6th Irish Shellfish Safety Scientific Workshop

Galway, 1st December 2005

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

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INTRODUCTION AND OBJECTIVES OF THE 6th IRISH SHELLFISH SAFETY WORKSHOP

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

On behalf of the Marine Institute (MI) and our co-sponsors, Bord Iascaigh Mhara (BIM), the Food Safety Authority of Ireland (FSAI) and the Irish Shellfish Association (ISA), I would like to welcome all the participants to this, our 6th annual Irish Shellfish Safety Workshop.

The workshop is part of the MI's role as the EU designated National Reference Laboratory for marine biotoxins and for shellfish microbiology in Ireland. This workshop has become an annual event since April 2000, where scientists, regulators and shellfish farmers meet to review developments in the monitoring and research of shellfish safety both in Ireland and internationally.

Objectives. Our specific objectives for the 2005 Workshop are to:

- Review the Irish Shellfish Monitoring System and the results during 2005
- Assess key developments since our last workshop in October 2004
- Summarise current Irish research work in Harmful Algal Blooms (HAB's), toxicology and microbiology
- Focus on research work under the BOHAB and ASTOX projects and early results from Clew Bay under the REDRISK project
- Take stock of EU and international developments on food safety and hygiene in 2005, including the progress on rapid test kits
- Provide a forum for debate/feedback.

Key Irish Developments in 2005

- The shellfish safety programme has evolved as a partnership between the MI, FSAI, DCMNR, BIM and the shellfish industry
- Within this, MI's role is to manage an integrated programme of monitoring with 7,000 tests pa.
- All results for phytoplankton, bioassay and chemistry are posted on MI and FSAI websites
- Rapid turnaround of MI results (90% + within 3 days)
- MI has continued to progress in laboratory accreditation and quality systems in the areas of phytoplankton, bioassay and chemical testing
- Toxicity has unfortunately been high in 2005, compared to 2003 and 2004 (23% of mussel samples tested by MI in 2005 were positive)
- The Molluscan Shellfish Safety Committee (MSSC) had 5 meetings, chaired by the FSAI, and a special session on DTX3 in July 2005
- The Management Cell was used frequently (80 decisions to date in 2005), to enable rapid decision making, in accordance to protocols which were drafted by MSSC members
- Major research projects have continued in the areas of biotoxins and Harmful Algal Events (BOHAB, BIOTOX, ASTOX and REDRISK projects).

I would like to convey our thanks to our MSSC colleagues, the samplers, the laboratory staff and all the members of the shellfish safety team for your efforts in 2005.

Key questions for research and debate

Some of the key questions that have been investigated in MI funded research, that will be debated at this workshop include:

Q What are the drivers for toxicity in mussels & oysters?
Q Can we link the oceanography with toxicity trends?
Q How toxic are azaspiracids and DTX 2 ?
Q Can we develop early warning systems for viruses in shellfish?

Communications with Stakeholders

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

- Collaboration with the FSAI in the development of the Codes of Practice, the online HAB database and the Management Cell.
- Weekly Reports by fax or email
- SMS Text message service by mobile phone relating to changes in bay status to over 90 industry and regulators
- Daily phone contact with DCMNR, samplers and industry members
- Participation at the 5+ MSSC meetings and its subcommittees
- Participation and advice to the Management Cell on a weekly basis
- Arranging conferences, workshops and regional meetings
- Issuing the proceedings of the annual Shellfish Safety Workshop to 400+ interested parties

Marine Environment Benefits

In addition to the direct relevance of the MI monitoring work to the scientists, regulators and shellfish industry, the Shellfish Safety Programme has brought important spin-off benefits in building our capacity to understanding the marine environment:

- MI phytoplankton dataset since 1990 (over 30,000 data points) can now be adapted as part of the Water Framework Directive Index of Water quality

- The MI programme detected and tracked a major bloom of *Karenia mikimotoi* in Summer 2005 and we are issuing a report on this event today

- The oceanography and phytoplankton data can provide a baseline for carrying capacity modelling of inshore aquaculture bays.

Irish Molluscan Shellfish Industry, 1999 – 2004

Our objective for the Irish Shellfish Safety Programme since the reforms in 2000 has been to support the continued development of the Irish shellfish industry and promote seafood safety, by building the best Shellfish Safety management system in the Northern Hemisphere

I am glad that the Irish shellfish industry has continued to show its resilience and potential over the past five years. As MI and other agencies have invested in building our shellfish safety programme, BIM and the Irish industry has been able to develop new exports markets. The main export markets for processed Irish mussels in the past five years are France, the UK, Italy, the USA and Germany.

BIM Aquaculture Production data show an encouraging trend in Molluscan Shellfish:

Year	Tonnage	Value, € million
1999	23,516	21.6
2000	31,100	21.5
2001	35,583	27.9
2002	37,704	37.9
2003	44,678	41.8
2004	43,091	43.6

We can see that Ireland's investment in shellfish safety has supported a strong resurgence in Irish output of mussels, oysters & scallops since the year 2000.
Working together, we can continue to build on that success.

REVIEW OF PHYTOPLANKTON MONITORING 2005

Siobhan Moran[1], Joe Silke[1], Rafael Salas[1], Tara Chamberlan[2], Josephine Lyons[1], John Flannery[1], Valerie Thornton[1], Dave Clarke[1], Leon Devilly[1]

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Introduction

A national phytoplankton monitoring programme, has been in operation in Ireland since 1986, and fulfils requirements of the EU Council Directive 91/492/EEC.

This programme provides an important part of the baseline data in the overall integrated shellfish monitoring programme. The analysis of samples received on a regular basis from a site can provide very important information in assembling a population profile for the area. This helps in crucial decisions, for example in Management Cell Decisions - conducted by representatives from the industry, MI, FSAI and DCMNR - when borderline toxin results are present.

Phytoplankton monitoring is also hugely important in the Water Framework Directive, which all EU countries must follow, in developing an index of water quality in Ireland and Europe. The Irish Monitoring programme also gives valuable public health information to County Councils, Environmental Health Officer's and the public during times of bloom events.

Overview

The following paper provides an overview of phytoplankton sampling, analysis and reporting in 2005. The occurrence of potentially toxic and harmful phytoplankton found in Irish coastal and shelf waters in 2005 is also reviewed and the quality scheme in operation is described.

Methodology

Sampling Sites

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

Throughout 2005, over 2100 samples from 61 shellfish sites and 42 finfish sites around the coast were submitted to the phytoplankton laboratories. Of these, almost 92% were processed as part of the National Monitoring Programme. The remaining were analysed as part of various research projects.

Sampling Protocol

The Lund tube sampling method accounted for almost 53% of samples collected in 2005, with 26% sampled from the surface. However, almost 18% of samples received by the laboratories gave no information on sampling method. Rejected samples accounted for 4% in 2005 - a reduction from 9.9% in 2004 and 12.6% in 2003. This drop is due to a combination of improvements made to both procedures and sampling strategies.

Sampling Analysis & Reporting
All samples analysed for the presence of toxin producing/ problematic phytoplankton are examined using the Utermöhl method (Trondsen, 1995) following INAB accredited procedures. The method has a sensitivity of 40 cells.l-¹. By the beginning of November 2005, the results of a total of over 1300 samples were reported back to the industry and related bodies, in over 246 phytoplankton reports, issued on a daily basis. The overall turnaround time from laboratory receipt to reporting is ~ 90% within one working day, and 98% in two working days, well exceeding the 80% within two working days requirement as stated in the service agreement between the MI, FSAI and DCMNR.

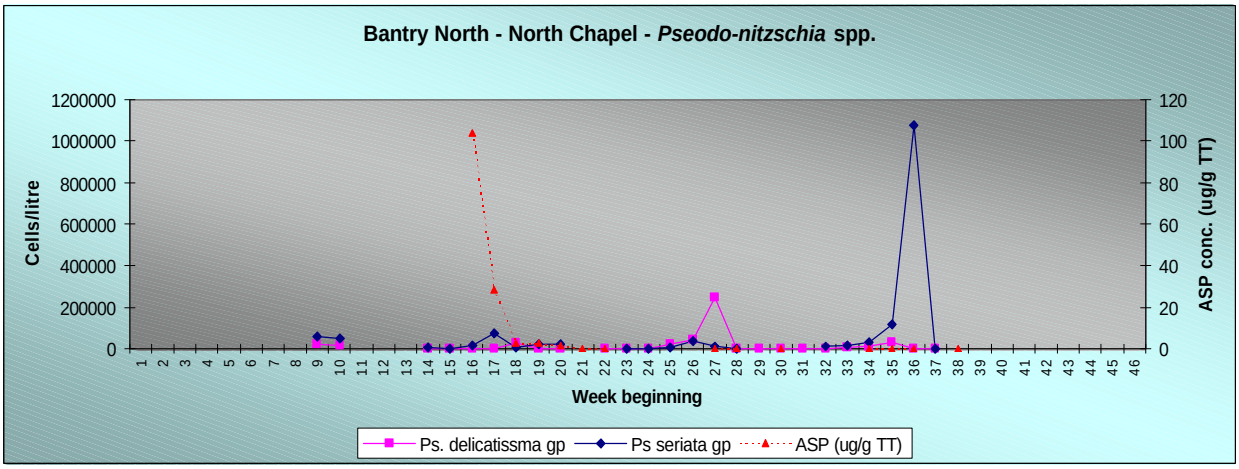
Toxic phytoplankton in Irish waters in 2005
There are four main toxic algal groups that occur in Irish waters. These are the phytoplankton species that produce the toxins that cause, Diarrhetic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP), and Azaspiracid Poisoning (AZP). In previous years closures in shellfish growing areas mainly resulted from DSP events, with localised closures in Cork Harbour due to PSP events. However, in 2005, prolonged closures also occurred due to ASP and AZP events.

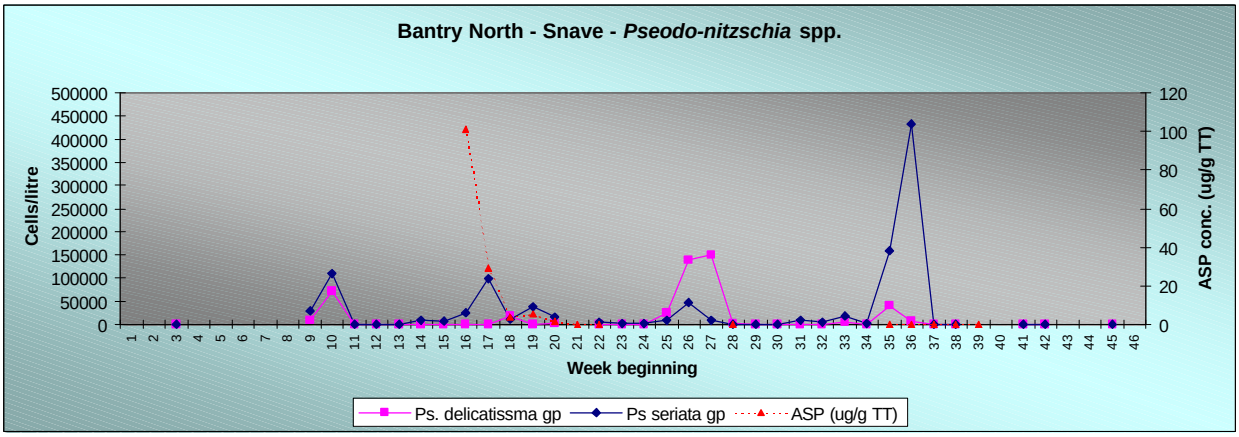
Figures 1, 2 and 4 to 7, show graphs for known or suspected toxin producing phytoplankton and their associated toxin profiles from January to November 19th (week 1 to 46), 2005. The profiles illustrated are chosen from among the sites which had consistent data-sets available, between phytoplankton counts and toxin results, due to the provision of regular water and shellfish flesh samples to MI laboratories for that time period. The toxins profiled are ASP, DSP and AZA.

