmental parameters such as rainfall, temperature and salinity will also be recorded. Data on the prevalence of NoVs in the population will also be gathered establish a link with the level of NoVs in the community and occurrence in shellfish at the site. This monitoring will allow an assessment and validation of the trigger values used to indicate high risk periods. During this period, the effectiveness of interventions and additional control measures will be assessed through microbiological monitoring of oysters before, during and after the treatment.

Summary

- Despite extensive regulatory controls, based on bacterial monitoring, outbreaks of viral illness associated with the consumption of sewage contaminated oysters can occur.
- Risk management procedures in shellfisheries are required to control the risk of viral illness associated with sewage contaminated oysters.
- Quantitative real-time PCR procedures provide a tool to identify environmental conditions responsible for NoV contamination in shellfisheries. This provides an initial step towards developing risk management procedures.
- A risk matrix approach based on hazard identification, risk analysis and risk management has been proposed.
- The Marine Institute and the Seafishery Protection Authority will undertake a joint project to develop and trial site specific risk management programmes in three commercial oyster areas. The programmes will be developed under the proposed risk matrix approach identified here.

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THE SHELLFISH WATERS DIRECTIVE

Kieran Burns, Coastal Zone Policy, Department of Agriculture, Fisheries and Food.

Shellfish Waters Directive

The EC Shellfish Waters Directive (79/923/EEC), was adopted on the 30th October 1979 and its aim is to protect or improve shellfish growing waters. It does this by specifying limits for physical, chemical and microbiological water quality requirements for designated shellfish water. The Directive applies to both bivalve and gastropod molluscs.

Enactment

In 1994, the Department of Environment published SI 200/1994, in which 14 Shellfish waters were designated.
In 2001 the Department of the Environment published SI 459/2001 which provided for the establishment of Action Programmes for the 14 designated waters.
In 2006 the Department of Communications Marine and Natural resources published SI 268/2006, which provided for the establishment of consolidated action programmes for the 14 areas and listed public authorities in the regulations. It furthermore provided that every public authority must exercise its functions, as far as practicable, to promote compliance with the Directive.

ECJ Cases

The ruling (11/9/2003) in Case 67/02 found that Ireland had failed to implement meaningful pollution reduction programmes. On foot of this, the action programs were strengthened with the publication of SI 268/2006. At the same time the interdepartmental Shellfish Waters Management Committee was also formed. The Commission has closed this case.
In case 148/05 the Commission ruled (14 /6/2007) that Ireland had failed to designate all waters needing protection. Ireland responded to this ruling in August 2007 with a commitment to designate all areas where bi-valves are taken commercially and further committed to establishing action programmes, implemented in a top down manner based on a strategic environmental assessment of the areas.

Shellfish Waters Management Committee

The interdepartmental Shellfish Waters Management Committee was established pursuant to Government Decision in 2006, in order to ensure a high degree of cross organisational co-ordination. The following organisations are represented:

Department of Environment Heritage & Local Government
Department of Finance
Department of Agriculture, Fisheries & Food
Department of Community Rural & Gaeltacht affairs
Environmental Protection Agency
Bord Iascaigh Mhara
Marine Institute

Through the committee a proposal to make further designations was developed and sent forward to Government on the 30th July 2007.

Government Decision
The Government agreed the following criteria which will trigger the designation of a shellfish growing area:

The Live Bivalve Molluscs (Production Areas) Designation Orders applies to the area or the area is a licensed aquaculture site; and the area is actively productive; and that the area is in need of protection or improvement with respect to the parameters of the Directive.

Also approved is the Minister's discretion to designate further areas as the need arises. In order to ensure that the action programs are effective, public authorities must feed information to the Department on developments and decisions which will impact designated waters. Permission has also been given to create an offence for causing a breach of the regulations, which will have an associated fine.

In order to quantify the potential infrastructural costs of making further designations, a preliminary list of potential designations was produced. This indicated the cost of upgrading the Waste Water Treatment infrastructure for these areas to be in the region of €533 million. The list of sites identified is only a preliminary indicative list, and work is ongoing to identify all areas which meet the criteria.

Project Plan
The following is an indication of the work involved in moving forward the designation process. This project is a major undertaking given that it will cover in excess of 50 designations.

Define waters meeting the criteria
Define the boundaries of the areas
Public & Stakeholder Consultation
Strategic Environmental Assessments
Bay Modelling
Testing & Analysis
Designate the areas by means of Statutory Instrument
Establishment of action programmes

The first deliverable from this project will be a definite list of areas for designation in January 2008. In accordance with a Government decision of October 2007, responsibility for the implementation of this Directive will shortly transfer to the Minister for the Environment, Heritage & Local Government.

REVIEW OF PHYTOPLANKTON MONITORING PROGRAMME AND RESEARCH ACTIVITIES

Rafael Gallardo Salas, Tara Chamberlain, Josephine Lyons, Paula Hynes and Joe Silke
Marine Institute, Rinville, Oranmore, Co Galway

This paper provides a review of the activities of the Phytoplankton Unit in the Marine Institute as part of the National Monitoring Programme for 2007 and compares the findings with those recorded during 2005 and 2006., It also presents an overview of the research activities carried out by the phytoplankton team during the year with a focus on culturing phytoplankton and the introduction of real time PCR techniques for phytoplankton identification. .

The National Monitoring Programme (NMP) for phytoplankton is an important element of the National Biotoxin Monitoring Programme in Ireland. The phytoplankton monitoring programme provides data and information on the distribution and occurrence of toxic and harmful algae around the coast of Ireland. The data are provided to the aquaculture industry, regulatory agencies, the scientific community and the general public. It provides key data for use in Management Cell decisions (see the paper by Devilly *et al*. in this volume). Additionally the data series will be used in other research programmes e.g. Climate Change and in fulfilling Ireland's obligations under the Water Framework Directive.

Phytoplankton species and related toxins

As shown in Figures 1 and 2 the *Dinophysis acuminata* cell numbers recorded in 2006 and 2007 were significantly lower than those recorded in 2005.

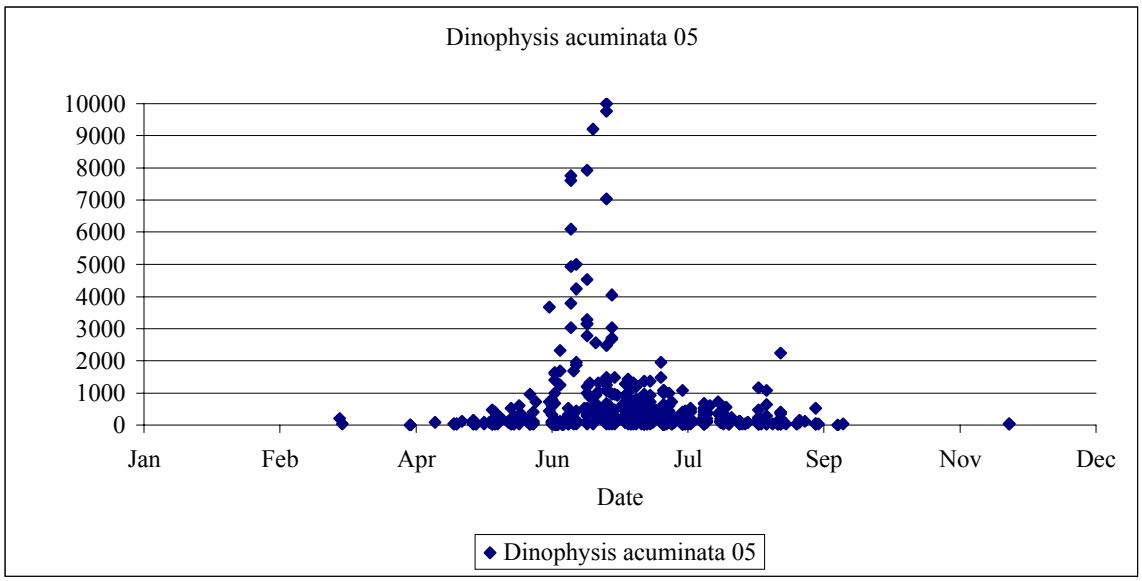

Figure 1. *Dinophysis acuminata* 2005

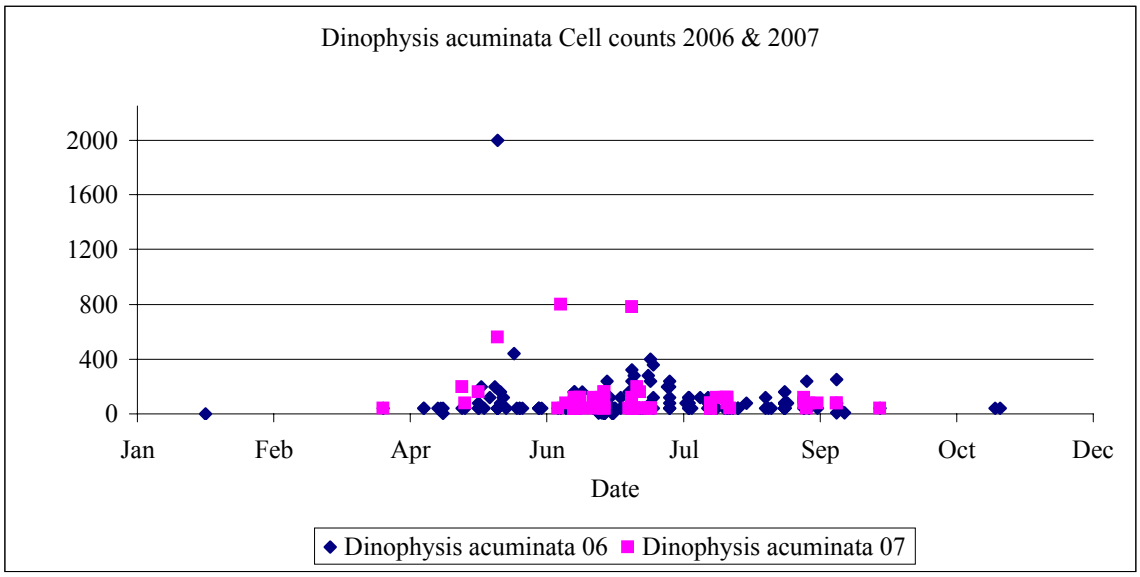

Figure 2. *Dinophysis acuminata* 2006 and 2007

The pennate diatom *Pseudo-nitzschia* spp. is known to produce Domoic acid, the primary toxin causing Amnesic Shellfish Poisoning. Figures 3 & 4 show the cell counts of *Pseudo-nitzschia* spp. for the last 3 years. . *Pseudo-nitzschia* spp. for practical reasons has been separated into two groups: the seriata group and the delicatissima group. Species from both groups can actually be toxic, but they are very difficult to identify to species level when using conventional light microscopy, so a pragmatic approach has been used to narrow down the species by grouping them in this manner.

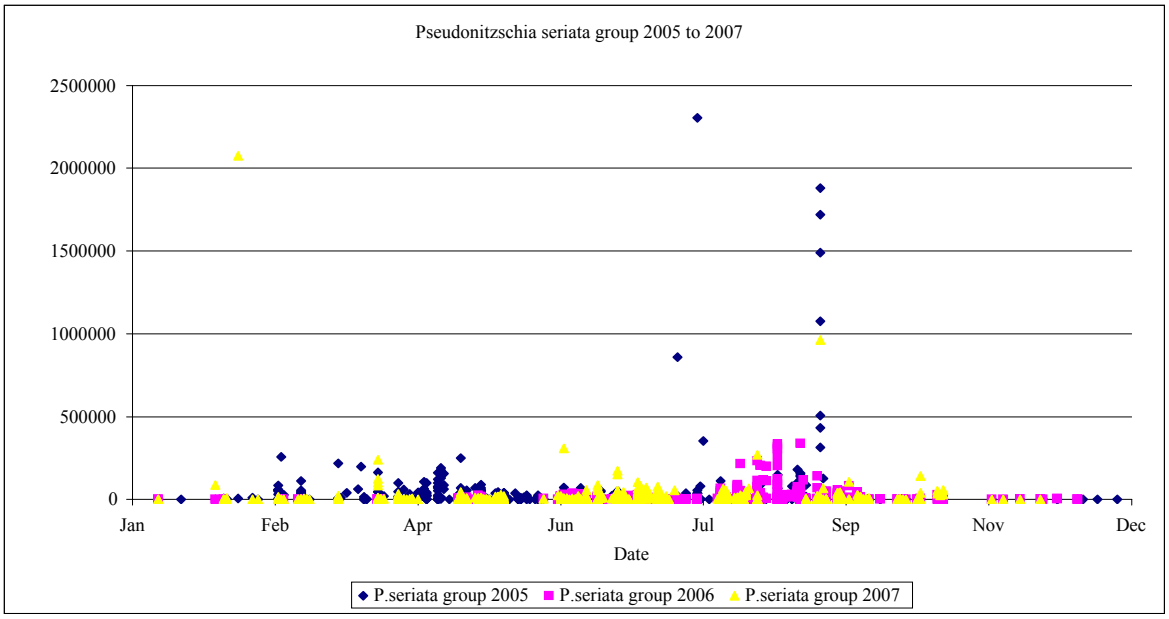

Figure 3. *P. seriata* cell counts 2005, 2006 & 2007

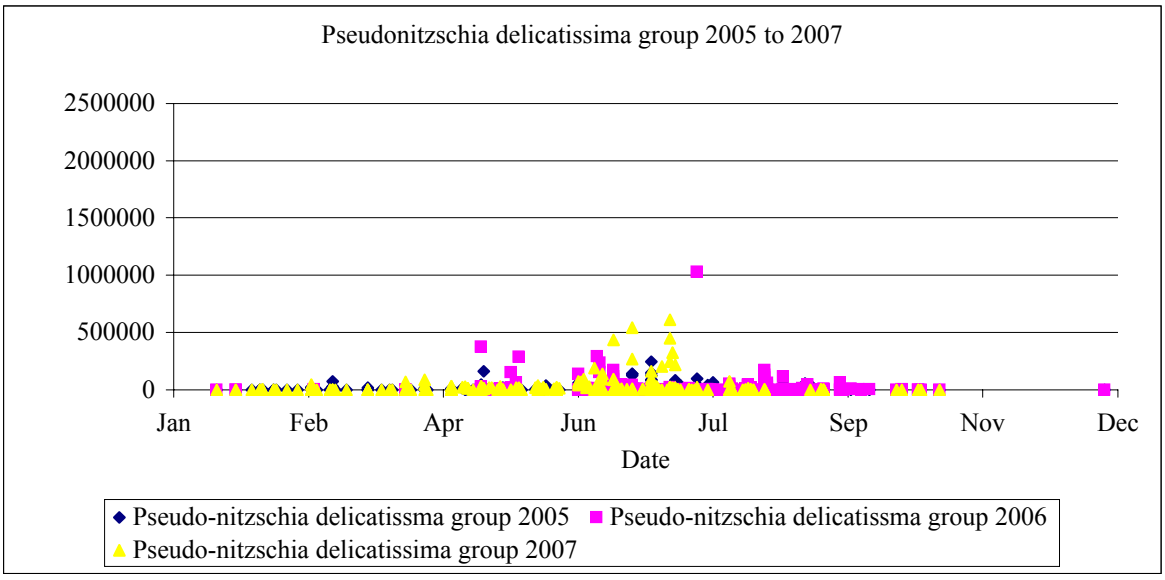

Figure 4. *P.delicatissima* group cell counts 2005, 2006 & 2007

The data presented shows that the density of these species over the last 3 years has not changed significantly. It shows that these species are very cosmopolitan and conspicuous, occurring throughout the year but in greater numbers typically during the spring bloom in April and during the summer months. However in 2005 the levels of Domoic acid in mussels for the SW coast exceeded the regulatory limit in early April (Clarke *et al.,* 2005) but this did not occur in either 2006 or 2007. This indicates that cell numbers of *Pseudo-nitzschia* spp. alone are not a good indicator of the onset of ASP toxicity and that it essential to identify the organism to species level in order to better evaluate the risk. To this end the Phytoplankton Unit is developing gene probes coupled with Real time PCR techniques to enable such data to be collected on a routine basis. (See the paper by Kavanagh *et al* this volume).

In Ireland the occurrence of PSP toxins in shellfish is mainly confined to PSP in to the Cork harbour area, where shellfish, principally mussels, become toxic usually for a short period of 1 -2 weeks in June . In 2007 PSP toxins were detected in mussels from Cork Harbour in late June and early July. The levels of *Alexandrium* cells in the water were quite low compared to 2006 (Figures 5 and 6).

