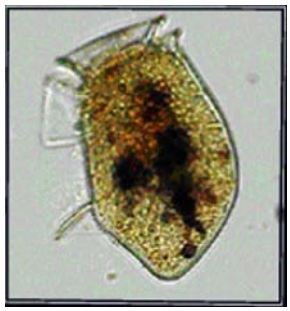
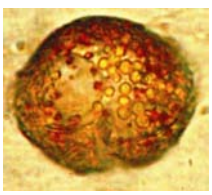
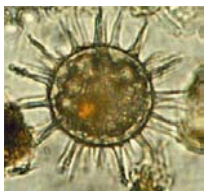
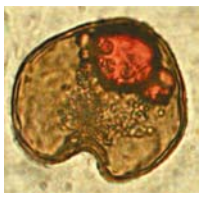
Proceedings of the Third Irish Marine Biotoxin Science Workshop

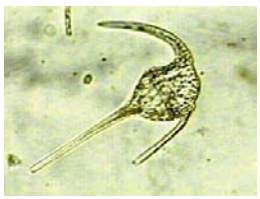


Galway 14th November 2002









3rd Irish Marine Science Biotoxin Workshop

Galway, Thursday 14th November 2002

Organised by the Marine Institute and Bord Iascaigh Mhara in association with the Food Safety Authority of Ireland

Contents

OBJECTIVES OF THE 3rd IRISH MARINE SCIENCE BIOTOXIN WORKSHOP

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

On behalf of the Marine Institute and our co-sponsors, BIM and the Food Safety Authority of Ireland (FSAI), I would like to welcome the participants to this, our 3rd annual Biotoxin workshop.

The **Marine Institute's objectives** for the Irish Biotoxin programme are:

- **Support the brand of Quality Irish Shellfish**
- **Promote food safety**
- **Work with our MSSC partners in the development of the Irish shellfish industry**
- **Develop the best Biotoxin management system in the Northern Hemisphere** (put another way, to be the "All Blacks of Europe".)

The workshop is part of the Marine Institute's role as the National Reference Laboratory for Marine Biotoxins in Ireland. This initiative was started in 2000 and was modelled on the Marine Science Biotoxin workshops, which have taken place in New Zealand since 1994.

This workshop is an annual event, where scientists, regulators and shellfish farmers meet to review developments in the monitoring and research of Biotoxins in Ireland and internationally.
The Institute's roles are to Monitor/Research/Advise/Communicate. These are inextricably linked. It is essential to carry out targeted research in order to answer the questions which are generated by the monitoring. As Minister of State Hugh Byrne said at the 2001 Workshop; "***Co-ordination and teamwork are crucial to progress in the biotoxin issue".***

<u>Objectives of the 2002 workshop:</u>

1. To review the Irish Biotoxin Monitoring programme in 2002
2. To summarise current and proposed new Irish research in the areas of Biotoxins and Harmful Algal Events (HAE's)
3. To provide an International view on Biotoxins issues, with invited speakers from France, Norway and the UK
4. To provide a forum for debate and communications.

Communications with Stakeholders.

The Marine Institute is committed to open communications with the many stakeholders, especially with industry, regulators and scientists. As part of the MI Biotoxin programme, we sought to promote communications in 2002 through the following eight channels:

- Weekly Reports by fax or email – **619** issued to date in the year 2002
- SMS Text message service by mobile phone re changes in bay status to over 90 industry and regulators
- Daily phone contact with samplers and industry members
- Participation at the MSSC meetings and its subcommittees
- Participation and advice to the Management Cell
- Arranging conferences, workshops and regional meetings
- Issuing the Proceedings of the annual Biotoxin workshop to 400+ interested parties
- Collaboration with the Food Safety Authority (FSAI) on an online HAB database.

Key Irish Developments in 2002

- Phasing in of the **Management Cell,** to facilitate rapid decision making, according to protocols which have been drafted by the Molluscan Shellfish Safety Committee (MSSC) members

- **Audit** of the efficacy of the national Biotoxin programme was carried out by the FSAI in Spring 2002.

- Provision of integrated weekly monitoring including Phytoplankton, Bioassay and Chemistry (LC-MS)

- Reduced level of toxicity (**3.4%** of shellfish samples positive) in 2002 compared to 2001 (16% positive)

- Appointment of a Shellfish Co-ordinator by the Department of Marine

- Scale up in the resources allocated to Irish **research** in the areas of Biotoxins and Harmful Algal Events (HAE's). There are active research groups in the Marine Institute, NUI Galway, Cork IT, Bioresearch Ireland, University College Dublin and Queens University, Belfast. Most of these groups are in regular contact and collaborate on projects.

International Best Practice / Research

Biotoxins and HAB's are a global issue. The scale of the natural processes underlying marine toxins means that international co-operation is essential.

The Marine Institute has worked closely in 2002 with the following and we look forward to building on these links in the years ahead:

- The EU Reference Laboratory in Vigo and the network of National Reference labs for Biotoxins in the EU
- International Gene Probe workshop in Galway, May 2002 with the support of MBARI (Monterrey) and Cawthron Institute, New Zealand
- Exchanges of staff with the Woods Hole Oceanographic Institution, USA which is a world leader in oceanography and HAB research
- Follow up contacts with biotoxin researchers in Japan, including scientists at both Tohoku and Chiba Universities.

REVIEW OF PHYTOPLANKTON AND ENVIRONMENTAL MONITORING 2002.

Caroline Cusack[1], Tara Chamberlan[2], Leon Devilly[1], Dave Clarke[1], Josephine Lyons[1] Joe Silke[1] Terry McMahon[3] & Michéal O Cinneide[1].

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[2]Marine Institute, Gortalassaha, Bantry, Co. Cork
[3]Marine Institute FRC, Snugboro Road, Abbotstown, Dublin 15.

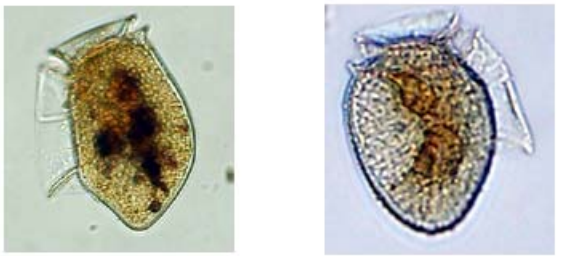
The Marine Institute has examined water samples off the Irish coast for the presence of phytoplankton since the early 1980s. In 2001, the phytoplankton monitoring programme was intensified, and today more then 2000 water samples are analysed on an annual basis.

1. PHYTOPLANKTON COMPOSITION AND ASSOCIATED TOXICITY, 2002

Every year, with few exceptions, periods occur when shellfish become contaminated from feeding on toxic phytoplankton and production areas are closed for shellfish harvesting because of the risk to human health. Significantly fewer toxic events occurred in Irish waters in 2002 than in 2001.

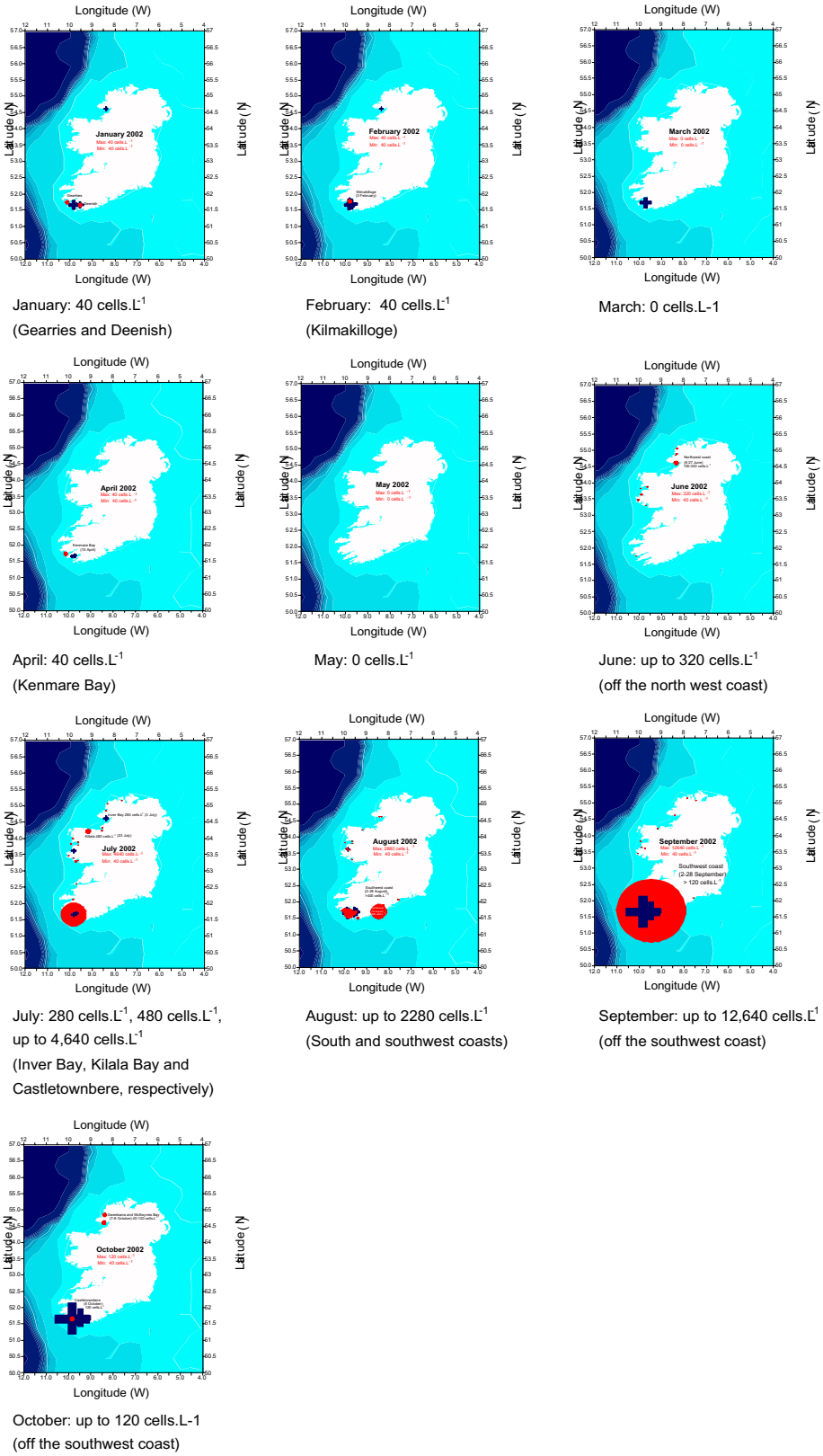
Data collected in 2002 showed that a lot of the closures in shellfish production areas were associated with presence of toxic species from the genus *Dinophysis* (Fig 1-2). Okadaic Acid (OA), the responsible chemical for Diarrhetic Shellfish Poisoning (DSP) in humans, was frequently detected in shellfish after the dinoflagellate *Dinophysis* was reported in water samples. For example, *Dinophysis* spp. were reported off the southwest coast (Castletownbere, Bantry Bay, Co. Cork) of Ireland during July 2002. Low cell densities (40 cells.L^{-1}) of *D. acuminata* (Fig. 1) were recorded on the 9th and 22nd July. Shellfish samples tested from this production area showed OA levels (0.2 μg.g^{-1}) well above the regulatory limit (0.16 μg.g^{-1}) on the 29th July, when cell concentrations of *D. acuminata* of up to 4,640 cells.L^{-1} were reported. A mixed population of *D. acuta* and *D. acuminata* (cell concentrations ranged from 40 to 760 cells.L^{-1}) were observed at this site throughout the autumn months (August, September, beginning of October) and OA concentrations ranged from 0.25 to >1.00 μg.g^{-1}.

Other potentially toxic phytoplankton species were also recorded off the Irish coast at high cell concentrations in 2002 and these included *Alexandrium* spp. *Pseudo-nitzschia* spp. and *Protoperidinium* spp. The highest recorded cell counts are given in Fig. 3 for these species.



Dinophysis acuta *Dinophysis acuminata*

Figure 1. Light micrographs of the DSP toxin producing *Dinophysis acuta* and *D. acuminata* from Irish waters.



January: 40 cells.L^{-1}
(Gearries and Deenish)

February: 40 cells.L^{-1}
(Kilmakilloge)

March: 0 cells.L-1

April: 40 cells.L^{-1}
(Kenmare Bay)

May: 0 cells.L^{-1}

June: up to 320 cells.L^{-1}
(off the north west coast)

July: 280 cells.L^{-1}, 480 cells.L^{-1}, up to 4,640 cells.L^{-1}
(Inver Bay, Kilala Bay and Castletownbere, respectively)

August: up to 2280 cells.L^{-1}
(South and southwest coasts)

September: up to 12,640 cells.L^{-1}
(off the southwest coast)

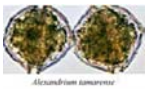
October: up to 120 cells.L-1
(off the southwest coast)

Figure 2. Areas off the Irish coast during 2002 (January-October) where the genus *Dinophysis* (red circles) and the DSP toxin, Okadaic acid (blue crosses) were detected. Size of bubble markers reflects the cell and toxin concentrations.

Dinophysis acuta
Dinophysis acuminata

Date	cells.L^{-1}	Site
29-Jul	4640	Castletownbere
12-Aug	2880	Oysterhaven
9-Sep	12640	South Chapel

Alexandrium tamarense



Date	cells.L^{-1}	Site
3-Mar	40	North Chapel
23-Apr	720	Roscarberry

DSP PSP
ASP AZP

Date	cells.L^{-1}	Site
8-Aug	1772960	Mc Swynes Bay
22-Aug	250920	Killary Harbour Inner
9-Apr	304960	Mc Swynes Bay
9-Apr	340000	Cuan Baoi

Pseudonitzschia spp.

Date	cells.L^{-1}	Site
21-Oct	69200	Kilmakilloge
2-Sep	5760	Oysterhaven
16-Apr	5680	Mc Swynes Bay
27-Aug	5760	Dungarvan
26-Sep	5120	Castletownbere***

Protoperidinium spp.

******Protoperidinium crassipes/curtipes*** 600 cells.L^{-1}

Figure 3. Highest cell concentrations of species from the genus *Dinophysis*, *Alexandrium*, *Pseudo-nitzschia* and *Protoperidinium* recorded in Irish waters during 2002.

2. Temperature and Phytoplankton Shifts, 2002

In addition to phytoplankton monitoring, environmental data may prove useful in the prediction of toxic algal events. An example of this can be seen from the temperature data collected at Roancarrig, in Bantry Bay during 2002 (Fig. 4). Temperature sensors were deployed at discrete depths (6 m intervals) at this site from the surface to 15 m and left in the water for 78 days. A temperature drop from 16°C to 13°C occurred throughout the water column on the 23rd July, 6 days prior to reported levels of OA toxin in the shellfish. During this period *Dinophysis* spp. were also observed in the water column. A second drop in temperature of 3°C was recorded on the 6th September, 3 days before the highest OA concentrations were detected in the shellfish. These changes in water column temperature indicate that cold water intrusions occurred at the times discussed above and the *Dinophysis* populations recorded at these times were more than likely transported into the bay with these water bodies.

Although the phytoplankton monitoring programme is primarily concerned with mapping the distribution of harmful algae, it is important that the species succession of non-toxic phytoplankton is also recorded. Collection of this type of data is invaluable, since it allows us to investigate interannual shifts in the

species composition of phytoplankton populations and intercomparisons between regions. Changes in the species spectrum of phytoplankton populations over long periods of time can be the result of anthropogenic effects (euthrophication) or climate change.

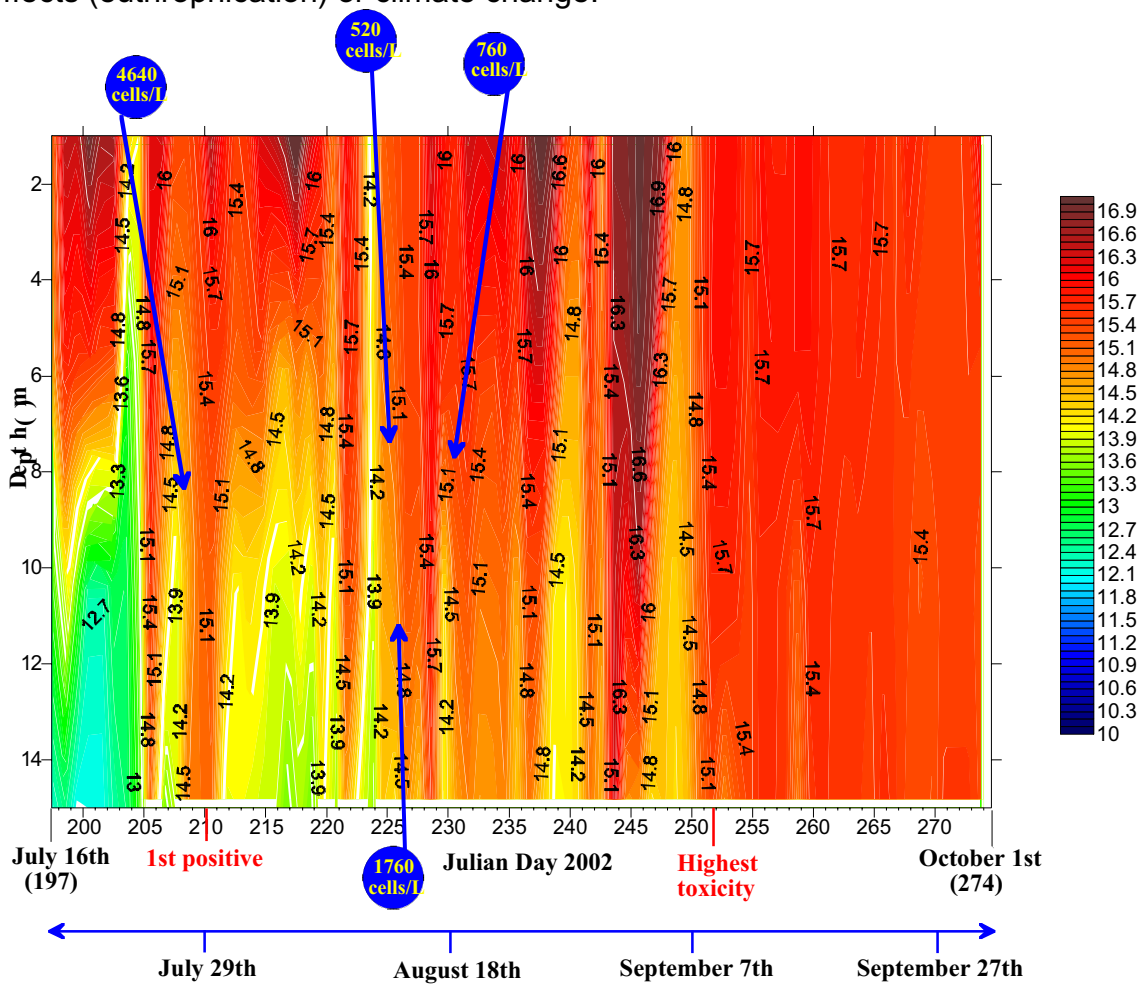


Figure 4. Surface to bottom (15 m) water temperature plotted against time (x-axis) and depth (y-axis) at Roancarrig, Bantry Bay in 2002. The temperature values from the surface to depth decreased from 16°C to 13°C on the 23rd July. On the 29th July, six days after a coldwater intrusion, the first record of OA was found in shellfish from the area. A large population of *Dinophysis* spp. (4,640 cells.L^{-1}) was also recorded after this sudden change in temperature. A second drop in temperature (3°C) was detected on 6th September, three days before the highest levels of OA were detected in shellfish in this area in 2002.

3. Differences between the phytoplankton populations recorded in 2001 and 2002.

Off the west coast of Ireland (Inishlaughill, Clew Bay) in 2002, the phytoplankton composition displayed a similar pattern to that reported in 2001 (Fig 5). The spring diatom bloom peaked on the 15th April, 2002, a week earlier than last year (22nd April, 2001) with cell densities of up to 2,443,000 cells.L^{-1} (more then double the cell concentration to that recorded in 2001, 917,780 cells.L^{-1}). The diatom population was predominated by *Chaetoceros* spp., *Skeletonema costatum*, *Thalassiosira* spp. and *Asterionella glacialis*. During July, 2002, dinoflagellate populations appeared a month earlier than last year (15th August 2001) with cell concentrations of up to 29,000 cells.L^{-1}. Non-toxic species from the genus *Scrippsiella* predominated at this time. A small autumnal bloom of diatoms (562,440 cells.L^{-1}) consisted of *Skeletonema costatum*, *Leptocylindrus danicus*, *Thalassionema nitzschioides* and *Skeletonema costatum*.