Amnesic Shellfish Poisoning
Amnesic Shellfish Poisoning (ASP) toxins (domoic acid), are produced by the diatom *Pseudo-nitzschia* spp. There are eight identified species in Irish waters – six of which are potential domoic acid producers. Amnesic Shellfish Poisoning toxicity has generally only been found in scallops in Ireland. However in 2005, mussel and a lower number of oyster closures occurred in the south west and to a lesser extent in the north west due to this toxin (Clarke *et al*., 2006). This occurred in April to mid-May and was associated with a low biomass monospecific bloom of *Pseudo-nitzschia* spp. By the end of May however, the toxicity had fallen and all areas had re-opened. Later, in August and September there were further increases in *Pseudo-nitzschia* spp. numbers at several sites, in particular Kenmare and Bantry. Numbers peaked at 2.3 million cells/l in two sites in Galway. However no toxicity was associated with these blooms.

Due to the limitations of light microscopy (Figure 3) in identifying minute, detailed morphological structures found in the *Pseudo-nitzschia* genus, cells are generally categorised into one of two groups. These are *Pseudo-nitzschia seriata* group – which contains the main toxin producing species, and *Pseudo-nitzschia delicatissima* group. Work in identifying the possible causative species for the April toxicity is continuing.

Figures 1 and 2 show this correlation between cell numbers of the two main *Pseudo-nitzschia* groups described above, and toxicity in the sites shown, for the toxic event early in the year, and the non-toxic bloom later in the year. There were differences in the toxicity of the individual blooms, most likely due to different species succession through the year.





Figures 1 and 2. Cell counts of *Pseudo-nitzschia seriata* group and *Pseudo-nitzschia delicitissma* group from North Chapel and Snave in Bantry North, for week 1 to 46, 2005. It also shows the toxin levels of ASP for the same time period.

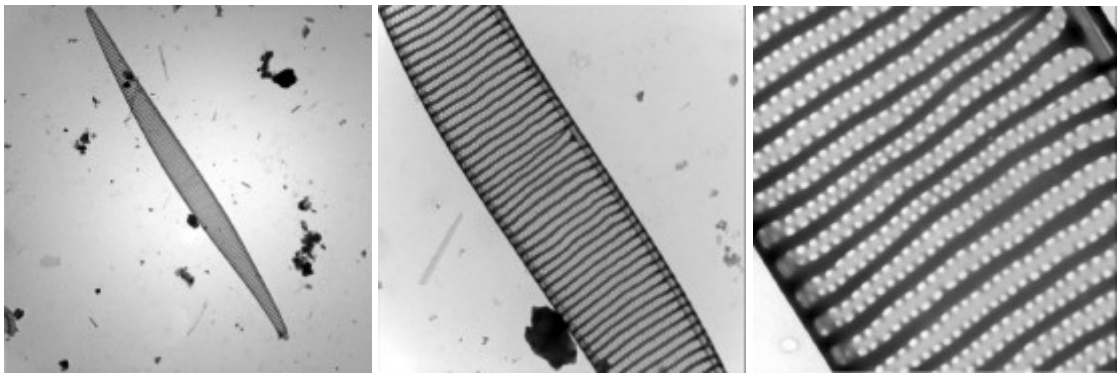


Figure 3. *Pseudo-nitzschia* spp. cell showing the level of detail required to identify it to species level, using electron microscopy.

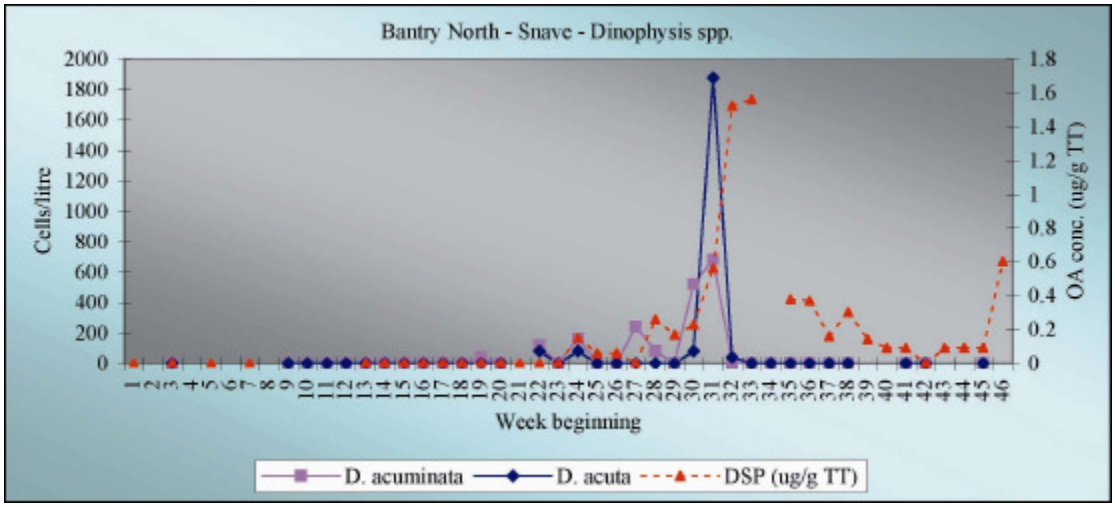
Paralytic Shellfish Poisoning
Paralytic Shellfish Poisoning (PSP) toxins - saxitoxins - are produced by *Alexandrium* spp. Due to the potential severity of the toxin, the presence of this species in water samples triggers increased testing of shellfish samples for PSP toxins.

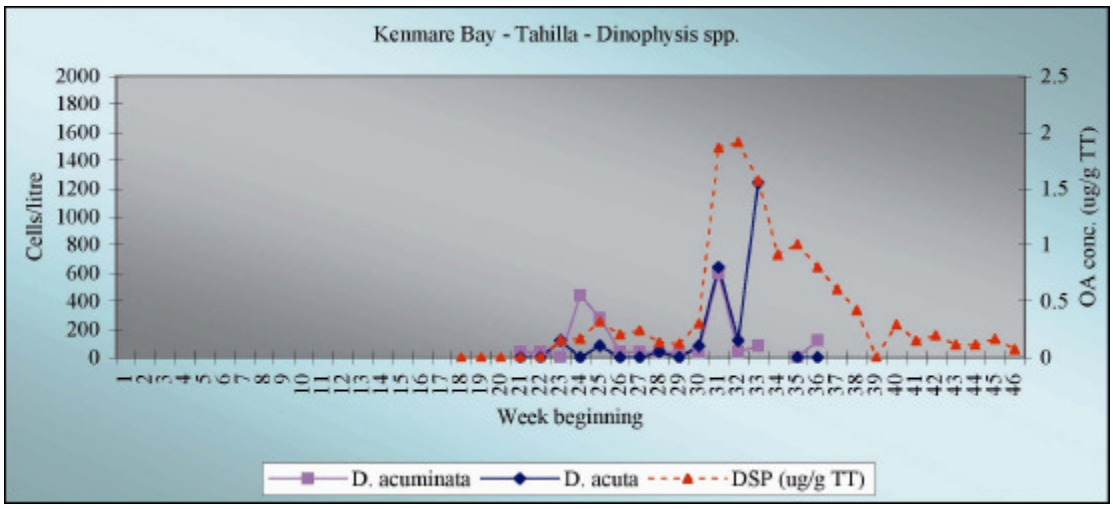
To date the main production area that has experienced closures due to PSP toxins is North Channel in Cork Harbour. Levels of *Alexandrium* spp. were generally observed at low levels all around the Irish coast throughout May to August (when compared to 2004), with the highest levels observed in North Channel, Kinsale, Oysterhaven, Loughras Beg & Greenore. Paralytic Shellfish Poisoning toxicity occurred in mussels in early to mid June in North Channel, Cork Harbour (Clarke *et al.* 2006), which corresponded a rise in *Alexandrium spp.* levels to 1080 cells/litre. Numbers subsequently decreased and rose again to 1640 cells/litre in early July. However, no toxicity was present at that time. The levels and distribution of *Alexandrium* spp. were observed to decrease further around the coast in September and were observed to be mainly confined to the south and south west at low levels in October.

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

During May, in the majority of west and southwestern locations, *Dinophysis* spp. were present at typically low levels. In June, levels were observed to increase in a small number of locations in the south west, but typically remained at the same levels as those observed in May. In the west and north west significantly large increases in cell numbers were observed, but generally decreased in July and decreased further in August.

This decrease was not observed in the south west, and significant cell counts occurred in all sites in August. In September, cell counts of *Dinophysis* spp. decreased rapidly in all localities - especially the south west. From October to date, no *Dinophysis* spp. have been observed.



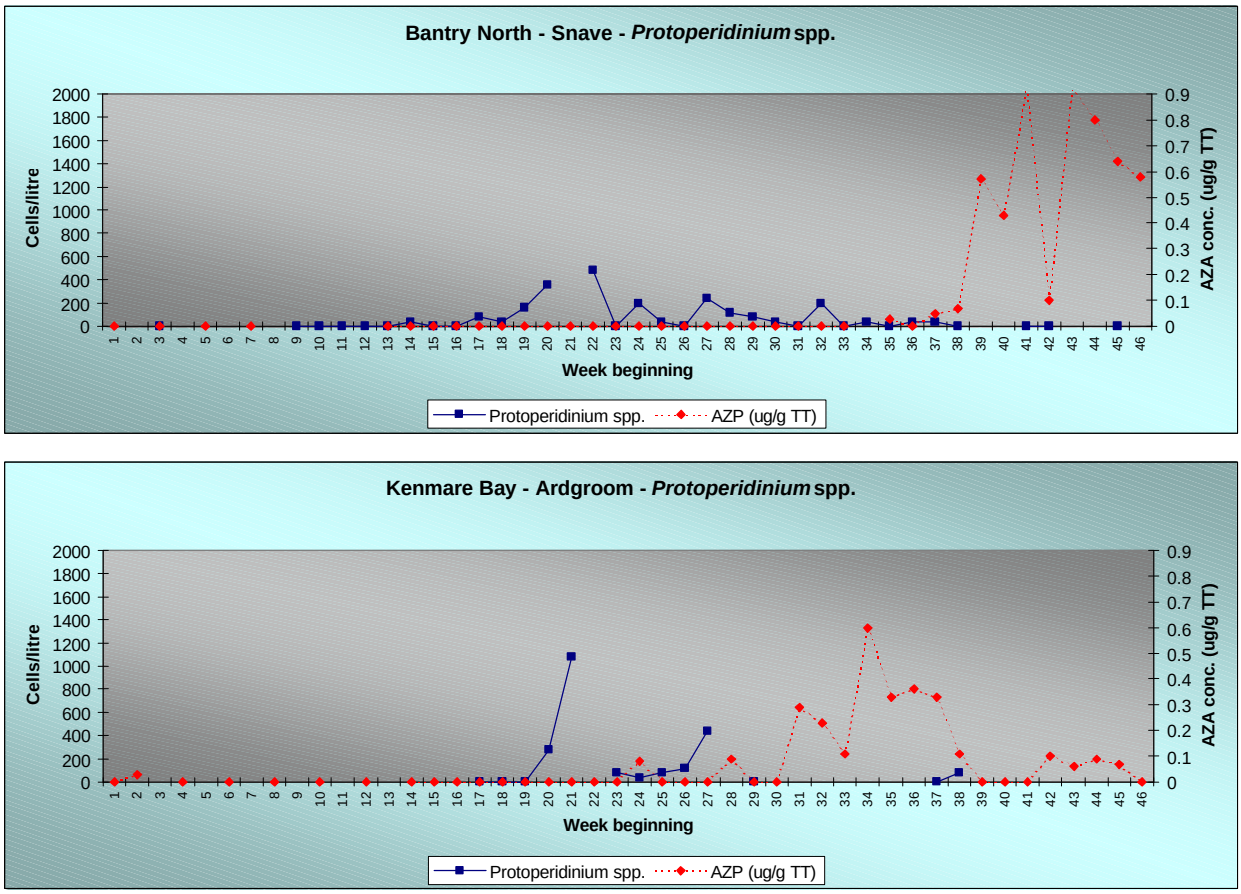


Figures 4 and 5. Cell counts of *D. acuminata* and *D. acuta* from Snave in Bantry North, and Tahilla in Kenmare Bay, for week 1 to 46, 2005. These figures also show the toxin levels of Total Okadaic Acid equivalents for the same time period.