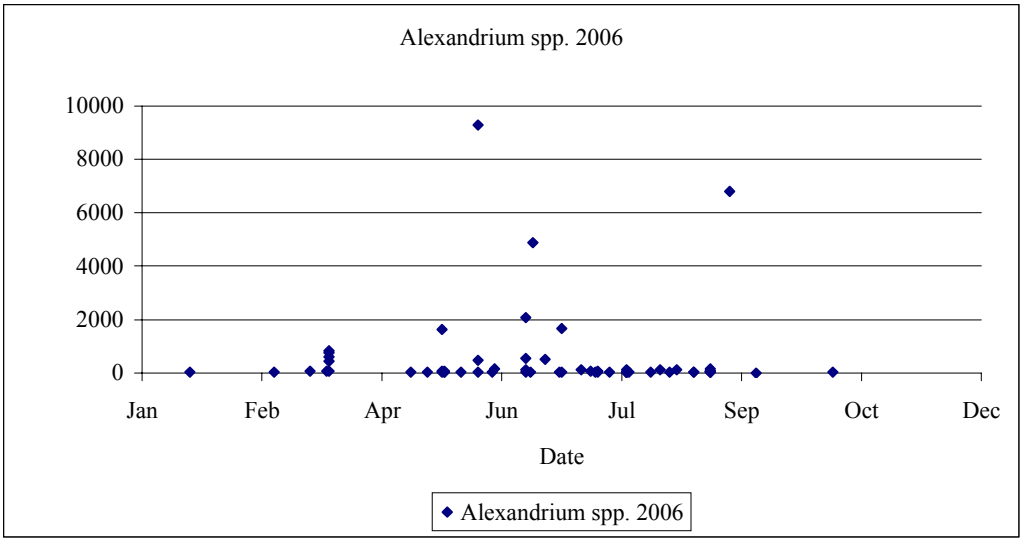

Figure 5. *Alexandrium* spp. cell counts in 2006

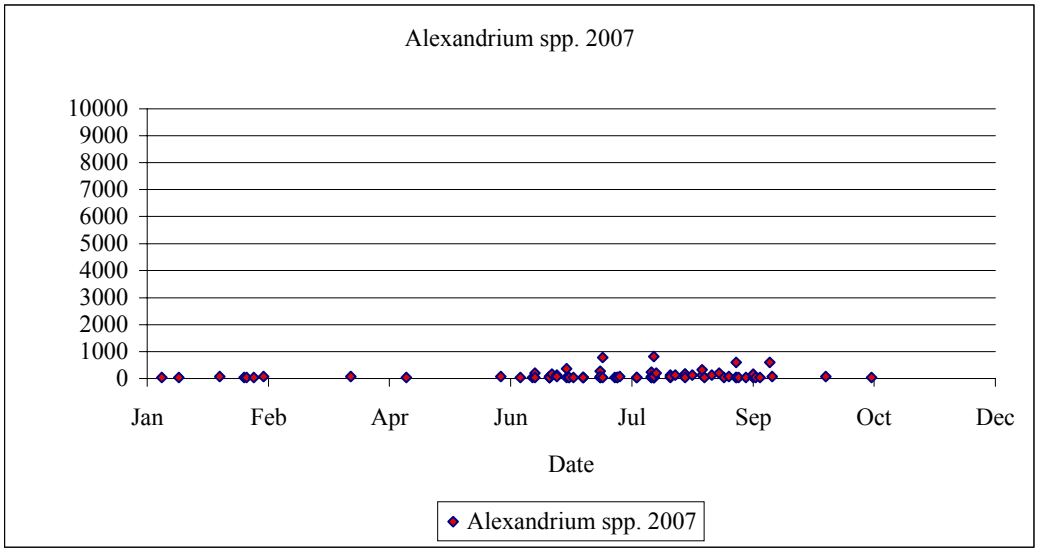

Figure 6. *Alexandrium spp.* cell counts in 2007

Azaspiracid (AZA) was first discovered in 1995 and *Protoperidinium crassipes,* taken from plankton net hauls off the SW coast of Ireland, has been reported to be the causative organism (James *et al* 2004). However, *Protoperidinium* spp. are heterotrophic organisms and could therefore accumulate the toxin through feeding upon the true progenitor, which could explain the poor correlation between the levels of *Protoperidinium* spp and the levels of AZAs detected in shellfish. Figures 7 & 8 show that *Protoperidinium* spp. are cosmopolitan but ubiquitous in Ireland, most times occurring in low cell numbers all throughout the year. In 2007 AZA was detected in shellfish at levels above the regulatory limit in October and November but no clear link is apparent between this AZA event and the cell numbers of *Protoperidinium* spp recorded at the same time.

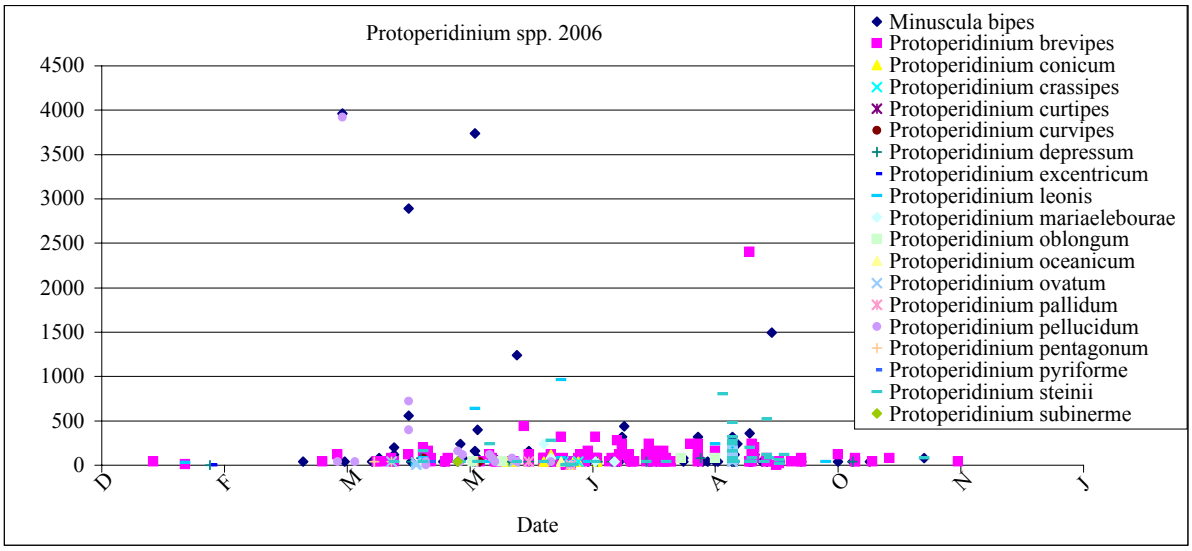

Figure 7. *Protoperidinium spp*. 2006

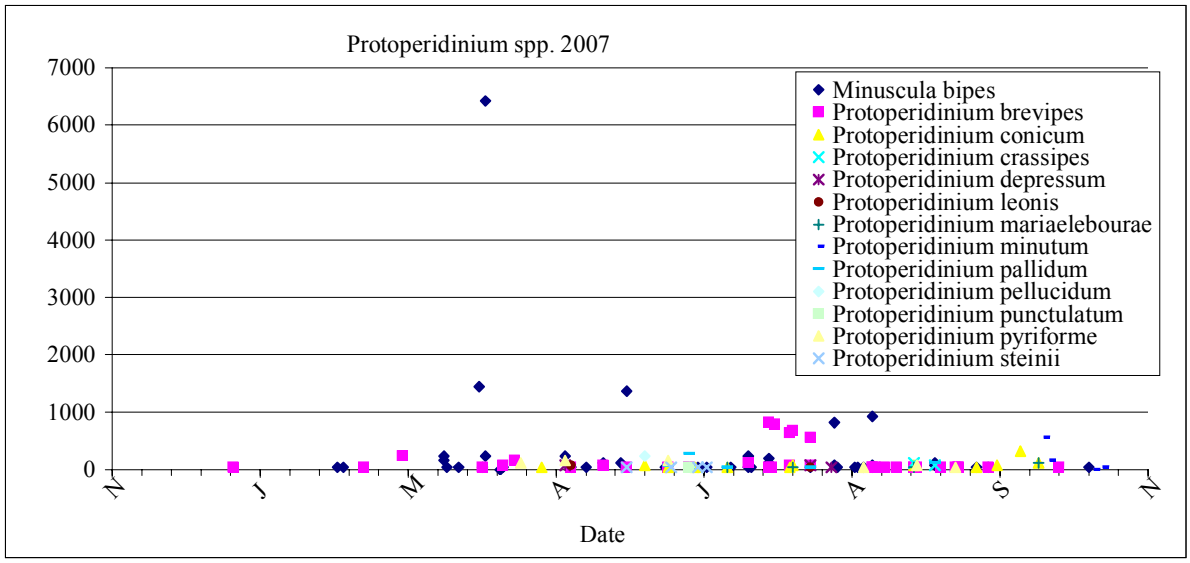

Figure 8. *Protoperidinium spp.* 2007

Temperature monitoring

The Marine Institute maintains a network of temperature probes (TidBits ™) at 11 aquaculture sites around the coast.. Each site has several sensors attached to nets or buoys at different depths and they measure temperatures hourly over a period of several months before the data needs to be downloaded. This data is a comprehensive time series of temperature data around the coast of Ireland, which can be accessed through our website www.marine.ie . Figure 9 shows an example of temperature time series for the past 4 years from Killary Harbour, County Galway. In 2007 water temperatures were generally lower than in previous years and only exceeded 14°C for a shorter period compared with the 2004 – 2006.

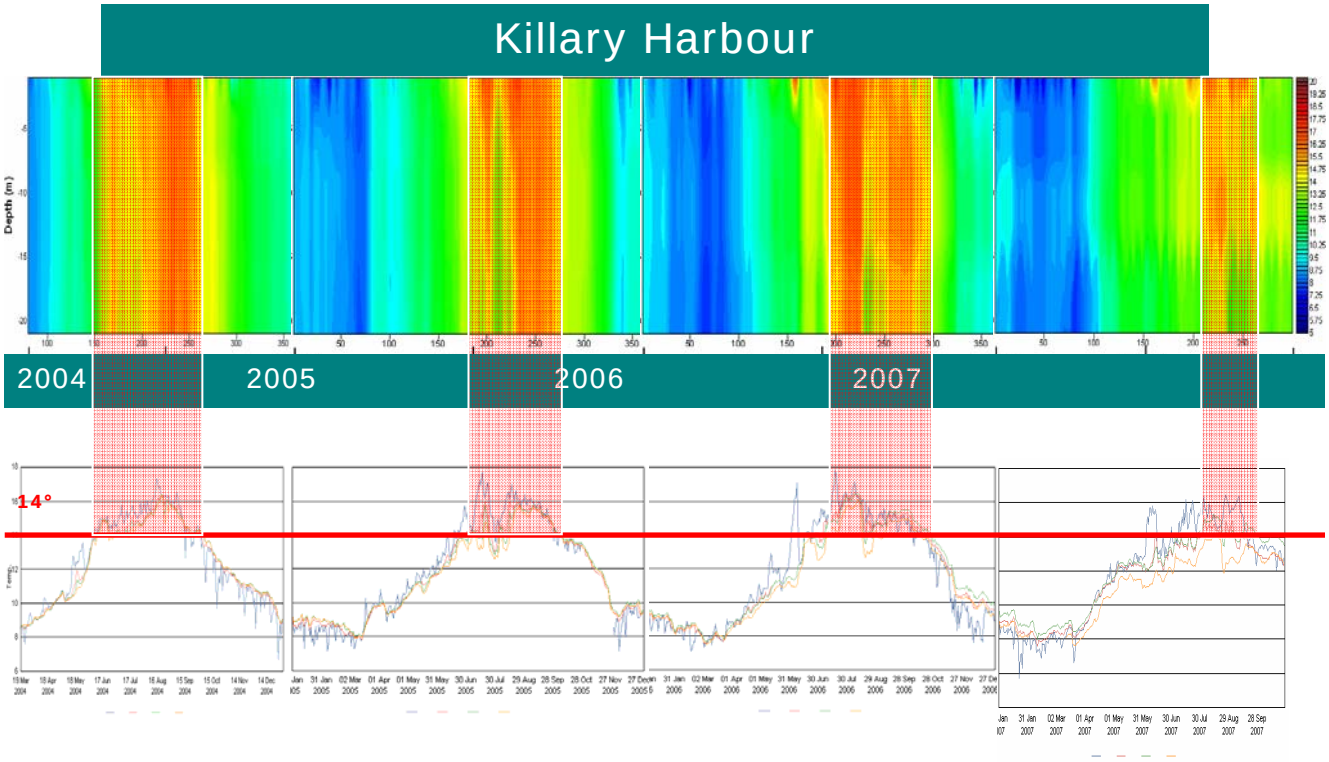

Figure 9. Killary Harbour temperature data since 2004.

Research Activities

The Marine Institute's Phytoplankton Unit has been involved over the years and continues its involvement in number of collaborative research projects at an international and national level. In the past we have been working closely in projects including BOHAB, MATSIS, METRIC and others. At present we are collaborating in a project called PHYTOTEST which involves the development of gene probes to assist in the identification of toxic/harmful marine phytoplankton species. Details of this project are presented in the paper by Kavanagh *et al* in this volume

As well as working in this project, the phytoplankton unit staff since the move to the new Headquarters at Rinville, Oranmore, County Galway, have been working and developing their skills in the new culture unit for phytoplankton. The new facilities include a walk-in incubator, several stand alone incubators, a laminar flow hood and a dedicated and fully operational culture lab (Plate 1)

Plate 1. MI Phytoplankton Culture facilities

A significant effort has been made over the last 2 years to culture ecologically important marine phytoplankton species. Some 30 strains of mostly toxic and harmful phytoplankton species have been established and used for a number of research projects. The cultures have been used in morphological studies, life history studies, toxicological studies, molecular studies but also for teaching and demonstration purposes.

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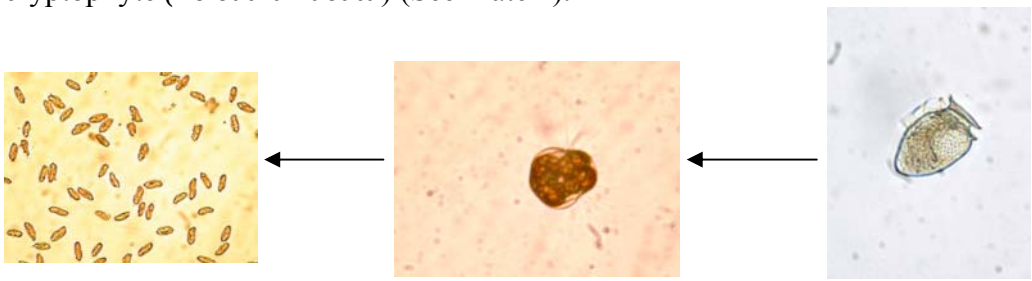
At present the culture unit is attempting to culture *Dinophysis* spp. Attempts at culturing *Dinophysis* spp. had failed over the years but in 2006 a Korean research group successfully cultured this species for the first time (Park *et al.,* 2005) The process involves feeding *Dinophysis* with a ciliate (*Myrionecta rubra*), this ciliate in turn would have to be fed with the cryptophyte *(Teleaulax acuta*) (See Plate 2).

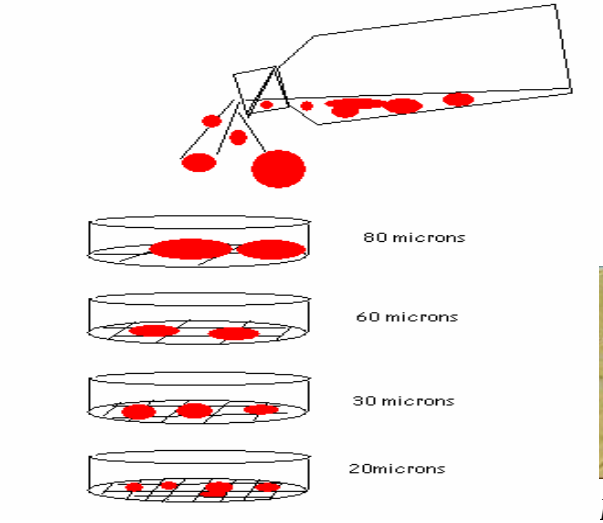


Teleaulax amphioexa *Myrionecta rubra* *D. acuminata*

Plate 2. Pictures of organisms involved in the culture of *Dinophysis acuminata*

In collaboration with colleagues D. Kulis and D. Anderson from the Woods Hole Oceanographic Institute work is ongoing to culture *D. acuta* and *D. tripos D. acuta* has been maintained in culture for the past 5 months and feeding and division has been recorded. The small cells of *D. acuta* are very similar to *Dinophysis dens* cells and it is possible that they are the same species at different stages of their life cycle. In addition we have a 3 month old culture of *D. tripos* and we have also observed feeding and reproduction giving way to small cells as has been observed with *D. acuta. It is likely that, in the near future,* the genus *Dinophysis* will have to be taxonomically completely revised.

The phytoplankton lab is also trying to isolate and culture organisms that produce Azaspiracid. This is done by obtaining live samples from AZA affected areas and carrying out fractionation of the sample to the smallest mesh possible (1µm). Cultures of the fractions are bulked up and analysed using advanced LCMS techniques (Figure 10).



Protoperidinium crassipes

Figure 10. Fractionation of live samples through mesh.

As well as culturing phytoplankton, the phytoplankton unit is part of the molecular biology facility in the MI. This unit was established recently, this year a real time PCRinstrument has been commissioned and is functioning at present. Phytoplankton personnel have started training in molecular techniques and our aim is to use these molecular tools to identify toxic phytoplankton found in the Irish coastal waters, create a database of Irish strains and ultimately develop gene probes as a risk management tool for the phytoplankton monitoring programme.

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REVIEW OF SHELLFISH TOXICITY IN IRELAND 2007

Leon Devilly[1], Olivia Fitzgerald[1], Joe Silke[1], Terry McMahon[2], Micheal Ó'Cinneide[1]

[1]Marine Institute, Rinville, Oranmore, Galway
[2]Marine Institute, Harcourt St., Dublin

Introduction

The National Marine Biotoxin Monitoring Programme for shellfish is co-ordinated by the Marine Institute's National Marine Biotoxin Reference Laboratories based in Galway. Samples of shellfish species are routinely analysed 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 (MI) as National Reference Laboratory (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 both internal and sub-contracted laboratories.