Off the southwest coast, at the Castletownbere site in Bantry Bay, the timing of the diatom spring bloom was a month later in 2002 (8th April) than in 2001 (5th March). Diatom cell concentrations recorded during the spring were also much lower in 2002 (70,520 cells.L^{-1}) than levels recorded the previous year (416,680 cells.L^{-1}). During the summer potentially toxic dinoflagellates from the genus *Dinophysis* predominated the dinoflagellate community that consisted primarily of *Prorocentrum micans*, *Protoperidinium* spp. and *Ceratium* spp.
A small bloom of diatoms (458,280 cells .L^{-1}) predominated by *Skeletonema* costatum, *Leptocylindrus* danicus and *Thalassionema nitzschioides*
occurred in the autumn (September).

Other blooms worth noting in 2002 included the nuisance pymnesiophyte, *Phaeocystis* off the south, southwest and west coasts in April. When present in very high concentrations this organism can sometimes clog and irritate (due to the production of acrylic acid) fish gills, although there were no such reports this year. A bloom of *Noctiluca scintillans* was evident off the east coast (in the vicinity of Howth, Co. Dublin) during July 2002 (Fig.6). This large heterotrophic dinoflagellate is not known to be toxic, however when blooms (water is an orange red colour) of this organism subside, microbiological activity can lead to oxygen depletion in the surrounding water and high concentrations of ammonia present in the vacuoles of *N. scintillans* can sometimes cause gill damage in fish. The photoautotrophic ciliate *Mesodinium rubrum* was responsible for water discolouration off the Waterford coast in August (Fig. 6). This organism is often responsible for “red tides” but does not seem to have any obvious harmful effects, although discolouration from such blooms often causes concern among local residents.

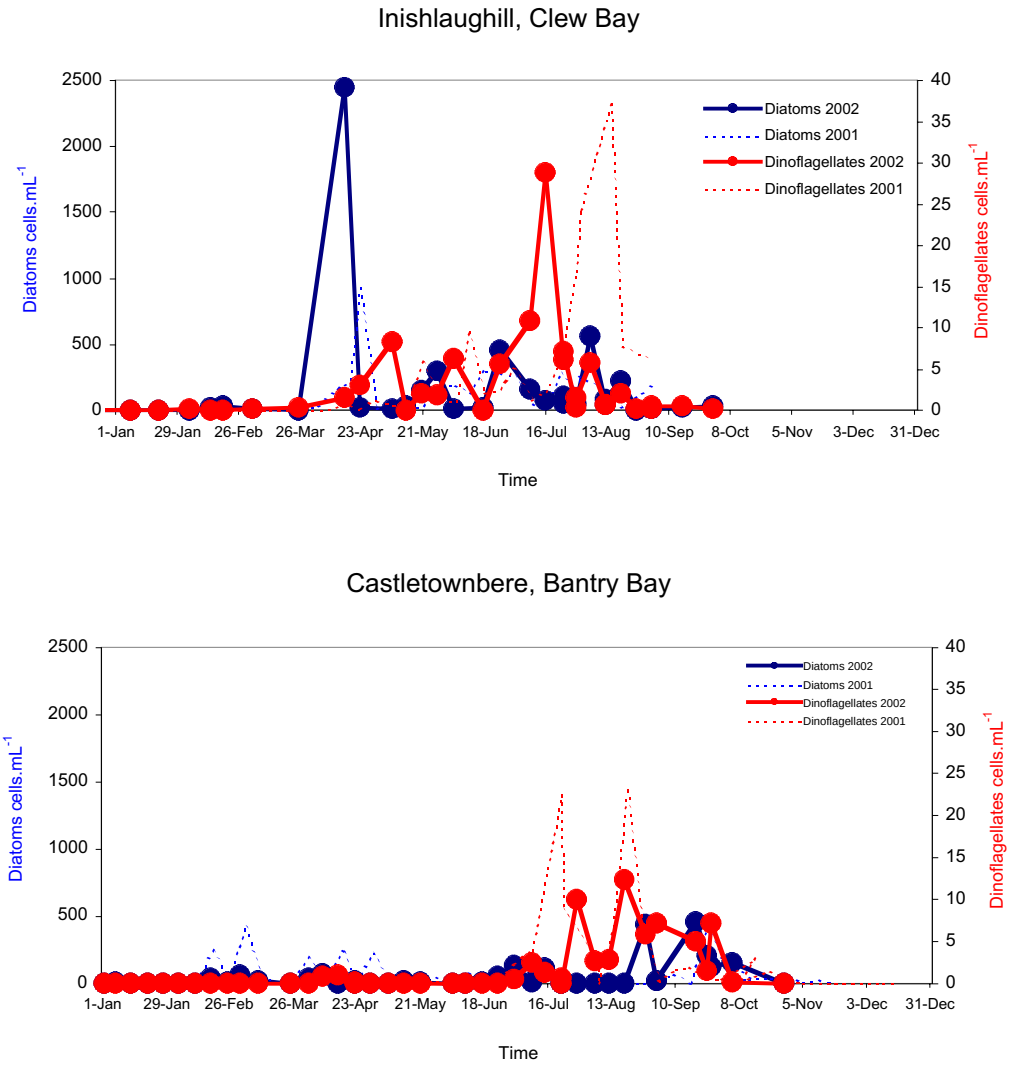


Figure 5. Diatom (primary y-axis) and dinoflagellate (secondary y-axis) cell concentrations (cells.mL^{-1}) recorded in 2001 (dotted lines) and 2002 (solid lines) plotted against time (x-axis) from Clew Bay and Bantry Bay. The diatoms are plotted on the left hand y-axis and the dinoflagellates are plotted on the right hand y-axis.

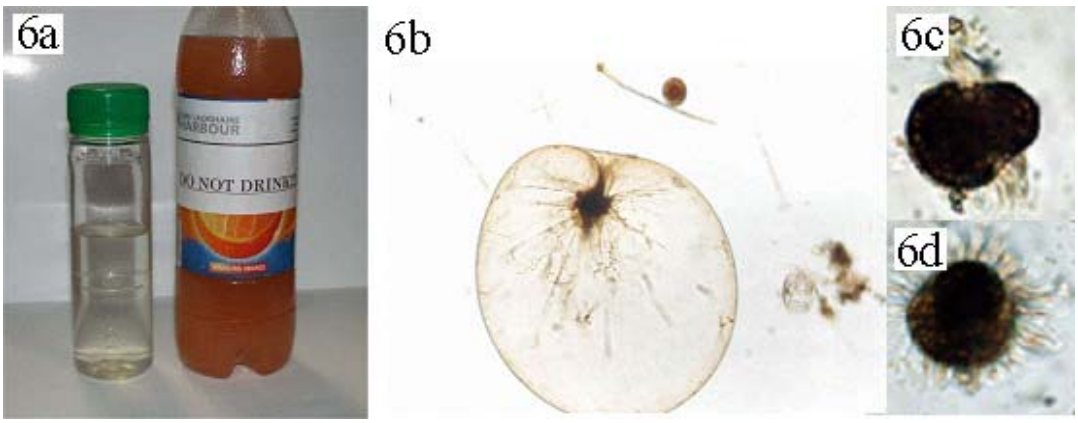


Figure 6.
6a. Picture of two unpreserved water samples taken off the east coast of Ireland (off Howth) during July 2002. The samples contained the phagotrophic dinoflagellate, *Noctiluca scintillans*, an organism that is frequently associated with finfish kills worldwide. Note: the sample bottle on the right hand side is a deep orange colour due to higher cell densities.
6b. Micrograph of the dinoflagellate, *Noctiluca scintillans* taken from a sample collected off the east coast of Ireland in July, 2002.
6c-d. Micrographs of the ciliate, *Mesodinium rubrum* from a sample collected off the Waterford coast in early August, 2002.

The primary goal of the phytoplankton programme is to gather and disseminate information on a weekly basis on the abundance of potentially toxic phytoplankton species present in aquaculture production areas around the Irish coast. The success of this programme is very much reliant on the sampling frequency at each site. If water samples arrive to the laboratory on a regular basis from each phytoplankton site then the data created is invaluable. A better picture on the occurrence of toxic species in a particular area can be determined and any obvious correlation between the presence of these organisms and variations in shellfish toxicity can be elucidated.

Since the phytoplankton monitoring programme is ongoing, the phytoplankton team will continue to improve the system. In 2001, a new sampling technique (Fig. 7) was introduced in order to aid in the detection of toxic organisms throughout the water column. Improvements were also introduced during the identification process carried out in the laboratory (Fig. 8).

Figure 7. The “Lund tube” sampling technique. Phytoplankton composition in the water column is better reflected with integrated samples (this is especially true when the water column becomes stratified in the warmer months of the year).

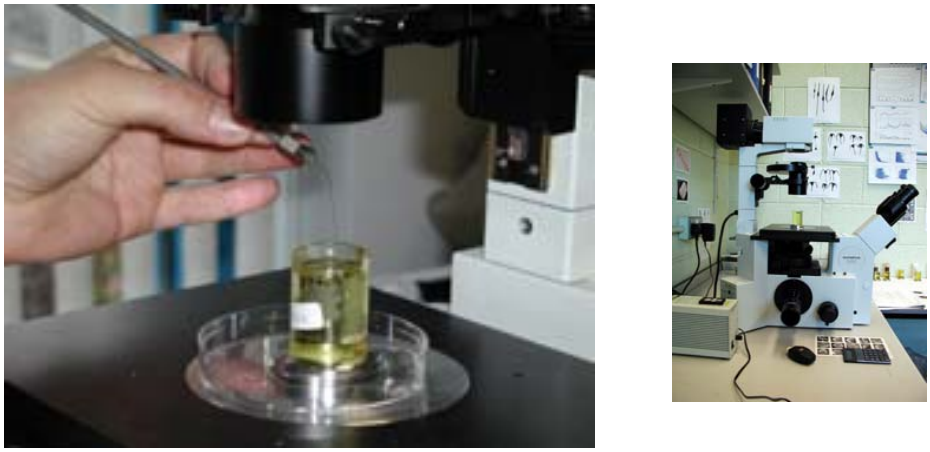


Figure 8. Picture of an inverted light microscope. The analyst is using an acupuncture needle to manipulate phytoplankton in order to help with the identification process.

Future plans for improving the system in 2003 will include the launch of a more user friendly database to input and receive results (Clarke et al., 2002). In addition to this, work has already begun to achieve quality assurance during the analysis of samples at the Marine Institute.

ACKNOWLEDGEMENTS:

Thanks to the summer bursar students, Sandra Lyons, Rafael Gallardo-Salas and John Flannery for helping out in the phytoplankton laboratory during the summer, 2002.

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A REVIEW OF SHELLFISHTOXICITYMONITORING IN IRELANDFOR 2002

Dave Clarke, Marine Institute, Parkmore, Galway

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

Resources for 2002

In 2002, new laboratories for phytoplankton analysis, phytoplankton culturing and cyst analysis, and bioassay analysis were transferred from Dublin to Galway. A new LC-MS and analytical team were introduced and implemented in Galway to complement existing laboratory facilities in Dublin for the chemical analysis of shellfish.

Analysis	Laboratory	Number of Samples (Jan – Oct 02)
Phytoplankton	MI (Galway & Bantry)	**2036**
DSP (Bioassay)	BLE (Ballina); BESU (Cork) & MI (Galway)	**2447**
PSP (Bioassay)	BLE (Ballina) & MI (Galway)	**125**
ASP (Chemical – HPLC)	MI (Dublin)	**609**
DSP / AZP (Chemical LC-MS)	MI (Dublin & Galway)	**2402**

Figure 1. Summary of the no. and type of samples analysed Jan – Oct 02

Bioassay analysis

During 2002 there was a decrease in the number of samples (*Figure 1*) submitted for DSP bioassay analysis (2854 projected in 2002 compared to 4030 samples in 2001). This was mainly due to the decrease in toxicity presence observed in all areas (reduction in twice weekly testing for mussels from 2001) and also due to the introduction of monthly testing of oysters during low risk toxicity periods (November – April).

Overall for 2002 the number of samples testing positive under mouse bioassay was 3.4% (based on 2402 shellfish samples submitted), compared to approximately 17% of samples observed testing positive during Summer 2001. No oyster, cockle, clam or razor clam samples submitted, tested positive for DSP Toxicity during 2002.

The toxicity observed in mussel samples in early 2002 (*Figure 2.*) was due to the carryover of toxicity in samples (due to presence of Okadaic Acid (OA) equivalents) from 2001 (6% in Jan 02 decreasing to 0.7% in Apr 02). In May/June 2002 no DSP toxicity was observed in all samples submitted. DSP Toxicity (OA equivalents) was observed to be present in mussel samples predominantly from the South West from July 02 (1.7% increasing to 5.8% in Sept 02). *Dinophysis sp.* was also observed to be present in these areas during this period. DSP toxins were observed to be decreasing from these mussel samples during the months of Oct 02 (4.8%) to 2% in Nov 02 of all samples submitted.

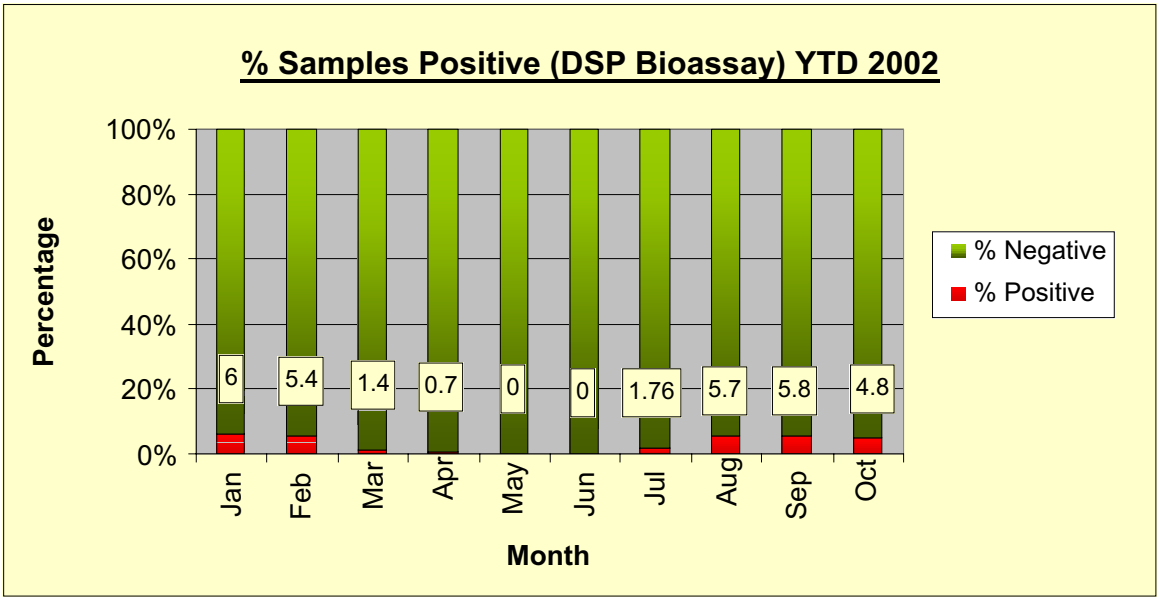

Figure 2. Number of positive samples (%) observed from Jan – Oct 2002

Azaspiracids - There were 3 occurrences during early 2002 (Jan 02 – Mar 02) where AZA's were observed to be present >0.16 µg/g Total Tissue in samples.

PSP toxicity was observed to be present above regulatory threshold levels in both mussels and oysters from Cork Harbour during a 3-week period in July 2002. 125 samples were submitted (Jan – Oct 02) for PSP analysis compared to 306 (Jan – Sept 01). The PSP toxin producing phytoplankton species *Alexandrium tamarense* was observed to have a lower presence in areas in 2002 than in 2001 accounting for the decrease in the number of samples submitted

ASP toxicity above recommended regulatory limits (> 20µg/g) was recorded for the time in a sample of mussels from County Donegal in June 2002.

Sample Analysis & Reporting

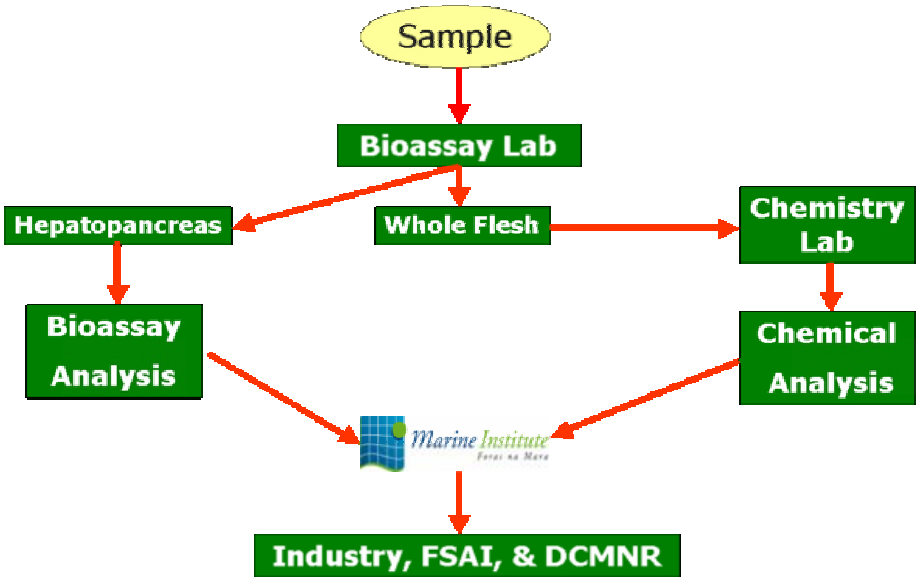

Figure 3. Flow Diagram Outlining Sample Analysis through to Reporting

Samples submitted are analysed and reported as per flow diagram (*Figure 3.)*, which allows for both bioassay and chemical analysis to be conducted on the one sample submitted. Presently all results are sent to and compiled by the Marine Institute. The status assigned to each production area, based on the results of samples submitted, is in accordance with the FSAI "Code of Practice". Results are issued by fax, email and SMS. To date (Jan - Oct 02) 614 individual DSP/ASP/Phytoplankton reports have been issued to industry, DCMNR and FSAI.

Off the 2402 DSP/AZP (Bioassay & Chemistry) samples analysed from Jan 02 – Oct 02, 83.5% of these samples were reported within 3 working days of the sample being taken (*Figure 4*.). In June 2002, FASI conducted an audit on the "Efficacy of the National Biotoxin Monitoring Programme" and concluded

' In general reporting is both efficient and timely'.

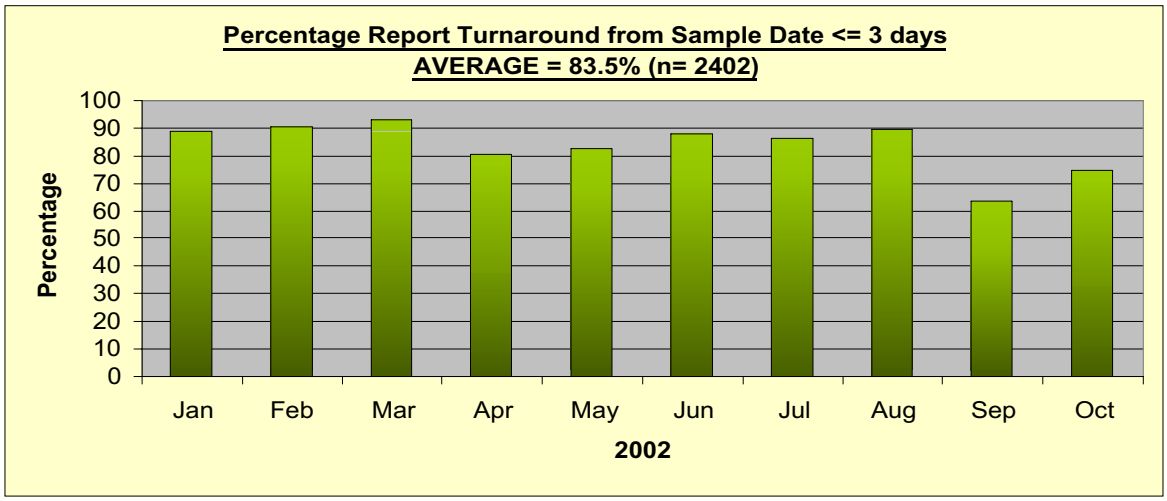

Figure 4. Turnaround time from sample date to report date for DSP / AZA (Bioassay and Chemical Analysis)

Of the 83 positive results obtained for Jan – Oct 02 (*Figure 5.*), 96.4% of samples were reported within 3 working days of the sample being taken (79.52% within 2 working days).