Azaspiracid

Azaspiracid (AZA) toxins, have been associated with the dinoflagellate *Protoperidinium crassipes.* In other genera as the production of toxins can come from several species, it may be the case therefore that more than one *Protoperidinium* species may produce AZA.

The cell counts and AZA levels are shown in the figures 6 and 7 and no apparent correlation was evident between the presence of *Protoperidinium* spp. in the water and the toxin level increases, which occurred. In all sites *P. crassipes* was present at levels averaging 40-80 cells/litre.



Figures 6 and 7. Cell counts of *Protoperidinium* spp. from Snave in Bantry North, and Ardgroom in Kenmare Bay, for week 1 to 46, 2005. It also shows the toxin levels of AZA for the same time period.

Other problematic species in Irish waters in 2005

Two main problematic species occurred in Irish water's during 2005 at bloom levels. *Noctiluca scintillans* occurs annually during the summer/early autumn months. In 2005 the highest concentration recorded was in Donegal Bay on 5th September at 7.2 million cells/litre, and Kilmakillogue, Co. Kerry on 4th August at 7 million cells/litre. No human health problems were associated with these blooms. A second more widespread and damaging bloom of *Karenia mikimotoi* also occurred during the summer months of 2005. A detailed report of this bloom and its impact on coastal communities is given in these proceedings and in Silke *et al.*, 2006.

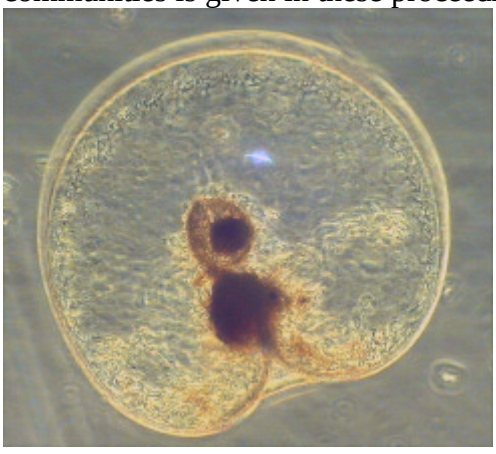


Figure 8. *Noctiluca scintillans*

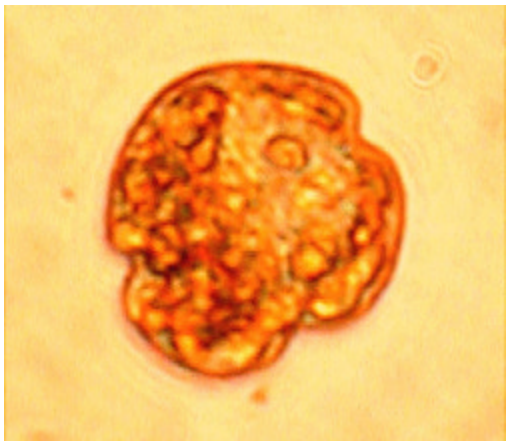


Figure 9. *Karenia mikimotoi*

Quality System

Towards the end of 2004, the phytoplankton laboratories of MI applied to the Irish National Accreditation Board (INAB) for ISO 17025 accreditation, in the test method used for the identification and enumeration of toxic and problematic species, using the Utermöhl method of analysis.

In March 2005, this award was presented to both the Galway and Bantry laboratories. The MI is the first national phytoplankton monitoring laboratory in Europe to receive accreditation for this analysis and had to meet the quality assurance and procedural guidelines under ISO 17025. One of the requirements included an audit trail for species identification, which was satisfied by introducing a system of recording a series of photo-micrographs for each species observed by an analyst over specified time period.

Irish National Accreditation Board accreditation ensures a high standard certification process, guaranteeing traceability, confidence and consistency in all samples analysed.

References

Clarke, D., Devilly, L., McMahon, T., O'Cinneide, M., Silke, J., Burrell, S., Fitzgerald, O., Gibbons, Hess, P., Kilcoyne, J., McElhinney, M., Ronan, J., Gallardo Salas, R., Gibbons, B., Keogh, M., McCarron, M., O'Callaghan, S., Rourke, B.,: A Review of Shellfish Toxicity Monitoring in Ireland and Review of Management Cell Decisions for 2005. (These proceedings)

Silke, J., O'Beirn, F., Cronin, M.,: *Karenia mikimotoi*: An Exceptional Dinoflagellate Bloom in Western Irish Waters, Summer 2005. *Marine Environment and Health Series*, **No. 21,** *2005*

Karenia mikimotoi: AN EXCEPTIONAL DINOFLAGELLATE BLOOM IN WESTERN IRISH WATERS, SUMMER 2005.

J.Silke, F.O'Beirn and M. Cronin, Marine Institute, Parkmore Galway.

Introduction

A bloom of the dinoflagellate *Karenia mikimotoi* (see Figure 1) originated along the west coast of Ireland during June 2005 and persisted for approximately two months. During that time, mortalities were reported of vertebrate and invertebrate species along the length of the western seaboard. The Marine Institute scientists conducted comprehensive surveys of the coastline. High cell counts were related to subsequent mortalities.

The Marine Institute agreed with (DCMNR) to investigate and produce a full report on the phenomenon and this was published in November 2005 (Silke *et al*., 2005). The following provides a summary of the findings of the investigation.

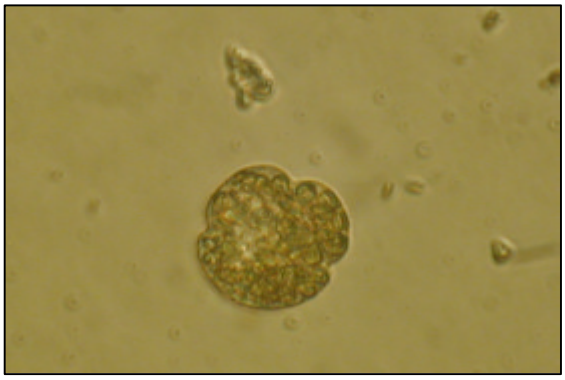


Figure 1. The dinoflagellate *Karenia mikimotoi*, observed at x200 magnification (MI)

Methodology

Methods for the investigation were many and varied. They can be divided broadly into 3 areas of assessment:

(a) Assessment of the scale of the blooms

- The scale of the bloom was determined from the satellite imagery and phytoplankton counts.
- A review was carried out of environmental conditions measured along the western seaboard during the bloom event.

(b) Assessment of the intensity of the blooms

- Samples of seawater were collected from along the western coast to identify and enumerate the phytoplankton present, resulting in seawater discolouration and marine mortalities.
- RV Celtic Voyager Survey of West Galway coast (July 13-14), for phytoplankton, fluorescence and oceanographic conditions.

(c) Assessment of the impact of the blooms

- The scale of the impact was evaluated by discussion with staff from public agencies, as well as private individuals concerned about, or affected by the blooms.

- Numerous information sources were investigated with a view to documenting the intensity of mortalities in areas as well as the geographic scale of the impacts.
- Shore sites visits in Donegal Bay (July 1).
- MI Dive investigations in Kilkieran Bay July (4-5)
- MI's RV Celtic Voyager Camera Survey of Donegal Bay (July 10-11).
- Phytoplankton and benthic grab survey of Killary Harbour (August 7).
- A survey of aquaculture installations and surrounding environment in Killary Harbour was commissioned by MI (Aqua-Fact International Services Ltd.).

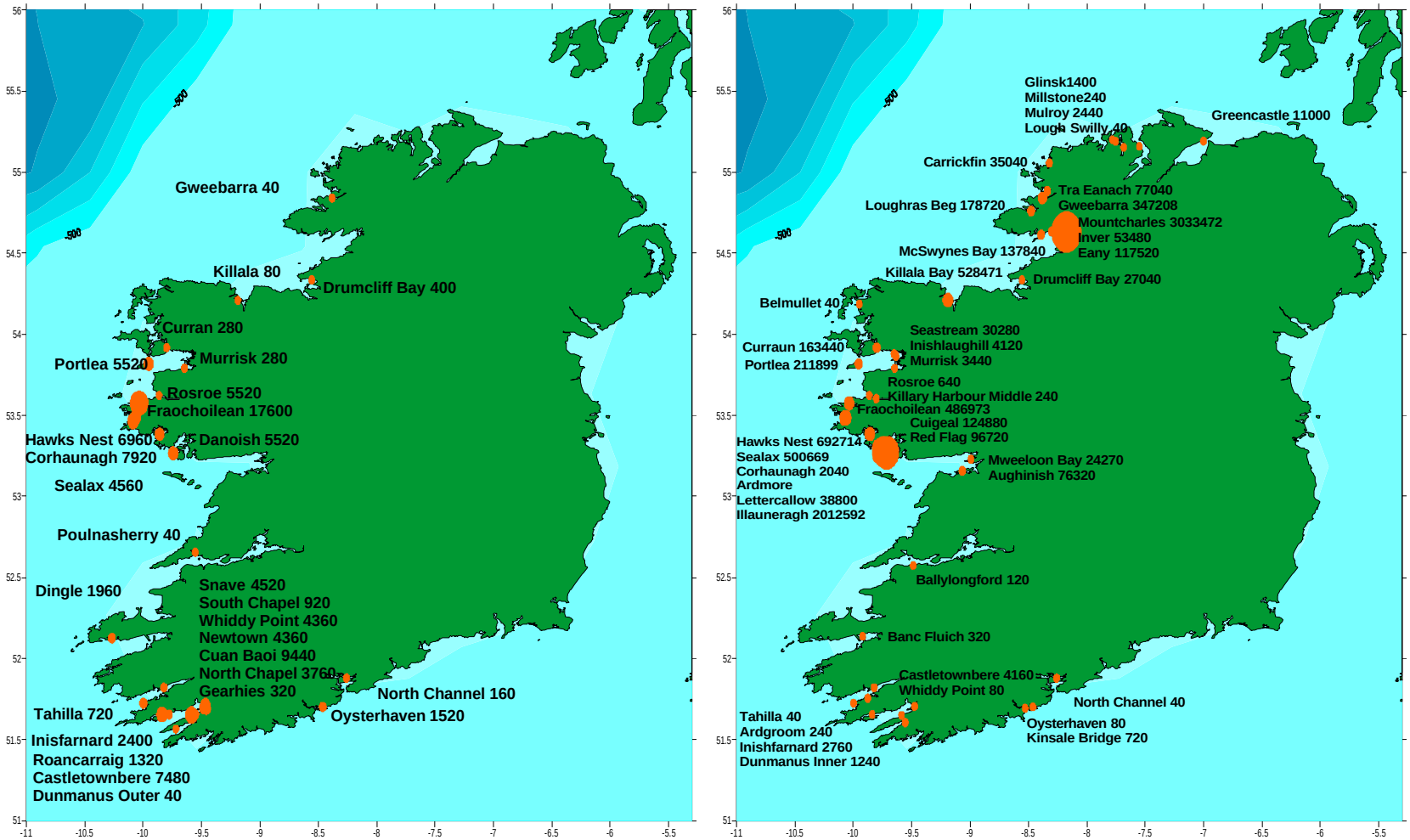
Specific investigations by MI were supplemented by additional input from a variety of other sources. The details of these additional investigations are summarised main report.