In 2007, shellfish were analysed as part of this monitoring programme from both wild fisheries and aquaculture production sites. These were analysed for Amnesic Shellfish Poison (ASP), Diarrhetic Shellfish Poison (DSP), Paralytic Shellfish Poison (PSP) and Azaspiracid Shellfish Poison (AZP).

Amnesic Shellfish Poisoning

The total number of scallop tissues analysed between January to the 15th November 2007 by HPLC for domoic and epidomoic acid was 460. The majority of samples received into MI are processed scallops hence most of the tissues analysed were adductor muscle (217) and Gonad (213). Additional analyses were carried out on remainder tissues (15) and total tissue (15). All adductor muscle analysis produced levels of ASP below the regulatory limit with the highest level observed from Mine Head Ground (32-E2) on the 12th of July 2007 at 18.6µg/g. Gonad tissues produced a highest level of 66.3µg/g in Valentia River from a sample taken on the 10th January. Of all gonad tissues analysed only 3.7% were above the regulatory limit. The remainder tissues and total tissue analysis both produced over 40% positives from the total number analysed. The highest concentration observed were 227.6µg/g in the remainder and 114.6µg/g in the total tissue from Clew Bay North scallops sampled on the 11th of September.

Further analysis was carried out on shellfish received as part of the monthly sentinel site testing programme. During 2007 there was 14 sample sites selected for the full suite of tests (ASP, DSP/AZP by bioassay and LC-MS and PSP by bioassay) on a monthly basis. The following species were analysed by HPLC for domoic and epidomoic acid, Oysters (*C. gigas*), Razor fish (*E. siliqua*), Mussels (*M. edulis*), Cockles (*C. edule*), Clams (*T. philipinarum*). Of the 114 samples analysed all concentrations observed were below the regulatory limit at <LOD and <LOQ.

The report turnaround from samples analysed between January and 19th November 2007 was 93.20% within 4 days of receipt into the Marine Institute laboratories. This was an improvement on last year's turnaround of 89% within the same number of days of receipt. Table 1.

Table 1. ASP Report Turnaround, January to 19th November 2007. n=579

Day	**1**	**2**	**3**	**4**	**5**	**>5**
No. of results	0	311	206	24	34	4
%	0	53.6	89.10	93.20	99.20	0.7

Paralytic Shellfish Poisoning (PSP)

The number of samples analysed for PSP toxins in 2007 was 158 and included analysis of Clams, Cockles, Razor fish, mussels, Pacific oysters and Native oysters. The majority of samples received were part of the monthly sentinel site monitoring programme. Additional analysis was carried out when *Alexandrium* species were observed by the Phytoplankton Unit in water samples. In this eventuality, a request for flesh samples is forwarded to sea fishery protection officers through the phytoplankton reports and by the shellfish coordinator. Of the 158 samples analysed, none produced positive results, however, 2 samples from Cork Harbour mussels sampled on the 26th June and the 2nd of July did show low levels of toxicity. The highest level observed at 39µg/STXdiHCL100g^{-1} whole flesh.

The Marine Institute are currently in the process of validating the Lawrence HPLC method for the detection of PSP toxins following the suspension of the Jellett Rapid test kits as a monitoring tool in early 2007 due to quality control issues . Currently the Lawrence method takes 3 days from sample arrival to the reporting of results in comparison to the bioassay method where results can be issued on the same day as sample arrival. As a result the initial aim is to use the Lawrence method as a confirmatory method of analysis along with the AOAC bioassay. The validation is due for completion by late 2008.

Lipophilic toxins (DSP and AZP) by Yasumoto 1984 bioassay and LCMS analysis (DSP)

Compared with previous years, 2007 was notable for the very infrequent and low level occurrence of DSP toxins in shellfish flesh. This resulted in a decrease in mouse bioassay numbers from 2384 bioassays in 2006 to 1803 to end of November (predicted <1900 by end of the year).

All positive bioassays were observed in mussel samples. A total of 17% of the 1080 samples analysed up to the end of November produced positive results. This was a decrease on 2006 when 29% of mussel samples were positive. In contrast all oyster samples analysed produced negative results and this has been the case since 2001 when only 5% positives were observed.

From LCMS analysis carried out up to the 27th November it is clear from Figure 1 that the year was dominated by AZP toxicity.

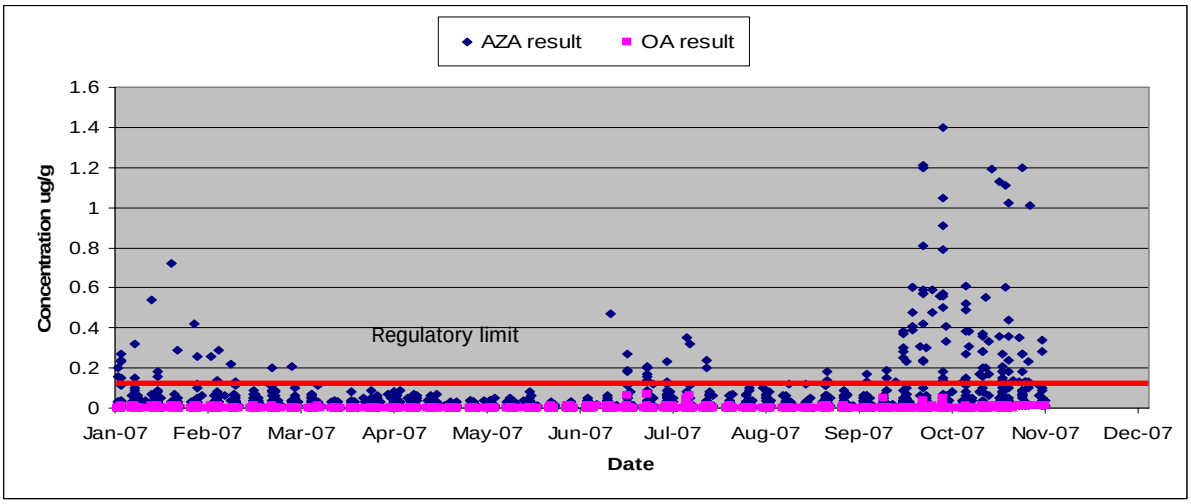


Figure 1. Concentrations of OA equiv. & AZA's equivalents in samples submitted January to November 2007

No levels of OA equivalents above the regulatory limit were detected. There were only 8 samples above <LOQ and all of these occurred in the South West. The highest value recorded was in Kilmakilloge on the 09th of July at 0.07µg/g, less than half the regulatory limit Figure 2.

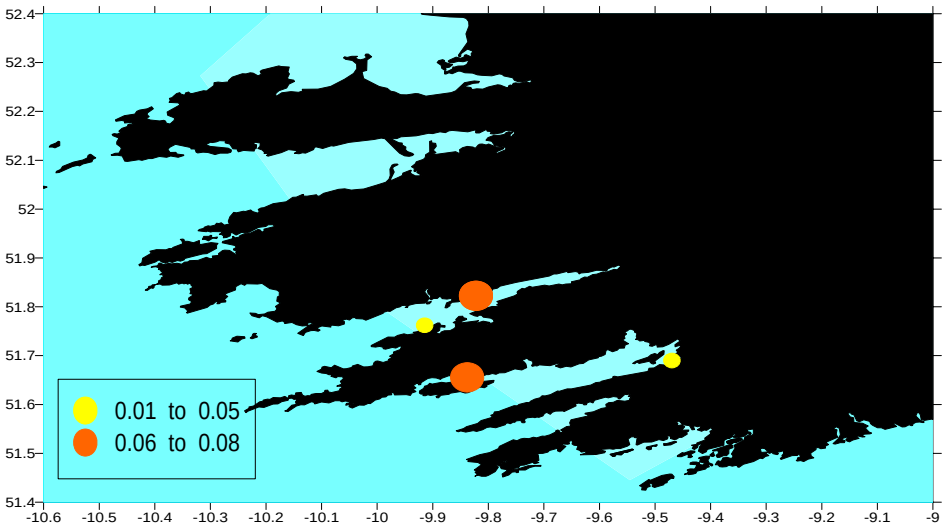


Figure 2. Concentration of OA equivalents in samples observed January to November 2007

Report Turnaround

The Report Turnaround for 1861 samples from receipt into the contract labs to the reporting of results by the Marine Institute was 94.85% in 3 days or less from January to October 2007.

Azaspiracid Shellfish Poisoning (AZP)

In contrast to the previous two years, 2007 was dominated by AZA toxicity with most production area closures occurring from early October through to the end of November, Figure 3**.**

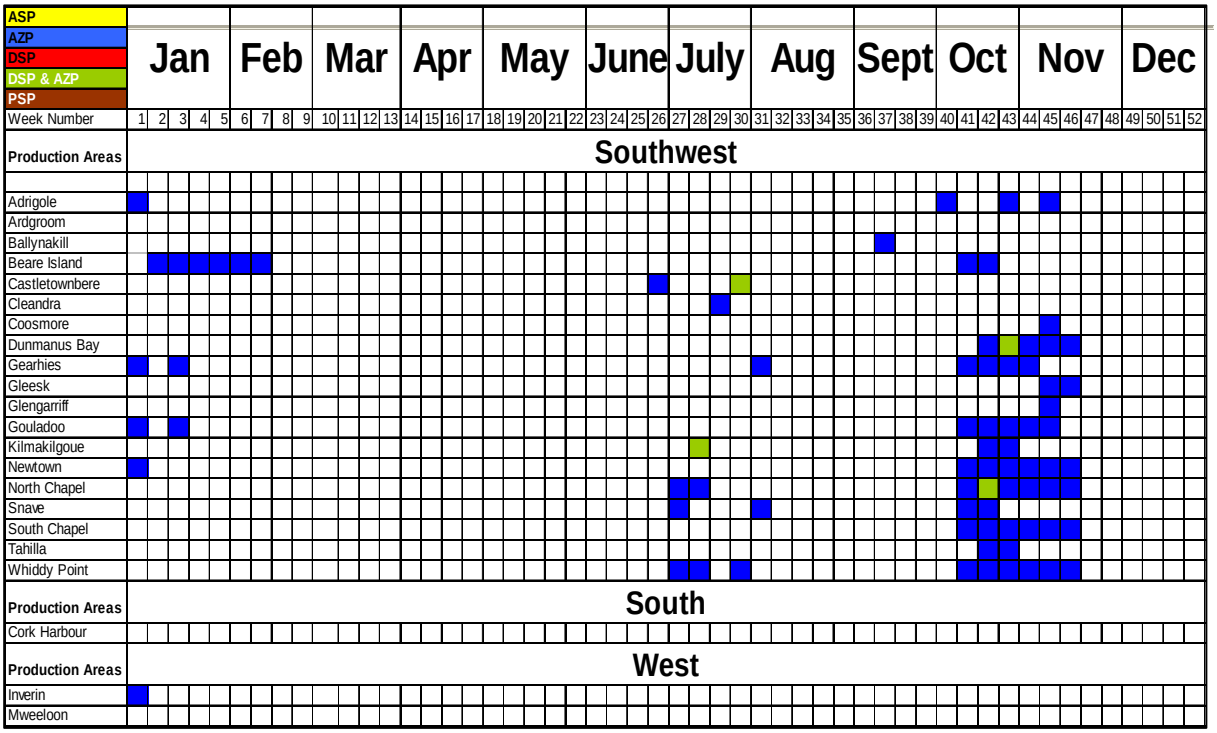


Figure 3. Closures during 2007 (n=93)

During 2005, a total of 450 closures were issued. ASP toxicity occurred during April and this was then followed by DSP dominated closures from the end of May through to September with AZA toxicity dominated closures from September to the end of the year. During 2006 a decrease to 282 closures was observed. AZA toxicity dominated from January through to May followed by a DSP event from June to early September. Again as in the previous year AZA dominated from September to the end of the year. Figure 4 & 5 illustrates AZA toxicity was present in the South West throughout 2007. From February to the end of June there were no closures. From the end of June to the beginning of August occasional closures occurred in the South West of Ireland and one occasion in Ballinakill in Galway in September. A large AZA toxification then occurred in October and carried on through to December. The highest level of AZA observed during this time was 1.4µg/g in Whiddy Point in Bantry Bay on the 22nd of October. In total 93 closures were enforced up to the end of November 2007.

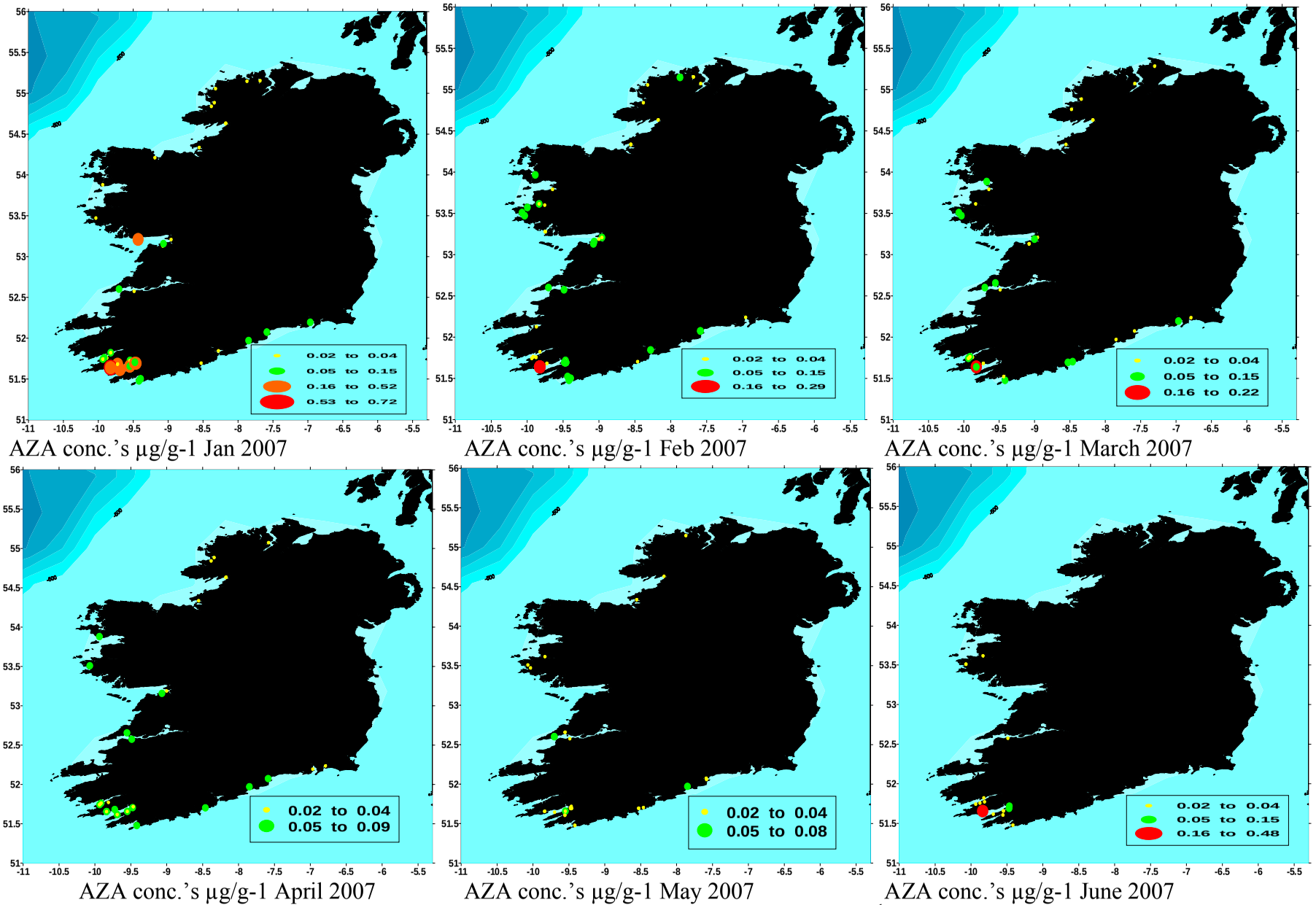
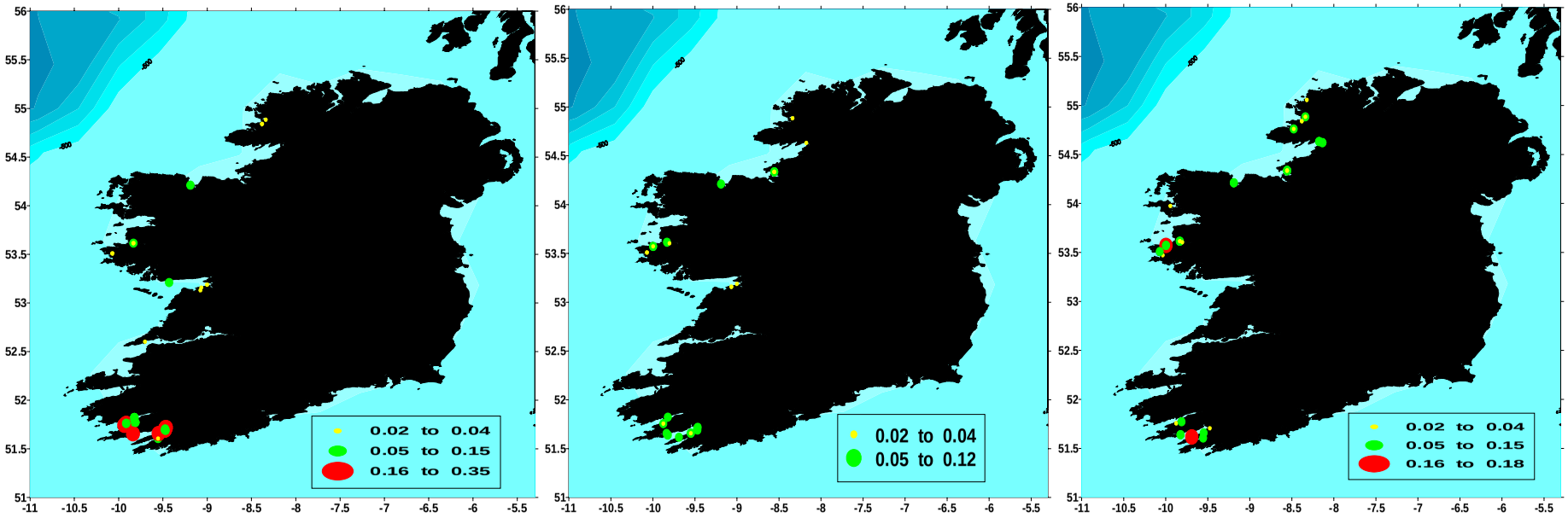


Figure 4. AZA Results January to June 2007. Total Azaspiracids AZA's 1, 2 & 3 in µg/g TT^{-1}



AZA conc.'s µg/g-1 July 2007 AZA conc.'s µg/g-1 Aug 2007 AZA conc.'s µg/g-1 Sep 2007

AZA conc.'s µg/g-1 Oct 2007 AZA conc.'s µg/g-1 Nov 2007

Figure 5. AZA Results July to November 2007. Total Azaspiracids AZA's 1, 2 & 3 in µg/g TT^{-1}

Management cells

The Management cell was set up by the Molluscan Shellfish Safety Committee to manage the potential risk presented by marine biotoxins. The Committee includes members from the Food Safety Authority of Ireland, Sea Fisheries Protection Authority, Marine Institute, Irish Shellfish Association and Shellfish Industry members. The aim of the Management cell is to enable rapid decision making in non routine situations. In the event the Management Cell does not reach a consensus opinion the Food Safety Authority view takes precedence.