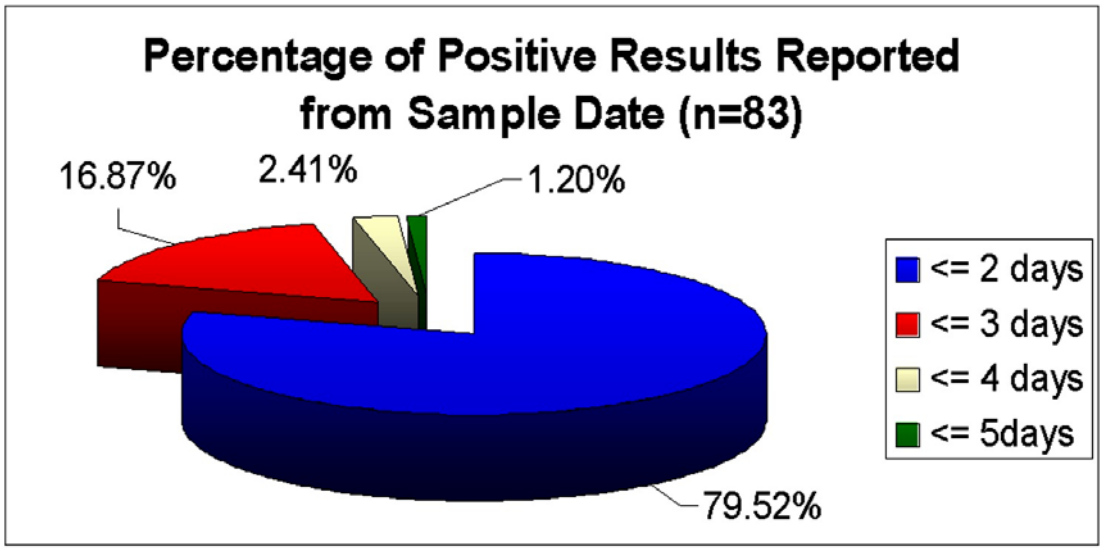

Figure 5. Turnaround Time from Sample Date to Report Date For Positive Samples

How can reporting turnaround be improved?

- Shellfish samples should arrive into Bioassay Labs before/on every Wednesday of each production week.

- All samples should be labelled correctly with Production site, sample code and date.

- Samples should be sufficient in both weight and size for both Bioassay and chemical analysis.

- DCMNR have appointed a sample co-ordinator to aid in improving the submission of samples into laboratories.

Reporting

In 2002 the FSAI introduced a website (**www.fsai.ie**) to allow users to access up to date shellfish toxicity results produced by the Marine Institute.
In 2003 a new database system will be available on line to the Shellfish Industry through the Marine Institute web site. The HABS (Harmful Algal Blooms) database allows for each lab involved in analysing samples to input the results obtained directly into the database. The Decision Platform Application process of the database compiles the reports, which are then viewed and the appropriate status to the production area assigned. The report will then be published via Web/Fax/SMS Text. An e-mail link is also sent informing and directing the recipient to the new report.
The database allows users for on line access for searches of published reports, updates on the status of production areas, and historical searches for Phytoplankton, ASP and DSP analysis for individual sites.

BIOTOXIN-CHEMISTRY MONITORING 2002

Deirdre Slattery, Marine Institute, Abbotstown, Dublin 15

The biotoxin-chemistry team is located in both Dublin and Galway. Two methods for routine analyses are carried out. ASP by HPLC is carried out in Dublin and AZP/DSP by LC-MS is carried out in both Dublin and Galway. For ASP monitoring, samples are received from shellfish production areas all over Ireland. For DSP/ASP monitoring, samples are received from the bioassay laboratories which are situated in Ballina (BLE), Cork (BESU) and Galway (MI).

- 609 ASP analyses have been performed to date in 2002 by HPLC-UV.
- 2357 AZP/DSP analyses have been performed to date in 2002 by LC-MS
- A new biotoxin-chemistry facility was introduced in Galway.
- A second LC-MS was put in place there and four new staff were recruited.

ASP:

There are two main parts to Council Directive 91/492 and Commission Decisions dealing with ASP. The first part states: “The total Amnesic Shellfish Poison content in the edible parts of the molluscs must not exceed 20µg/g of domoic acid using the HPLC method”
The second part states: “Member states may authorise the harvesting of bivalve molluscs belonging to the species *Pecten maximus* with a concentration of domoic acid in the whole body not exceeding 250 mg/kg”

The Marine Institute analyze domoic acid in the adductor muscle and gonad separately in order to ensure that these are not above the level of 20µg/g. This analysis satisfies the first part of the directive. The Marine Institute also analyze domoic acid in the remainder tissues. From the results of domoic acid in the tissues, the total tissue can be calculated for domoic acid. This satisfies the second part of the directive.

In 2002, approximately 10% of gonads tested for ASP had levels of domoic acid greater than 20 µg/g. Approximately 2% of adductor muscles tested for ASP had levels of domoic acid greater than 20 µg/g. (Ref: Figure 1)

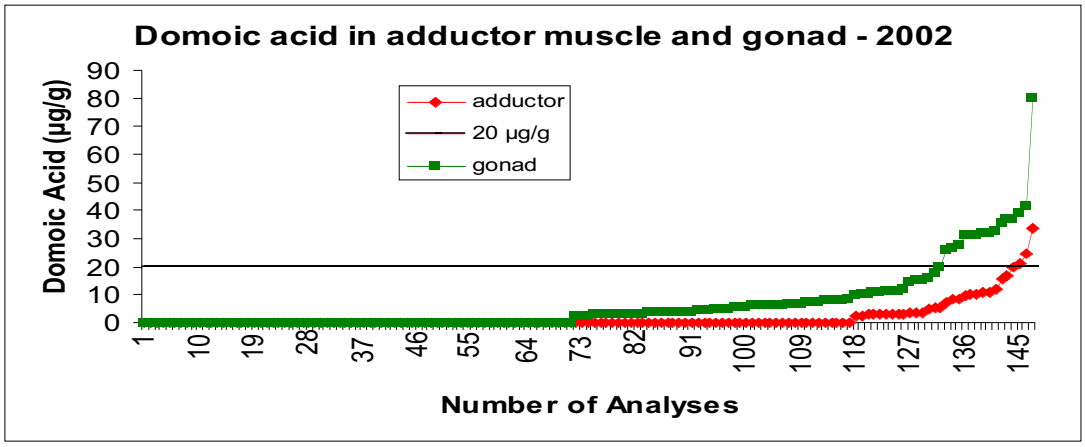

Figure 1: Domoic acid levels in adductor muscle and gonad

The maximum value of domoic acid in the adductor muscle in 2002 was 33.8µg/g. 2% of the analyses in 2002 were greater than 20µg/g domoic acid as compared to 1.1% in 2001.
The maximum value of domoic acid in the adductor muscle in 2002 was 79.9µg/g. 10.1% of the analyses in 2002 were greater than 20µg/g domoic acid as compared to 8.1% in 2001.
The maximum value of domoic acid in the total tissue in 2002 was 574µg/g. 31.7% of the analyses in 2002 were greater than 20µg/g domoic acid as compared to 38.6% in 2001.
There was an incident in July 2002 where mussels were analysed by the Public Analyst's Laboratory and they obtained levels greater than 20µ/g in the whole flesh. Since this incident, the Marine Institute have been testing mussels and oysters for ASP. Initially, in July, one sample had a value of domoic acid greater than 20µg/g. Since then domoic acid levels in both mussels and oysters have been below the regulatory level. (Ref: Figure 2)

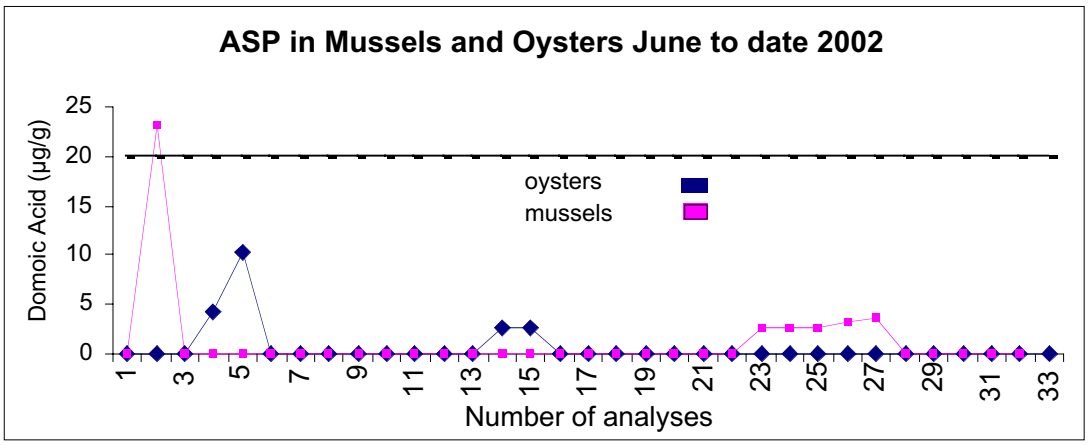

Figure 2: ASP in Mussels and Oysters 2002

The Marine Institute have submitted their ASP method for ILAB Accreditation. ILAB stands for the Irish Laboratory Accreditation Board. ILAB accreditation would 'formally recognise the competency of the body to carry out a specific test'. It would also ensure recognition internationally.

DSP/AZP:

Council Decision 2002/225/EC states: "The maximum level of okadaic acid or dinophysistoxins in the edible part of molluscs shall be 160 µg OA equivalents/kg" and "The maximum level of azaspiracid in the edible part of molluscs shall be 160 µg AZP equivalents/kg".

Okadaic acid was not detected in 96% of oyster samples. The highest value for okadaic acid was 0.03µg/g which is well below the regulatory level. Azaspiracid was not detected in 75% of oyster samples. The highest value for azaspiracid was 0.04µg/g which again was well below the regulatory level.

Okadaic acid was not detected in 70% of mussel samples. Approximately 7% of samples tested for OA were above the regulatory level. Azaspiracid was not detected in 75% of mussel samples. Approximately 1% of samples tested for AZP were above the regulatory level.

The biotoxin-chemistry results for OA and AZP were compared to bioassay results in 2002. 98.8% correlation was obtained between the two methods.

- 96.54% of all results had a negative bioassay and negative biotoxin-chemistry result.
- 2.5% of all results had a positive bioassay and positive biotoxin-chemistry result.
- 0.25% of all results had a negative bioassay and positive biotoxin-chemistry result.
- 0.96% of all results had a positive bioassay and negative biotoxin-chemistry result.(Ref: Figure 3)

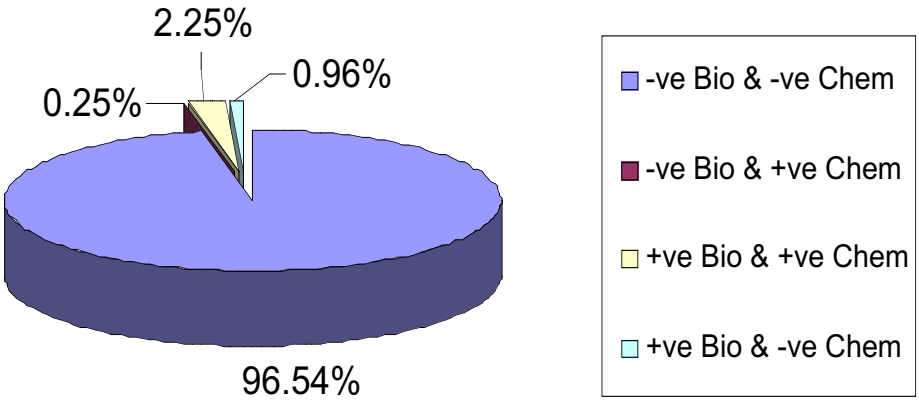

Figure 3: Comparison Chemistry vs mouse testing Jan – Oct 2002

BIOTOXIN AND PHYTOPLANKTON TRENDS

Irish Shellfish Monitoring Programme since 1984

Joe Silke, Marine Institute, Parkmore, Galway.

In the period up to the early 1980s, shellfish toxicity leading to diarrhetic shellfish poisoning (DSP) occurred so rarely in Ireland that it failed to provoke any action on the part of either the public health authorities or the Department of Fisheries. However in 1984 in response to a rapidly developing shellfish industry coupled with observations of *Dinophysis* detected at Sherkin Marine Station, the Irish Biotoxin and Phytoplankton Monitoring programme commenced. *Dinophysis* had been implicated in poisoning incidences in Holland (Kat 1983), and therefore was of great concern when detected in Ireland. The initial testing programme consisted of observations of phytoplankton samples on field trips made by Department of Fisheries staff to the southwest and the use of rat bioassay for testing the shellfish. This testing continued through the late '80s in the southwest, mainly in the summer months. With the expansion of the industry in the early part of the 1990s up along the west coast and into Donegal, testing also increased to cover these areas supplemented by HPLC. From the mid 1990's year round testing was adopted to take account of uncharacteristic winter toxic episodes and a switch was made to the mouse bioassay. The Marine Institute also took over the role of co-ordinating the testing from the Department around this time. By the late 90's up to 4000 bioassays and corresponding LC-MS analyses were carried out, and a phytoplankton monitoring programme was also in place around the coast (Fig 1).

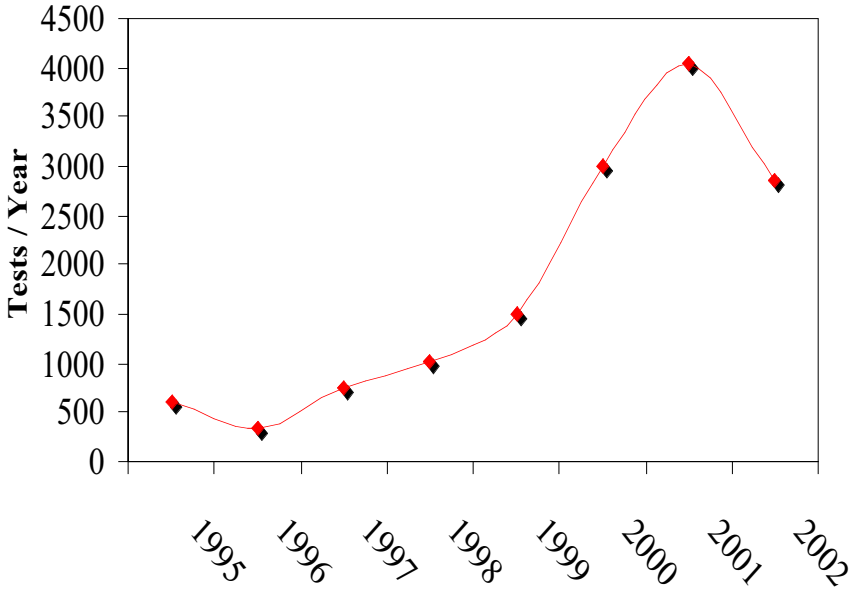

Figure 1 Increase in the number of DSP bioassays per annum in response to the expansion of Irish shellfish production

Since the early 1990's the most significant toxicity that has resulted in closures of shellfisheries was due to DSP toxins and from 1995 onwards some of these closures were also attributed to AZP toxins. The detection methods employed to detect these toxins have evolved and the Marine Institute has changed both bioassay methods and chemical methods a number of times since monitoring commenced (Table1). This included the switch from the rat bioassay to mouse bioassay in 1997, with a further refinement in 2000 by incorporation of a di-ethyl ether stage into this extraction method, and the provision of state of the art LC-MS analysis and quantification.

Year	Methods Employed	Method Reference
1984 –1996	DSP Rat Bio Assay OA + DTX2 by HPLC	*Kat 1983* *Lee 1987*
1997	DSP Mouse Bio Assay (Acetone extract)	*Yasumoto 1978*
2000	DSP Mouse Bio Assay (Di-ethyl ether step)	*Yasumoto 1984*
2001	OA, DTX-2, AZA Chemical Analysis LC MS-MS	*Hess 2001*

Table 1. DSP/AZP Monitoring methods used by the programme

With this data-series gathered in the course of this programme, we can begin to examine trends and try and answer some questions that are frequently asked. In some cases these trends point to research that is necessary to try and fill the gaps in our knowledge in order to explain the episodic nature of HABs and Shellfish toxicity. However, it must be remembered in examining trends, that there were a number of different protocols employed in terms of the areas tested, the time of year tested and methods employed in the lab. Nonetheless, there are some interesting patterns evident from the data.

***Why are some years more toxic than others*?**

Table 2 shows the percentage positive bioassays observed for all species since 1994. The earlier years have high percentages, but this is due to the emphasis of testing the southwest mussels alone and only in the summer months. The overall picture here however does show that there are some years when there is very little DSP toxicity present, as low as 1.5% for all species but other years can be much higher, and especially in the summer months. No obvious pattern is present for these variations such as correlation with high rainfall years, warmer summers etc. A similar pattern is evident, albeit at a lower level with PSP toxicity.

These figures are bases on all shellfish species tested, and as rope-grown mussels are more prone to toxification (see later) than the other commonly farmed species such as oysters and clams, there may therefore be a much higher incidence of toxicity in these shellfish.

Year	Total DSP Bioassay	% Positive		Total PSP Bioassay	% Positive
1994	778	61.3			
1995	611	29.3			
1996	343	13.1			
1997	755	1.7		140	1.4
1998	1010	1.5		93	1.1
1999	1488	6.9		17	0
2000	2991	18.1		27	7
2001	4030	16.3		217	0
2002	2494	3.4		124	3.2

Table 2. Percentage of Positive Bioassays since 1994

The question remains, why are these inter annual differences present, and in the absence of geographical, or inter-annual patterns the finger points at ecological attributes mainly in the phytoplankton. The most important of these is probably the subtle switch from diatom dominated phytoplankton in the early summer to a dinoflagellate dominated community. The time of this switch and the ratio is different each year. Observing this however demands a high frequency phytoplankton monitoring programme with many more sampling points and some offshore sampling. The proposed BOHAB programme described later in this publication will attempt to resolve some of these subtle phytoplankton issues. This is imperative to try and forecast the possibility of good years from bad years, in particular for the mussel industry.

Why does toxicity occasionally appear patchy in adjacent areas?

On examination of the DSP toxicity data there are occasional occurrences of high variability within bays, leading to conflicting results within single production areas.

For example, a study published by Carmody et al (1995) looked at toxin variability in mussels from inner Bantry Bay A total of 11 sites were sampled on 12/8/91and the levels of DTX-2 and OA were measured. Results from sites inside Whiddy ranged from 0.21 to 6 ug/g DTX2 and 0.05 to 0.37 OA Hepatopancreas. These data show that levels of DSP toxins were up to 29 times higher between sample locations within a 1 mile radius in Inner Bantry (Figure 2)

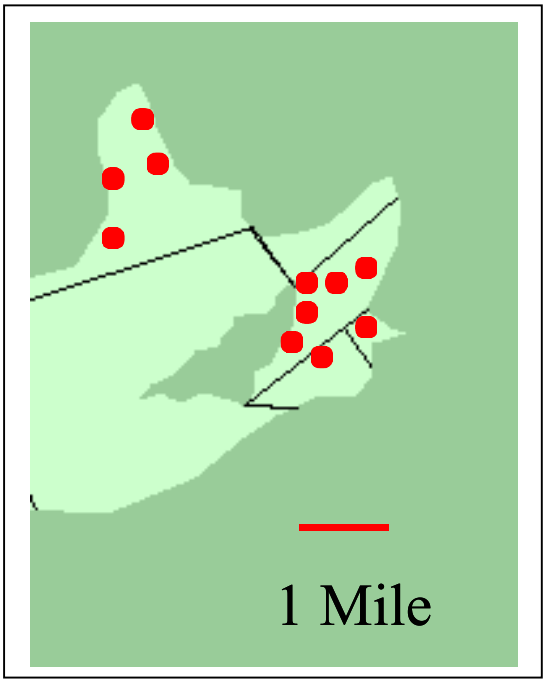

Figure 2. Carmody *et al.* study sites

One possible explanation for this variability may be due to the nature of phytoplankton transport into bays from offshore. Figure 3 shows a photograph taken in the South West of a water discolouration due to a bloom of *Noctiluca scintillans*.

Fig3. Phytoplankton Blooms (Photo C O'Shea)

It can be seen in this photo that there is a clear difference in phytoplankton concentration across very short distances. A similar pattern of phytoplankton distribution is present with *Dinophysis* and other toxic species, and this to some extent, explains the reason why shellfish from adjacent ropes or areas within close proximity to each other may display very different toxicity. The reasons for these streaky distribution of phytoplankton distribution is due to the fact that many of these populations are streamed off high concentrations at the mouths of bays and get blown or shifted with currents up the bay. This is why phytoplankton monitoring stations at the mouths of bays or upstream of shellfish growing locations may be a key to developing early warning capacity.

Are mussels more likely to be toxic than oysters?