Results and discussion

Samples analysed as part of the National Phytoplankton Monitoring Programme identified the onset of the bloom from the end of May to the beginning of June. This first bloom was present in the northern and north western part of the country and a subsequent bloom developed in the southwest. The monthly maximum cell counts at the locations of the sample points taken as part of this programme are given in Figures 2 (a-d). Early development of the bloom during the month of May showed highest counts observed in western County Galway (Fraochoilean 17,600 cells/l). The bloom continued to develop in this area during the month of June up to 692,714 cells/l recorded in Hawks Nest, but extended northwards into Donegal Bay where exceptionally high counts in the inner part of the bay reached over 3 million cells/l. The bloom dissipated in Donegal during August.

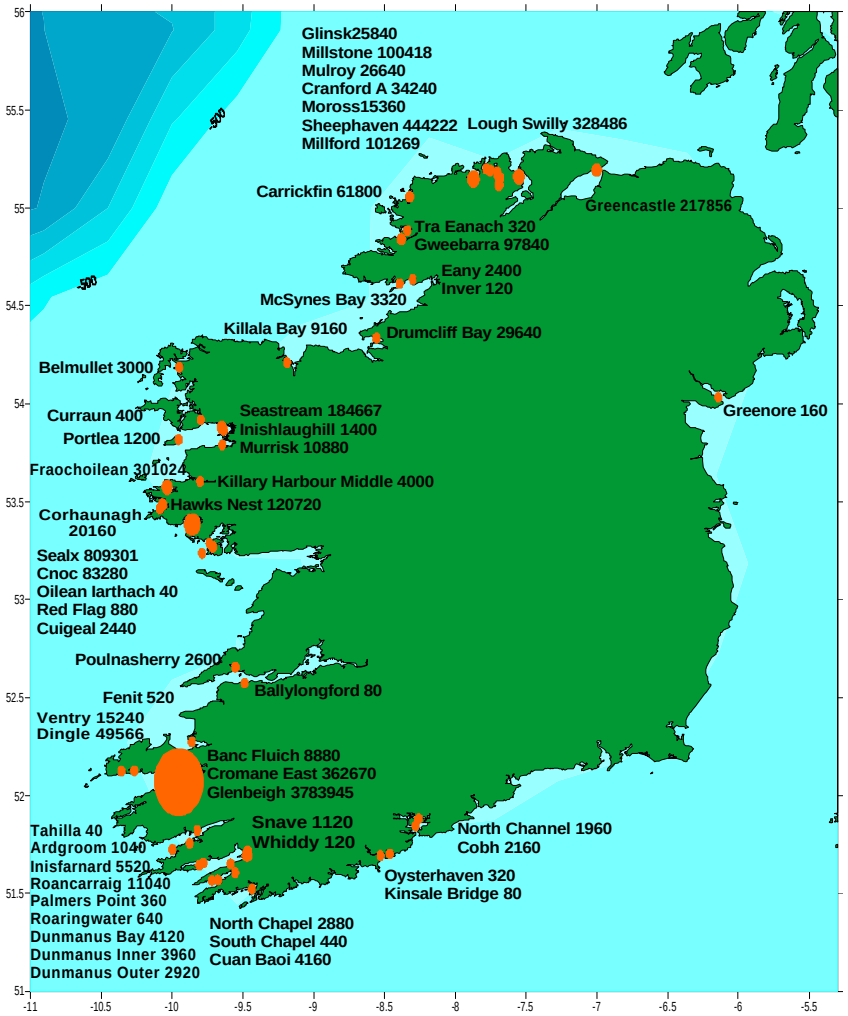
Meanwhile in the south west, a second bloom had established during July where high concentrations of up to 3.7 million cells were observed in the Glenbeigh area of Dingle Bay. The bloom in the south west was not as persistent as in the north and had significantly decreased by the start of August. Castlemaine Harbour showed the highest levels of the month at only 2000 cells/l on the 2nd August. However typical levels were between 40 and 200 cells/l along the north west, west and south west coasts, apart from 840cells/l found in McSwynes Bay on 22nd August. The bloom continued to dissipate through the month of August back to background levels by the end of the month (Figure 2a-d).

The extent of these blooms was also apparent from satellite images. A suite of images of 7 day composite images (Figure 3 a-e) taken from the MODIS aqua sensor revealed the presence of elevated sea surface chlorophyll levels, greater than 9 mg m^{-3} during the first half of June. This bloom was very extensive covering the western coastline north of Slyne Head, and developing as the month progressed to an area west of Donegal extending to approximately 100 km offshore. The levels were concurrent with the high cell counts observed in the area of *K. mikimotoi*. Elevated chlorophylls were also visible in Dingle Bay in August, although not as extensive and not as striking in the images due to its localised presence in a coastal area where the land effects interfere with satellite imagery. A photomontage of the shore investigations along Donegal Bay is seen in figure 4.

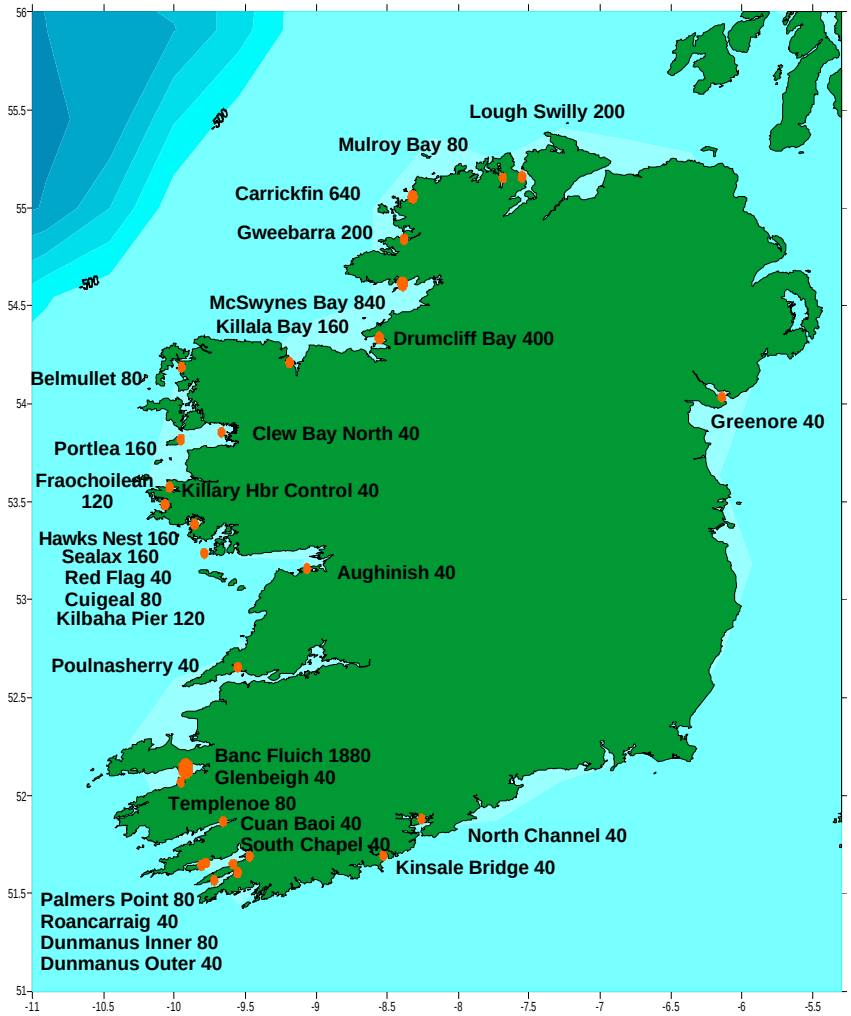


(a) May (b) June

Figures 2: (a-b). Monthly maximum cell counts observed in national monitoring programme locations (cells/litre) May /Jun



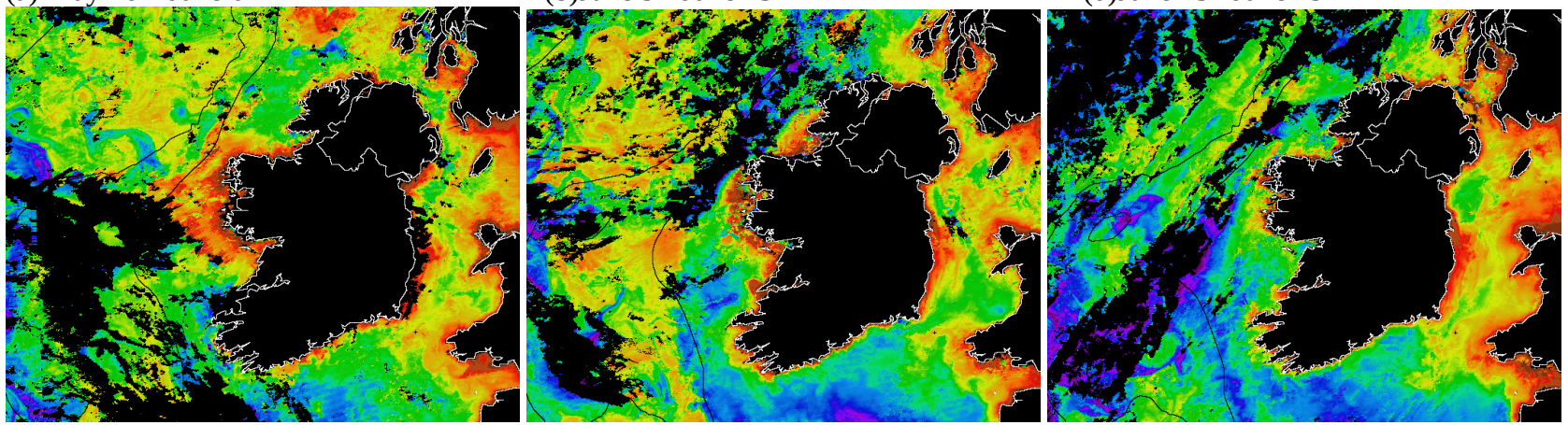
(c) July



(d) August

Figures 2 (c-d). Monthly maximum cell counts observed in national monitoring programme locations (cells/litre) Jul – August

(a) May 26 – June 01 (b)June 9 - June 15 (d)June 23 –June 29



(d) July 28 – August 3 (e)August 4 – 10

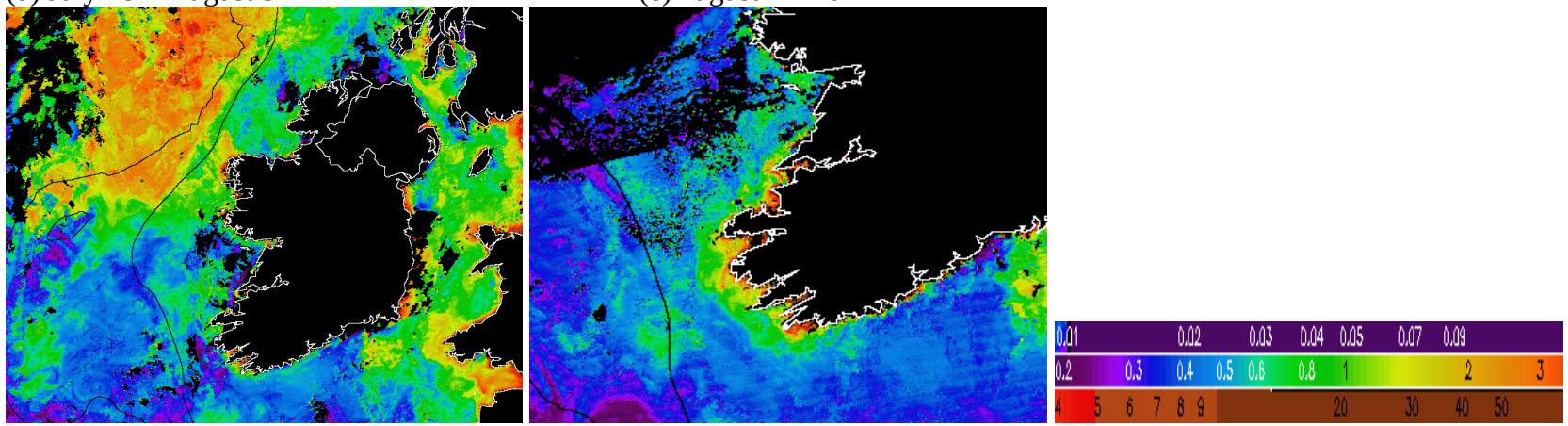


Figure 3: (a-e) Sea surface chlorophyll taken from MODIS Aqua satellite sensor.