During 2007 there were 35 Management Cells raised up to the end of November. Of these, 12 production areas that were previously open remained on an open status and only two went from open to closed. When compared to the number of Management cells raised since 2004 it can be seen from table 2 that the number of Management cell raised reflects the low toxicity during 2007

Month	Number raised 2004	Number raised 2005	Number raised 2006	Number Jan to Nov 2007
January	3	10	28	0
February	1	1	26	2
March	2	1	4	2
April	3	14	3	1
May	2	1	9	0
June	7	13	13	10
July	4	12	4	11
August	5	10	2	0
September	4	9	3	1
October	7	13	3	0
November	7	5	2	8
December	4	0	6	N/A
Total	**49**	**89**	**103**	**35**

Table 2. Management cells raised from 2004 to November 2007

Quality Control

The test methods used in the Marine Institute to monitor for biotoxins are all accredited by the Irish National Accreditation Board. The DSP bioassay was accredited in 2004 and the PSP bioassay and LCMS for OA, DTX-1, DTX-2, AZA 1, 2 and 3 were accredited in 2005. These methods have maintained accreditation due primarily to the ongoing internal audits and QC checks carried out by Marine Institute staff. Part of the ongoing competency also includes the participation of the MI in Intercomparisons organised by the Community Reference Laboratory each year on the 3 toxin groups (ASP, PSP, DSP). Additionally annual audits are carried out on the bioassay labs and Intercomparisons are also conducted each year.

Summary

In comparison to the previous two years there was significantly less toxicity recorded in 2007. The contrast in toxicity between 2005 to 2007 can be viewed in the production area closures for those years. These ranged from 450 in 2005, 282 in 2006 and just 93 closures from January to the end of November 2007.

The most notable feature was the almost complete absence of DSP toxins and their associated phytoplankton (*Dinophysis sp.*) observed over the summer period in shellfish production areas. The only levels of DSP toxicity as illustrated in figure 2 were detected in the South West of the country and were found to be below the regulatory limit. On three occasions, mussel production areas were placed on closed / closed pending statuses due to a combination of results including positive bioassays and levels of DSP and AZP toxins below the regulatory limit. These closures occurred in Tahilla sampled on the 02/07/07, Kilmakilloge sampled on the 09/07/07 and Kenmare Outer sampled on the 23/07/07. Due to the presence of DSP toxicity in the samples and a positive bioassay it was determined by the Management cell that the reference method (mouse bioassay) be used to determine the status of those production areas.

AZP toxicity was present during the year but extended over a considerably shorter time period than in the previous two years. The main AZP event took place between October and November when levels as high as 1.4µg/g were detected by LCMS in the Marine Institute. Despite this very sudden toxicity of mussels in the South West it was observed to decrease very rapidly with most of the effected production areas re-opening before the end of the year.

Acknowledgements
Laboratory staff from MI Bioassay and Biotoxin Chemistry Units, Oranmore.
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DEVELOPMENT & IMPLEMENTATION OF THE PHYTOTEST PROJECT

Siobhán Kavanagh[1], Claire Brennan[1], Josephine Lyons[2], Tara Chamberlain[3], Rafael Salas[2], Siobhán Moran[2], Joe Silke[2] and Majella Maher[1]

[1]DNA lab, National Diagnostics Centre, NUI, Galway, Co. Galway.
[2]Phytoplankton Unit, MEFS, Marine Institute, Rinville, Oranmore, Co. Galway.
[3]Biotoxin Unit, MEFS, Marine Institute, Bantry, Co. Cork.

Introduction

The Irish shellfish industry, worth approximately €60 million to the economy in 2006 (Browne *et al.,* 2007), is adversely affected by the presence of harmful microalgae such as *Dinophysis* and *Pseudo-nitzschia* species. Several *Dinophysis* species have been shown to produce okadaic acid and dinophysistoxins (DTXs), which are associated with Diarrhetic Shellfish Poisoning (DSP). The DSP toxin producing species *D. acuta* and *D. acuminata* occur in Irish coastal waters throughout the year, primarily from late Spring through to early Autumn, and the majority of closures of Irish mussel-farms during the summer months are attributed to their presence. Members of the *Pseudo-nitzschia* genus are the causative agents of Amnesic Shellfish Poisoning (ASP) in scallops. In 2005, an extended toxicity period caused by ASP and DSP resulted in prolonged closures at many sites. The first closures due to ASP toxicity in rope mussels occurred in that year.

In order to satisfy EU legislative requirements pertaining to the production and export of shellfish (EC Regulations, No. 853/2004 and No. 854/2004, which replaced the EU Shellfish Hygiene Directive 91/492/EEC in January 2006), the Irish Marine Institute (MI) have put a programme in place to monitor the presence of harmful algal species and biotoxins in coastal waters. This monitoring programme currently relies on microscopic identification of phytoplankton species and biochemical analysis of shellfish tissue for toxins. Microscopic identification of phytoplankton species is time consuming and requires a high level of expertise (Penna *et al.,* 2007). While *Dinophysis* may be identified to species level by a trained taxonomist using light microscopy, this is not the case for *Pseudo-nitzschia* species. Intensive electron microscopy investigation is required for species identification and this technique cannot be easily integrated into a routine monitoring programme.

Molecular techniques utilise unique sequence signatures within genomes for identification and discrimination between closely related species. Molecular identification can be performed on a variety of platforms and therefore provides a rapid alternative to laborious morphological investigation. Nucleic acid-based diagnostic assays have been developed and applied to the identification and quantification of toxic phytoplankton species (Scholin *et al.,* 1997; Saito *et al.,* 2002; Galluzzi *et al.,* 2004). Real-time PCR based assays have been developed to detect and/or quantify species including *Alexandrium, Pfiesteria*, *Heterosigma*, *Lingulodinium* and *Chattonella* (Bowers *et al.,* 2000; Hosoi-Tanabe & Sako, 2005; Moorthi *et al.,* 2005; Coyne *et al.,* 2005).

Phytotest is a 3-year research and development project funded through the Marine Institute Strategic Research Programme in Advanced Technologies as part of the National Development plan 2000-2006. The project is a collaboration between the National Diagnostics Centre at NUI Galway and the MI and involves the development of real-time PCR assays for *Dinophysis* and *Pseudo-nitzschia* species that are important in Irish waters. In the current final phase of the project, the real-time PCR assays are being transferred to the MI to support the phytoplankton monitoring service.

The potential for real-time PCR assays in HAB detection

What potential do these real-time PCR assays have for the detection of HAB species? *Dinophysis* can be identified to species level using light microscopy. However the morphology of many *Dinophysis* species can be variable (Edvardsen *et al.,* 2003) and extensive taxonomic experience is required for accurate species identification. The examination of samples is time-consuming when a large amount of biomass is present, limiting sample through-put. Conversely real-time PCR is automated, rapid and allows for a high-throughput of samples. A real-time PCR assay for detection and differentiation of the key Irish *Dinophysis* species, *D. acuta* and *D. acuminata*, is useful as a research tool, enabling the analysis of multiple spatial or temporal samples.

The availability of real-time PCR assays for the identification of *Pseudo-nitzschia* to species level, in particular key toxic species such as *P. australis*, adds significant value to the monitoring of HABs. Currently, light microscopy classifies *Pseudo-nitzschia* species into two groups, the *delicatissima* group or the *seriata* group, based on size. In the event of the occurrence of high numbers of *Pseudo-nitzschia* cells in a sample, a number of questions arise. Is it a unialgal bloom? Are toxic or non-toxic species present? Real-time PCR assays provide a rapid alternative to Electron Microscopy (EM) with the capability to answer these questions. Additionally, if required, sequencing of the PCR product generated in the assay can be performed to confirm species identity.

Methodology

Development of real-time PCR assays

The first stage in the development of our assay method was to obtain single cells (SCs) of target species from wild samples and to set up phytoplankton cultures. During the course of the project, *Pseudo-nitzschia* (*P. australis*, *P. fraudulenta*, *P. delicatissima* and *P. pungens*) and indigenous phytoplankton species cultures were maintained at the MI for sequence generation and assay testing. *Dinophysis* species cannot be readily cultured so isolation of SCs from preserved phytoplankton samples was required to obtain sequence information for indigenous *D. acuta* and *D. acuminata* cells.

Sequence information was generated for selected targets from indigenous *D. acuta* and *D. acuminata* and for *Pseudo-nitzschia* species established in culture. Analysis of the sequence information identified the large ribosomal subunit (LSU) in *Dinophysis* species and the internally transcribed spacer regions (ITS) in *Pseudo-nitzschia* species as the most suitable regions for the design of PCR primers and probes (Figure 1). For Phytotest, the real-time PCR assays incorporating these primers and DNA hybridization probes were designed to be run on the LightCycler®. A pair of fluorescently labelled probes hybridizes to single-stranded DNA from the target region as it is generated in the PCR reaction. When the two probes hybridize in close proximity to each other, energy is transferred between them and emitted in a process known as Fluorescence Resonance Energy Transfer (FRET). This energy is proportional to the increasing amount of PCR product generated and is read by the real-time PCR instrument (Anon, 2000). By combining hybridization probe technology with an analysis function of the LC® software, melt peak analysis, it is possible to generate species-specific melt peaks, which can distinguish target species from non-target species within a sample. In Phytotest, the real-time PCR assays were designed to operate under a common PCR protocol, providing the potential to identify a range of different *Dinophysis* and *Pseudo-nitzschia* species in the same analytical run.

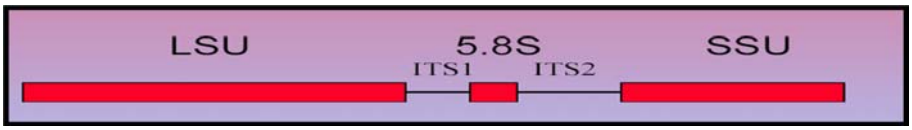

Figure 1. The large ribosomal subunit (LSU) and internal transcribed spacer (ITS) regions of ribosomal DNA gene.

The selection or development of a DNA extraction method is another critical step in the assay method. The primary requirement of a DNA extraction method is that all species present within the sample are represented within the final DNA extract. Initial sample processing involves filtration of 25 ml samples onto a 1 µm cellulose nitrate filter to catch the maximum biomass present within the sample. Samples may be processed immediately or stored preserved with Lugol's iodine for at least 1 year, enabling the method to be applied to study archive samples.

The release of nucleic acids from cells is another important step in the DNA extraction procedure. To ensure detection of target cells that are present in low numbers, it is also crucial to obtain the purest DNA possible for real-time PCR, free from protein, salt and humic acid contaminants. *Dinophysis* cells are easily broken open but *Pseudo-nitzschia* cells possess a hard silica frustule so a physical pre-extraction step, freezing of cells with liquid nitrogen and thawing to 80 ^{o}C, is required. In Phytotest, DNA extraction was performed using a chemical extraction procedure.

Following real-time PCR assay design and optimisation of the nucleic acid extraction method, evaluation of the assays is performed initially using SCs or DNA extracts from pure cultures and plasmids containing the target (LSU or ITS) regions. Assay parameters including specificity and sensitivity are determined. Evaluation of wild samples containing target species is performed, to determine the detection capabilities of the assays using real samples with a background microbial and eukaryotic community.

Results

Dinophysis species real-time PCR assay

Initial evaluation of the *Dinophysis* species real-time PCR assay involved testing with SCs of *D. acuta* and *D. acuminata* and with plasmids incorporating the target regions from both *Dinophysis* species. Melt peak analysis of the probes consistently yielded a melt peak at 61^{0}C for SCs of *D. acuta* isolated from different Irish coastal locations and for plasmids containing the assay target region for *D. acuta* (Figure 2). Melt peak analysis of the *D. acuminata* SCs with the probes produced melt-peaks at 48^{0}C, approximately 13^{0}C lower than for *D. acuta*. This melt peak temperature for *D. acuminata* was confirmed when a plasmid containing the target region for *D. acuminata* was included in the real-time PCR assay (Figure 2).

The next step in the assay evaluation process involved testing of wild samples containing *Dinophysis* cells. Samples from the MI phytoplankton-monitoring programme were selected for testing, as they are routinely examined by light microscopy for the presence of HAB species. Fifty-five Lugols iodine preserved samples from the 2006 MI phytoplankton-monitoring programme were tested with the assay. Thirty-three of these samples were reported to contain *Dinophysis* species cells based on microscopic analysis, with cell numbers in the range of 1-8 in 25 ml. Twelve samples contained only 1 cell of either *D. acuta* or *D. acuminata*. The real-time PCR assay detected the presence of *Dinophysis* species in all samples reported to contain *Dinophysis* cells. The real-time PCR assay identified *D. acuta* or *D. acuminata* in DNA extracts from all samples reported to contain either species by microscopy. The percentage occurrence of *Dinophysis* species within these samples is illustrated in Figure 3.

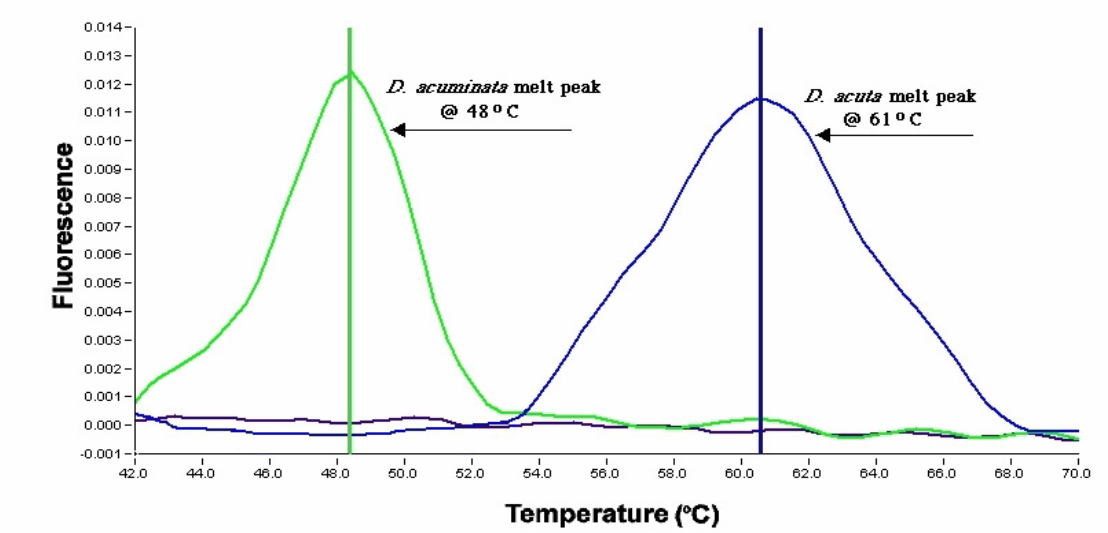

Figure 2. Real-time PCR assay for the detection of *D. acuta* and *D. acuminata* using Hybridization Probes. Both species are simultaneously identified with the assay.