It has been often observed that in areas where both mussels and oysters are grown together, that the mussels seem to be more prone to toxicity. Figure 4 shows the difference in positive bioassays for mussels and Pacific oysters.

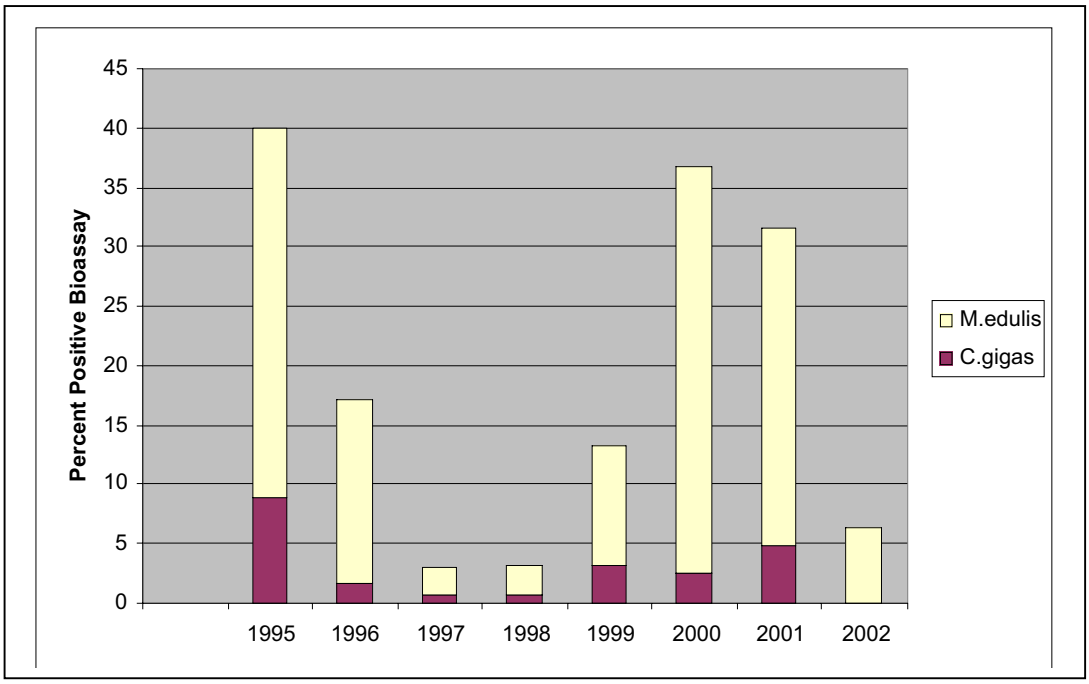

Figure 4. Percent Positive Bioassays for Pacific oysters and Mussels 1995 to 2002

It can be seen that Pacific oysters were responsible for a much lower incidence of toxicity than mussels, this has implications for the risk associated with the Pacific oysters, and the frequency of testing these can be reduced in the low risk times of the year. The highest year for positive mussels was 2000 with 34% of samples tested positive, while in the same year only 2.6% of Pacific oysters were positive. Overall the mean percentage of positive mussels were 16% compared to 2.8% of Pacific oysters.

Is there more widespread toxicity now than before?

A global spreading hypothesis maintains that the geographical extent, frequency and intensity of novel and nuisance algal events is increasing (Wyatt 1995). A similar perception is sometimes held that shellfish toxicity is more prevalent in Ireland now than in previous years. While the nature of this argument may be well founded based upon the increased amount of testing, increased awareness of toxicity, global warming and others, there has been little discussion on the counter-arguments, and little if any rigorous testing carried out.

Figure 5 shows the increase in Irish shellfish production based on figures from BIM, and the corresponding increase in annual numbers of bioassays carried out for monitoring purposes. The percent positive tests observed since 1995 is shown on the pink line. While there are obvious fluctuations expected in interannual prevalence of toxicity due to natural causes, methodology and sampling differences, the expected overall increase in percentage positives is not evident in this data. The spreading hypothesis is therefore not observed, perhaps due to a requirement for a longer time-series to observe human impacts on the coastal environment, climatic or tidal cycle change. A possible explanation of the perceived increase is due to the increased samples being tested, and also the expansion of the testing to cover more of the coastline and consequently there is the potential for a higher number of positives. Further analysis of this data will look at specific comparison of seasonal, geographic and species data to attempt to compare like with like, as the overall dataset is inadequate to observe subtle increases evident in more detailed comparisons.

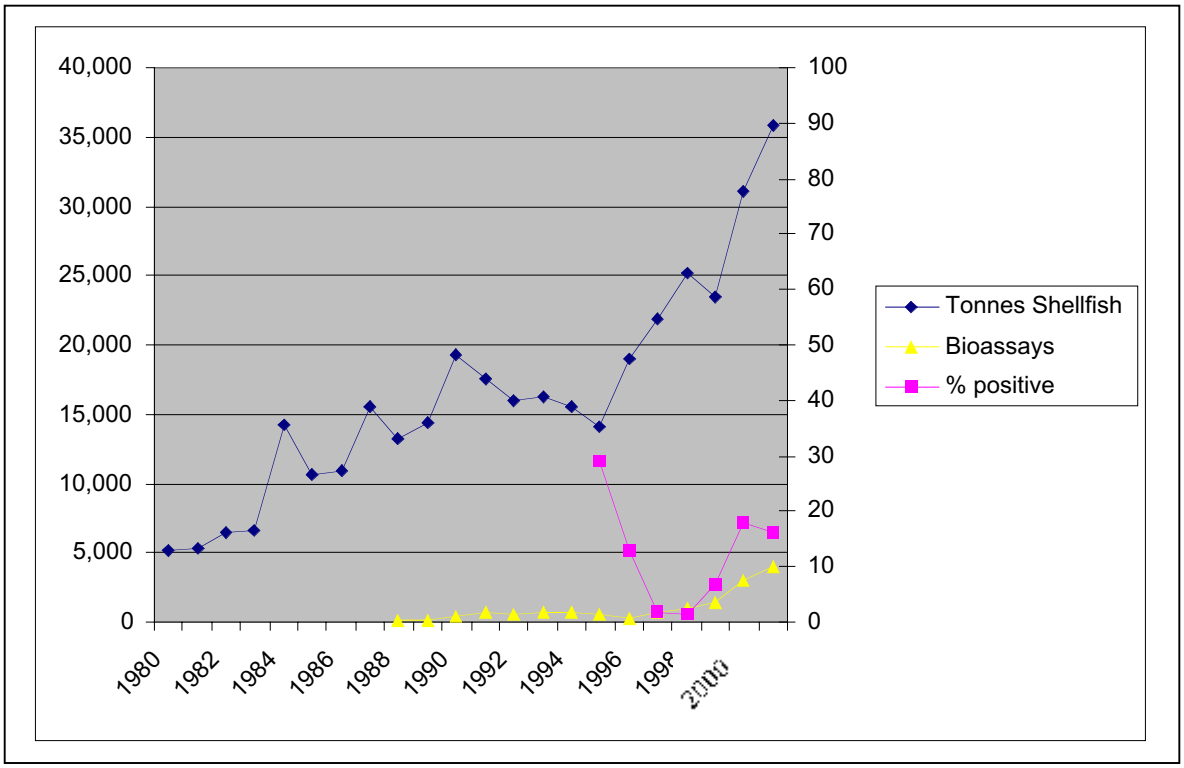

Figure 5: Increase in national shellfish Production, number of bioassays and percentage positives recorded

Discussion

The preceding account suggests that there are no obvious trends in interannual variability, spatial variability, or in geographic spreading. There is however an observable inter-species difference. The interannual and geographic spreading variability are possibly part of a much greater scale of episodic regime. This point is not gratuitous, there is an obvious trend between pre 1980 when toxicity didn't merit monitoring and after this period when it has increased to a point where it has almost decimated the shellfish industry out of existence. There have been parallel drastic trends in many other areas over similar time scales, such as changes in the North Atlantic phytoplankton bloom extent and period based on the continuous plankton recorder programme (Reid et al 1998), zooplankton change include in the eastern North Atlantic including a shift from *C. finmarchicus* with the slightly smaller *C. helgolandicus* (copepod species) which has had an observed effect on various life stages of redfish, haddock, herring, mackerel, salmon, cod, and capelin (ICES 2002). This zooplankton shift may also have an effect on prey phytoplankton species, allowing a shift in phytoplankton dominance to occur.

The changing pattern of toxicity in shellfish is most likely part of a process that is affecting all trophic levels, and not just a small group of dinoflagellates. There are still gaps in our knowledge of the processes that cause variability in the harmful effects of harmful blooms. Programmes such as BOHAB (described later) will attempt to address these.

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INDUSTRY PERSPECTIVE ON BIOTOXIN MONITORING PROGRAMME

Richie Flynn, ISA Executive Secretary

ISA Members, and especially those who have worked closest with the biotoxin issue are not attending this biotoxin workshop and have asked me to explain the Association's position to the attendees. In particular they would be anxious that our foreign speakers are aware that the non-attendance by the industry is no reflection on their particular work.

The contents of the previous presentation explain in themselves why the industry is so frustrated with the ongoing defensive attitude being taken on biotoxins. Statistics can be used in any form – for example the numbers of time bays were closed. However, from an industry point of view it is how the system is managed which is crucial. For instance, to suggest that 2000 and 2001 were relatively good years for biotoxins is astonishing when one considers the heartbreak endured by farmers and the subsequent payment of £2.5 million by the State for the extended closures during that period.

There was not sufficient consultation with ISA on the content of the presentations in advance of this meeting. In the context of last year's conference where the content of some of the presentations infuriated the industry because science was being used to score political points, it was felt that more advance notice of any similar "messages" should be given to the ISA, especially in light of the fact that we are supposed to be a "co-organiser".

In reality, not much progress has been made on industry issues within the biotoxin management regime. We are still reliant on the mouse bioassay, despite the huge amount of information gathered from chemical, & plankton sources. Things could be said to have become even worse in light of the fact that we could now potentially be closed on the basis of a second mouse bioassay carried out by the processors as part of their HACCP plans. Have we found out yet what is behind the strange closures of Bruckless and Castletownbere? Why are we still dumping product from open bays? Where is the promised co-ordination of sampling? We still have a long way to go.

When the industry wanted its voice properly recognised as part of a fully working and representative Management Cell, we had to approach Minister Browne to get our full rights established. We are grateful for the sympathetic hearing he gave to us on the day. We hope to see his commitments honoured in full by all parties. However, we have been down this road before and it seems that the industry always has to push to the extreme to get any recognition for its views.

In the MSSC our priority is to have equal status in the Management Cell for decision making on "grey areas". That's where a small organisation with few resources can concentrate our efforts on behalf of our members. It is a huge credit to the small band of voluntary shellfish farmers that we have made any progress whatsoever on biotoxins to date.

The biotoxin workshop is not the forum within which to express these views as it does not give us the scope for a real debate. This is made even more dangerous by the fact that the proceedings will probably be published and presented as an accurate reflection of the situation on the ground.

Industry is angry that we have to scrape and beg for every last cent – and then they freeze the NDP programme (our "ring fenced" Brussels –funded entitlement). They even stopped the paltry sum going to some farmers in multi-user bays for collecting samples. Yet money is always available for yet more people and more machines whenever the phrase "food safety" is attached to the financial request from State Agencies.

We hear of an extension of the biotoxin monitoring to bacteria and virus monitoring. What an appalling vista it is if we are to repeat or even triple the chances of the same people managing the industry on these parameters. The sad fact is that yet another expensive state machine will be hooked up to the ailing body of industry to monitor the disease without curing the cause – pollution. Just look at the only bay currently monitored for viruses – Cork Harbour – to see what the future holds for the industry.

Members are not interested in PR exercises but in being given the ability to carry out their jobs and market their fish. We have our representatives at the MSSC to put forward our views and work to improve the system if there is a willingness on the state side to co-operate. Fora such as this, operating in a context where there remains a lot of ground to make up to have a biotoxin system, are of little worth to the industry. The fundamentals will be lost in the fog of detail and industry will be back at square one. This industry does not have scientists or statisticians to call on. We have to work to keep our businesses afloat so we are at a supreme disadvantage of the only scientists, statisticians and officials we can rely on have a different agenda to our own and that is the nub of the problem.

I see we have a conference of Doctors next door and I wonder if I'm in the right room. Our shellfish industry is like a patient on permanent starvation diet. Meanwhile the number of consultants hovering around the bed and expensive machines being hooked up to the patient get more numerous and more expensive. Constantly the patient's appeals to be allowed to feed and look after itself are ignored while reports are written and conferences held to recommend how best to employ yet more consultants and buy more machines.

Thank you.

Azaspiracid toxins – current and proposed research projects

Philipp Hess, Terry McMahon, Micheal O'Cinneide

1. Abstract

This presentation discusses the past, present and proposed future research work on azaspiracid toxins (AZAs). The fields of research discussed include occurrences of the toxins since its discovery in 1995, toxicology, chemical synthesis as well as structure elucidation and isolation studies. The proposed future studies also include the international component of research and the possibilities of extending existing research fields to more applied topics, such as the management and mitigation of azaspiracids. The presentation does not attempt to give a comprehensive overview of the research conducted but summarises the milestones of work achieved this far.

2. Historic Overview

2.1 Occurrence

The initial occurrence of a new toxin in shellfish from Ireland was reported by McMahon and Silke (1996), following an incident of food poisoning in the Netherlands in 1995. During the winter 1995/1996 prolonged closures were enforced in Killkary Harbour due to the presence of toxicity as detected by both the rat bioassay and the mouse bioassay, Yasumoto 1978. A further food poisoning incident in 1997 (Arranmore Island, northwest Ireland), for which AZA contamination was responsible was reported by McMahon and Silke (1998). The occurrence of the toxin was only recorded on Arranmore island while during 1997 very little toxicity was detected in the other shellfish production areas in Ireland. During 1999, AZAs occurred again at concentrations detected by the mouse bioassay in France, in fact ca. 600 tonnes were retested (after export) and 5 out of 12 batches tested positive for toxins. During 2000, relevant levels of AZAs occurred in shellfish from Irish waters, again leading to prolonged closures. During 2001, the Marine Institute introduced chemical testing for AZAs specifically, and showed that levels of AZAs were relevant (> 0.16 µg/g) but okadaic acid and DTX-2 were the main toxic components resulting in the closure of shellfish harvesting areas. In 2002, James *et al.* presented a study, which demonstrates the presence of AZAs in other European countries, i.e. Norway, UK and France. Independently, Hess *et al.* (unpublished) found low levels of AZAs in shellfish from Norwegian and Swedish waters. This means that AZAs are a more wide-spread problem than initially believed.

2.2 Toxicology

The initial studies on acute toxicology of AZA-1 were conducted by Satake *et al.* (1998). This work showed that the acute effects in mice were different to those resulting from okadaic acid (OA) but that LD50s were similar to those of OA. Ito *et al.* (2000) studied the chronic effects of AZA-1 and found that multiple organs are damaged following repeated exposure of mice to AZA-1. The acute effects of AZA-2 and –3 on mice were published by Ofuji *et al.*

(2001) demonstrating that these two congeners are slightly more toxic than AZA-1. Cytotoxicity and PP1 inhibition was studied by Flanagan *et al.* (2001), again demonstrating that AZA-1 has a mode of action different to OA. These studies were summarized in a risk assessment conducted by the Food Safety Authority Ireland, (Anderson *et al.*, 2001) which was presented to an EU working group in May 2001. Ito *et al.* (2002) subsequently found evidence that AZA-1 may be carcinogenic, however, current work is continuing in this research due to the low numbers of mice initially exposed. Roman *et al.* (2002), also studied the subcellular effects of AZA-1 and demonstrated the non-apoptotic nature of AZA-1. In summary, we can say that the acute effects of AZA-1 result from doses similar to those of OA, justifying the current levels in the legislation. Further studies on the chronic effects of AZAs may lead to the revision of the current levels as outlined in the minutes of the EU working group from May 2001, and the current legislation (225/2202/EC).

2.3 Analytical methodology

The initial characterization of AZAs was carried out by HPLC-UV, NMR and LC-MS, (Satake *et al.*, 1998). Ofuji *et al.* (1999), developed a method based on LC-MS detection of AZA-1, -2 and -3, including an SPE clean-up. Draisci *et al.* (2000) developed an isocratic method for AZA-1, using LC-MS-MS detection. Quilliam *et al.* (2001) developed a gradient method for these three toxins, which can be incorporated in a general multitoxin method. During 2001, the Marine Institute also introduced routine testing for AZAs, using tandem MS (Hess *et al.*, 2001). Moroney *et al.* (2002) extend the initial data on SPE cartridge clean-up by a study of five different manufacturers' stationary phases. Overall, the method development has progressed well and the work remaining in the area will mainly focus on validation of existing methods.

2.4 Chemical Synthesis

Carter & Weldon (2000) published the synthesis of three fragments of the main AZA skeleton, namely the C1-C12, C13-C19 and the C21-C25. Aiguade *et al.* (2001) constructed a putative precursor to a fourth fragment (C28-C40). A further milestone was achieved by Carter & Graves (2001) with the construction of the C1-C19 fragment. Nicolau *et al.* also construct fragments, namely C1-C19 and C26-C47 (both 2001). Finally, Forsyth *et al.* (2001) constructed the C26-C40 domain of the azaspiracid skeleton. In total, a complete synthesis of the whole azaspiracid chain has not been achieved to date, thus the production of standards by chemical synthesis has not been possible to date.

2.5 Structure elucidation and isolation

The initial isolation and elucidation of the structure of AZA-1 (formerly referred to as Killary Toxin 1 = KT-1) was accomplished by Satake *et al.* (1998). The same group also isolated AZA-2 and AA-3 (Ofuji *et al.*, 1999). Quilliam and Hess postulated a stereo-isomer of AZA-1, as determined by LC-MS analysis of naturally contaminated samples, named AZA-1b in 1999 (unpublished data). Ofuji *et al.* (2001) isolated AZA-4 and –5, two hydroxylated homologues of AZA-3. James *et al.* (2002) postulate AZA-6 to –11, as

determined by LC-MS analysis. From July 2001 to march 2002, the Marine Institute conducted a pilot project on the isolation of azaspiracids to develop some expertise in this area and guarantee a longer term supply of these toxins. After initial isolation of ca. 2 mg of AZA-1 during a visit to the group led by Dr. Satake, the necessary equipment was also purchased and installed at the Marine Institute, resulting in the necessary infrastructure to conduct research in this area.

3. Current Projects

3.1 RASTA

This project is carried out collaboratively between the Marine Institute and Queen's University Belfast and is sponsored by the Food Safety Promotion Board. Its aims include, the isolation of standards from the phytoplankton and naturally contaminated shellfish , the development and validation of confirmatory analytical methods and the development of rapid screening tests via the use of antibodies. An overview and update on the progress of this project has been provided in 2 companion papers by McEvoy et al and Moran et al in these proceedings. In summary, the project has focused on the culture of Protoperidinium crassipes, which had previously been found to contain AZAs selectively in a sample of nethauled phytoplankton. Furthermore, this project has allowed the 2 institutes involved to further their knowledge in the isolation of AZAs from contaminated shellfish. During the project vital equipment was installed in Galway (Fig. 1) Immunisation of mice have not resulted in the production of antibodies so far (2 repeat injections of 2 mice) but the work is ongoing. This project will continue until August 2004.

Figure 1. Large-scale rotary evaporator and operator after installation of equipment in the MI facility, Galway Technology Park

4. Proposed Future Research

4.1 Cellular toxicity of AZAs

The Marine Institute (MI) has initiated this collaboration with scientists from NOAA following a visit of the lead scientist from NOAA, Dr. Gregory Doucette, to the MI Gene-Probe workshop in May 2002. NOAA has funds from its parent organization to conduct this study and indicated their interest in this work earlier this year. The Marine Institute has supplied the standard reference material necessary to conduct the initial toxicity studies while NOAA, with the help of a postdoctoral researcher, carry out the toxicity studies. This project has shown already initial results for the test of seven different cell cultures and the results will be published and integrated in any further research projects in which the Marine Institute will be involved.

4.2 In-vivo toxicity of AZAs

The Marine Institute is collaborating with the scientist who has conducted most of the initial studies on the toxicicty of AZAs, Prof. Emiko Ito of the

University of Chiba, Japan. Similar to the above, project, the results from this collaboration will be published in due course and integrated into any further research work conducted at MI or with MI input.

4.3 Azaspiracid isolation and toxicology (Marine Research Measure)
This project is a proposed collaborative study between the Marine Institute and the Conway Institute UCD. The work is scheduled over three years and will integrate the ongoing studies on isolation and toxicology in Ireland, US and Japan. The ultimate goal in this project is to carry out sound toxicological studies to evaluate the acute and chronic effects of AZAs, their mode of action and the No-Observable-Adverse-Effect-Level (NOAEL). For this purpose, a major part of the project will also be focused on the isolation of pure azaspiracids required for the toxicology work.