Figure 4. Photomontage of the shore investigations along Donegal Bay detailing large numbers of dead heart urchins, dead cockles and lugworms and discoloured water and break-up of the bloom along the shoreline.

Discussion

Blooms of *Karenia mikimotoi* are mostly unpredictable events, occurring intermittently in Irish coastal waters. The life cycle of *Karenia mikimotoi* is not fully elucidated and the cyst stages (if any) have not been described. It is most likely that the species over-winters as a motile cell in low numbers, awaiting a return to favourable conditions to bloom. These conditions do not occur every year and the species has variable success in achieving dominance among the phytoplankton community. On the occasions it does achieve dominance, it can form dense and extensive blooms, such as were observed to the west of Ireland in summer 2005.

This harmful algal event was more extensive and persistent than previously reported *Karenia* blooms in Irish waters. The scale of the benthic mortalities was also more severe than in previous blooms.

Coincident with the bloom were reports of mortalities of vertebrates (fish) and invertebrate organisms along the western seaboard. Mortalities were reported from Dingle Bay to Loughros Beg (Co. Donegal). The habitats impacted ranged from muddy environments (McSwynes Bay, Co. Donegal and Killary Harbour Co. Galway), sandy habitats (Co. Sligo coastline) to rocky substrates (Gurraig Sound, Kilkieran Bay, Co. Galway). This broad range of habitats impacted and the geographic spread suggest a widespread water borne causative agent.

All of the mortality reports described large megafauna (> 4mm). These observations were validated by the follow-up grab survey of Killary Harbour, which included a quantitative analysis of macrofauna (>1mm). Given that smaller macrofauna were found alive in the Killary survey, it would appear that large macrofauna (echinoderms, cockles) were worst affected. Potential explanations for the observed mortalities include:

- That the organisms were particularly sensitive to perturbations; for example, mortalities were reported in echinoderms from Donegal to Galway. Echinoderms are generally considered sensitive to pressures in the marine environment, for example, physical disturbance and organic loading (Budd, 2002; Hill, 2000).

- Megafauna have greater oxygen demands than smaller animals due to their size. In addition, the timing of feeding activity may have coincided with periods of low oxygen in the water. (This is speculative, as information on the timing of feeding in echinoderms is scant.)

- Mortalities of cultured cod in Bertrabui Bay, Co. Galway consisted mainly of larger animals, e.g. finfish (cod). This may be due to the more aggressive feeding behaviour of the larger animals, leading to higher oxygen demands during feeding (Paul Casburn, Taidge Mara Teo, personal communication). Under normal circumstances this would not appear to pose a problem, but under the low oxygen conditions experienced during the bloom, the increased demand resulting from feeding could have resulted in mortalities.

- Aquaculture animals may have been compromised as a consequence of their culture and life stage; for example, anecdotal information suggests that substantial mortalities in oysters were in those that were being 'hardened' i.e. moved in to the intertidal zone to facilitate greater closing of shells and longer shelf life upon transport to market. Mortalities were also recorded in seed animals that were put out in the intertidal zone during 2005. However, it should be noted that no mortalities were noted in seed oysters in the Castlemaine Harbour where high mortalities were recorded in adult oysters.

The second bloom began to dissipate by the start of August. Although the break up of the bloom is usually associated with a change in weather conditions, it is not a definite marker to signify the end of the bloom. As recorded by Evans (1975) gales only served to intensify and extend its distribution in waters of the North coast of Wales. His paper suggests that the possibility that if heavy precipitation (with associated land drainage, low salinities and high nutrients) occurs simultaneously with gales, then the gales can actually aggravate the situation, once the organism has gained a good 'hold' on the water mass. In the case of the 2005 blooms, there were no significant changes in the weather to affect their break ups. Most likely the natural succession to a different phytoplankton community was affected by a change in nutrient and temperature conditions.

Records of long-term data sets indicate the blooms of phytoplankton are becoming more common and persistent. Continuous Plankton Recorder (CPR) data have also shown that many of the variables influencing phytoplankton standing crop are governed in by the prevailing weather. It is believed that phytoplankton changes may well be a consequence of the general deterioration of North Atlantic weather since 1940 (Reid, 1977).

Changes in phytoplankton may be attributed to an amelioration of climate in recent decades. In several areas it has been reported that phytoplankton season length and abundance seem to have increased. In the more than 50 years that the CPR survey has operated in the north east Atlantic and North Sea, large changes have been observed in the abundance and distribution of some plankton taxa (Reid, 1998). The long-term trend in the abundance of the plankton is believed to be responding to hydroclimatic variability.

Conclusions

The marine mortalities observed in coastal fauna, along the west coast in 2005 were undoubtedly associated with the presence of this exceptional bloom of *Karenia mikimotoi*. This conclusion is drawn on the basis that the specific organisms impacted were benthic megafauna and fish species previously reported as sensitive to this species. Furthermore, the patterns observed were consistent with those observed as a consequence of previous *Karenia* sp. blooms.

In addition to the susceptibility of some organisms to mortality a number of mechanisms of mortality have been suggested:

- Evidence of an ichtyotoxic compound associated with *K.mikimotoi* has recently been reported (Satake *et al.,* 2002 & 2005.). Although the mechanism of toxicity of *K.mikimotoi* is poorly understood, the toxins Gymnocin A and Gymnocin B have been suggested as a potential cause of mortality in marine vertebrates and invertebrates.

- Deoxygenation has usually been suggested as effecting mortalities of caged fish both in Ireland (Parker, 1982) and in Norway (Tangen, 1977). Tangen (1977) considered two possible causes of de-oxygenation, either oxygen depletion during hours of darkness due to dinoflagellate respiration or, alternatively, aerobic bacterial breakdown during decomposition of dinoflagellate cells. (However, in previous observations by Pybus (Pybus, 1980) extremely high oxygen concentrations were recorded during a *Karenia* bloom and associated fish mortalities, therefore casting doubt on the role of oxygen depletion as the principal cause of these mortalities. It may, however, contribute as a secondary indirect cause, adding to stresses exerted by other features of the bloom.)

- Diurnal fluctuations in dissolved oxygen concentrations may induce mortalities. Oxygen super-saturation during daylight hours followed by oxygen depletion during the late hours of darkness (oxygen sag) may also result in mortalities. The EPA recorded DO supersaturation of 164% in waters along Mullaghmore Strand, Co. Sligo, on the 20th June 2005. Hundreds of dead heart urchins (*Echinocardium chordatum*) washed up on the strand were observed on the same day. The impact of oxygen fluctuation may be particularly significant if the organisms are compromised either as a consequence of their conditions (high-density culture) or their location (high in the intertidal zone or the stage of culture (newly planted seed oysters or clams). Other possible causes including irritation and clogging of gill membranes have been suggested (Parker, 1982) as inducing mortalities in caged fish.

The events described above have resulted in a substantial elimination of certain benthic communities along the western seaboard. The Marine Institute carried out a major survey of conditions and impacts associated with the event, and are committed to monitoring the recovery of the impacted areas.

Recommendations

In summary, recommendations for minimisation of future impact associated with harmful algal blooms include:

- The present state of technology therefore has not as yet provided a suitable solution to preventing red tides, and the best that can be attempted is to prevent mortalities on a small local scale. In caged fish farms it has been sometimes useful to provide aeration to the cages during periods when the depletion of oxygen is causing stress to the fish. However, this may sometimes exacerbate the situation if there is a deep layer of low oxygen water, which may be brought to the surface by aeration. It is therefore important to examine the oxygen profile of the water column before deciding to aerate fish cages.
- The toxins of *Karenia mikimotoi* in Irish water have not been studied, but it is presumed that the Irish strain may have the toxin profile identified by Satake *et al*. The severe and rapid mortality observed by *Karenia mikimotoi* in several previous events in Ireland suggests that it is not simply due to low oxygen levels. To further understand the reasons for these mortalities it is important to carry out a toxicological and characterisation of the toxins involved.
- Use of technology such as screens and bubble curtains might help protect caged fish from such high densities of algae. This technology should be further investigated in the Irish context.
- Reducing fish farm husbandry activities may limit stress to stock. For instance, reducing stocking densities of caged fish would result in less stress for the animals in low oxygen conditions, as the overall demand for oxygen would be lower. In addition, stressed fish are more susceptible to secondary infections, and this could be minimised.
- Mitigation strategies used in other countries (China, Korea, Japan) to dissipate dense blooms of red tide organisms include the dispersal of large quantities of clay into the sea to flocculate the algae, resulting in it sinking to the seabed (Hepeng, 2004). However, the method is too costly and technically difficult to prevent and terminate massive red tides. This is only suitable to break up blooms in small sheltered embayments, and usually used in these countries to prevent a bloom causing mortalities in caged fish farms. This solution however, may have other more severe and as yet unknown ecological consequences and as such, is not a suitable solution (Archambault *et al*, 2003).
- The public information strategy used appeared to function very effectively. In future the same model of public information provision should be adopted as was used on this occasion. It led to a rapid turnaround of accurate information, which is essential in the management of public interest in such events.

The occurrence of more frequent blooms such as the *Karenia* bloom of 2005 may be part of the effects of climate change on plankton at ocean-wide scales (Reid, 1998). Research is recommended into the area of potential effects of climate change on phytoplankton communities in order to predict, to mitigate, to minimise and potentially to prevent the harmful effects associated with such algal blooms.

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A REVIEW OF SHELLFISH TOXICITY MONITORING IN IRELAND & REVIEW OF MANAGEMENT CELL DECISIONS FOR 2005

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Introduction

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

The Marine Institute as National Reference Laboratory (NRL) for Marine Biotoxins is required as part of their NRL duties under Council Decision 93/383/EEC, *of 14 June 2003 on reference laboratories for the monitoring of marine biotoxins,* to co-ordinate the activities of the National Laboratories in respect of biotoxin analysis under the National Biotoxin Monitoring Programme which includes the organisation of intercomparison exercises and the regular auditing of the National Laboratories, En-Force Laboratories and Charles River Biological Laboratories.

Extended toxicity periods have been seen in 2005, which have resulted in prolonged closures of many sites, where some sites have been closed for several months, which have led to economic losses for producers and processors.