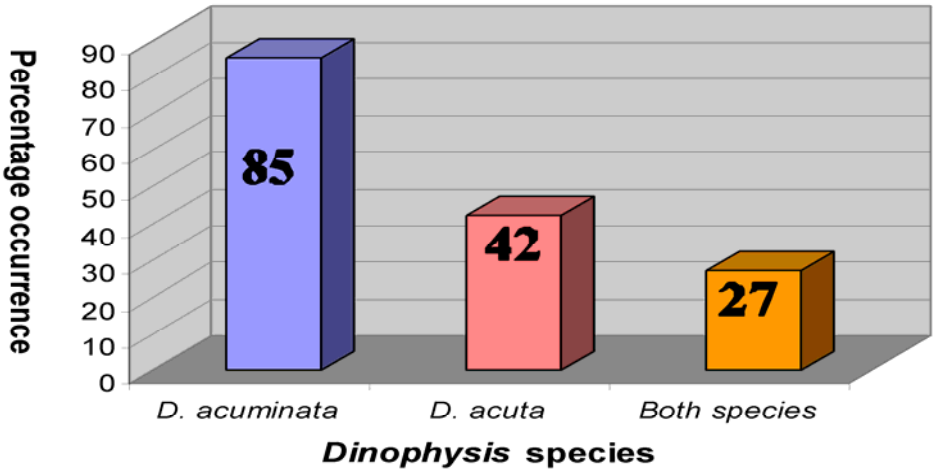

Figure 3. Percentage occurrence of *Dinophysis* species in 2006 MI phytoplankton monitoring programme samples.

An advantage of hybridization probe technology is the potential to detect and distinguish between two or more species with a single probe set, using melt-peak analysis. Specific-melt peaks were observed for *D. acuta* and *D. acuminata* with the real-time PCR assay for four samples reported to contain both species by light microscopy, illustrating that this assay simultaneously detects and discriminates between species, even when occurring at low numbers. There was no detection of *Dinophysis* by the assay in the MI samples where *Dinophysis* cells were not observed by light microscopy. A specificity panel, including a range of phytoplankton species commonly found in Irish waters (Table 1) was tested against the assay and no cross-reactivity was observed. Phytotest successfully developed a real-time PCR assay capable of identifying the two important *Dinophysis* species in Irish waters when present at 1 cell/25ml.

Table 1. Phytoplankton specificity panel tested with the real-time PCR assays.

Phytoplankton species	
Pseudo-nitzschia australis [a]	*Fragilariopsis* cf. species [b]
Pseudo-nitzschia fraudulenta [a]	*Gymnodinium* cf species [a]
Pseudo-nitzschia delicatissima [a]	*Lingulodinium polyedrum* [a]
Pseudo-nitzschia pungens [a]	*Leptocylindrus danicus* [b]
Pseudo-nitzschia multiseries [a]	*Licmophora* cf. species [b]
Pseudo-nitzschia calliantha [a]	*Myrionecta rubra* [a]
Pseudo-nitzschia seriata [a]	*Navicula* cf. *erifuga* [b]
Dinophysis acuta [a]	*Nitzschia lecointei* [b]
Dinophysis acuminata [a]	*Pleurosigma* cf. species [b]
Phalacroma rotundata [a]	*Prorocentrum dentatum* [a]
Alexandrium minimum [a]	*Prorocentrum lima* [a]
Alexandrium tamarense [a]	*Prorocentrum micans* [a]
Alexandrium fundyense [a]	*Prorocentrum minimum* [a]
Alexandrium catenella [a]	*Protoperidinium brevipes* [b]
Akashiwo sanguinea [a]	*Prorocentrum reticulatum* [b]
Asterionellopsis glacialis [b]	*Rhizosolenia fragilissima* [b]
Ceratulina pelagica [b]	*Scripsiella* cf. species [b]
Chaetoceros debilis [b]	*Skeletonema costatum* [b]
Chaetoceros cf. species [a]	*Skeletonema marinoi* [b]
Coscinodiscus wailesii [a]	*Striatella unipunctata* [b]
Cylindrotheca closterium [b]	*Teleaulax* species [a]
Cyclotella meneghiniana [b]	*Thalassiosira rotula* [b]
Dinobryon pellucidum [b]	*Tintinnid* cf. species [b]
Ditylum brightwellii [b]	

[a] denotes phytoplankton species that were directly tested with the real-time PCR assays as single cell templates or DNA extracts of pure cultures

[b] denotes phytoplankton species that were present in MI phytoplankton monitoring programme samples

Pseudo-nitzschia species real-time PCR assays

For initial evaluation of the *Pseudo-nitzschia* species real-time PCR assays, DNA extracts from pure cultures of *Pseudo-nitzschia* species and plasmids incorporating the target regions were tested. Evaluation of the *P. australis* assay, determined a species-specific melt peak at 56-57 ^{0}C for *P. australis* (Figure 4a). The *P. fraudulenta* assay produced a species-specific melt peak at 55 ^{0}C for *P. fraudulenta* (Figure 4b) and the *P. delicatissima* assay produced a species-specific melt peak at 58 ^{0}C for *P. delicatissima* (Figure 4c). Evaluation of the *P. pungens* assay determined a species-specific melt peak at 60 ^{0}C for *P. pungens* (Figure 4d). The *P. pungens* real-time PCR assay also detected and identified other *Pseudo-nitzschia* species including *P. australis* with a melt peak at 49 ^{0}C and *P. fraudulenta* with a small melt peak at 42 ^{0}C. In addition, *P. multiseries* produces a melt peak at 53 ^{0}C with this assay (Figure 4e). All *Pseudo-nitzschia* species assays were tested for specificity using a panel of *Pseudo-nitzschia* species and indigenous phytoplankton species (Table 1). No cross reactivity was observed for any of the assays with non-target species.

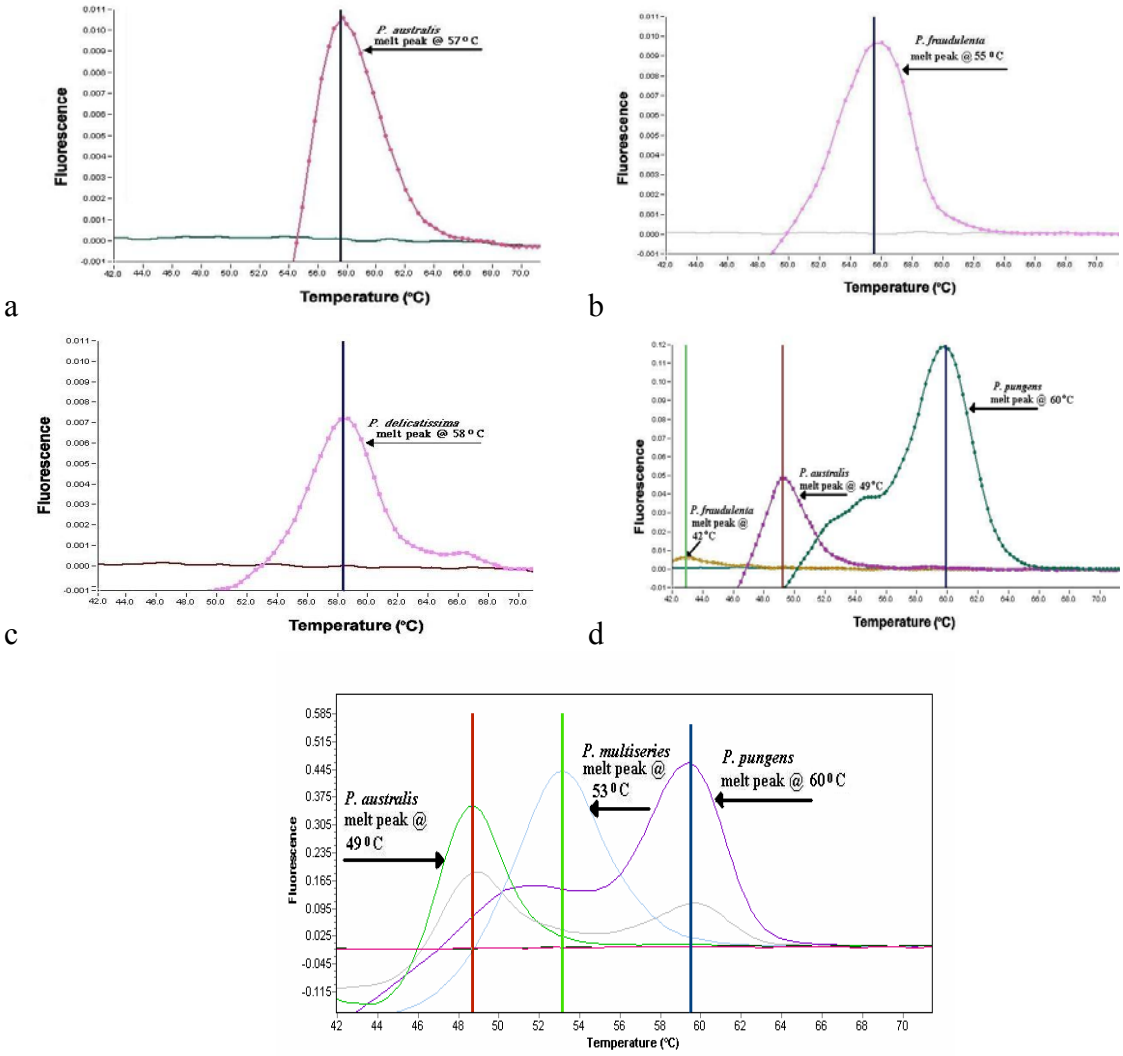

Figure 4a-e. Real-time PCR assays designed for the detection of *Pseudo-nitzschia* species.
a. The *P. australis* assay yields a melt peak at 56-57 ^{0}C with *P. australis.*
b. The *P. fraudulenta* assay yields a melt peak at 55 ^{0}C with *P. fraudulenta.*
c. The *P. delicatissimsa* assay yields a melt peak at 58 ^{0}C with *P. delicatissima.*
d. The *P. pungens* assay yields a melt peak at 60 ^{0}C with *P. pungens*. Melt peaks are also detected at 49 ^{0}C with P. australis and at 42 ^{0}C with *P. fraudulenta*.
e. Melt peak analysis P. pungens assay probes yields a peak at 53^{0}C with *P. multiseries.*

Evaluation of the *Pseudo-nitzschia* species assays was performed using wild samples that had been examined for the presence of *Pseudo-nitzschia* cells by light microscopy. Fifty-eight Lugols iodine preserved samples from the 2006 MI phytoplankton-monitoring programme were tested with each of the *Pseudo-nitzschia* species (*P. australis*, *P. fraudulenta*, *P. delicatissima*, *P. pungens*) real-time PCR assays. Forty-one of the samples contained *Pseudo-nitzschia* cells, with numbers ranging from 40-3889 cells in 25 ml preserved sample. In addition to these samples, seven 2006 Lugols iodine preserved samples were received for testing from Dunstaffnage Marine laboratory. All seven samples contained *Pseudo-nitzschia* cells, with numbers varying from 31-2750 cells in 25 ml.

All of the 2006 samples reported to contain *Pseudo-nitzschia* cells produced melt peaks with at least one of the *Pseudo-nitzschia* species real-time PCR assays. The assays identified the presence of two or more species in 78% of the samples, supporting the reported co-occurrence

of *Pseudo-nitzschia* species from the literature (Hasle *et al.*, 1996, Cusack *et al.*, 2004). Figure 5 illustrates typical real-time PCR assay results obtained for a MI phytoplankton-monitoring programme sample. Melt peaks, indicating the presence of *P. australis*, *P. fraudulenta*, *P. delicatissima, P. multiseries* or *P. pungens,* were absent from the seventeen samples where *Pseudo-nitzschia* cells were not observed by light microscopy.

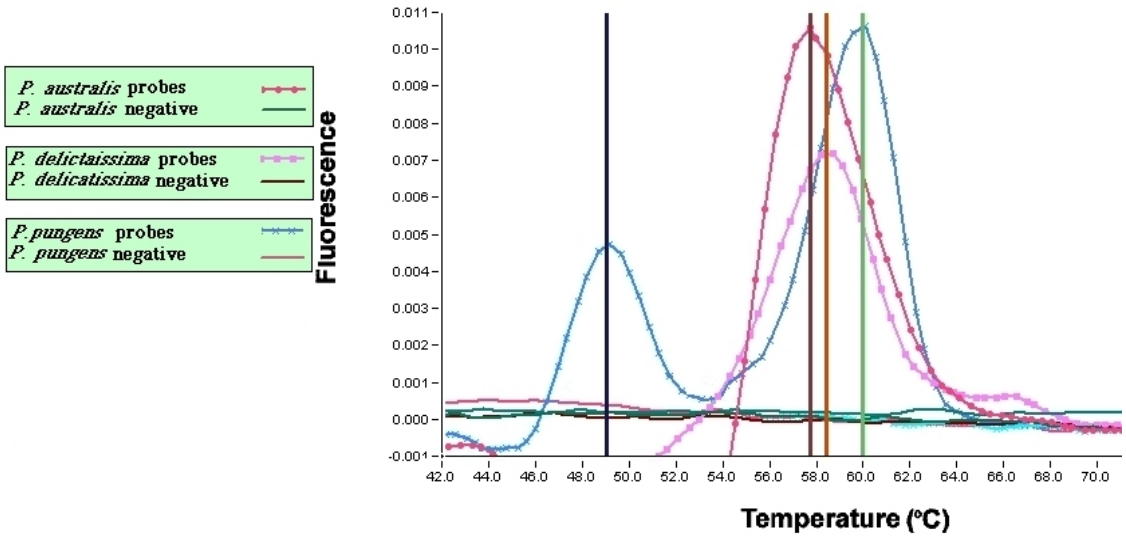

Figure 5. Real-time PCR assays results from a DNA extract of the 2006 Hawks Nest, Mannin Bay sample. This sample was reported to contain 140,760 Pseudo-nitzschia cells/L by light microscopy. Three Pseudo-nitzschia species were detected within the sample

In addition to the 2006 samples, a DNA extract of 173 cells isolated from a preserved seawater sample was tested with all of the assays. This seawater sample was taken from Castlemaine Harbour as part of the phytoplankton-monitoring programme and was associated with a toxic bloom event in April 2005. The *P. australis* real-time PCR assay detected the presence of *P. australis* in the DNA extract. This result was further confirmed by sequencing of the PCR product and Transmission Electron Microscopy (TEM).

Implementation of Phytotest

Since July 2007, the technology transfer phase of the project has begun with the purchase and installation of the LC® 480 instrument. Training of MI Phytoplanton Unit staff in real-time PCR and nucleic acid extraction methodologies has commenced. Initial testing of the assays with positive controls indicated that the real-time PCR assays for *Dinophysis* and *Pseudo-nitzschia* species are working successfully on the LC® 480 instrument. Currently, the specificity and limits of detection for these assays are being verified. A performance evaluation of the assays for the identification of the relevant toxic species in wild samples is being performed between the MI (LC® 480 instrument) and at the NDC (LC® 2.0 capillary machine) as part of a validation of the real-time PCR assays. It is expected that the real-time PCR assays will be used by the MI Phytoplankton unit to support the monitoring programme after March 2008.

Acknowledgements

Thanks to the Dunstaffnage Marine laboratory, Oban, Argyll, Scotland and the EU SPIES Project, NUI Galway for the provision of wild samples and phytoplankton culture material for testing. This project (Grant-aid Agreement No. AT/04/02/02) is carried out with the support of the Marine Institute and the Marine RTDI Measure, Productive Sector Operational Programme, National Development Plan 2000-2006.

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RECENT DEVELOPMENTS IN UK SHELLFISH BIOTOXIN MONITORING

Myriam Algoet[1], Lorna Murray[2] Claudia Martins[3], Cowan Higgins[4] and Susanne Boyd[5]

[1] CEFAS Weymouth Laboratory, Weymouth, Dorset
[2] Food Standards Agency Scotland, St Magnus House, Aberdeen
[3] Food Standards Agency, Aviation House, London
[4] Agri-Food & Biosciences Institute, Belfast
[5] Food Standards Agency Northern Ireland, Belfast

In the UK, the central Competent Authority responsible for the implementation of European food safety regulations is the Food Standards Agency (FSA), with its office in London and also in Cardiff, Belfast and Aberdeen for the devolved administrations. The requirements of the national official control biotoxin monitoring programmes, as set out in EC regulation 854/2004 (Commission Regulation (EC) No 854/2004) and associated texts, are delivered by a number of organisations. In 2007, these are:

- CEFAS (The Centre for Environment, Fisheries and Aquaculture Science) which delivers, for the FSA, the coordination of the English and Welsh programmes and the required flesh and water testing, the Diarrhetic and Paralytic shellfish poisoning testing (DSP and PSP) for FSA Scotland. It also carries out import control testing for shellfish originating from third countries and follow up testing on samples from England, Wales and Scotland suspected to be responsible for human intoxication occurances.
- Integrin Advanced Biosystems Ltd., which is responsible for the co-ordination of the Scottish biotoxin programme, sample timetable delivery, assistance with programme management and amnesic shellfish poisoning (ASP) testing of Scottish samples, whilst SAMS (The Scottish Association of Marine Sciences) is responsible for the delivery of the Scottish water monitoring programme.
- AFBI (the Veterinary Sciences Division of the Agri-Food and Biosciences Institute) which delivers the co-ordination of the Northern Irish programme and all associated water and flesh testing.
- Local food authorities, which collect flesh and water samples and send these to the testing laboratories and are responsible for local implementation of delegated responsibilities which include administration of Temporary Closure Notices.
- The UK National Reference Laboratory is the Fisheries Research Directorate Marine Laboratory at Aberdeen. Their main role is set out in regulation EC 882/2004 and includes advising official control testing laboratories and co-ordinating testing activities between these.

This paper focuses on the current biotoxin flesh programmes, describes how these have evolved in the past few years and offers a few insights on what further changes may be expected in the next two years.