4.4 Management and mitigation projects
These project proposals have been put together with the input of Brendan O'Connor from Aquafact, and Cilian Rodan (Consultant) and Michael Irwin (Oyster Creek). The projects, once active, will investigate the possibilities to depurate toxins from shellfish through relaying and/or tank experiments with clean seawater-supply tanks. These projects depend heavily on the fresh occurrence of relevant concentrations of AZAs in shellfish, which was not the case during 2002. Therefore, they will only be carried out once these levels occur.

4.5 European Research Projects
An Expression of Interest has been prepared for June 2002. The consortium agreeing to participate in this work comprised ca. 25 scientists from Europe and overseas. Further work will be necessary to make the proposal attractive to the EU research program. This should also include a strong input from the shellfish industry, and the Marine Institute currently encourages interested industry bodies to participate.

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RAPID AZASPIRACID SHELLFISH TOXIN ANALYSIS – RASTA UPDATE

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This paper gives a brief overview on azaspiracids, describes the background to the RASTA project and reports the results generated during the first year for one of the partners in the project (VSD). A companion paper from the Marine Institute given as part of this presentation at the Third Irish Biotoxin Workshop describes the progress made by the Marine Institute during the first year of the project.

Introduction

Azaspiracids (AZA) are relatively new shellfish toxins which were first detected in mussels harvested from Irish waters in 1995 and exported to the Netherlands (1). The unlucky Dutch consumers suffered symptoms typical of Diarrhetic Shellfish Poisoning (DSP) intoxication which included nausea, vomiting, diarrhoea and stomach cramps. Satake et al (2) first purified and identified the toxin (AZA 1). Subsequently several other analogues have been structurally elucidated and described (3,4) – see Figure 1 below. Recently, AZA have been reported from other European countries including England and Norway (5), underlining the widespread nature of these toxins.

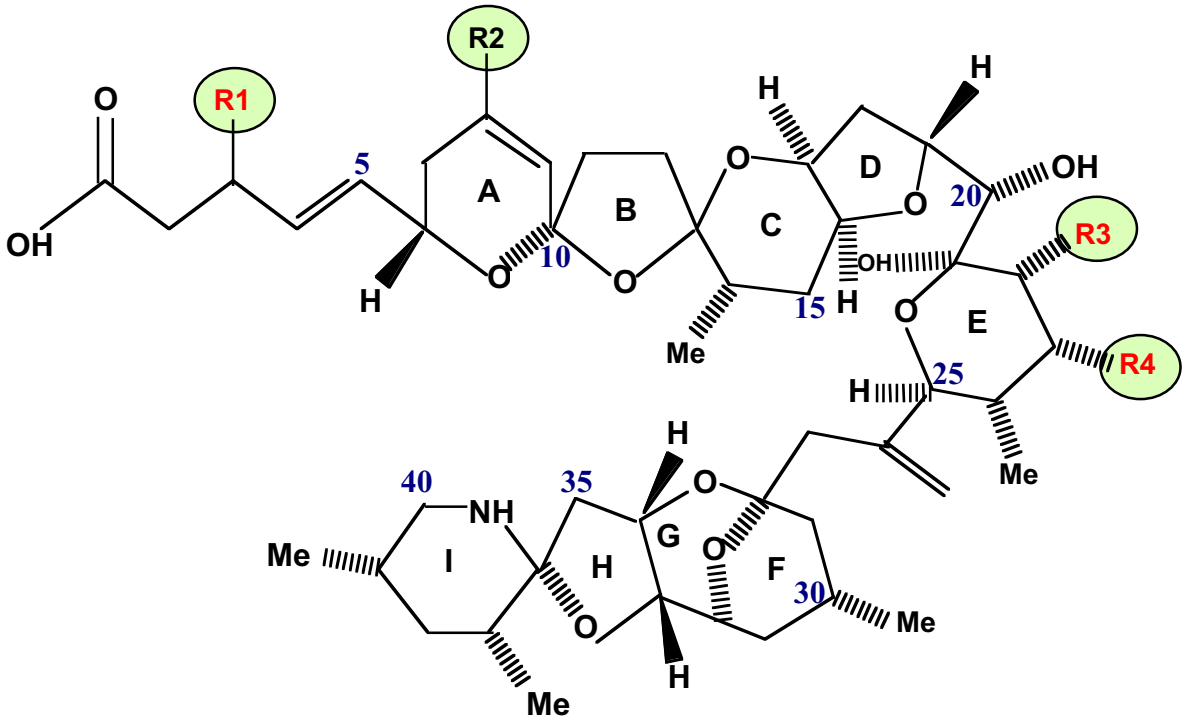

Figure 1. Structure of AZA analogues identified to date.

		R1	R2	R3	R4
AZA-1	azaspiracid	H	H	CH_3	H
AZA-2	8- methyl azaspiracid	H	CH_3	CH_3	H
AZA-3	22- demethyl azaspiracid	H	H	H	H
AZA-4	3-hydroxy-22-demethyl azaspiracid	OH	H	H	H
AZA-5	23-hydroxy-22-demethyl azaspiracid	H	H	H	OH

The (algal) source of AZA remains to be proven conclusively and this point will be addressed in the companion paper to this overview. Although the clinical signs of human intoxication are similar to DSP, the precise mode of action of the AZAs remains to be determined. Toxicity studies have concentrated on elucidating the mode of action in mice following acute oral (6) and chronic oral dosing (7). Multiple organ damage was observed in both studies. Recently cell culture studies (8) with AZA 1 have shown that the toxin may act by several different mechanisms.

With regard to the regulation of this toxin group in the EU, the recent Commission Decision 2002/225/EC (9) has established a regulatory limit of 160 µg total AZA / kg shellfish flesh. The Decision specifies that both the rat bioassay (10) and various mouse bioassays (11, 12) are suitable for the detection of AZAs at the regulatory limit. However, the use of animals in bioassays has attracted increasing criticism from both anti-vivisectionists and the shellfish industry, albeit for different reasons. In the UK, all procedures with animals are subject to the concepts of the three 'R's – *reduction* (of the number of animals used), *refinement* (of animal procedures to minimise suffering) and *replacement* (with non-animal alternatives). In addition, recent work by James et al 2002 (13) has suggested that the mouse bioassay may be susceptible to a higher proportion of false negative results with AZAs compared to 'traditional' DSP toxins (okadaic acid and the dinophysis toxins) because of the differential distribution of the different analogues in shellfish tissues. AZA 1 was the predominant toxin detected in the digestive glands of contaminated Bantry Bay mussels whereas AZA 3 was predominant in the remaining tissues.

A number of chemical assays for AZA analysis have also been described which utilise liquid chromatography/mass spectrometry and tandem mass spectrometry (LC-MS and LC-MS-MS) (14-19). However, the lack of availability of pure toxin standards has precluded the widespread adoption of this methodology. The equipment is also expensive and there is a clear need to develop cheaper and more rapid non-animal alternative tests for AZA.

The RASTA project

Given these problems, VSD in collaboration with the Marine Institute applied to the Food Safety Promotion Board for funding for the RASTA project. The project was launched in September 2001 with four principal aims:

- to develop rapid and cheap non-animal based tests (immunoassays);
- to extract and purify analytical standard material from contaminated shellfish and from potentially causative algal species;
- to refine and fully validate existing LC-MS / LC-MS-MS methods according to current EU criteria (20) and;
- to disseminate the results to industry and regulators.

Within these four discrete areas of work (so called Workpackages – WP), each partner has a specific number of tasks to carry out. VSD is responsible for:

- Extraction of AZA from contaminated shellfish (WP1)
- Production of AZA-protein conjugate(s) for immunisation (WP1)
- Production and characterisation of conventional polyclonal and monoclonal antibodies in laboratory animals (rabbits, mice) (WP1)
- Development and validation of immunoassay for AZA (WP1)
- Examining the correlation between immunoassay results and MS-MS results in positive mussels
- Carrying out method inter-laboratory comparisons by the blind analysis of positive mussel samples (WP3)

The Marine Institute is responsible for:

- Extraction of AZA from toxic phytoplankton (WP 1)
- Validating MS-MS methods for the confirmation of AZA in mussels to current EU criteria (Commission Decision 2002/657) for identification and quantification. (WP2)
- Examining the stability of AZA in mussels stored prior to analysis and in mussels following cooking. (WP2)
- Disseminating the information to the shellfish industry and consumer groups throughout the island by the holding of a Technology Transfer workshop. (WP4)

Progress to date at VSD

Purified AZA 1 (16 µg) was received from the Marine Institute in late 2001. This was conjugated to a carrier protein (human serum albumin –HSA) and to a horse radish peroxidase enzyme label through the terminal carboxylic acid moiety (Fig. 1) by the mixed anhydride method of Erlanger et al 1957 (21). Given the small amount of starting material, two mice were immunised twice (10 µg Azaspiracid-HSA immunogen) by intraperitoneal injection (Jan and March 2002) and were test bled following the second injection to check for an anti-AZA-1 immune response. No response was observed and further purified AZA 1 will be required to ‘boost’ these mice.

In response to this requirement, a second phase of purification of Bantry Bay mussels for AZA 1 was carried out in collaboration with staff from the Marine Institute in September/October 2002. The nine step protocol (see below) is complex, time consuming and requires specialist equipment.

i) Extraction of bulk sample;
ii) Partitioning of concentrated extract between ethyl-acetate and water;
iii) Partitioning of concentrated extract between 80 % methanol and hexane;
iv) Normal-phase chromatography on silica using a gravity column;
v) Medium-pressure size-exclusion chromatography on HW40;
vi) Medium-pressure reverse-phase chromatography on Develosil Lop C18;
vii) Medium-pressure anion-exchange chromatography on DEAE-Toyopearl;
viii) Medium-pressure cation-exchange chromatography on CM650-Toyopearl;
ix) High-pressure reverse-phase chromatography on a polymeric C18 column.

The exercise was partly successful and several critical control points were identified e.g. the ion exchange chromatography steps where ~ 50% loss of AZA1 was seen. Further extraction and purification work is now targeted for early 2003.

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Rapid Azaspiracid Shellfish Toxin Analysis - RASTA: An update on culturing of *Protoperidinium spp*.

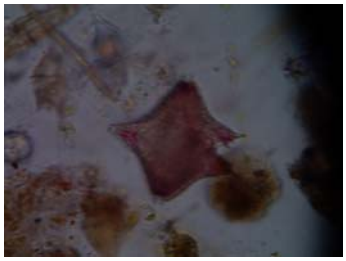
Siobhan Moran, Terry McMahon, Joe Silke, Caroline Cusack and Dave Clarke
Marine Institute

BACKGROUND

In a companion paper in these proceedings McEvoy *et al* gave an overview of the RASTA project whose main aim is the development of a rapid assay for the detection of Azaspiracid (AZA) toxins in shellfish. This project, funded by the Food Safety Promotion Board, involves collaboration between the Marine Institute (MI), and the Veterinary Sciences Division (VSD) of the Department of Agriculture and Rural Development (DARD) in Northern Ireland.

Under Work Package 1 of the RASTA project the Marine Institute is responsible for the extraction of AZA from toxic phytoplankton. This can be done in two ways:

1. By collecting samples of bulk phytoplankton and extracting AZA toxins present.
2. Collecting and culturing the algal species producing AZA toxins. Recently published work by Yasumoto et al (2002) has indicated that the armoured heterotrophic dinoflagellate *Protoperidinium crassipes* produces AZA toxins.



Protoperidinium crassipes

Phytoplankton Sample

So the question arises: Why culture cells if toxins can be extracted from bulk harvesting?
The collection of bulk phytoplankton samples, which can be time consuming and costly, may not always yield a supply of toxins. Chemical analysis carried out on phytoplankton samples collected from 59 stations along the Irish West Coast, from North Donegal to Cork, during a survey onboard the R.V Celtic Voyager in July / August 2001, showed the presence of AZA at varying levels at all stations (Hess et al, 2002). However AZA was not detected in any samples taken during a similar survey in July / August 2002. This highlights the advantage of culturing cells rather than relying on bulk phytoplankton harvesting as a source of toxins.
Successful isolation and culturing of the genus *Protoperidinium* in the laboratory, would provide a continuous and reliable source of the toxin. This method is also species specific and will help us to understand the role of food sources and culture conditions, in the toxin production process. Furthermore,

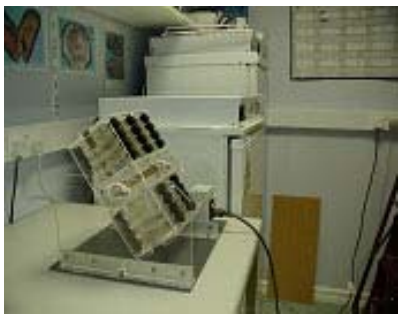
since it is likely that more than one species of the genus *Protoperidinium* may have the potential to produce AZA toxins, the culturing method allows this to be investigated more fully. For these reasons the Marine Institute is concentrating it's efforts on the culturing aspect of the project. Also, *P.crassipes* is not the only organism targeted but all species within this genus are subject to investigation.

Separate to the RASTA project, but running parallel with it, is collaboration with Dr. Don Anderson and his lab in the Woods Hole Oceanographic Institute in Cape Cod. His staff – and especially his student Kristin Gribble -- have been extremely generous with their time and knowledge, especially in regard to their experience in dealing with dinoflagellates. Staff from the Marine Institute have visited the Woods Hole laboratory for training in phytoplankton isolation and culturing techniques and Kristin Gribble, from the Woods Hole laboratory participated in the survey onboard the Celtic Voyager in 2002. Collaboration between both laboratories is continuing.

CULTURING HETEROTROPHIC PROTOPERIDINIUM

With regard to culturing of phytoplankton it is important to distinguish between autotrophic and heterotrophic species. In general most culturing work deals with autotrophic species. Such species photosynthesise and mainly require suitable conditions of light, temperature and nutrients for growth and division. However the species belonging to the *Protoperidinium* genus are generally heterotrophic. This means that in order to successfully grow and divide they must prey on an external food source and use what is called a 'pallium' or feeding veil to engulf and digest their prey. In laboratory culture is it is important therefore to keep the prey cells – diatoms or other dinoflagellates – in suspension, to allow the free-swimming heterotrophs to come into contact with them. To achieve this, a Plankton Wheel was designed to fit into an incubator. This wheel holds 40 x 60ml tissue-culture flasks containing the cultures and media, and revolves continuously at 1 r.p.m.

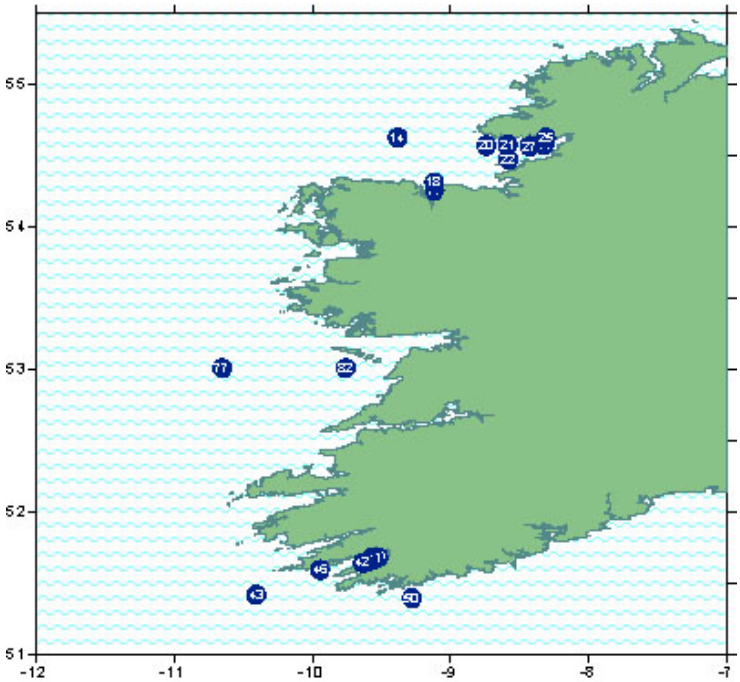
Plankton Wheel revolving inside incubator



Side Profile of Plankton Wheel

HARVESTING *Protoperidinium*

Harvesting of cells of *Protoperidinium* was carried out during the R.V. Celtic Voyager survey in July / August 2002. The aspect of the survey, of interest to the RASTA project was the collection of cysts from sediment samples and the collection of live cells from which to establish cultures of *Protoperidinium* species for subsequent chemical analysis for AZA presence.



From 85 stations sampled on the cruise, 21 were targeted for live phytoplankton sampling. Of these 3 produced sufficient numbers of cells for sustained cultures to be established. These were Stn 27 in Donegal Bay, Stn 46 in Bantry Bay and Stn 50 off the Cork coast. Viable cells were shared with WHOI and similar cultures established in their lab.

The samples collected were held in 60ml tissue-culture flasks, and stored onboard in an incubator at 15°C, with 12 hours light: dark cycle at a salinity of 35. On returning to the lab *Protoperidinium* cells were isolated into fresh flasks containing prey species, using a capillary pipette technique. To further help eliminate contamination, washing steps of the cells were also included. Prey species were initially selected by researching historical data. Other species were included, and either accepted or rejected based on feeding observations.

RESULTS

Protoperidinium have been successfully isolated and maintained in culture for more than 3 months, albeit at low numbers. Similar findings were recorded in the WHOI lab, although their cultures have now ceased to be viable. The knowledge gained from this work however will be invaluable when setting up new cultures in the future.

Species isolated have included: *P. depressum, P. crassipes/curtipes, P. ovatum, and P. oblongum.* The most continuously sustained culture has been *P. depressum.*

The prey species used with the best success have mainly been *Ditylum brightwellii* and *Chaetocerous affinis,* but also *Leptocylindrus danicus* and *Ceratium spp*.




D.brightwellii *C. affinis* *L. danicus* *Ceratium tripos*

Also extensive video recordings of both cell divisions and use of the pallium (feeding veil), have been made. A potentially important result from the study was that the primary suspected AZA producer, *P. crassipes,* changed colour depending on the diatom diet provided as prey. This may be a significant discovery since this feature (i.e. colour) is one of the few characteristics that differentiates this species from *P. curtipes*, using light microscopy for identification. However further work is required to determine the significance of this finding.

FUTURE WORK

Cultures will continue to be maintained and increased. International and national links are being developed with scientists working in the same area. Experimentation with new designs of plankton wheel and culturing techniques is ongoing. Procedures have been put in place to ensure that when *Protoperidinum* and/or AZA is detected in high concentrations in shellfish, bulk water samples can be collected from that area for isolation of *Protoperidinium*.

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MOLECULAR PROBES FOR TOXIGENIC PHYTOPLANKTON

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The worldwide increase in the incidences of HAB's (Harmful algal blooms) has led to an increased frequency of related illnesses and has had an adverse impact on natural resources. To date sixty species capable of producing toxins have been identified (Pierce & Kirkpatrick, 2001). As a result of the potential impact on public health and the environment, monitoring programmes have been put in place in regions affected by HABs. The development of new and innovative analytical techniques for the identification of these species and their toxins has been stimulated by the increased requirement for monitoring. One such development, molecular probes is finding a role in monitoring and identifying toxic species.

Nucleic acids (DNA and RNA) provide the unique molecular genetic to information required for protein synthesis in all organisms. Nucleic acids have a uniform stable chemical structure consisting of a linear sequence of bases along the length of the molecule which is common in prokaryotic and eukaryotic organisms. The complimentary nature of the base sequences arises due to the structure and binding properties of its four bases (adenine, thymine or uracil, guanine and cyostine) which comprise nucleic acids. This complimentarity enables the hybridization of short specific sequences of DNA known as molecular probes to their complimentary DNA target in a diagnostic assay thereby enabling the detection and identification of a specific target sequence and consequently a specific organism in a sample (Smith et al, 2000). Molecular probes are short specific sequences of DNA usually 20 to 40 bases in length comprising the 4 bases arranged in a sequence that is complimentary to the sequence of the target genomic DNA that one wishes to detect or identify in a sample. DNA probes can be commercially synthesized and labeled with a fluorescent, chemiluminescent or colorimetric tag to facilitate the detection of the nucleic acid-probe hybrid in a sample. Molecular probes described to date in the literature for the identification of toxigenic phytoplankton species target the small ribosomal subunit (SSU), the large ribosomal subunit (LSU) or the intergenic spacer region (ITS) (Miller & Scholin. 1998) (Simon *et al,* 2000) & (Rhodes *et al*, 1998). These genetic targets are chosen because they are multicopy targets that contain both conserved and variable sequence regions which make them very suitable for the design of genus or group specific and species-specific probes as the application requires.