There have been 3 separate toxic events that have led to these closures during 2005; ASP observed in the south west in April/May, DSP observed nationally from end of May to September, and AZP observed nationally from September to December

Amnesic Shellfish Poisoning

During January to August, 375 analyses for ASP were conducted on scallop tissues (*Pecten maximus*) typically, gonad and adductor muscle tissues where the levels observed on adductor muscle tissues (169 analyses) were all below the regulatory limit (highest level 18.7 µg/g in August from Wexford Ground). Twenty of 169 gonad tissues (169 analyses) analysed were observed to be > 20 µg/g, and the highest level observed was 123.8 µg/g in May from Portmagee Channel. Report turnaround time within 3 days of laboratory receipt for ASP analysis of scallops from January to October was 98.4%.

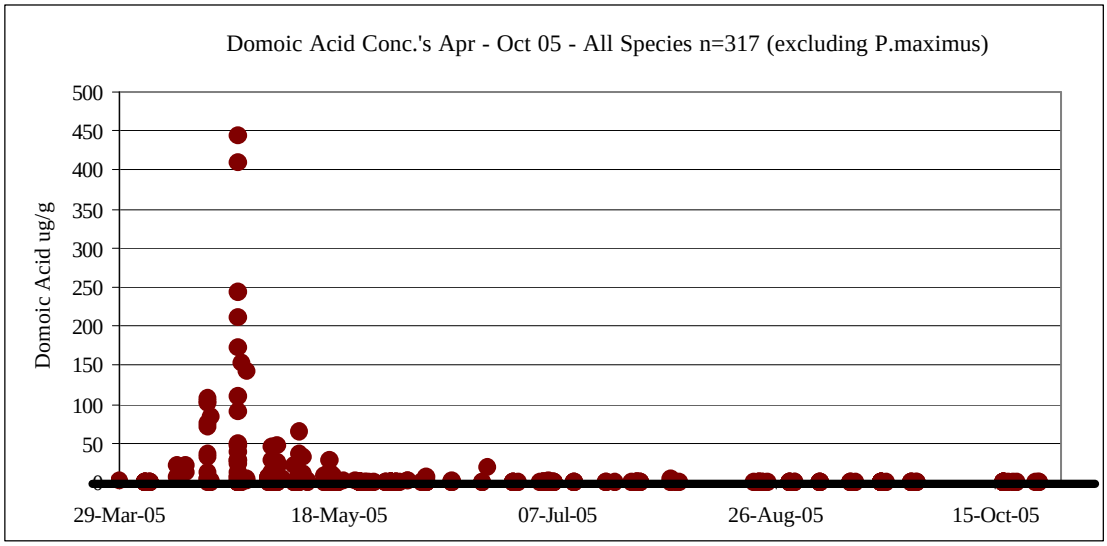


Figure 1. Domoic Acid concentrations in mussel and oyster samples for 2005

Additionally during this time period, 354 samples of *M. edulis, C. gigas, C. edule, E. siliqua & T. philipinarium*, were analysed for the presence of domoic & epi-domoic acid (DA). For the first time in Ireland, a major ASP event was recorded in both samples of *M. edulis* and *C. gigas*, where levels were observed above the regulatory limit (Figure 1). Previously, there had only been one recorded incident of ASP levels slightly above the regulatory in a sample of *M. edulis* in 2002 from Co. Donegal.

Typically levels were observed to be < the Limit of Detection (LOD) until April. During April to mid May, 35 samples were observed to be above the regulatory limit, predominantly *M. edulis* in all Bantry sites and in both *M. edulis* & *C. gigas* samples from Kenmare. Highest level observed was 444.9 µg/g whole flesh. During this toxicity period testing was scaled up to analyse all samples from the south west. Domoic Acid concentrations are illustrated in Figure 2. By the end of May all areas were re-opened and testing was reduced. Only one sample has been observed to be at the regulatory limit since, in June in *M. edulis* from Castletownbere (20 µg/g whole flesh). A level of 4 µg/g whole flesh was observed in samples of *E. siliqua* in early August from Gormanstown.

From August to September, dramatic increases in *Pseudo-nitzschia spp.* had been observed in Bantry and Kenmare, where cell counts were observed to be >1,000,000 cells/litre. Samples of *M. edulis* from these areas were analysed during this period and levels of DA typically observed were <LOD. From mid September to October there was a dramatic decrease in the numbers and distribution of *Pseudo-nitzschia spp.*, where very low levels were observed.

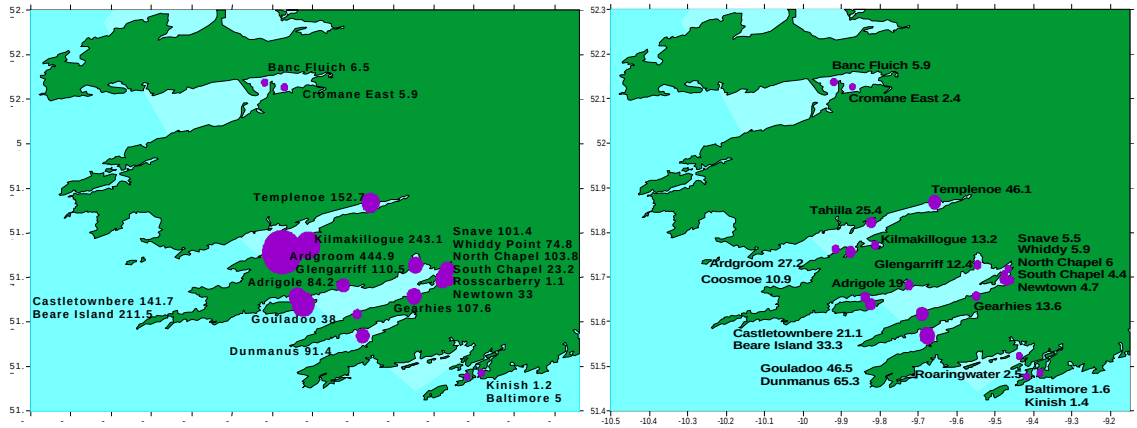


Figure 2. Domoic Acid µg/g TT south west April to May 2005

Diarrhetic Shellfish Poisoning (DSP)

For 2005 (to end of October 2005) 2133 samples (2450 samples projected year end 05 compared to 2196 samples submitted for 2004) were submitted for DSP Bioassay analysis and chemical confirmatory analysis for the presence of Okadaic Acid equivalents (OA, DTX-1, & DTX-2) and Azaspiracids (AZA's 1, 2, 3). Figure 3 illustrates the number of samples submitted from 1988 to 2005. The increase in the number of samples submitted for 2005 was primarily due to the increased toxicity observed in samples nationally, where some sites submitted samples on a bi-weekly basis to facilitate affected areas to re-open in a shorter time frame during the shoulder toxicity period. Secondly, due to the extended toxicity period observed the reduced sample frequency for species during low risk periods which has operated in previous years was delayed until December. Mussel (*Mytilus edulis*) samples were submitted on a fortnightly basis from January to April, and then weekly for the remainder of the year. Razor and clam species (*Ensis siliqua, Ensis ensis, S. solida, T. philippinarium*) were submitted on a monthly basis during January to April and a fortnightly basis during the summer months, reverting back to monthly frequency from October. Oysters (*Crassostrea gigas* and *Ostrea edulis*) were submitted on a monthly basis during January to April and a fortnightly basis during the summer months, reverting back to monthly frequency from December. The report turnaround time for samples submitted for DSP/AZP analysis was 88.1% within 3 days from laboratory receipt.

Overall for 2005, (to end of October 05) the total number of all samples testing positive under DSP Mouse Bioassay was 15% (n = 2133) compared to 3.2% over the same time period for 2004 and 3.6% for 2001. A breakdown of percentage positives by species for *M. edulis* reveals 23.9% of samples tested positive (of 1130 samples) compared to the same period for 2003, 5.8% samples tested positive (n = 1122). No cockle or razor clam samples submitted and analysed were positive for DSP/AZA Toxicity during the same time period. Whilst no samples of oysters tested positive via bioassay, samples of *C. gigas* analysed showed the presence of Azaspiracids above the regulatory limit in samples from Donegal Harbour, Tra Eanach and Killala.

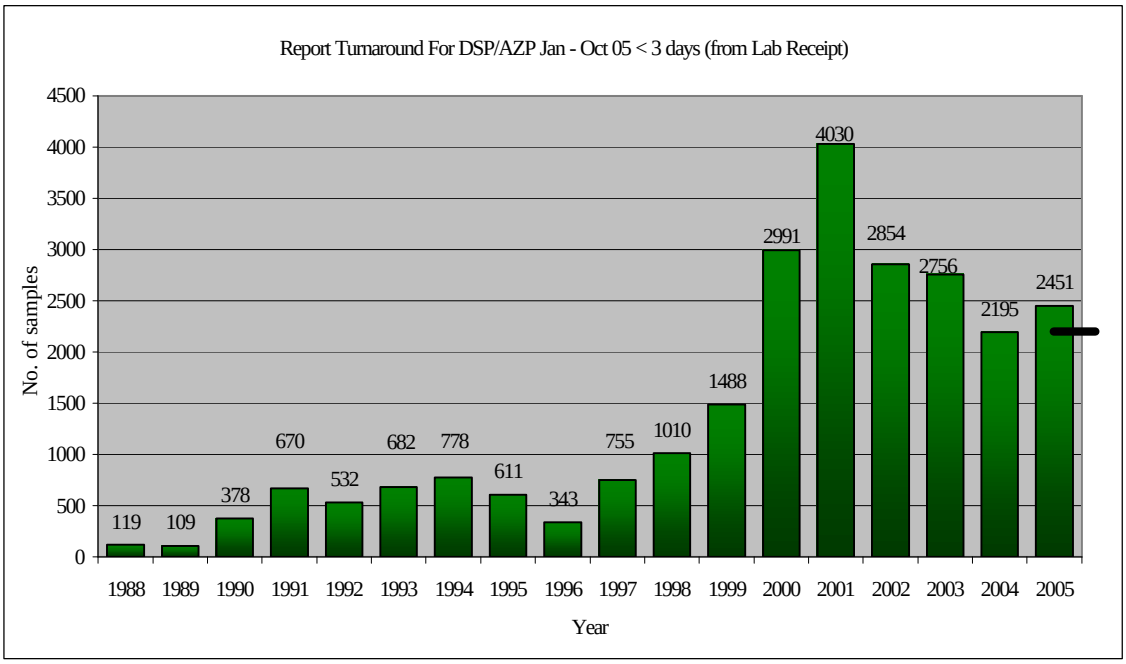


Figure 3. Number of all samples submitted for DSP/AZP analysis for 2005

For the first time, DSP toxicity (total concentration present in the form of OA esters) was detected clams (*S. solida, T. philipinarium*) from Galway & Sligo in July, where the highest concentration observed was 0.27µg/g total tissue (post hydrolysis) where corresponding positive bioassays were observed. Figure 4 illustrates the percentage of mussel samples testing positive/negative via DSP Bioassay from 1998 to 2005. Positive DSP bioassays were first observed in samples from Galway at the end of May. Chemical analysis showed levels of Okadaic Acid equivalents present at vary levels above and below the regulatory limit in June, where positive bioassays were observed in Donegal, Galway and the south west. During July further increases in Okadaic Acid concentration were observed in Donegal, decreases in OA concentration in samples submitted from Galway (except 1 site which had increases in concentration), increases in OA concentration above the regulatory limit in one site in Mayo and on average remaining at the same concentration levels in the majority of sites in the south west (2 sites showed increases) as was observed for the region in June.