Current biotoxin flesh monitoring programmes

Size of the programmes:

A total of over 80 representative monitoring points (RMPs) and 170 associated harvesting areas (AHAs) are monitored in Scotland, covering 186 classified production areas. In England and Wales, the flesh monitoring programme involves the monitoring of over 110 sites, representing 68 classified production or relaying areas and in Northern Ireland, over 35 classified beds are monitored across six loughs.

Frequency of monitoring:
The frequency of monitoring of each Scottish and Northern Irish site has been established based on the results of recent risk assessments which were conducted on historical biotoxin data for the sites. Shellfish from Scottish RMPs are monitored weekly for PSP throughout the year. The DSP and ASP monitoring of these shellfish areas are conducted on a risk based approach with weekly testing during the high risk season (for DSP: April to November and ASP: May to November) and fortnightly or monthly testing outside of these periods. A new risk assessment is currently being conducted by FSA Scotland and may result in changes to the programme. In Northern Ireland, each bed is monitored monthly and each water body monitored fortnightly for all three toxins.

A risk assessment is being conducted for English and Welsh sites, which are currently monitored on a minimum of monthly basis for all three toxins. Monitoring is increased (to weekly or fortnightly) in areas known, from historical data or phytoplankton data, to be at higher risk and in areas where low levels of toxins are detected.

Where positive results occur, the affected sites continue to be tested, until two consecutive negative or below maximum permitted level results are obtained, allowing the subsequent lifting of any harvesting restrictions applied on the sites. Samples for closed sites are usually collected 7 days apart.

Species monitored:
Shellfish chosen for monitoring are representative of the species regularly harvested from that area. Typically this would be mussels, oysters, cockles, scallops and clams of commercial size. In Scotland, for the majority of harvesting areas, mussels are used as the representative species. Shellfish samples collected by sampling officers are sent chilled, by next day delivery to the official control laboratories. The use of thermally validated Biotherm boxes (allowing temperature during transport not to exceed 10°C over a 48h period) was implemented in early 2006 across the UK, at the recommendation of the UKNRL, the aim being to ensure, as far as possible that animals taken for analysis arrive at the laboratories in the best possible condition and that the possibility if total toxin content changing during transport is minimised. Where the duration of transport of samples to the laboratory does not exceed 12h, the use of coolboxes is allowed. All samples are assessed against set criteria to ensure freshness before being processed for testing.

Flesh methodology:
The methods employed by UK official control laboratories are standard and internationally recognised, that is:

- the AOAC official method 959.08 (AOAC Official Method 959.08) mouse bioassay (MBA) which, in certain shellfish species is used in combination with a high performance liquid chromatography (HPLC) screen based on the AOAC 2005.06 official control method for PSP,
- a modification of the Yasumoto *et al.* (1984) method for the DSP MBA and
- a HPLC method for ASP. At AFBI, this is preceded by a screen using a biosensor method in some species.

Laboratory methodology follows UKNRL protocols where these exist and are accredited to ISO17025 standards where possible. The action limits used in the national programmes are those defined by the EU regulations, which are 80µg STX equivalence/100g shellfish flesh, 20µg domoic acid/g shellfish flesh and presence of lipophilic toxins by MBA. Results are reported to the relevant FSA offices usually within 24-30 hours of sample receipt and communicated to stakeholders by the FSA. The FSA is immediately alerted when toxins are detected or when signs indicative of DSP are observed.

How the programmes have evolved

A number of drivers have impacted on the UK national official control biotoxin monitoring programmes in recent years. These have included the implementation from January 2006 of the Consolidated European food hygiene legislation (particularly EC regulation 854/2004), which led to changes in the frequency at which classified production areas are monitored for biotoxins and to a major revision of the Scottish wild *Pectinidae* monitoring programme, which now relies on inshore verifications at first ports of landing, processors or fish markets instead of off-shore sampling of shellfish and systematic testing, as applied in previous years. The adoption in November 2006 of EC regulation 1664/2006 (Commission Regulation (EC) No 1664/2006) allowing the use of the AOAC 2005.06 method (AOAC official method 2005.06) (the Lawrence method) for the detection of PSP toxins in shellfish flesh allowed speedy progress to be made towards the replacement of the MBA for such toxins. Further methodology changes were made possible with the adoption of EC regulation 1244/2007 in October 2007 (Commission Regulation (EC) No 1244/2007) which allows the use of the 2006.02 ELISA method for the screening of ASP in shellfish flesh.

Simultaneously, there has continued to be a strong emphasis, both in EU food safety legislation and in animal ethics, on the need for reduction, refinement and replacement (the 3Rs principle) of animal use for scientific purposes. At the same time, the UK Competent Authorities and the biotoxin official control laboratories have continued to demonstrate their commitment to the 3Rs by pursuing and implementing alternative methodologies for biotoxin testing. Finally, the withdrawal in 2005 of two of the UK official control laboratories from the biotoxin monitoring programmes led to a major re-structuring of the UK biotoxin testing programmes.

Overall, these national and international pressures led to a significant increase in the total number of shellfish samples monitored between 2006 and 2007 (as illustrated in Table 1), mainly as a result of the review of the Scottish monitoring programme. The past three years have also been characterised by major changes in the testing methods used, notably the refinement of MBAs and the implementation of analytical methods (such as the HPLC screen for PSP) enabling negative samples to be screened out, prior to MBA testing. These changes have required the rapid development of laboratory resources and processes to enable the delivery of evolving programmes.

Table 1 Total number of PSP, DSP and ASP tests performed in the UK. Numbers in brackets indicates the number of samples found to exceed EC regulatory limits.

		2006	2007	2008 forecast
England and Wales				
	PSP	1038 (2)	1084 (0)	975
	DSP	968 (6)	1008 (9)	975
	ASP	954 (0)	975 (0)	975
Scotland				
	PSP	1074 (15)	2443 (8)	3564
	DSP	1064 (74)	2233 (84)	2997
	ASP	1052 (2)	2169 (7)	2835
Northern Ireland				
	PSP	333(0)	410 (0)	480
	DSP	344(3)	427 (9)	480
	ASP	330(0)	411 (6)	480

Implementing the 3Rs – new methodologies

In the UK, the use of animals in laboratory testing is regulated by the Animals (Scientific Procedures) Act 1986. This Act is enforced by the Home Office with Project licences specifying permitted animal usage required by any laboratory undertaking animal testing. The official control laboratories and the FSA have worked towards the 3Rs through the refinement of current MBA methods, use of non-animal screens and analytical method development. Both the DSP and PSP MBA have been refined.

First the end point of the DSP MBA was replaced by defined clinical signs rather than mouse death, with the aim of reducing animal suffering. The use of these signs introduced a more precautionary approach to the assay in terms of public health. Further the number of mice was reduced from 3 to 2 mice per DSP test with a third mouse only permitted when inconclusive clinical signs were observed.

The PSP MBA is performed on two mice and the duration of the test has been reduced from 1 hour to 20 minutes. At 20 minutes the MBA test performed in the UK still detects PSP toxins at levels well below half the regulatory limit of 80µg STX equiv/100g shellfish flesh. The number of animals used in the calibration of the PSP MBA and the frequency of calibration were also reduced. More significant reductions in numbers used have been allowed through the implementation of screens, which remove the need to test negative samples by MBA. Two screening methods were introduced in the monitoring programmes. The first screen employed was the Jellett Rapid Test (JRT). The JRT was implemented in selected Scottish shellfish samples from April 2005, but its use was suspended from official control testing in May 2006 due to internal quality control failures. This screen was replaced by an HPLC screen implemented from late October 2006 in England, Wales and Scotland and from December 2006 in Northern Ireland.

Use of HPLC for the determination of PSP in shellfish samples

The PSP HPLC screen in use at AFBI and CEFAS is based on the AOAC official method 2005.06 (also known as the Lawrence (2005) method). The method relies on the acid extraction of toxins from the shellfish tissues (by HCl extraction at CEFAS (in compliance with and to retain compatibility with the AOAC method 959.08) or acetic acid extraction at AFBI (as specified in AOAC method 2005.06)) followed by oxidation (by periodate and/or peroxide) and LC analysis, as illustrated in Figure 1. The method was subjected to a programme of in-house validation during which its performances, fitness for purpose, robustness and potential for bioassay reduction were assessed according to EC Regulation 882/2004. The method which was approved by the UK Competent Authority is used as a qualitative screen in validated shellfish species (at CEFAS: mussels, native and pacific oysters, cockles and whole king scallops). Only those samples found positive by the HPLC screen are further tested by MBA to establish the PSP content of the samples. Samples of shellfish species for which the screen has not been validated continue to be tested by MBA alone.

During the first 10 months of implementation of the method at CEFAS, a total of 2,569 official control samples were submitted for PSP testing. Of these samples, 95.6% were screened by HPLC and 587 out of these were found positive and therefore forwarded on for MBA. The implementation of the screen therefore resulted in a 76% reduction of bioassay usage for PSP in this period. During the same period, bioassay usage for PSP was reduced by 96% at AFBI.

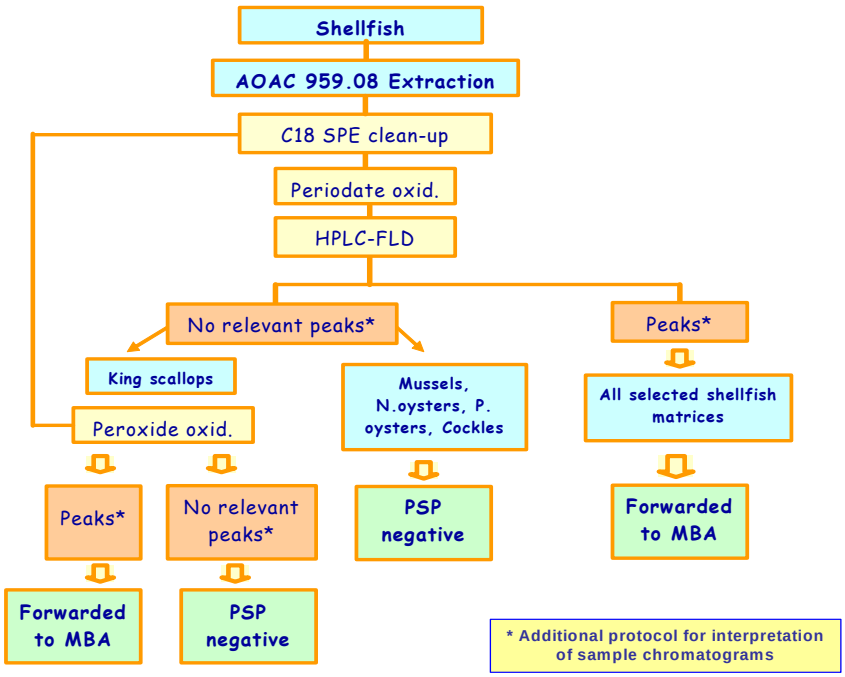

Figure 1. PSP HPLC screen at CEFAS: methodology summary and decision tree

Whilst implementation of the method resulted in excellent results in terms of reduction of bioassay usage, there are clear logistical differences between the analytical and bioassay approaches. The former requires a higher skill base and greater flexibility in staff working times, greater laboratory investment, costly equipment maintenance and servicing schedules as well as equipment repair support and more complex contingency measures than the MBA (Plate 1). The method is also more time consuming than the biological assay and therefore can impact on the timeliness of results. However, not withstanding these issues, laboratories can be set up to screen large numbers of samples (up to 35-40 samples per day at CEFAS), with results generally available within 30 hours of sample submission.

Plate 1. HPLC suite at CEFAS

A UKNRL procedure is being drafted for this method and the methodology and method validation report have been presented to the European Commission and the Community Reference Laboratory for Marine Biotoxins, in an effort to persuade other European members to adopt the HPLC screen to reduce the need for MBA.

Work continues on the implementation of the quantitative AOAC HPLC method. A programme of in-house validation is underway at both CEFAS and AFBI. The method performance characteristics are being assessed, in compliance with 882/2004, in 8 shellfish matrices of relevance to the UK and in 14 toxins, including some such as dcNEO and dcGTX2, 3, which were not covered by the AOAC method. The first matrix to be tested was the mussels, which constitute over 70% of all shellfish samples submitted to CEFAS. The method, pending successful validation and demonstration of its fitness for purpose is intended to be introduced in the UK biotoxin monitoring programme from April 08. Implementation, which may be for one species at a time, will be subject to a period of consultation with stakeholders. The UK experience with both the screen and the full quantitative method is being made available to other EU members through participation in EU working groups.

What next for the UK shellfish biotoxin monitoring programmes

The next few months will bring a few more changes to the UK biotoxin monitoring programmes. Firstly, the contracts for the role of National Reference Laboratory and for the coordination and delivery of the Scottish flesh programme are both due for renewal in April 2008. The organisations involved in the current programmes may therefore change. Further changes may also occur in 2009/10 when the English and Welsh programmes are put out to tender. Further methodology changes are also to be expected in the next couple of years. As previously mentioned, the AOAC 2005.06 quantitative method is expected to be introduced in the UK in early 2008. An increased use of the ELISA screen for ASP detection may also be expected. Significant progress has been made on the replacement of the PSP bioassay, similar approaches would be expected for the DSP bioassay. A number of methods have been available in official control laboratories, but none have yet been demonstrated to be useable within the framework of routine official control monitoring. A programme of liquid chromatography-mass spectrometry (LC-MS) method validation is being funded by the FSA from January 2008 onwards, with a view to lead to an implementable method within the next two years.

Whilst the scope of the programmes may not change, the way in which they are delivered may be further amended, as a result of new risk assessments and implementation strategies. In this context, the need for phytoplankton data and use of the current water monitoring programme (based on weekly, fortnightly or monthly sampling) may come under review, particularly at sites where weekly flesh data is being collected.

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IRISH, EUROPEAN AND INTERNATIONAL RESEARCH ON HARMFUL ALGAL BLOOM

Robin Raine, The Martin Ryan Institute, National University of Ireland, Galway.

It is useful to start any discussion on international co-operation in the field of Harmful Algal Bloom (HAB) research by asking "What is the goal we are trying to achieve?" "Why are governments throughout the world sponsoring costly programmes on HABs?" Few other branches of science research have an answer that is so straightforward. We are trying to understand how HABs arise in order that they can be predicted so that their damage to the aquaculture economy and public health can be curtailed or mitigated.

This mantra of understanding, prediction and mitigation is the underlying theme of GEOHAB[†] which is the UNESCO project on harmful blooms. This project, the most globally encompassing programme currently in operation, seeks to foster international co-operative research on HABs in ecosystem types sharing common features, comparing the key species involved and the oceanographic processes that influence their population dynamics. In doing so, there is a recognition that no one institution, and very likely no one country, has all the resources necessary to tackle any one of the outstanding problems in HAB research.

The GEOHAB programme has at its core four core research projects; on HABs in upwelling systems, eutrophic areas, coastal bays and fjords and, finally, stratified environments. An additional project on toxic epibenthic dinopflagellates is being considered. Ongoing research projects are affiliated to one of these areas, and cooperation between them is enabled. A number of framework activities are also sponsored, such as workshops, of which the recent HABWatch workshop was one.

The European Commission also sponsor international research. In the recent Framework 6 (FP6 2003-2010) three cooperative projects on HABs were sponsored. Their acronyms are ESSTALL, SEED and HABIT and are studying, respectively, molecular biological techniques for HAB species, their lifecycles, and their occurrence in high cell densities in sub-surface thin layers. An additional dimension to this EC funding call is that partnerships with the US (funded through their National Science Foundation) are promoted. All three projects are affiliated to GEOHAB, and NUI, Galway is centrally involved in HABIT and is a partner in SEED.

One of the activities in HABIT during 2007 was a research survey on the Marine Institute's *Celtic Explorer*. All the European partners in the programme including Spain, France and Britain took part, each participant contributing specialist equipment and expertise. The value of such collaboration is witnessed by the vertical profiles of *Dinophysis acuta*, one of the organisms responsible for contamination of shellfish with Diarrheic Shellfish Poisoning toxins, off the south coast of Ireland. Stations were sampled within 10 km of the coastline and high density subsurface layers containing up to 55,000 cells per litre were found (Figure 1).

[†] Global Ecology and Oceanography of Harmful Algal Blooms. This project is funded through IOC and SCOR.