The current application of molecular probes for the identification of toxigenic species includes their use in whole cell assay, sandwich hybridization assay and real-time detection using an environmental processor. The whole cell assay has been developed to assist conventional microscopic identification of toxic species. The cells are mounted on a microscope slide and treated to render the membranes permeable to a fluorescently labeled probe designed to identify a specific toxic species. This probe binds to the ribosomal RNA

target and unbound probe is washed away. The probe – RNA hybrid can be visualized using a fluorescent microscope thereby identifying the presence of the toxic species of interest in the sample) (Miller & Scholin. 1998) & (Simon *et al,* 2000). Saigene® Corporation have developed a microtitre plate based assay for the identification of a range of toxic species. For this assay the probes are immobilized on a solid support and the sample is treated to release the nucleic acid which is hybridized to the probe on the solid support and then detected (www.saigene.com). Another development where molecular probes are currently being used to identify the presence of toxic species in the ocean in real-time is the Environmental Process Sampler developed by Chris Scholin and his colleagues at the Monterey Bay Aquarium Research Institute in California. This device is designed to periodically collect samples from the ocean, to treat the samples to release the nucleic acids. The nucleic acids are hybridized to a series of species-specific probes which are immobilized on a membrane support and a signal is obtained when the species of interest is present in the sample. This device may serve as an important early warning system for the presence or increase in number of toxic species in marine waters. A more recent application of molecular probes for the identification of toxic species has been their application in real-time PCR-based assays (Bowers *et al,* 2000).

At the National University of Ireland, Galway, the Martin Ryan Institute in collaboration with the National Diagnostics Centre and with an input from the Marine Institute have just commenced a research programme funded under the PRTLI (Programme for research in third level institutions) aimed at developing molecular probes and immunological based assays for toxic species and their toxins respectively. Molecular probes will be developed for Pseudonitzschia, Dinophysis and Alexandrium sps. that cause problems in Irish waters. DNA probes currently available for these species will be evaluated and DNA sequencing of ribosomal genes from a representative number of Irish strains will be undertaken to determine if there are sequence differences between Irish strains of these species and species from other geographic locations. A long-term aim of the project will be to develop rapid molecular diagnostic assays for these species using a PCR-based approach. PCR is an *in vitro* technique used to enzymatically amplify, in an exponential manner, a specific fragment of DNA, starting from either a DNA or RNA template, through a series of repetitive reaction cycles. During each cycle the number of copies of the target sequence doubles and newly synthesised copies also serve as templates for subsequent rounds of synthesis increasing the amount of DNA generated exponentially to several million molecules after 30 cycles of amplification (Smith *et al*, 2000).

At the National Diagnostics Centre we have developed a DNA probe colorimteric membrane assay technology which enables DNA probe-based detection of PCR products. PCR is performed using biotinylated primers and the PCR is heat denatured and hybridised to the membrane bound probes. The PCR-DNA probe hybrid is detected by the addition of Streptavidin alkaline phosphatase and chromogenic substrates which generate a purple coloured signal where there is a positive reaction of the DNA probe and its

target PCR product on the membrane. This technology has been successfully used to develop assays for a range of food-borne pathogens (O Sullivan et al, 2000, O Connor et al, 2000 & Collins et al 2001).

A significant technological development in recent years has been the introduction of real-time PCR technology. Real-time PCR which has the ability to detect PCR amplicons by measuring fluorescence while they are being synthesised in the PCR reaction vial. Real-time instruments like the Light Cycler (Roche) and the TaqMan LS-50B PCR Detection System (PE Biosystems) have recently become available, with the former having the ability to perform and detect PCR amplification in twenty- five minutes, while the latter has the ability to perform and detect PCR in a 96-well format which offers high-throughput capabilities (Smith *et al,* 2000).

Given the current advances in technology and the increasing amount of research being undertaken in the area of HAB's, along with the continued accumulation of sequence data for these toxic species, the coming years should see the development of a plethora of probes for the detection of species of importance in Irish waters.

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REPORT ON MOLECULAR PROBE TECHNOLOGY FOR THE DETECTION OF HARMFUL ALGAE WORKSHOP

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Between the 20th and 24th May, 2002, a workshop entitled "Molecular Probe Technology for the Detection of Harmful Algae" was held at the Martin Ryan Institute, National University of Galway, Ireland (MRI, NUIG). The workshop was jointly sponsored by the Marine Institute (MI) and Bord Iascaigh Mhara (Irish Sea Fisheries Board) with the support of GEOHAB (Global Ecology and Oceanography of Harmful Algal Blooms. Figure 1). Co-hosted by the Marine Institute and NUIG, 45 delegates from 15 countries worldwide participated (Fig. 2). The workshop opened with a welcome address by a representative from the Marine Institute, Michéal O' Cinneide. This was followed by an address by John Patching (MRI) and Jim Browne (NUIG).

The primary objectives of the workshop were to build on the earlier initiative of Chris Scholin (who convened a similar workshop at the Monterey Bay Aquarium Research Institute, California in 2001), to disseminate current knowledge on the subject of genetic probes and to improve the understanding of the methods currently in use.

The workshop benefited from the involvement of several invited speakers (John Tyrell, Laurie Connell, Santiago Fraga, Siobhan Kavanagh, Holly Bowers, Chris Scholin, Rick Gordon, Linda Medlin, Ann Sofie Rehnstam-Holm, Lesley Rhodes, Melissa Gladstone, Donal Eardly and Jose Cordova) who gave detailed lectures and practical demonstrations. There were also numerous oral and poster presentations given by the participants. Subjects discussed ranged from the type of molecular techniques to use, the role of gene probes in current phytoplankton monitoring programmes and the use of these tools in automated systems in the field.

During the practical sessions light microscopic analysis (taxonomic investigation and isolation techniques) were performed by the participants on cultures from the Instituto Español de Oceanografia Apdo, Spain. Hybridisation techniques demonstrated included real time PCR (polymerase chain reaction), whole cell hybridisation, sandwich hybridisation and DNA microchip arrays. In addition, tutorials on Bioinformatics, denaturant gradient gel electrophoresis (DGGE) and toxin detection assays were given.

Several roundtable sessions took place during the workshop and the participants felt that there was a need to standardise sampling protocols and hybridisation methods. While the Cawthron Institute in New Zealand has pioneered the validation of the whole cell and sandwich hybridisation assays for the use in their existing phytoplankton monitoring programme, there is no widespread use of these methods worldwide. In order for this type of technology to progress it was felt that inter-laboratory calibrations of the

assays is needed, and that the assays need to become more commercially available with the assurance of quality control. It was also pointed out that there is a need to increase the network of people involved in the design and development of molecular probes and the methods used. This in turn brought up the topic of financial support and the difficulties involved in fundraising.

A website (www.geneprobes.org) has been established (co-hosted by the Cawthron Institute, Marine Institute and NUIG) and its purpose is to serve as a focal point where knowledge can be exchanged between probe users (Fig. 3). The website will be updated periodically with new material as it becomes available.

Since there was an obvious demand for periodical meetings of this nature, the next probe workshop will be held in Mexico in 2004.

The Marine Institute will continue to establish and maintain contacts with international research groups on the subject of Gene Probes. The Marine Institute are currently collaborating with other experts in the field by providing samples of potentially toxic algae from Irish waters. The Marine Institute will assist in the development of molecular probes for the detection of harmful blooms within Ireland.

More details about the above workshop can be found on the website www.geneprobes.org

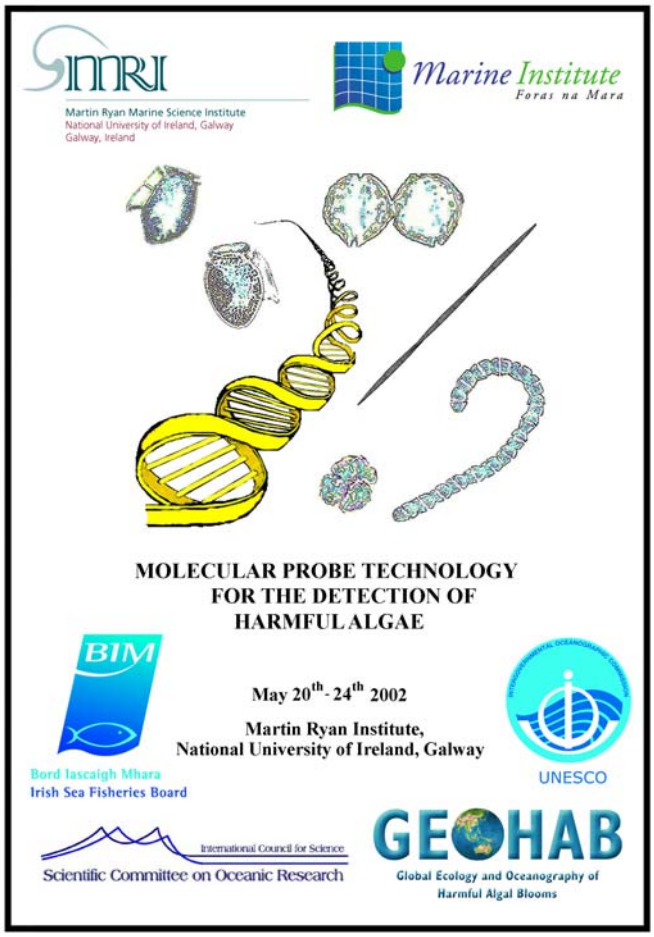

Figure 1. Logo for the workshop "Molecular Probe Technology for the Detection of Harmful Algae" designed by Siobhan Kavanagh, NUI,Galway.

Figure 2. Photograph of participants attending the workshop.

1. Morten Wiuf, 2. Melissa Gladstone, 3. Ben Sandee, 4. Jesus Perez-Linares, 5. Martin Whittle, 6. Eileen Bresnen, 7. Alice Ilaya Gedaria, 8. Elizabeth Smith, 9. Allison Haywood, 10. José Córdova, 11. Mårten Flø Jørgensen, 12. Linda Medlin, 13. Wiebe Kooistra, 14. Luisa Orsin, 15. Mary Hensey, 16.Christopher Scholin, 17. Antonella Penna, 18. Greg Doucette, 19. Holly Bowers, 20. Jason Kempton, 21. Majella Maher, 22. Ian Lucas, 23. Laurie Connell, 24. Siobhan Moran, 25. John Tyrrell, 26. Silke Kröger, 27. Nina Lundholm, 28. John Slater, 29. Wayne Litaker, 30. Bente Edvardsen, 31. Mark Vandersea, 32. Hanne Ramstad, 33. Santiago Fraga, 34. Anna Godhe, 35. Ann-Sofi Rehnstam-Holm, 36. Ingela Dahllöf, 37. Richard Gordon, 38. Elisabeth Antoine.

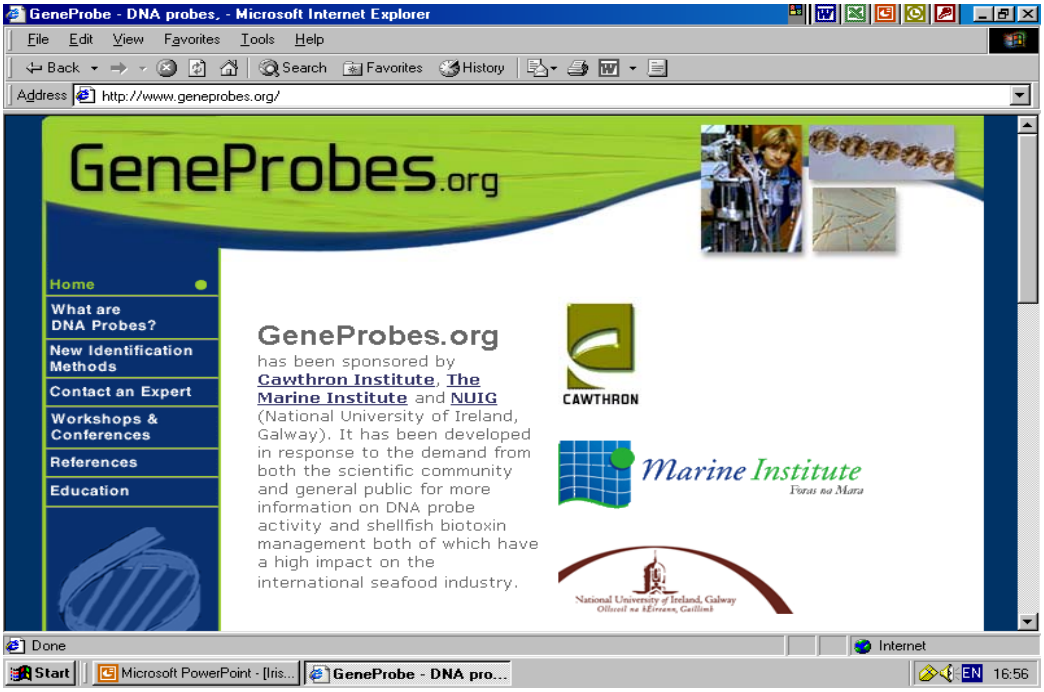

Figure 3. Geneprobe Website (www.geneprobes.org)

THE USE OF RECOMBINANT PHAGE-DISPLAYED ANTIBODIES (RPA) FOR THE DETECTION OF ALGAL TOXINS

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Research at the National Diagnostics Centre is aimed at producing antibodies specific for algal toxins for use in rapid detection systems. The resulting antibodies will be used in the development of a range of assay formats, which would be suitable for applications in different situations. These include (i) microtitre plate assays, robust laboratory-based assays that can be used manually for analysis of relatively small sample numbers or with the aid of robotics for large sample numbers; (ii) biosensor-based assays, suitable for high-throughput situations and (iii) one-step membrane-based or 'lateral-flow' assays suitable for very rapid, single, on-site analyses.

The affinity of an antibody determines its detection limit, with high affinity antibodies able to detect very low concentrations of toxin. If a successful, rapid assay for the minute amount of toxins in algae is to be developed then the production of very high affinity antibodies is critical.

But why use antibodies?

Antibodies are incredibly versatile tools; they are cheap to produce on a large scale and can be packaged into rapid detection systems that can be used at the point where they are needed, rather than sending samples to laboratories for toxin detection. These detection systems are not labour-intensive and a relatively unskilled worker can produce reliable and reproducible results. The technology employed in the membrane-based assay format (similar to that of a high-street, pregnancy test kit) means that at point of test there is no need for electrically powered equipment for sample application or test reading.

How are antibodies produced?

Milstein and Kohler first described the production of monoclonal antibodies in 1975, for which they were awarded with a Nobel Prize in 1984. Monoclonal antibodies are produced by fusing spleen cells from an immunised mouse to a tumour (myeloma) cell line, which is cloned to produce cell populations that are monoclonal, producing an antibody having a single specificity.

The procedure relies on the immunisation of an animal to increase the percentage of cells whose antibodies are potentially reactive against the immunogen. The fused spleen cell-myeloma hybrid is incubated in hypoxanthine-aminopterin-thymidine (HAT) containing medium. Spleen cells from the mouse encode an enzyme HPRT enabling the spleen cells to survive in the presence of HAT. Myeloma cells lack HPRT and so any that are not fused to a spleen cell will be killed. Spleen cells that remain unfused will not

be killed by HPRT, but will die as they only have a short duration of life *ex vivo*.

Recombinant Phage Antibody (RPA) Technology

Recombinant antibodies are produced in a totally different manner compared to that of conventional antibodies. Whereas the conventional antibody technology relies on the immortalisation of an antibody-producing cell, recombinant antibody construction relies on isolating the genetic material from antibody producing cells from an immunised animal.

When constructing the recombinant antibody, the DNA regions encoding the heavy and light chain of the antibody variable region, responsible for the recognition of the target molecule are isolated and artificially joined with a short DNA region, encoding a flexible linker. This artificially linked antibody is known as a short chain antibody fragment, or ScFv. The remaining DNA encoding for the 'framework' of a complete antibody is not necessary for construction of a recombinant antibody.

The linked antibody chains are then placed into a phagemid vector, transferred to bacteria and packaged and expressed on the surface of a filamentous phage in a near native format. The phagemid allows for the insertion of the antibody DNA into a stretch of DNA encoding the gp3 coat protein of phage (producing a gp3-ScFv hybrid). However the phagemid is deficient in the DNA necessary for full construction of a phage particle, but relies on a helper phage being present in bacteria at the same time as the phagemid to produce a fully assembled, replicative phage particle. As the phage particle is being assembled in bacteria, the gp3-ScFv hybrid is transcribed and expressed on the surface of the phage. The phage particle then acts like a full antibody, with the framework of the antibody replaced by the body of the phage particle. The main advantage of this approach is that you now have a phage antibody, with the recognition properties of a conventional antibody, but which also contains the DNA that encodes for the antibody as shown in figure 1.

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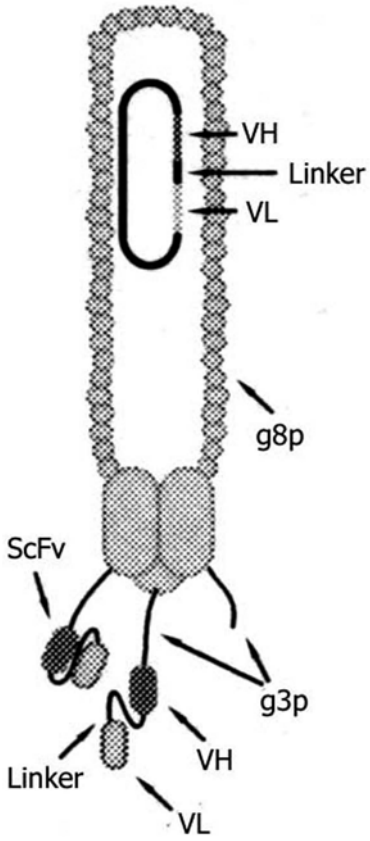

Figure 1: The gp3-ScFv hybrid is expressed on the tip of the phage particle. The phage particle, consisting mainly of the outer coat protein, gp8, also contains the phagemid containing the linked Heavy Chain (VH) and the Light Chain (VL) DNA encoding for the ScFv expressed at the tip.

When constructing the recombinant antibody, the starting pool of genetic material will not just encode for the antibody of interest, but potentially a large proportion of the immunised animals antibody repertoire that is expressed at the time. Therefore it is necessary to use selection procedures to isolate the antibody of interest, and produce a monoclonal phage population. Selection is carried out in a procedure known as 'biopanning', where the entire phage population, also known as the 'phage library' is washed over a plastic surface to which the toxin has been bound. Phage specific for the toxin will bind to the molecule whereas other phage will not. A degree of non-specific binding of phage is always observed, although the proportion of specific binding phage is much higher. Washing of the plastic will result in most weak non-specific binding phage, and unbound phage to be washed away. Bacteria capable of being infected by phage are then added to the plastic support and will take up any bound phage. Incubation of the infected bacteria in the presence of helper phage will result in the completion of the phage life cycle, and the production of phage-ScFv hybrids being released into the culture media. Hybrid phage will have increased in proportion compared to the original phage library. By carrying out several cycles of biopanning it is possible to produce a phage

population high enriched for anti-toxin antibody activity. By isolating phage from individual bacterial colonies, it is possible to identify monoclonal phage recognising the toxin.

What is the best animal to raise antibodies?

It has been convention to use mice for antibody production, mainly because of the availability of a stable myeloma cell line to allow immortalisation of antibody producing spleen cells. The range of observed affinities of monoclonal antibodies produced in mice ranges 10^{-5} to 10^{-10} M. Recently, several reports have shown that antibodies raised in sheep have affinities that can range as high as 10^{-12} to 10^{-14} M which would be ideal for sensitive detection assays. As no stable, suitable cell lines exist that can reliably immortalise sheep antibody producing cells, it has not been easy to harness the high affinities of sheep antibodies. As the recombinant antibody technology does not require cell fusion and immortalisation it is now possible to construct phage libraries from sheep immunised with toxins, and hopefully improve our chances of isolating high affinity antibodies. However, the higher affinity antibodies which provide adequate sensitivity for many analytical applications are quite rare and difficult to isolate.

How can recombinant antibodies be improved?

As previously mentioned, one of the main advantages of a recombinant, phage-displayed antibody is that they carry the DNA that encodes for the ScFv. Any manipulation of that DNA will alter the ScFv produced and potentially its specificity and affinity. The effect of any mutation of DNA on the ScFv is then assessed, and those antibodies where there is an improvement are kept, whereas those whose affinity is reduced are rejected. By repeated mutation it is possible to significantly increase an antibodies affinity. Several approaches to mutation can be used. Firstly the DNA can be mutated using enzymes that produce random mutations. Secondly, the regions of the antibody responsible for direct contact with its target molecule have been identified, so inducing mutations in these regions will obviously have dramatic effects on the binding of the antibody, however regions outside of the contact points can also contribute to affinity by affecting the folding of the antibody fragment and these have to be considered as well. Finally, it is possible to replace entire regions of DNA in a process known as chain shuffling which again will alter the affinity of the antibody.