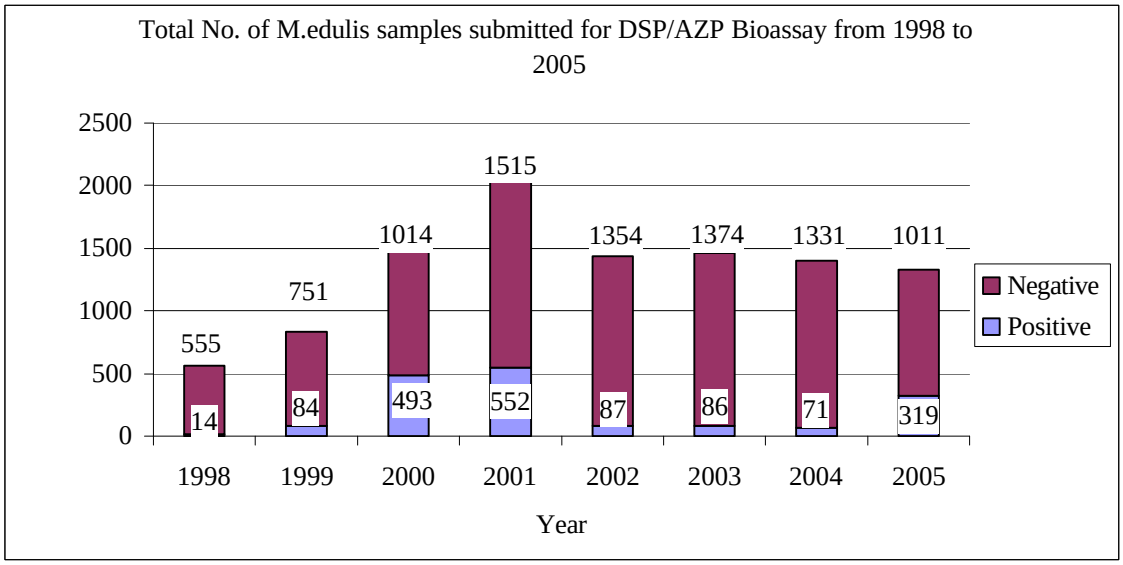
For August, decreases in OA concentration were observed in the west and the north west, however dramatic increases in OA concentration were observed in all sites in the south west, where the highest level observed was 4.0µg/g total tissue. For September, OA levels decreased in the south west, where an increase was observed in the south east, but was below the regulatory limit. OA levels in the west & north west remained on average consistant with the levels observed in August. OA levels continued to further decrease in all areas during October, where the majority of sites in the south west were below the regulatory limit, with further decreases in OA concentrations observed for November. Figures 9.1 to 9.6 illustrate OA concentrations from May to October 2005

The predominant toxin observed in samples from the end of May to the beginning of August was Okadaic Acid, whereas from this time levels of OA decreased, where levels of DTX-2 increased to become the predominant toxin present in samples. The levels of DTX-2 peaked in early August and were observed to decrease throughout

the remainder of August through to early November to below regulatory levels in the majority of samples submitted.

Hydrolysis extractions (as agreed by the Molluscan Shellfish Safety Committee September 2004) to determine the presence of Okadaic Esters (DTX-3) were conducted on samples submitted from Sentinel Sites, where there was a discrepancy between bioassay and chemical results, and as required by the Management Cell. Figure 6 illustrates the detection of Okadaic Acid esters present in samples from the end of May (predominantly Hydrolysed OA) reaching a peak in early August, and decreasing throughout September (predominant toxin observed was hydrolysed DTX-2) to levels below the regulatory limit in the majority of samples in October.

Management Cell Decisions were taken in all cases where a discrepancy was observed between bioassay and chemical results (including the Hydrolysed Okadaic Acid Ester result). Following a recent MSSC meeting (November 2005), hydrolysis extractions will only be conducted on samples at the request of the Management Cell.



Year	1998	1999	2000	2001	2002	2003	2004	2005
%Positive	2.5	11.2	48.6	36.4	6.43	6.26	8.33	23.9
Highest OA µg/g Total Tissue	N/A	N/A	N/A	N/A	3.81	0.57	3.83	4.00
Highest AZA µg/g Total Tissue	N/A	N/A	N/A	N/A	0.23	0.19	0.02	>0.9

Figure 4. Percentage of Mussel Samples Positive for 2005

Figure 5 illustrates the patterns and trends observed in the presence and relationship of Okadaic Acid & DTX-2 in samples analysed.

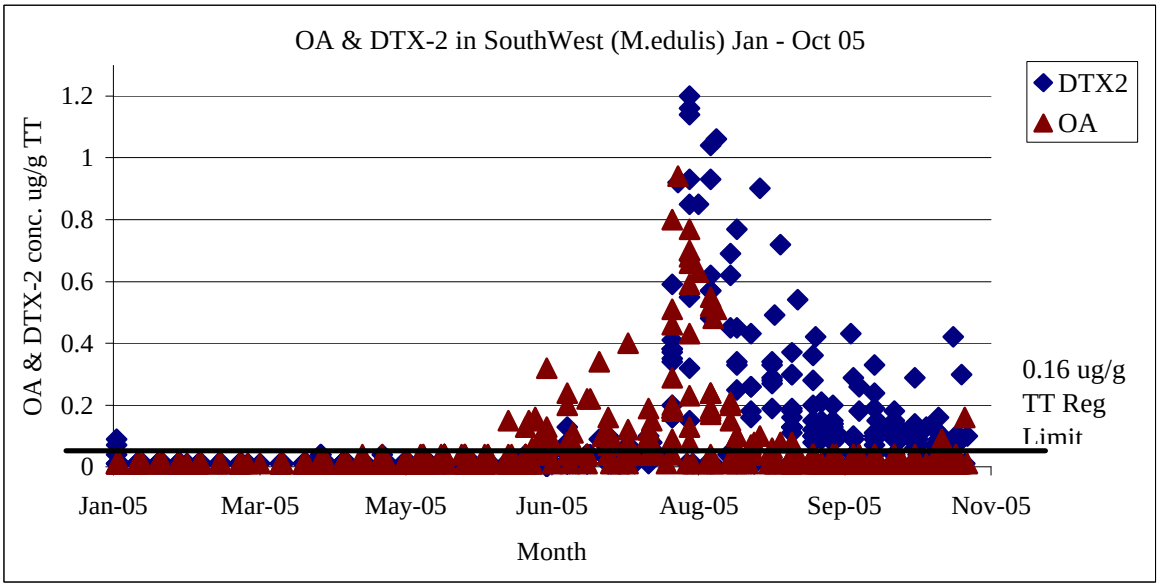


Figure 5. Concentrations of OA & DTX-2 in samples submitted

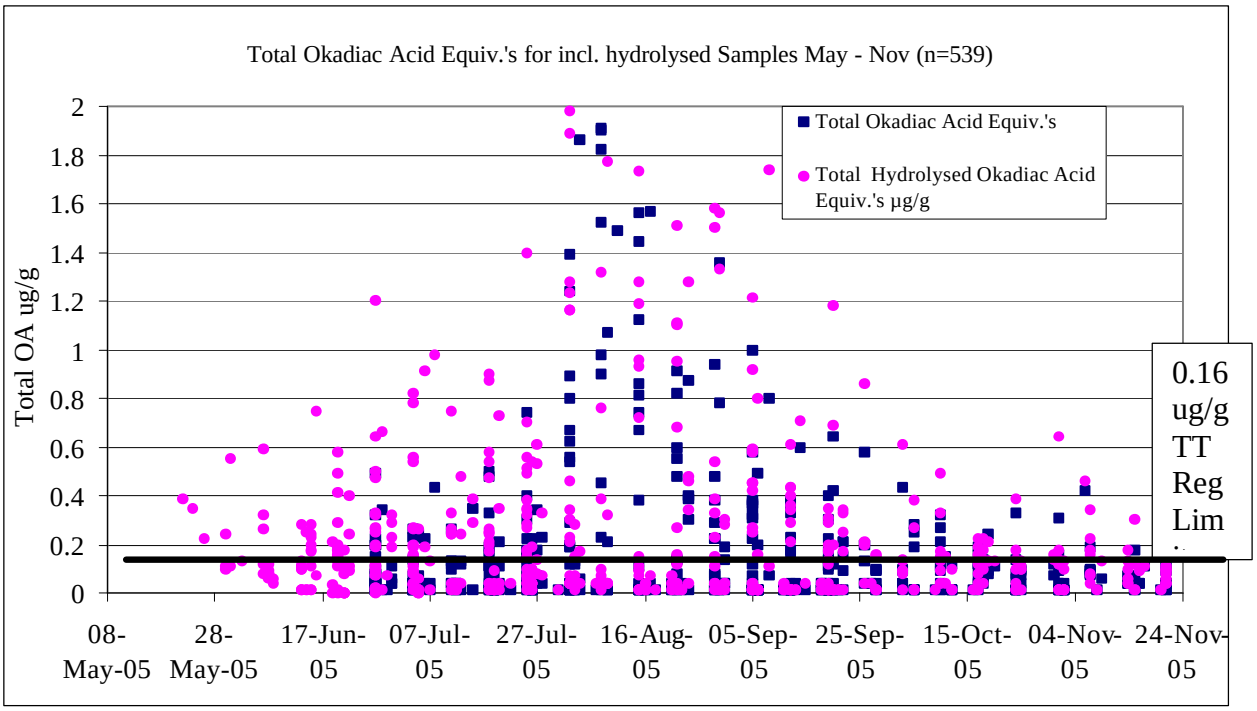


Figure 6. Concentrations of unhydrolysed OA & Hydrolysed OA in samples analysed for 2005

Azaspiracid Shellfish Poisoning Summary (AZP)

Figure 7 illustrates the trends observed in concentrations observed in samples for OA, DTX-2 & AZA's 1, 2, & 3 for 2005. Figures 10.1 – 10.6 illustrate the distribution and concentrations of Azaspiracids in samples observed from June to October 2005.Typically the levels observed from Jan – June were < LOD, all except one result in late June for Bruckless where 0.08 µg/g were observed.

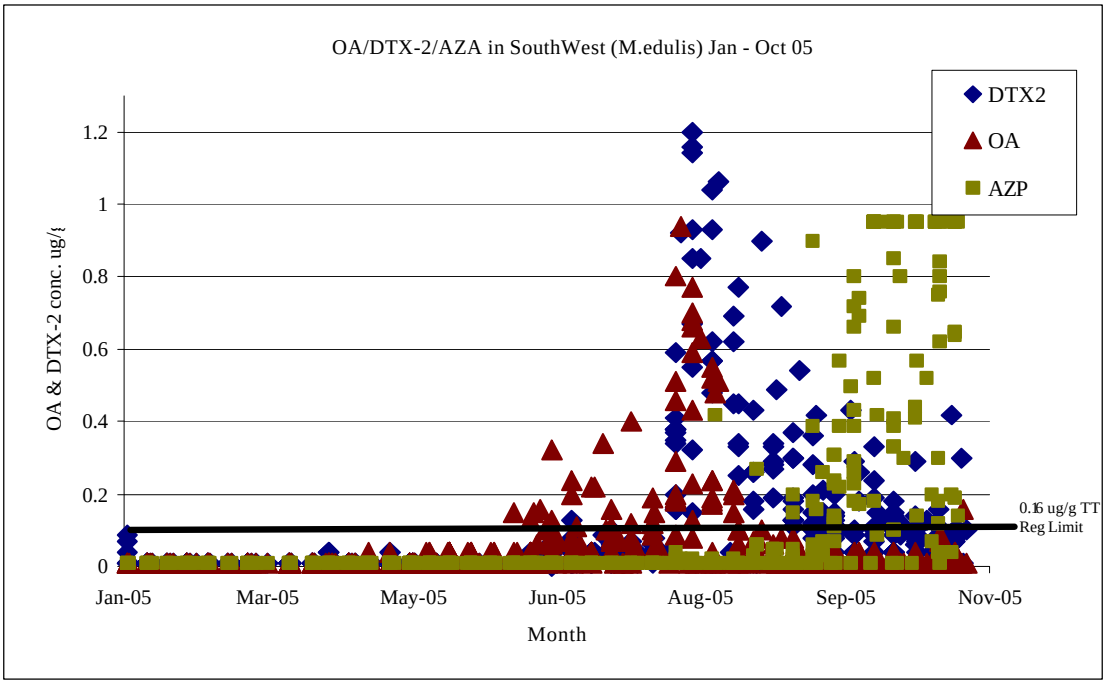


Figure 7. Concentrations of OA, DTX-2 & AZA's 1, 2 & 3 in samples submitted

During July AZA's were observed to be present in a number of areas in the west and north west, in particular levels above the regulatory limit were observed in McSwynes, Mountcharles and Tra Eanach. During August, AZA's were observed to increase and spread to a number of localities, mainly in low conc.'s in mussels in the West and NorthWest, levels above regulatory limit observed in Bruckless, Mountcharles, & Killary. AZA levels above the regulatory limit were also observed in *C. gigas* samples from Killala, and just below the regulatory limit in oysters from Gweebarra & Drumcliffe. AZA's were also observed to be present mainly in low conccentrations in the south west (levels above the regulatory limit observed in Castletownbere).