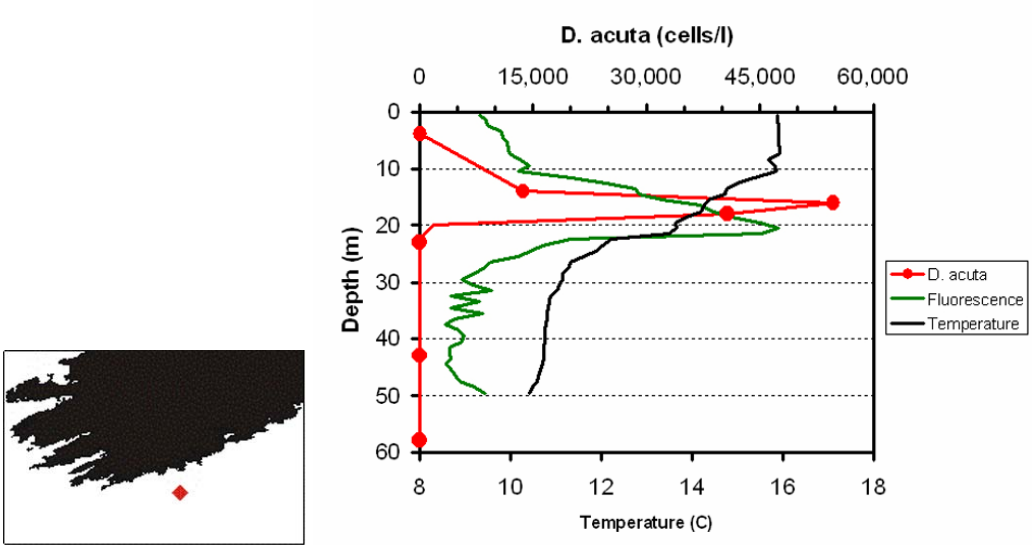
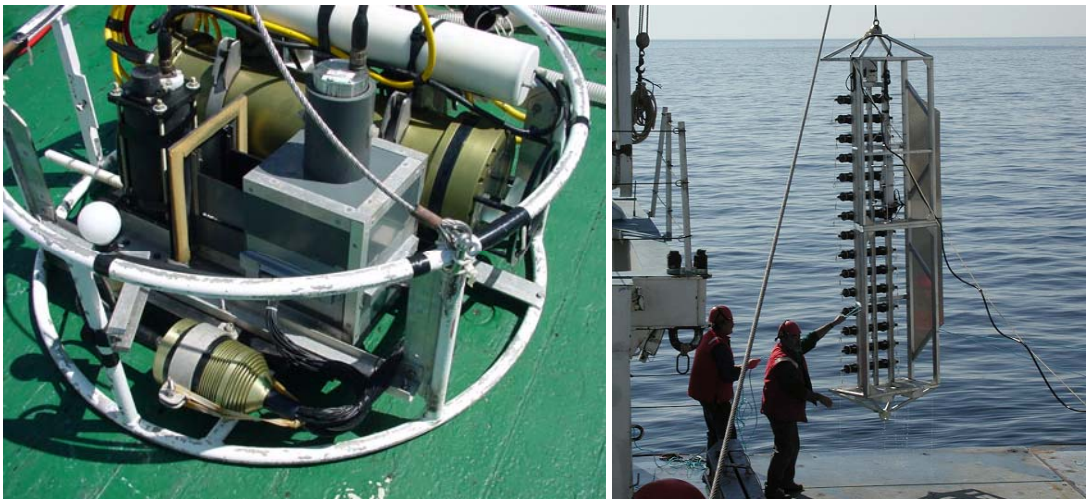


Figure 1. Vertical profiles of temperature, chlorophyll fluorescence and *Dinophysis acuta cell density* found off the south coast of Ireland on 23rd July 2007. The location of the sampling point is shown on the inset map. Note the very high cell density at a depth of ca. 15 m, and that this is offset from the chlorophyll fluorescence maximum.

The important point to make is that the thin layers would not have been detected without equipment belonging to IFREMER (France; Figures 2a and 2b), the state of the cells (which were found to be very active) would not have been discerned without the input from the Spanish participants from IEO, Vigo, and the observations made on the vessel of the *Dinophysis acuta* population being carried along in a coastal jet flowing parallel to the coastline would not have been possible without the input from physicists from CEFAS (Lowestoft). In fact, the *D. acuta* thin layer existed as a patch of 3 km diameter being carried along at a speed of ca. 7 km per day. The origin of this patch has not yet been elucidated, but results indicated that it arises in a similar mechanism to the *Dinophysis* populations of northwestern France (see McGrane *et al.*, 2004). At the end of the field research study, the population of *Dinophysis* was just to the east of Clear Island. There is no indication from the data of the national monitoring programme that this population existed, but if the weather (wind) had had a particular pattern, it could have ended up in Bantry Bay within 1-2 days.

There are two conclusions to be made from this. First, to predict infestations of *Dinophysis*, particularly in the southwest, there is a need for (upstream) offshore observatories. Secondly, if attempts are to be made to model these blooms, then the physical component of the model has to have a high enough resolution to encompass these relatively small scale coastal processes.

A large component of the project SEED focuses on the life cycle of *Alexandrium.* Participation in the programme was enabled through knowledge of *Alexandrium* blooms in Cork Harbour gained through a Marine Institute NDP PhD fellowship, and further funding through resources made available from the Higher Education Authority PRTLI (Cycle 3). Through this funding, it has been possible to ascertain the nature and population dynamics of a mixture of toxic and non-toxic species (*A. minutum, A. tamarense* and *A. ostenfeldii* have so far been identified) which are found there. This could not have been possible without molecular biological techniques (Touzet and Raine, 2007) and in addition, the toxic blooms can now be predicted well in advance with some accuracy (Figure 3; Ní Rathaille 2007).



a) b)

Figure 2. IFREMER instrumentation used on the *Celtic Explorer* research expedition, July 2007. a) The high resolution particle size profiler, with CTD, pump, videocamera and fluorometer; b) the fine scale sampler, capable of taking 15 samples over a distance of 3 metres.

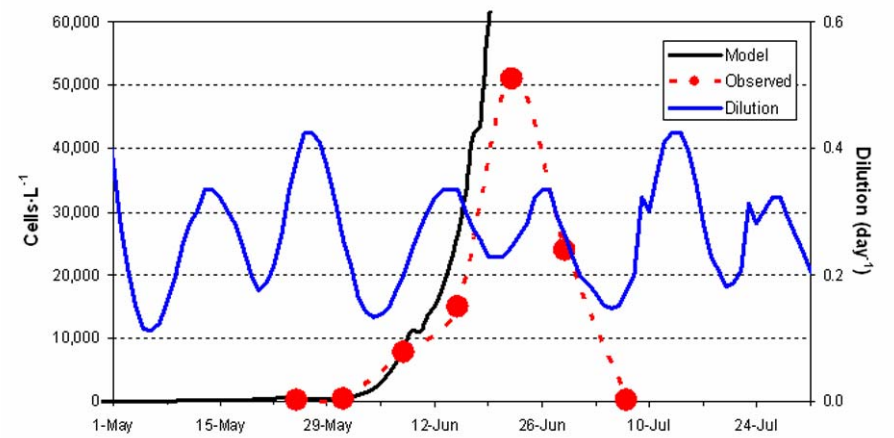


Figure 3. A comparison of the output from an *Alexandrium* growth model for Cork Harbour (black line) to observed cell densities (red dashed line) in 2006. The bloom was predicted to have occurred on or about the 16th June three months prior to its occurrence (see Ní Rathaille, 2007).

Thus the combination of national and international funding has reaped many rewards, but there is still much left to do. Those problems still outstanding and relevant to Ireland can be summarised, probably over succinctly, in Figure 4. However, with upcoming programmes of FP7, INTERREG, and national NDP funding the outlook is promising that resources can be obtained to tackle these.

DSP **The origin of *Dinophysis* populations off the south coast of Ireland still has to be sourced**

AZP **Planktonic organisms responsible for this toxin still have to be identified with certainty**

ASP **The plethora of *Pseudo-nitzschia* species extant and their diversity in toxin production will probably require new approaches to monitoring the toxin**

PSP **There has been recent success in modelling the occurrence of *Alexandrium* in Irish waters. Results have now to be converted into a management tool for industry.**

Figure 4. Some of the major outstanding problems in HAB research relevant to Ireland.

Acknowledgements.
The cell density results shown in Figure 1 were carried out by Hazel Farrell, MRI, NUI Galway. The author would like to acknowledge resources made available through the Higher Education Authority, PRTLI Cycle 3, the Marine Institute NDP programme (2000-2006), the EU FP6 programmes SEED (CT 2005 3875) and HABIT (CT 2005 3932), and the NTERREG NWE IIII programme FINAL.

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PHARMACOLOGICAL CONCEPTS AND CHEMICAL STUDIES RELEVANT TO EVALUATING THE TOXICITY OF AZASPIRACIDS

Philipp Hess[1] and Nils Rehmann[1,2]
[1] Marine Institute, Rinville, Oranmore, County Galway, Ireland
[2] University College Dublin, Belfield, Dublin 4, Ireland

Abstract
Azaspiracids (AZAs) are a group of shellfish toxins that were first discovered in mussels from Irish waters in 1995. Up to the year 2000 some 5 incidents of human poisoning associated with the consumption of shellfish containing this toxin were recorded. Since 2000, however, following the introduction of a regulatory limit and enhanced monitoring controls in Ireland, no cases of human poisoning have been reported. The occurrence of toxins from the Azaspiracid group was also demonstrated in shellfish from other EU Member States and in 2002 an EU regulatory limit was established. Due to the infrequent occurrence of poisoning incidents, the human toxicity of these compounds is a continued matter of debate.

In this paper, progress to date of toxicological research on Azaspiracids focussing on a recently completed multi-disciplinary, international research project funded under the Irish National Development Plan, 2000 – 2006, is summarised briefly. Pharmacological concepts of absorption, distribution, metabolism and excretion are introduced, and the importance of recent chemical studies on the toxins is evaluated in the context of these concepts. While AZAs have been shown to be unstable in hydrochloric acid, evidence from experiments simulating human stomach conditions suggests that the shellfish matrix acts in a protective fashion towards AZAs. Similarly, initial experiments on the stability of AZA under weak basic conditions suggest that AZAs are likely to survive in the human intestine. The discovery of novel shellfish metabolites (dihydroxy-, carboxy- and carboxy-hydroxy-metabolites) presents potential metabolic pathways that may also be relevant to human metabolism.

Finally, the paper also identifies gaps in the existing knowledge on the toxicity of AZAs, particularly the lack of statistically valid *in-vivo* data on the various Azaspiracid analogues, bioavailability of the toxins when consumed as part of a shellfish meal, and the effects of combined exposure to different toxin groups, e.g. co-occurrence of Azaspiracids and Dinophysistoxins.

Review of Regulatory Limits and Toxicological Studies on Azaspiracids
Azaspiracids (AZAs) are a group of natural compounds produced by planktonic micro-organisms, which as yet have not been unambiguously identified AZAs accumulate in filter-feeding bivalves, the consumption of which has led on several occasions to human poisoning. Following the identification of Azaspiracids as a new causative agent for an illness very similar to Diarrhetic Shellfish Poisoning (DSP) (Satake 1998), the Food Safety Authority of Ireland (FSAI) introduced a regulatory limit for these compounds in shellfish of 100 µg/kg in 2001 (Anderson *et al*., 2001). In 2002 the EU Commission set a limit of 160 µg/kg (Anon. 2002, superseded by Anon. 2004). These limits were based on relatively crude estimates of toxicity and with limited information available on the epidemiology or the chemical behaviour of Azaspiracids. In 2006, the FSAI re-evaluated the earlier risk assessments in the light of new information available on chemical behaviour and occurrence of analogues of AZAs, and concluded that a limit of 160 µg/kg was an appropriate limit to protect human health (Anon. 2006).

Toxicological studies had been hampered for a number of years by the lack of available purified AZAs. However, following work undertaken in a recent project (ASTOX) funded under the Irish NDP 2002-2006, progress has been made and some limited amounts of AZA1 have become available (Hess *et al*., 2007). In the same project, a number of studies were also conducted which were aimed at elucidating the molecular mechanism of action of Azaspiracids. These show how the toxicity of AZAs can be better understood when results are available from studies ranging from the symptoms in humans through effects observed in animals and cell cultures to the effects at molecular level in cells (Figure 1).

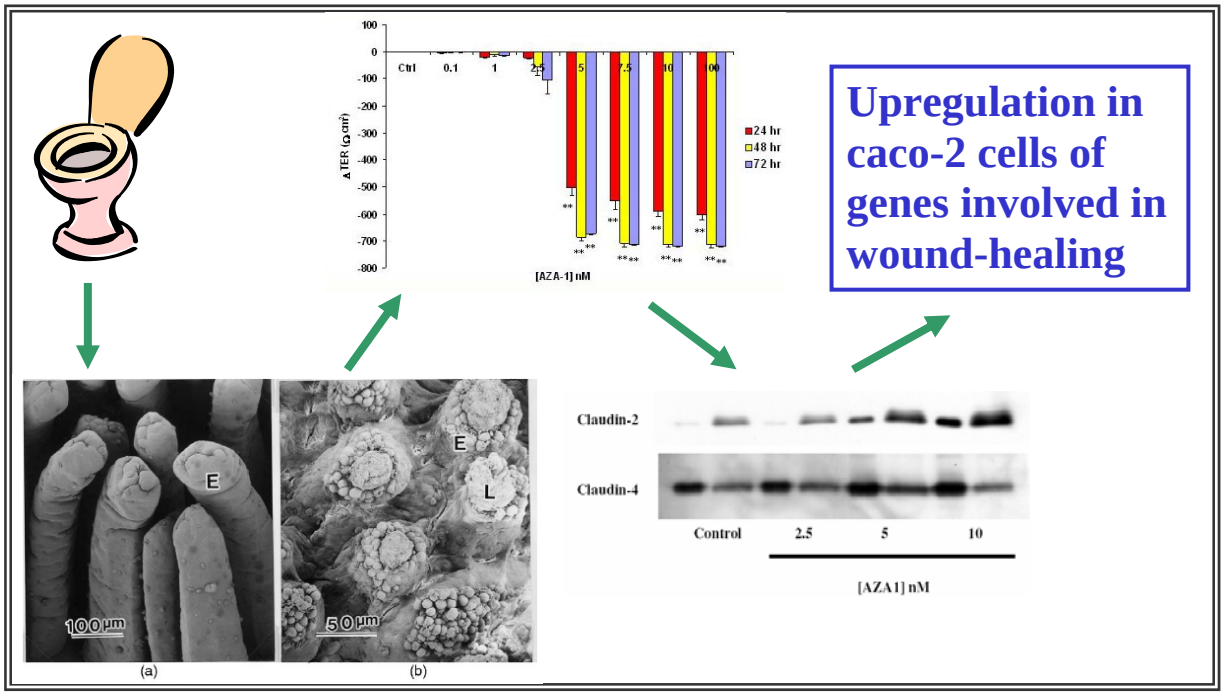

Figure 1. Toxic symptoms of AZA investigated at different effect levels; top left: symbol for diarrhoea in humans; bottom left: microphotograph of damage to villi on mouse intestinal wall (a) unexposed (b) orally exposed to AZA; top middle: AZA causes decrease in cell adhesion of caco2 cells measured as decrease of transepithelial electrical resistance (TEER); bottom right: AZA causes the overexpression of Claudin-2 (a protein involved in cell adhesion); top right: genes involved in wound healing are upregulated following exposure of caco2 cells to AZA.

Studies by other groups have also focussed on the cellular and molecular effects of AZAs, and these studies were summarised by Vilariño, 2007, and Twiner *et al*., 2008a. A study by Twiner *et al*., 2008b, has taken a systematic approach to the identification of molecular pathways through the evaluation of genetic changes triggered by exposure of a cell line to AZA1. The analysis of changes (up regulation / down regulation) in 37,000 genes was accomplished at different time points, and the results showed that pathways that intervene in cascades of wound healing and lipid metabolism, specifically cholesterol biosynthesis, were affected by exposure to AZA1. Although these studies have identified routes to determine the mechanism(s) of action of AZAs, none of the direct molecular targets of AZAs have been definitively elucidated to date.

Pharmacological Concepts in Toxicology

Pharmacology is the science of the action of drugs in the human body (Kenakin, 2004). In the evaluation of the toxic potential of compounds, toxicology leans very heavily on some basic pharmacological concepts. In broad terms, the pharamcological approach distinguishes between two major phenomena: dynamics of drug action and kinetics of drug action.

- The study of ***toxicodynamics*** of a compound qualitatively examines the interaction of the toxin with the body, i.e. effects caused by the interaction of the toxin with the molecular targets.
- The study of ***toxicokinetics*** is aimed at describing the concentration of the toxin as a function of time, in various places in the body.

While toxicodynamics aims to describe the action where it happens, toxicokinetics is more concerned about describing how the compound gets to the place of action, and at what concentration. Therefore, toxicokinetics is interested in all the chemical and biological phenomena that may lead to degradation or transformation of a toxin. In particular, before a compound can reach its target location it needs to be available to the body (in the case of food through the digestive process), and survive for some time under the physiological conditions in the human body (various levels of acidic and basic environments, 37°C as a temperature and a vast array of enzymes that may be involved in the transformation and degradation of chemicals). Furthermore, if a food constituent is suspected to exert an effect in a place other than the intestinal tract, it needs to be absorbed into the blood stream and be distributed throughout the body in order to reach the target place of action (Figure 2). The processes involved in making a compound available to the body, absorbing it into the body, metabolising, degrading and excreting it, all lead to specific concentrations at various places in the body and the extent of any effect observed directly depends on these concentrations. It is the understanding of the combination of dynamics and kinetics that will lead to a complete understanding of the toxicity of any compound.

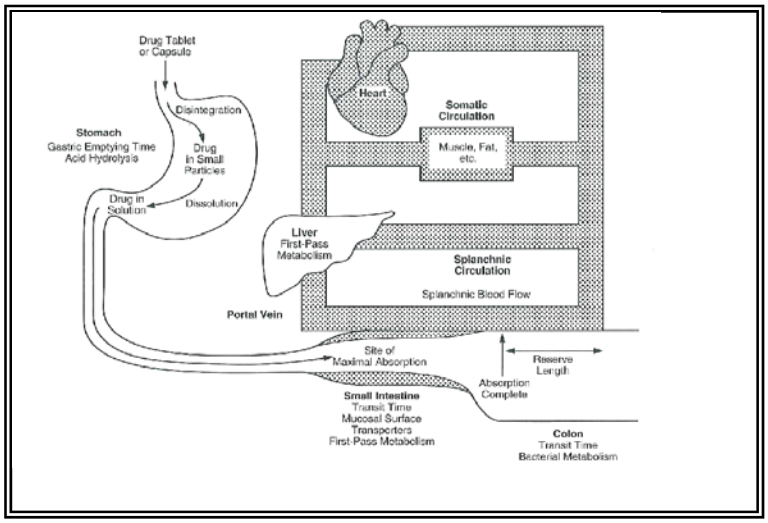

Figure 2. Schematic of the human digestion and blood circulation systems: food enters the digestive tract through the mouth and reaches the stomach where strong acidic conditions aid protein hydrolysis to start digestion. Subsequently, food passes to the small intestine where higher pH further aids digestion. Absorption from the intestine into the blood stream and on to other target organs is governed through passage through the intestinal barrier and the liver.