Recombinant antibodies in production at the National Diagnostics Centre.

Currently, recombinant antibodies raised in sheep are being constructed which are targeted against domoic acid. Of the three phage antibody libraries constructed so far, over 200 weakly binding antibodies, and 11 very strong binding antibodies have been identified, with 4 of these being carried forward for further investigation as very good candidate antibodies. Also in construction at the National Diagnostics Centre are recombinant antibodies raised in mice against domoic acid, and okadaic acid.

This work has been supported by a Marie Curie Host Development Fellowship, and is currently being supported by an Enterprise Ireland, Advanced Technologies Research Programme.

DOMOIC ACID POISONING IN SHELLFISH

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Background

Amnesic shellfish poisoning was first identified in 1987 in the Cardigan River region of Prince Edward Island, Canada, when cultured mussels were implicated in 107 cases of poisoning and 3 deaths (Todd 1993). The causative agent was identified as domoic acid, a naturally occurring compound previously unknown as a source of shellfish poison (Wright *et al*. 1989). It was found that the toxin is derived from diatoms of the genus *Pseudo-nitzschia*, a common member of the phytoplankton community not previously known to produce toxins (Bates *et al*. 1989).

Since 1987, domoic acid has been found to be responsible for the death of brown pelicans (*Pelecnus occidentalis*) and cormorants (*Phalacrocorax penicillatus*) at Monterey Bay in 1990, Santa Cruz in 1991 and Cabo San Lucas in 1996 (Wekell *et al*. 1994; Sierra Beltran *et al*., 1997). These outbreaks were attributed to the consumption of planktivorous fish (anchovy, mackerel) containing high levels of domoic acid. In 1998, the death of 400 sea lions (*Zalophus californianus*) along the central Californian coast was attributed to the consumption of contaminated anchovies (*Engraulis mordax*) (Scholin *et al*. 2000).

In addition to these high profile deaths, shellfish have been found contaminated with DA in most countries. These countries include Canada, USA, Australia, New Zealand, Japan, Spain, Portugal, Scotland, Ireland and France.

Symptoms of Amnesic Shellfish Poisoning.

The principle effects are gastrointestinal manifesting as nausea, vomiting, abdominal cramps and diarrhoea. However there are neurological effects such as dizziness, disorientation, lethargy, seizures and permanent loss of short term memory.

There appears to be a close association between memory loss and age. Younger patients (under 40) were more likely to have diarrhoea, whereas older patients were more likely to have memory loss and require hospitalisation (Perl *et al.,* 1990).

What is domoic acid?

Domoic acid is naturally occurring, belonging to the kainoid class of compounds. It was originally isolated some forty years ago from the red macroalgae *Chondria armata* (Takemoto & Daigo 1958). Administered orally

in low quantities, this substance was found to be markedly effective in expelling worms from young children without any observable side effects.

Domoic acid (figure 1) is a crystalline, water soluble amino acid that can be purified by a variety of chromatographic methods and contains a strong chromophore that facilitates detection by UV spectroscopy (λ_{max} = 242nm). It has a molecular weight of 311.34 and is very soluble in water at 7.6g/l (Wright *et al.* 1989; Falk *et al.* 1991).

Domoic acid is a glutamate agonist that displays marked neurotoxic properties in the mammalian central nervous system (Bird & Wright 1989). It is the most potent member of a group of amino acid analogues called excititoxins which bind to specific membrane–bound kainite receptors and _-amino-3-hydroxy-5-methyl-4-isoxazolepropianic acid (AMPA) receptors found in certain neuronal cells of the brain (Hampson *et al.* 1992). This results in increased firing of the neurons and eventual rupture of the cell (Bird & Wright 1989).

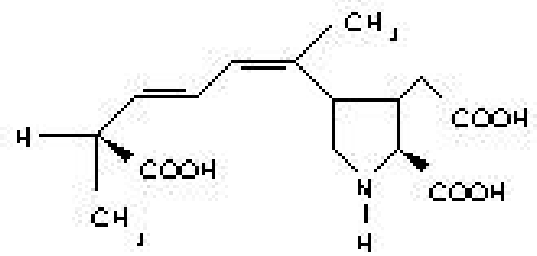

Figure 1 Domoic Acid

Domoic acid Producers.

Domoic acid has been isolated from 2 species of red macroalgae *Chondria armata* and *Alsidium corallinum*, a Mediterranean species. However, the principle source, which also causes the most concern are the diatoms. These include *Pseudo-nitzschia multiseries*, *P. pungens*, *P. delicatissima*, *P. seriata*, *P. australis*, *P. fraudulenta*, *P. pseudodelicatissima*, *P. turgidula* and *P. multistriata*. However, *Pseudo-nitzschia* is not the only genus of diatom that produces domoic acid. Recently a species *Nitzschia navis-varingica* was found to produce domoic acid in shrimp ponds in Vietnam.

The production of domoic acid in the diatom *Pseudo-nitzschia* (figure 2) is variable between species. It occurs at late exponential and stationary phase of the growth cycle. The concentration per cell can vary from 0.04pg cell^{-1} to 37.0pg cell^{-1}.

ASPOX Project

The ASPOX (ASP toxicology) project is a study concerning amnesic shellfish poisoning in Ireland. This is a collaborative study involving the National University of Ireland Galway (NUIG), University College Cork (UCC) and Cork Institute of Technology (CIT). The study involves the isolation and

culture of toxic *Pseudo-nitzschia* spp, the effects these species have on the shellfish, and the development and improvement of the analytical techniques.

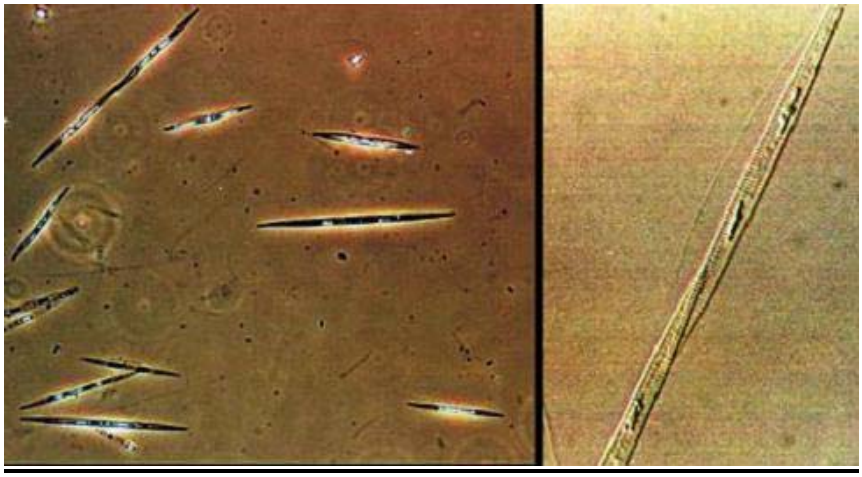

Figure 2. The diatom *Pseudo-nitzschia*

Aims of project –Domoic acid poisoning in shellfish.

1. Metabolism of domoic acid in shellfish (*Crassostrea gigas, Mytilus edulis, Pecten maximus*).

a) Domoic acid enzymatic breakdown assay found no breakdown or bio-transformation in the mantle, gill or digestive gland of the oyster and mussel.

b) A subcellular fractionation assay will be carried out on the digestive gland of the oyster, mussel and scallop to determine if domoic acid is organelle or membrane bound.

2. *Pseudo-nitzschia* culture.

3. Feeding Experiments

a) Zooplankton and shellfish will be exposed to toxic and non-toxic *Pseudo-nitzschia*.

b) Feeding rates, clearance rates, accumulation of domoic acid and distribution between tissues will be determined.

4) Physiology and Behaviour

a) Responses such as shell valve closure, filtration rate, byssus production, oxygen consumption, cardiac activity, swimming etc will be monitored.

Proposed results.

The combination of all areas that will be looked at will further help in the understanding of domoic acid and its effect on shellfish. This will further aid any research that may take place on depuration.

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BIOLOGICAL OCEANOGRAPHY: CURRENT AND PROPOSED RESEARCH

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2) Marine Environment and Health Services Division, The Marine institute, Galway technology Park, Parkmore, Galway.

Summary

There is a vast global investment in researching harmful algal events. The international effort can be gauged by the existence of programmes such as GEOHAB, run under the auspices of SCOR and the IOC, and EUROHAB funded through the European Commission[1]. National programmes researching HAB events, such as ECOHAB in the US, are a result of substantial funding from government agencies. It should be stressed that these are research activities. They require funds in addition to those provided for the crucially necessary phytoplankton and biotoxin monitoring programmes carried out in states where aquaculture operations exist.

HAB research can only progress along the path (outlined below):

- thorough understanding of the scientific issues
- ability to model and predict HAB events
- mitigation of the problem

There can be no progress along this path unless the previous step has been fully evaluated. It is a reflection of the degree of difficulty that exists in investigating HABs that despite the substantial resources that have been made available, the global scientific community still does not have a thorough appraisal of the problem. There are enormous gaps in our knowledge of HAB species, whether this relates to their life cycles, their ecology or even their production of toxins. Yes, huge advances have been made in these areas. To illustrate the size and scope of the problems remaining it is useful to consider the species and/or biotoxins which have caused substantial socio-economic damage to the aquaculture industry in Ireland, and what we urgently need to know about them.

DSP. Species of *Dinophysis* are implicated. It is only within the past year or so that it has been realised that this organism can exist in very high densities in thin layers sub-surface. There are serious implications for monitoring: does a bucket sample from the surface actually tell us anything? The other problem with these organisms is that it has proved impossible to culture, so we still

[1] GEOHAB : Global Ecology and Oceanography of Harmful Algal Blooms
SCOR : Scientific Committee on Oceanic Research
IOC : Intergovernmental Oceanographic Commission (UNESCO)
EUROHAB : European Initiative on Harmful Algal Blooms

know virtually nothing about its life cycle, particularly environmental conditions which promote excystment.

ASP. Unknown questions here are i) how many different species of *Pseudo-nitzschia* are there in Irish waters; ii) is the production of the toxin domoic acid confined to this genus or if it can be produced by other pennate diatoms; iii) why *Pectenidae* are so susceptible to contamination relative to other bivalves; iv) is there a link between points ii) and iii).

AZP. The causative organism is still unknown, despite links between azasparacid and *Protoperidinium crassipes*.

PSP. There is a known link between *Alexandrium* and PSP toxins (saxitoxins). However, we still cannot say with any certainty whether the toxic species is *A. tamarense* or *A. minutum*, or even both. Neither do we have an accurate idea of the distribution of these species around the Irish coast. The problem is exacerbated in that it is quite likely that some strains of the same species may produce toxin whereas others do not.

All of these problems are being researched at present. Indeed, an attempt to bring these research efforts into a cohesive whole has been launched by the Marine Institute, funded through the National Development Plan. The programme, BOHAB[2], will take place over the period 2003-2006, and contains the following elements.

1. Distribution Studies.	Map harmful species as a function of water column parameters and to establish a measure of variability and patchiness of these species to differing environmental conditions,
2. Intoxication studies.	Compare the susceptibility of different types of farmed shellfish to toxic species
3. *Alexandrium* toxicity.	Investigate the episodic nature of *Alexandrium* blooms, the environmental limits that control the germination of *Alexandrium* cysts and the development of blooms, Investigate the variability in toxicity of strains of *Alexandrium* in a variety of locations around the Irish coast,
4. HAB life cycles.	Determine the distribution of cyst beds of *Alexandrium* and *Protoperidinium*, investigate substrate preference for cyst deposits, and population dynamics and process rates.
5. Near-bottom toxicity.	Resolve the mechanisms involved in near bottom toxicity within the *Pseudo-nitzschia,* domoic acid,

[2] BOHAB : Biological Oceanography of Harmful Algal Blooms off the west coast of Ireland

and *Pectenidae* triangle. Where are the cells in relation to shellfish, and is secondary toxicity significance?

6. Biophysical Interactions. Study oceanographic events in relation to infestation of aquaculture areas. Are upwelling or downwelling events significant mechanisms for transporting cysts and/or vegetative stages and what is the link between oceanographic and meteorological conditions and HAB events.

7. Remote Sensing. Investigate the suitability of satellite imagery to the remote study of HAB events. Does cloud cover preclude satellite technology in Irish waters and are airborne sensors a viable option ?

8. Mooring Systems. Rationalise the use of in-situ monitoring systems on fixed moorings to observe HAB events in Irish waters.

The programme is funded for three years and the main partners within the project are the Martin Ryan Institute NUI Galway, the Marine Institute and the Woods Hole Oceanographic Institution in the Massachusetts, USA.

The structure of the work programme is such that a number of ongoing programmes can feed into the project. Linkages between BOHAB and existing programmes are portrayed in Figure 1.

Aside from the linkages shown in Figure 1, there is a strong focus on the interaction between harmful phytoplankton and physical oceanography in BOHAB. This aspect, often referred to as biophysical interactions, is likely to be very important in the onset of harmful events. It is already understood that local meteorology affects water and phytoplankton exchanges within Bantry Bay. Changes in wind direction have been associated with both red tides and toxic blooms in the bay (Raine et al., 1993; McMahon et al., 1999). This allows a certain predictive component of harmful events in this location, a feature which will be investigated in BOHAB through the link with the HABES[3] programme. However, the role importance of coastal jets, driven by density gradients in the water column near the coast, in transporting potentially harmful phytoplankton to regions of aquaculture has only recently been understood (Nolan et al., 2001; O'Boyle et al., 2002). The relationship between meteorological forcing of toxic events and the role of coastal jets driven by density gradients will also be investigated for Killary Harbour in BOHAB.
When the end of the BOHAB programme approaches, there will exist a coherent scientific framework for the next phase of HAB research : modelling and prediction.

[3] HABES: Harmful Algal Blooms Expert Systems

BOHAB Workpackage	Linkages and Inputs from other Research Programmes
Distribution Studies	Phytoplankton Monitoring Programme (Marine Institute; ongoing) Killary phytoplankton Programme (BIM, Killary CLAMS; 2000 - ?)
Intoxication Studies	Local Bay Hydrographic Studies (Marine Institute))
Alexandrium toxicity	Modelling *Alexandrium* in inshore environments (Martin Ryan Institute; 2002-2005. Marine Institute funded fellowship) Detection of toxigenic phytoplankton (Martin Ryan Institute, NUI Galway; 2002-2005. Funded by the HEA)
HAB Life Cycles	Rapid Azasparacid Shellfish Toxin Assay (RASTA) project (Marine Institute, 2002-2005) Detection of toxigenic phytoplankton (Martin Ryan Institute, NUI Galway; 2002-2005. Funded by the HEA)
Near bottom toxicity	Kinetics of domoic acid production - ASPOX (Martin Ryan Institute, NUIG, with CIT, UCC; 2001-2004. Funded by the HEA)
Biophysical Interactions	Harmful Algal Bloom Expert System (HABES) (Martin Ryan Institute, NUIG; 2001-2004. Funded by the EC under EUROHAB) Coastal HydrodynamicsPprogramme (CEFAS UK/Marine Institute/Martin Ryan Institute, NUIG; ongoing
Remote sensing	Coccolithophorid blooms and their impact (Martin Ryan Institute, NUI Galway; 2000-2004. Funded by the HEA) BIOCOLOR Programme (Martin Ryan Institute; 1997-2000. Funded by the EC)
Mooring Systmes	

Figure 1. Links between BOHAB and other ongoing Research programmes

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CURRENT ISSUES IN ENGLAND AND WALES IN THE MONITORING OF BIOTOXINS

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History of the biotoxin monitoring programme in England and Wales:

Regular monitoring of shellfish flesh for algal biotoxins has taken place in England since 1968, following an incident when 78 people became ill with Paralytic Shellfish Poisoning (PSP) after consuming mussels originating from the north east coast. This instigated a programme of sampling covering shellfish beds in the NE for PSP. In 1991 the Shellfish Hygiene Directive 91/492/EEC was put into force. This legislation requires EU member states to monitor for the possible presence of toxin producing plankton in production and relaying areas, and biotoxins in live bivalve molluscs. It was therefore necessary to review the current monitoring programme to include testing for Diarrhetic Shellfish Poisoning (DSP) and instigate a water monitoring programme. However the programme was still restricted to the NE coast.

During 1995 a visible bloom of *Alexandrium* spp. was observed in Milford Haven and the Fal estuary. Shellfish from these areas were found to contain PSP in excess of the action level. In response to this a new 5 year rolling programme of sampling was introduced in 1996. The shellfish production areas selected were divided between the flesh and water monitoring so that the whole of the country would be covered by one or other of the programmes within 2 years and after 4 years every site would have been covered by both.

In 1999 monitoring for Amnesic shellfish poisoning (ASP) was included as a result of an amendment to Directive EC 97/611/EC. After a large scale DSP incident in the Solent in 2000 it was realised that a zoned approach separating shellfish beds would allow easier control of open and closed shellfish areas. All classified active shellfish areas were reviewed to determine if other areas would benefit from a similar approach. Two other locations were deemed suitable in addition to the Solent, these were the Thames and West Mersea. Following consultation with the local authorities and the Food Standards Agency, zoning was introduced in two further areas, the Wash and the Burry Inlet following the incidence of atypical DSP positives in these areas.

To maximise the coverage of shellfish production areas the biotoxin monitoring programme was split into two: the flesh monitoring programme and the water monitoring programme with samples being collected on a monthly basis from the selected sites and weekly where toxicity was detected. These continued as rolling programmes until 2000, when the number of shellfish production areas monitored for the flesh programme was increased over two years so that by 2001 all active shellfish production areas were being monitored. This revised programme resulted in a five-fold increase in the coverage.

Summary of the current monitoring programme

At present in England and Wales the monitoring programme for algal biotoxins is divided into two separate programmes: the flesh monitoring programme and the water monitoring programme.

The current flesh monitoring programme includes all areas in England and Wales where commercial harvesting takes place. Samples are collected on a monthly basis except in areas with a historic occurrence of algal biotoxins or toxic algae. In these areas samples are collected fortnightly during weeks 14 - 39 (April 1 to September 28). The analysis of samples for PSP toxins is carried out by the method described by AOAC 1990. The analysis for DSP toxins is by a modified method described by Yasumoto *et al* (1894). The HPLC analysis of ASP toxins is carried out by the method described by Quilliam *et al* (1995). On the detection of algal biotoxins at levels exceeding the maximum permitted levels (MPL) the affected sites are tested on a weekly basis until two consecutive clear tests (negatives for DSP or below the MPL for PSP and ASP) are obtained. The water monitoring continues as a rolling programme where monthly samples are collected from 20 sites selected each year (including those sites with a history of toxic algae). On detection of algal cell concentrations in excess of the MPL shellfish samples are collected from the affected area for biotoxin flesh testing.

Enforcement

The Food Standards Agency (FSA) has overall responsibility for ensuring that the monitoring programme is effectively carried out, and the CEFAS Weymouth Laboratory is responsible for identifying the sampling areas and co-ordinating the programme. The regional Food Authorities are responsible for collecting the water and shellfish samples from the designated sites, which are then sent to the CEFAS Lowestoft laboratory for water analysis and the CEFAS Weymouth laboratory for flesh analysis. On detection of algal cell concentrations in excess of the maximum permitted levels (MPL) shellfish samples are collected from the affected area for biotoxin flesh testing. On the detection of algal biotoxins at levels exceeding the MPL the affected sites are tested on a weekly basis until two consecutive clear tests (negatives for DSP or below the MPL for PSP and ASP) are obtained. Where biotoxin action limits are exceeded, the FSA determines the necessary course of action, and the appropriate Local Authority carries out this action.

Advice regarding any enforcement action necessary in the event of a toxic bloom is be co-ordinated by the Local Authority Enforcement Support Division (LAESD). In general, protection of potential casual gatherers is undertaken by placing warning notices on the shore in the area of the bloom. Control of commercial harvesting is best approached by seeking the co-operation of the industry. The preferred course of action is to seek a Voluntary Closure Agreement (VCA) and this can often be obtained with shellfish farmers who usually have exclusive rights to cultivate shellfish in certain areas. Where this cannot be achieved, for example in public fisheries, then the Food Authority

can place a temporary prohibition order (TPO) under the Food Fishery Products and Live Shellfish (Hygiene) Regulations 1998, or for the Minister to make an order under the Food and Environmental Protection Act 1985. The latter action is the most stringent measure. However, it enables a wider range of controls to be applied, in particular it enables controls to be placed on species which would not be covered by a TPO and also covers non-commercial gathering. It may be the case that explicit action, other than perhaps the posting of warning notices, will not be required if the area is not subject to commercial harvesting at the time.