The studies mentioned in section 2 above have focussed on the effects of AZAs, attempting to identify the exact place in the body where the toxic action takes place, and the molecular target with which the toxin interacts. While to date only symptoms in the digestive tract have been reported in humans (diarrhoea, stomach cramps, vomiting, associated nausea), a study by Ito *et al.,* (2000), using oral administration of AZA1, has reported effects of AZAs in

multiple organs of mice, including the liver, lung, spleen and thymus. These effects can only be reasonably explained if AZA1, or its metabolites, have been transported via the blood stream to these organs. In addition, a series of exposures of AZA1 to mice over prolonged periods indicate some potential for chronic effects of AZAs, in particular slow recovery of damaged organs (Ito *et al*., 2002). As a result of these findings, a number of toxicologists have urged risk evaluators and managers to be cautious in the regulation for AZAs. Therefore, although no further acute poisoning incidents have been reported since 2001, the regulatory limit is questioned for its appropriateness in protecting the consumer from chronic effects.

Thus it becomes clear that knowledge of the potential distribution of AZAs within the human body following oral exposure e.g. through consumption of naturally contaminated shellfish, is important in the evaluation of the risk AZAs pose to the consumer.

Recent Chemical Studies at the Marine Institute

The stability of AZA is particularly important when modelling its concentration at different places of distribution. Studies in the Marine Institute investigated the stability of AZAs under various conditions, including different acid concentrations, temperature, and the influences of MeOH as solvent and of shellfish as a matrix. Initial studies used HCl as a strong acid, which had been reported to destroy AZAs effectively (Yasumoto T., personal communication). HCl is also a relevant acid as it occurs in the human stomach when food is digested. The results showed that AZA1 is destroyed within minutes when exposed, in a MeOH solution, to HCl at pH 0.5 (Figure 3). The time taken for almost complete destruction was 2hr when the pH was set at 1.5 (similar to human stomach), suggesting that AZA1 may not withstand physiological conditions in the stomach.

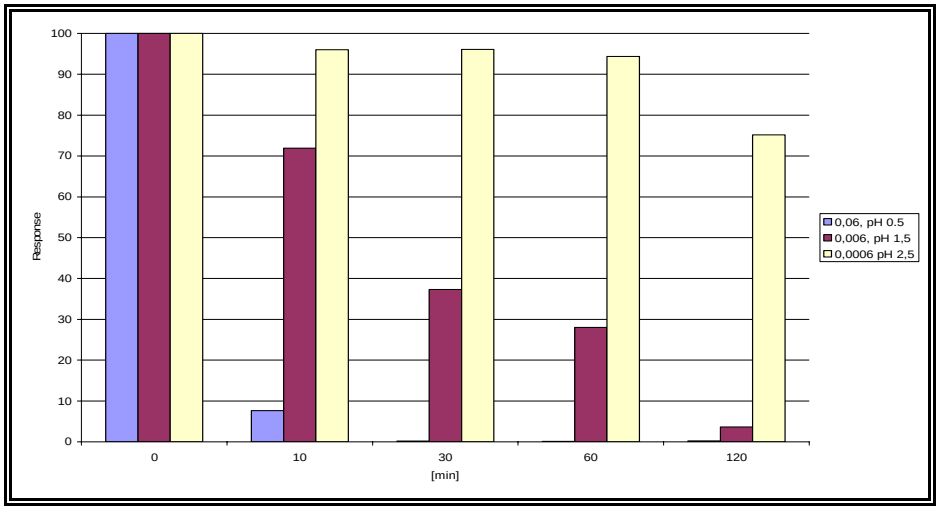

Figure 3. Stability of AZA1 in acidic MeOH solutions of different pH, 37°C; blue bars represent pH 0.5 (60 mM HCl), red bars represent pH 1.5 (6 mM HCl), yellow bars represent pH 2.5 (0.6 mM HCl). The condition in the human stomach may have a pH as low as 1.5 suggesting that AZAs maybe degraded in the human stomach.

However, since AZAs are consumed as shellfish contaminants and not as pure compound, we also investigated mixtures of naturally contaminated shellfish tissues with aqueous HCl (6 mM). The shellfish matrix clearly had a protective influence on AZA1, -2 and 3 (Figure 4). Even over prolonged exposure of the acid-tissue mixture to 37°C no degradation of any of the three AZA-analogues was observed, compared to the control material (LRM 1289), which was stored without acid at room temperature. The same effect was also observed when pepsin, which is an enzyme present in human stomach, was added in addition to the acid (data not shown). These results indicate that the start of food digestion in the stomach is unlikely to lead to destruction of AZAs.

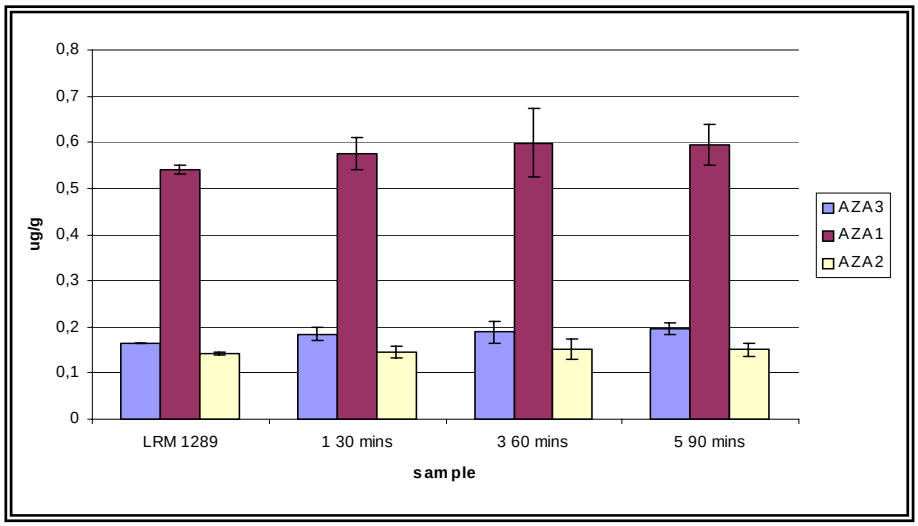

Figure 4. Stability of AZA1, -2 and -3 in shellfish tissue mixed with 6 mM HCl and kept at 37°C for ca. 0.5, 1 and 1.5 h (n=4, error bars are 1 SD). Neither analogue degrades over the study duration, demonstrating the protective effect of mussel matrix towards acidic conditions similar to those encountered in the human stomach.

This protective influence of the shellfish tissue has also been observed in other studies on the stability of AZAs, using γ-irradation as a technique to stabilise shellfish tissues for the production of reference materials (McCarron *et al*., 2007). The mechanism of the protective effect is not clear, but may be related to either specific binding of AZAs to shellfish matrix components, e.g. proteins, or simply due to AZAs, as lipophilic compounds, being unspecifically bound to any lipophilic pockets in cells, e.g. liposomes.

In contrast to the conditions in the stomach where the pH can be very low, the physiological pH in the human intestine may raise to as much as 9. Therefore, we also investigated the stability of AZAs under basic conditions. AZA1 in MeOH solution was exposed to either sodium hydroxide (NaOH) or ammonia (NH_4OH) at pH14 and 9, respectively. Clear degradation of AZA1 was seen at pH 14 at both 24h and 48h time points (Figure 5). The effect was dramatically accelerated by the rise from room temperature to 45°C. However, over the same period, AZA1was relatively stable at pH 9, with no significant degradation observed at room temperature and only very slight degradation at 45°C. Although we have not yet examined the influence of shellfish matrix under basic conditions, it is likely that AZAs are not destroyed at weakly basic pH, such as occurs in the human intestine.

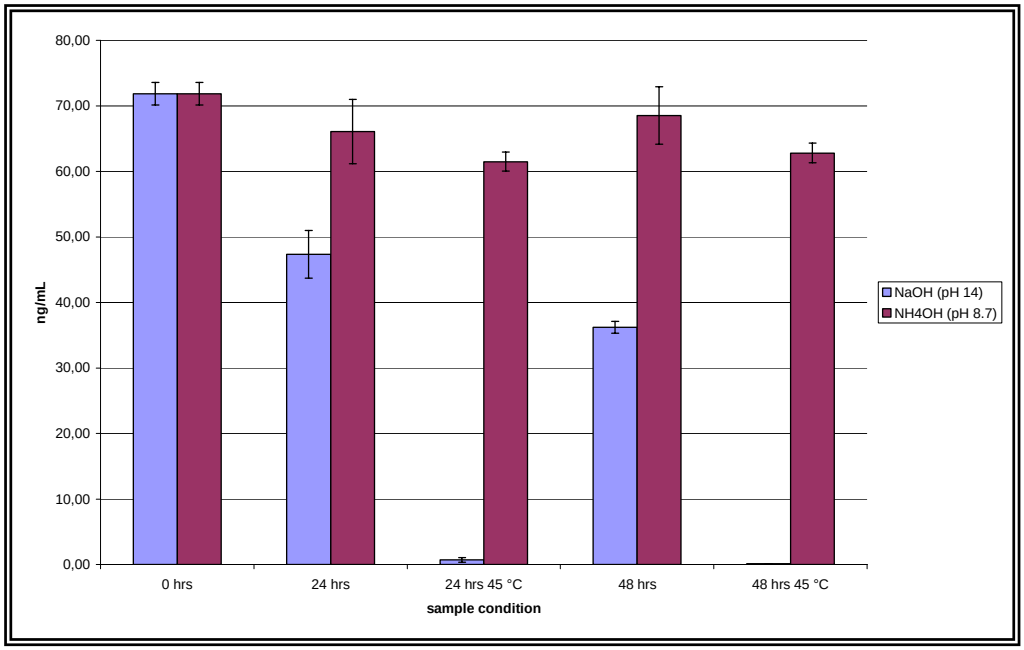

Figure 5. Degradation of AZA1 in MeOH at varying pH (n=4, error bars are 1 SD): blue bars represent treatment with NaOH (pH 14), red bars represent treatment with NH_4OH (pH 8.7). While strong alkaline conditions degrade AZA1 within 24h at 45°C, weaker conditions at a pH of 8.7 (similar to human intestine) do not result in significant degradation, even over a 48h period at 45°C

These studies at various pH levels also raise the question of how available AZAs are for absorption in the intestine. As the matrix appears to provide significant protection to AZAs from the acidic conditions encountered in the stomach, it is reasonable to ask whether AZAs can be absorbed into the blood stream when they enter the body following consumption of naturally contaminated shellfish, or whether they are actually so strongly bound to the matrix that they can not be absorbed, and only exert toxic effects in the digestive tract where direct surface interactions of AZA-shellfish matrix complexes are possible with the intestinal lining.

A further factor influencing the concentration of toxins in various body parts is their metabolism. As the causative organism of AZAs has not yet been unequivocally identified it is not clear exactly which AZAs are produced by the primary organism and which compounds are metabolites. AZA1, -2 and to a lesser extent -3 have been observed in plankton and water samples from Ireland and Norway (James *et al*., 2003, and personal communication Elie Fux, Chris Miles). Hydroxylated AZAs have been isolated only from mussels (*M. edulis*) suggesting that the hydroxylation takes place in the shellfish. We have recently isolated further metabolites in shellfish, including doubly hydroxylated AZAs, carboxylated AZAs and hydroxy-carboxy-metabolites. This suggests that AZAs are preferably metabolised via an oxidative pathway, possibly catalysed by the P450 family of enzymes. Further studies need to be conducted in mammalian systems to demonstrate degradation kinetics of AZAs, preferably *in-vivo*.

Conclusions and Requirement for further Studies

From the review of previous toxicological studies and the recent chemical studies outlined in this paper, it is clear that a number of questions remain to be answered regarding AZAs. These questions can be categorised into those relating to toxicodynamics, those relating to toxicokinetics and those relating to the causative organism:

- Toxicodynamics
  - Which is/are the molecular target(s) for AZAs?
  - Is there any organ-specificity in these targets?
- Toxicokinetics
  - How does digestion of mussels affect concentration of AZAs in different body compartments (bioavailability, distribution)?
  - How do lipophilicity, chemical characteristics and metabolism of AZAs affect absorption and distribution?
  - How does co-occurrence of different toxin groups affect absorption and distribution of AZAs?
- Causative organism
  - Which AZA analogues are produced by phytoplankton and which are produced in shellfish?

Due to the number and complexity of these questions, the authors are of the view that a multi-disciplinary effort from several research groups will be required to advance knowledge in this field. We invite parties interested in progressing such collaborative studies to contact the corresponding author of the paper.

Acknowledgements

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Appendix 1- List of attendees and speakers

Attendees

Surname	Forename	Company/Institute
Allen	Damian	Sea Fisheries Protection Authority
Allison	James	Sea Fisheries Protection Authority
Bire	Ronel	Marine Institute
Boyd	Suzanne	Food Standards Agency, Northern Ireland
Brennan	Claire	NUIG
Butter	Tim	En-Force Laboratories
Carney	Jimmy	Sea Fisheries Protection Authority
Chamberlain	Tara	Marine Institute
Costello	Patrick	Marine Institute
Condon	Jim	SFPA
Curran	Liam	Enterprise Ireland
Curran	Siobhan	Sea Fisheries Protection Authority
Cusack	Caroline	Marine Institute
Daly	Patrice	BIM
De Burca	Stiofan	Sea Fisheries Protection Authority
Deegan	Bryan	Altemar
Donlon	Pete	BIM
Duffy	Conor	Marine Institute
Falvey	John	SFPA
Fitzgerald	Olivia	Marine Institute
Flannery	John	Marine Institute
Furey	Ambrose	CIT
Fux	Elie	Marine Institute
Gilmartin	Maeve	Marine Institute
Greally	Maura	NUIG
Guilfoyle	Fegal	BIM
Guyader	Aurelie	Oyster Creek Seafoods Ltd,
Harrington	John	Irish Shellfish Association
Hensey	Mary	Glan Uisce Teo
Henshilwood	Kathy	Marine Institute
Hernan	Robert	Enterprise-Ireland
Higgins	Cowan	Agri-Food & Bioscience Institute, Northern Ireland
Hugh-Jones	David	Atlantic Shellfish
Hynes	Paula	Marine Institute
Irwin	Michael	Oyster Creek Seafoods Ltd.
Keady	Evelyn	NUIG
Kennedy	Simon	Killary Fjord Shellfish
Kerr	Marie	En-Force Laboratories
Kilcoyne	Jane	Marine Institute
Kinneen	Andrew	Sea Fisheries Protection Authority
Laffey	Chris	Public Analysis Lab Galway
Leitch	Jack	Charles River Labs
Lydon	Kevin	Killary Harbour Ltd.
Lynch	Gerard	Sea Fisheries Protection Authority
Lyons	Josie	Marine Institute
Lyons	David	FSAI
Lyons	Vicky	Bord Iascaigh Mhara
Maher	Majella	NUIG
Malavenda	Roberta	Student
Martin	Richard	Atlantic Shellfish Ltd
McCarron	Maria	Marine Institute
McCarron	Pearse	Marine Institute
McCleary	Stephen	Marine Institute
McDermott	Georgina	Environmental Protection Agency
McElhinney	Mairead	Marine Institute
McElwee	Joe	IFA Aquaculture
McFarland	Lynn	Environment & Heritage Service, Northern Ireland
McGowan	Niamh	BIM
McKinney	April	Agri-Food & Bioscience Institute, Northern Ireland
Mhic Ghiolla Chuda	Clíona	Meitheal Trá
Mulcahy	Diarmuid	Aquatask Enterprises Ltd.

Mullery	Alan	Sea-Fisheries Protection Authority,
Nicholson	Gearoid	Marine Institute
Norman	Mark	Taighde Mara Teo.
Nulty	Ciara	Marine Institute
O'Boyle	Niall	Coastal Zone Services Ltd.
O'Carroll	Terrance	BIM
O'Connor	Rebecca	Galway/Mayo Institute of Technology
Olden	Jamie	Atlantic Shellfish
O'Sullivan	Finian	Irish Shellfish Association
Rehmann	Nils	Marine Institute
Skeats	Joe	Environment & Heritage Service, Northern Ireland
Smith	Aisling	NUIG
Stack	Edwina	DCU
Swan	Sarah	Scottish Association for Marine Science
Touzet	Nicolas	NUIG
Tully	Oliver	BIM
Welsh	Jennifer	Galway/Mayo Institute of Technology

Speakers and Chairs

Surname	Forename	Company/Institute
Algoet	Myriam	CEFAS
Burns	Kieran	DAFF
Devilly	Leon	Marine Institute
Dore	Bill	Marine Institute
Ellard	Ray	FSAI
Heffernan	Peter	Marine Institute
Henson	Marie	SFPA
Hess	Philipp	Marine Institute
Kavanagh	Siobhan	NUIG
Keaveney	Sinead	Marine Institute
O'Cinneide	Micheal	Marine Institute
Raine	Robin	NUIG
Salas	Rafael	Marine Institute
Silke	Joe	Marine Institute
McMahon	Terry	Marine Institute