Algal Biotoxin Monitoring Programme 2002-2003

- water collections from 20 areas
- flesh samples from 62 areas
  - 503 analysed for PSP
  - 745 analysed for DSP
  - 436 analysed for ASP
- PSP detected in 13 samples from 2 areas, Ross links and Salcombe estuary and exceeded the action limit in 3 samples
- DSP detected in 1 samples from Solway firth
- atypical DSP detected in 107 cockle samples from 5 areas, mainly in the Thames, the Wash and the Burry Inlet
- ASP detected in 8 samples none at concentrations above the action level

Atypical DSP in the algal biotoxin monitoring programme

During the 2001 and 2002 algal biotoxin monitoring programmes atypical DSP positives were detected by the Diarrhetic shellfish poisoning (DSP) mouse bioassay (MB) in cockles from several areas around the coast of England and Wales. The most significant areas affected were the Thames estuary, the Burry Inlet and the Wash. All areas have extensive commercial cockle industries and the subsequent closure of these fisheries has had a major impact on the shellfish industry.

The symptoms being observed during the atypical DSP MB were neurotoxic with rapid onset. These were atypical and did not correspond to clinical signs induced by any known DSP toxin in mice.

Historical and International occurrences

It is only during the last two years of the monitoring programme that any extensive testing of cockles has been undertaken. Previous to this testing was usually limited to mussels or oysters. However, although limited, some cockles have been tested and subsequently closures were enforced in the Burry and Thames for DSP positives in cockle during 1992 and 1995 in the Burry and 1995 and 2000 in the Thames. Unfortunately no clinical sign data is available for these tests so it is not known whether these positives were atypical.

The problem of atypical DSP positives has also been experienced in several other countries. In Northern Ireland fast acting DSP positives with identical signs to those experienced in England and Wales have been noted in cockles from Mount Stewart, and these atypical DSP positives in cockles are still occurring. In Norway a similar fast acting positive in the DSP MB has been noted, which also occurs during the winter. This initiated a research effort co-ordinated by Professor Yasumoto and although the causative agent has not been identified it has be found to be unstable in frozen form (Prof. Tore Aune pers. com.). In Scotland and Canada during 1996 a sample of shellfish sent to Japan for analysis produced very similar reactions in the MB, again with no cause being identified (Prof. Yasumoto pers. com.).
The initial investigations of all these occurrences are characterised by the length of time it has taken to find the cause, and in most cases the toxin responsible remains to be identified. The situation in England and Wales has proved to be no exception, and although an investigation into the problem began in August 2001 and much useful work has been undertaken, the responsible chemical has not been identified.

Analytical Investigation

Analytical investigation by CEFAS, which included assistance from the NRC (Canada) and Prof. Yasumoto (University of Tohoku, Japan) of samples has eliminated many algal biotoxins as being the cause of the atypical DSP MB, these include: OA, DTX's, YTX, AZA's, spirolides, gymnodimine, oxazinins, prorocentrolides, PTX's, STX, NEO, GTX's and C-toxins. Further work by CEFAS has also eliminated a range of suspect metals and fatty acids.

Professor Yasumoto has also investigated several cockle samples and has detected similar atypical positives. On further examination by fractionation of samples toxic hydrophilic and lipophilic fractions were found. The hydrophilic toxin was adsorbed on an active charcoal column and eluted with 1% acetic acid-ethanol solution, analogous with PSP and tetrodotoxin. But the extractability and speed of action on the mice was not consistent with either of these toxins. He concluded that the cockle toxin differed from any known polyether toxin of dinoflagellate origin.

Further Work

Investigation into the causative agent is ongoing and it is hoped that liaison with national and international experts will continue. A toxicological study of the causative agent is planned to begin early next year by the FSA in the hope of determining the oral toxicity of the toxin and subsequently the potential health risk to the consumer.

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PHYCOTOXINS: THE FRENCH SYSTEM

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Historical aspects

In the early 80 's, *Dinophysis* « invaded » several French production areas. Okadaic acid in mussels was responsible for many phycotoxin outbreaks involving thousands of people. The first network was built by IFREMER in 1984 (REPHY)

French production

- 90,000 tons of oysters and 5,2000 tons of mussels (1999)
- 5,000 tons of clams and cockles
- 483 production areas (54,200 parcels given to 6,000 "responsibles" who employ 12000 people)
- 3,800 establishments including vessels, within 1200 purification centres
- 7 regions : Normandie-mer du Nord ; Bretagne Nord ; Bretagne Sud ; Pays de Loire ; Poitou-Charentes ; Bassin d'Arcachon ; Méditerranée
- 5 "hatching" centres

Administrative organisation

- A centralised system : the Ministry of Agriculture, Food, Fisheries and Rural Affairs (MAAPAR) in Paris, involving 2 divisions : the division for seawater fisheries and aqua culture (DPMA) and the general division for food (DGAl)
- 95 « départements » (+4 overseas) in which the government is represented by the « préfet ».
- In each « département », every application services are present. Each one must implement and return the results concerning the orders and the regulations sent by its central administration, and under the authority of the préfet, who co-ordinates, informs the concerned ministries and takes in account the local contexts before giving his orders.
- Dealing with shellfish and its sanitary security, 2 services : the maritime affairs departmental direction (DDAM) and the veterinary services departmental direction (DDSV).
- Also the concurrency services (DDCCRF) in retail and the public health services (DDASS) if food outbreaks.
- Coordination in a departmental security workgroup under the prefet's authority.

Scientific and technical support

- The French Institute for the Research and the Exploitation of the sea (**IFREMER**) is the public institute in charge both of R&D and of collecting and analysing the samples for official controls in the production areas.
- Supports of the DDAMs.
- Major lab in Nantes and 11 other sites in charge of phycotoxins around the French shores (8 practising bioassays).

- The French Agency for Food Sanitary Security (**AFSSA**) includes a division for risk evaluation and labs for all kind of items concerning animal and health problems.
- The microbiological toxins Unit in Maisons-Alfort is the French reference lab for marine bio toxins.
- The official analysis for the DDSVs are mainly done in departmental labs. **For each family of biotoxins, networks are under the scientific control of the NRL and the recognition of DGAl.**
- All labs must be accreditated (complete for NRL and some labs of the networks, the others are engaged in).

Procedures for the official survey and closures

- The French system has been built on the principle of observing the suspected toxic algae's and to practice tests on shellfish only when their presence is detected or a certain number of cells is reached.
- Each IFREMER lab director is in charge of managing the sampling and the analysis and of providing interpreted results.
- The REPHY is co-ordinated by the "responsible" of the network in Nantes who produces the Quality assurance procedures and organises the annual meetings of the labs. (inter comparison by NRL).
- 242 sampling points (166 for seawater and 176 for shellfish).
- IFREMER takes water samples (in specific points determined by IFREMER, taking in account water flows and risk analysis) each week to count the cells *(unless the sea is too rough or it is not a critical period).*
- Concerning **DSP**, the REPHY procedure states that **presence** of Dinophysis, *unless local historical data permit upper levels*, must be followed by a Yasumoto 84 mouse bioassay (aceton/dichloromethan/hepatopancreas - 24 hours).
- Concerning **PSP**, the count of *Alexandrium* must be below ***5,000*** cells/litre (*or more,* depending of local data), if not, an AOAC 1990 bioassay is engaged.
- Concerning **ASP**, the count of *Pseudonitzschia* must be below ***100,000*** cells, if not, analysis by Quilliam *et al* 1995 HPLC method is practised.

Consequence of any shellfish positive test :

1. Local IFREMER lab sends the results and its comments to a network of departmental and central administrations.
2. The departmental security workgroup (in fact generally under the responsibility of the **DDAM**) recommends the closure to the prefet (an official act - arrêté préfectoral - is submitted for his signature).
3. This act is then sent to every mayor whose city limits are concerned, to the professionals' representatives and **to the press.**

An order is being adopted to enforce all the principles of the closure procedures and associate the professionals for a better efficiency (tracing, batches…).

Re opening procedures

- Water and shellfish samples are taken and analysed by IFREMER every week during the closure.
- It's necessary to get 2 successive conforming results to decide to re open. **The results must be separated by at least 7 days**. In certain restricted circumstances 48 hours might be accepted (still considered as a risky procedure in France).
- IFREMER sends its results and comments to the same network and an act is taken for lifting.

Placing on the market controls

- Annual veterinary services control plan. Samples are collected by the DDSV in the dispatch or purification centres. This represents more than 500 samples to be analysed, each for the 3 types of toxins. The origin of shellfish is registered when sampled.
- If results are positive, the departmental lab (which is included in the network) gives information to the concerned DDSV and the NRL which receive the remains of the sample for further analysis (bioassay and, if useful, chemical analysis).
- The DDSV informs DDAM, the producer and DGAl.
- If still on the market, seizure of the batch occurs.
- DDAM ask IFREMER to check the suspect production area if it was still open.

Consequences of the new regulation (Decision EC 2002/225)

- The spirit of the EC decision 2002/225 is to guarantee the consumers that the shellfish placed on the market conform to the limits introduced for all the families of "DSP" toxins listed.
- The decision defines methods that can be combined to reach this objective. The Commission wants all member states to guarantee the same security level.
- Adaptations in France : IFREMER has changed the time for mice observation from 5 to 24 hours. Afssa has quit the Yasumoto 78 method to apply the 84 method.

Consequences observed in 2002. 2002 was not a typical year for Dinophysis (less in spring, more in autumn). More tests were carried out by IFREMER (350 before, doubled in 2002).

The development of the *Pectinidae* test was due to:

- positive DSP check on a batch of *Chlamys varia* in Spain (origin Normandy Islands) confirmed by our NRL (Y84 and traces of okadaïc acid under the level fixed in the decision EC/225/2002).
- the introduction in the future regulation on specific hygiene rules of obligations of monitoring the biotoxins on pectinidae.

140 tests were carried out in 2002 (DGAl plan) with the Yasumoto 84 method in the departmental labs. By early November 8 positive tests were seen on hepato-pancreas analysis but negative in the NRL on total flesh or, for *Pecten* and on muscle with gonads. The network will be developed in 2003 with association with IFREMER in the Seine estuary areas. There has been no health declaration till now.

Some potential limits with the French system:

- The tests are not systematic and they are mainly done on the hepatopancreas *so the monitoring of AZP does not seem to be guaranteed*.
- The tests are decided depending on the concentration of algae so there is delay to take a closure decision.
- Oysters are not considered to be contaminated by DSP.

The current French answers concerning the AZP :

1. Until it is not demonstrated that the "new" toxins have invaded our waters, it is no use practising systematic shellfish analysis. The weekly algae observation has until now demonstrated its efficiency, if we refer to the recorded consumption outbreaks linked to shellfish produced in the French waters (very rare cases). The same for not taking in account oysters if DSP.
2. This is reasonable only if a **vigilant system** can reveal the outcome of new toxins. 2 currently means :

- The **veterinary services control the plan** in all species.
- A 2002/2003 **study to monitor** the presence of **AZP** has begun in 16 suspect production areas (systematic water and shellfish samples taken every 15 days and analysed). The shellfish of those production areas used to give positive results with the Yasumoto 78 method (24 h) but negative with the Y 84 method (5 h).

Concerning the delays for closure decision, the samples of water and shellfish are taken at the same time, so the delay is very short (the time to observe and count the cells). *Quality assurance procedures have been regularly updated to give better security to our system*.
So the answer is there is no problem with the current system, but it must be ready to change because AZP might develop in the French waters (traces have been already found in 2000).

A major objective is to develop chemical analysis and, if possible, to limit the use of bioassays. Validation is engaged between AFSSA (NLR) and IFREMER (2 years study before expecting developing an operational network for chemical analysis).
The limit for developing chemical methods is in the difficulty to have the standards of toxins for which France wants to co-operate with Ireland.

Conclusions

- The crises involving Irish mussels in 1998 and 2000 have resulted in major changes in the principles of surveying and controlling DSP in the EC.
- Ireland has shown to the other member states its capacity to organise a secure system when facing a problem that could have affected for a long time its shellfish trade.
- The French authorities consider that Ireland represents an example for other member states who cannot exclude the development of new toxins in their waters.
- Even if it is obvious that surveying algae would not be sufficient while facing AZP, the improvement of the knowledge of the AZP algae would give other means of vigilance. The improvement of the representativeness of the sampling in each area is another major objective for us.
- Like Ireland, France is engaged in the development of chemical methods for replacing bioassays. It is a priority to ask the Commission to co-ordinate the production of chemical standards so that we can expect to manage toxins together.

Useful links for maps:

http://www.ifremer.fr/envlit/documentation/dossiers/toxines10ans/rephy-c5.htm : here you will find links to access maps showing the coasts that have been contaminated during the 10 past years by the different phycotoxins. For example, the DSP are in this address:
http://www.ifremer.fr/envlit/documentation/dossiers/toxines10ans/flash/carte_zones_dsp.htm.

http://www.ifremer.fr/envlit/documentation/dossiers/toxines10ans/rephy-c1.htm : here you will find the links to access to the maps that give all the REPHY sampling points.

BIOTOXIN MONITORING AND RESEARCH IN NORWAY- COMMENTS ON RECENT EU REGULATION

Tore Aune, Norwegian School of Veterinary Science

Norwegian national surveillance program (on behalf of National Food Control Authority):

- Algal samples are taken weekly from early spring through late fall from 26 locations along the coast.
- Public are advised against consuming self-picked shellfish when warning levels of algae are exceeded.
- The stations are reopened after verification of toxin levels in shellfish below tolerance levels.

Warning levels for algae

(subject to adjustment each year, based on experience):

Dinophysis acuta, 1 week	>300 cells/litre
Dinophysis acuta, 3 weeks	>100 cells/litre
Dinophysis acuminata	>900 cells/litre
Dinophysis norvegica	>2,000 cells/litre
Alexandrium spp.	>300 cells/litre
Alexandrium tamarense	>200 cells/litre
Pseudonitzschia spp.	>1 mill cells/litre*
Gonyaulax grindley	observed*
Lingulodinium polyedra	observed*

*triggers evaluation

Toxin analysis of shellfish in the surveillance programs on behalf of the National Food Control Authority (26 stations) and Directorate of Fisheries (50 stations):

DSPs (OA/DTXs, DTX3 upon hydrolysis): LC-MS
YTXs: LC-MS
PTXs and AZAs: LC-MS (semiquantitative)
PSPs: HPLC (post-column)
ASPs: HPLC
At 3 “routine” stations, mouse bioassays are performed biweekly, whole year.

Toxin analysis of shellfish intended for commercial marketing:

“DSP” toxins: mouse bioassay (Yasumoto 1984, modified), and in addition, LC-MS for OA/DTXs, YTXs, PTXs, and AZAs.
PSP toxins: mouse bioassay (AOAC), and in addition, HPLC (post column).
ASP: HPLC

Phytotoxins detected in Norway, by analytical methods:

Lipid soluble toxins:
OA, DTX1, DTX2, and DTX3 (after hydrolysis), periodically high levels.
Several YTXs (YTX, 45-OH-YTX, homo-YTX …), periodically high levels.
Several PTXs (PTX1, PTX-SA), mostly low levels.
Several AZAs (AZA1, AZA2, AZA3), low levels.

Water soluble toxins:
PSP toxins: STX, neoSTX and GTX1-4.
ASP toxins: domoic acid, low levels (scallops).

Distribution of phycotoxins in shellfish in Norway, general trends:

- Higher levels are seen in the south compared with northern parts.
- Lower toxin levels are found at the coast, compared with deeper in the fjords (DSP)
- Patchy distribution of PSP toxins
- Seasonal variations: PSP toxins mostly in the spring, but also detected in late fall. YTXs mostly in early summer, OA/DTXs mostly in summer/late summer and fall (sometimes through winter.)

Research project:

"Marine algal toxins; ecology, analysis and toxicology".
Financed by Norwegian Research Council (14.8 mill Norwegian Crowns). September 2000 to end of December 2004.
Participating institutions: Norwegian School of Veterinary Science, Norwegian Institute of Marine Research, Norwegian Veterinary Institute.
Project manager: Tore Aune.
Major research tasks:
-Introduce and further develop analytical methods for known relevant toxins
-Isolate and identify "new" toxins
-Study occurrence and ecology of potential toxic algae
-Toxicological/pathological studies of toxins from mussels
-Develop ELISA methods.

Comments concerning EU Commission Decision 15 March 2002,
Detailed rules for implementing the Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins (the lipophilic toxins) in bivalve molluscs, echinoderms, tunicates and marine gastropods have been created.
It was a significant step in the right direction when the Commission recognised that the DSP toxin complex in the future, should only comprise the true diarrhetic DSP toxins, OA and the DTXs. Yessotoxins and pectenotoxins were considered as individual toxin groups, outside the DSP complex. But, unfortunately, the Commission maintained the pectenotoxins together with OA/DTXs in the mouse bioassay at a tolerance level of 160 µg/kg, as OA equivalents. The tolerance level for YTXs was increased to 1 mg/kg shellfish meat, while the tolerance level for AZAs was established at 160 µg/kg.

Concerning methods of detection: The Commission acknowledged alternative methods to the mouse bioassay, requiring that they provide an equivalent level of public health protection, and that they are internationally validated before taken into use. Furthermore, *when the results from different methods demonstrate discrepancies, the mouse bioassay should be considered as the reference method*.

The Commission says that a series of mouse bioassays, differing in the test portion and solvents, can be used. However, what they do not say is, that if yessotoxins are present, they may lead to positive results in the mouse bioassay, even below the tolerance level. Professor Yasumoto has realised this, and has proposed a new protocol which necessitates *two* mouse bioassays for the lipophilic fraction, to correctly analyse all the relevant toxins.

In my opinion, this underlines the urgent need for preliminary steps towards accepting alternative, analytical methods, even before full international validation of alternative methods is completed.
Compared with the wide variations in the mouse bioassays, the state of the art for analytical methods (a combination of LC-MS and HPLC methods, as described in recent publications and discussed in scientific meetings) this presents better protection of the consumers. Furthermore, introducing a third mouse bioassay as a requirement for testing before marketing shellfish will meet strong opposition, both based on ethical considerations, cost and time.

Suggestions

The issue should be discussed by the CRL/NRL working group for marine algal toxins. The aim of this should be to persuade the Commission to accept established analytical methods (LC-MS/HPLC) for DSP-, YTX-, PTX-, AZA- and PSP-toxins as equal tests to the mouse bioassays, upon documentation of safety in use, even before whole scale international validation is completed.

International collaboration is already in operation in this field, involving scientists from among others Ireland (Marine Institute), Japan, New Zealand, Canada, Spain and Norway. This group should follow up the EU Working Group (2001) and Commission (March 2002) urge for method development and validation. Collaboration should lead to the production of necessary standards and certified reference materials. In the meantime, collaborative studies should be undertaken with the toxin standards available, between laboratories already active in the field (work along these lines is already in operation).

CONCLUSION

Dr. Patrick G. Wall, CEO, Food Safety Authority of Ireland.

Food safety, consumers' health and the reputation of the Irish Shell fish industry are inextricably linked and it is only by all parties working together that we will achieve the best outcome for all. Biotoxins are a natural phenomena and no blame is attributable to anyone when they occur in shellfish. However, if people fall ill, Irish producers, processors and the regulatory agencies quickly come under fire.

Biotoxins are a global problem. It is understandably frustrating for shellfish farmers when, due to biotoxin levels, they are unable to harvest their shellfish at the optimum time when the shellfish are mature. It is equally frustrating for processors when they have processed and shipped product that subsequently turns out to be contaminated. The challenge is to get the correct balance between protecting consumers' health and allowing shellfish farmers and processors make a living. The FSAI, the shellfish farmers, the Marine Institute and the Department of Communications, Marine and Natural Resources are working together to develop the monitoring programme to cope with the problem and ensure consumer health is adequately protected.

With 94 production areas around Ireland producing around 35,853 tonnes of shellfish, the industry constitutes a major national resource, contributes significant revenue to the economy and provides local employment in rural areas. The reputation of Ireland as a centre of excellence for shellfish depends on the confidence of international purchasers in our product and our controls. Much progress has been made and foreign purchasers of Irish shellfish and the EU auditors recognise that Ireland is addressing the issue and has a credible monitoring programme.

Research is important to develop better and more rapid tests and early warning systems so that shellfish can be harvested when they are clear and left in the sea when they are not.

The annual biotoxin workshops provide an opportunity for sharing information and pooling knowledge generated nationally and internationally which will lead to more informed policy and management decisions. A bit of "creative tension" can energise initiatives and give them the necessary urgency, however, conflict between parties is non productive and impedes progress. I am confident that working together we will get the best results for consumers and the Irish industry.

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