In September, levels of AZA's increased in a number of sites in the south west above the regulatory limit (sites in Bantry, Kenmare & Castlemaine). Levels of AZA's increased during September in the north west in a number of sites, but were observed to decrease in the west. Further sharp increases in AZA levels were observed in the south west (in many sites, levels were > Upper Limit of Quantification (ULQ =0.9 µg/g)) in October, whereas in the west and north west no further increases were observed in AZA concentrations and remained at approximately the same levels as those observed for August. From July to December, AZA levels have remained consistently high in McSwynes Bay, where levels in a number of sites from the south west were observed to decrease to levels below the regulatory limit in December, illustrated in figures 8.1 and 8.2.

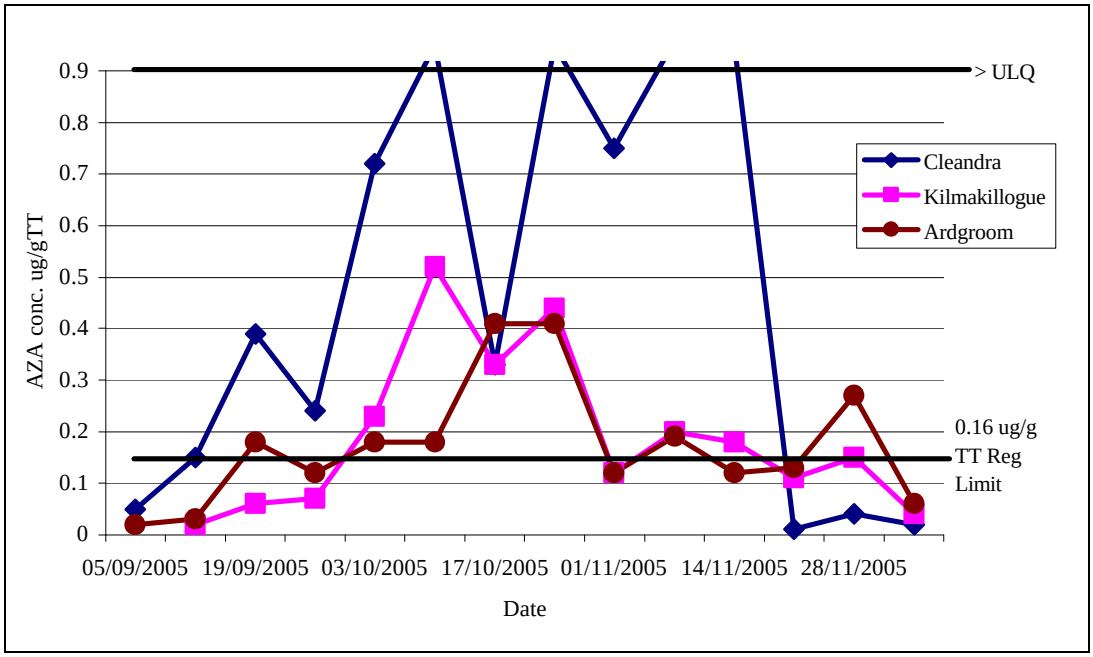


Figure 8.1. AZA concentrations Kenmare September to December 2005

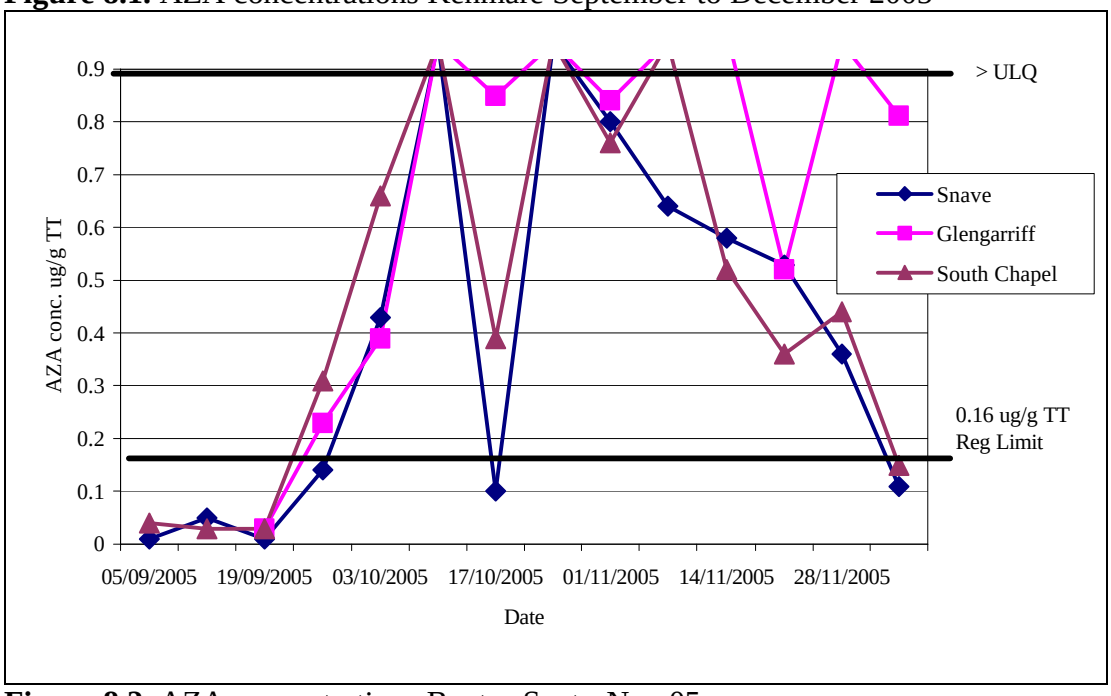


Figure 8.2. AZA concentrations Bantry Sept - Nov 05

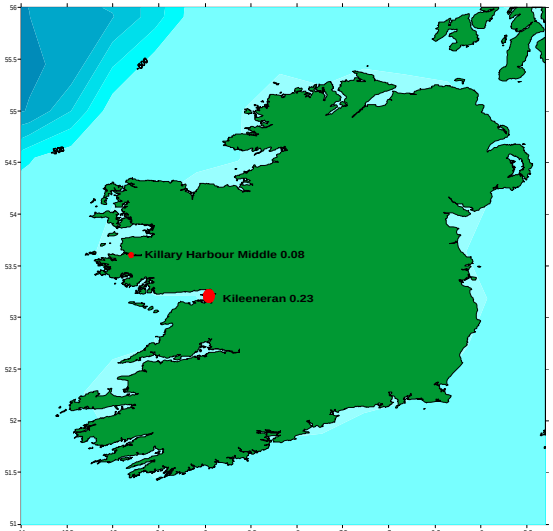


Figure 9.1 OA levels MAY

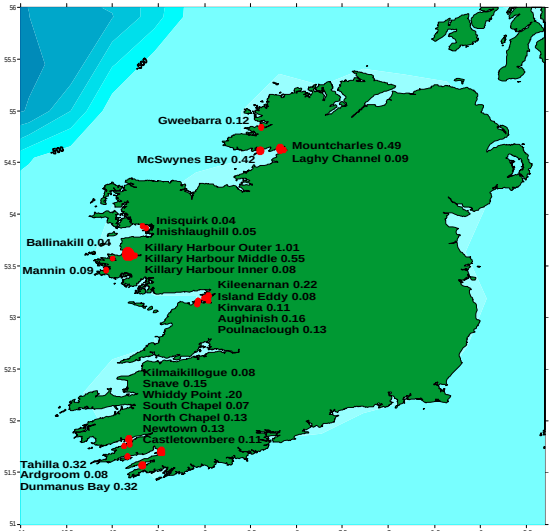


Figure 9.2 OA levels JUNE

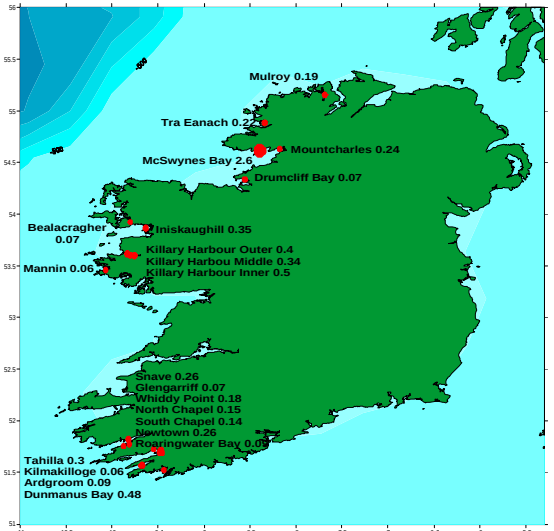


Figure 9.3 OA levels JULY

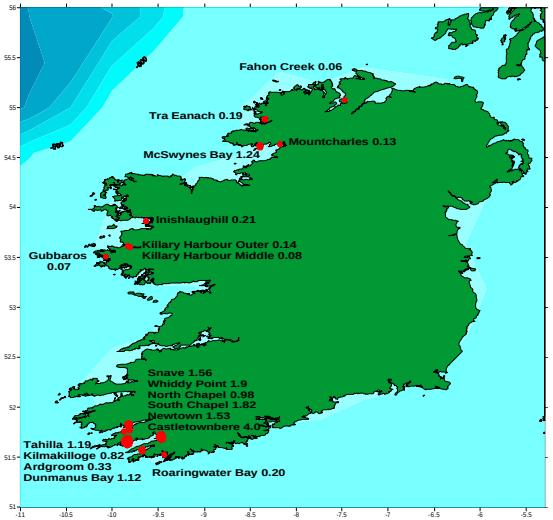


Figure 9.4 ***OA levels*** *AUGUST*

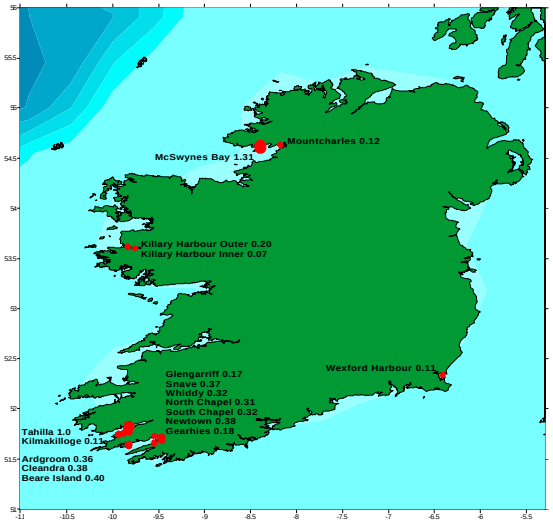


Figure 9.5 ***OA levels*** *SEPTEMBER*

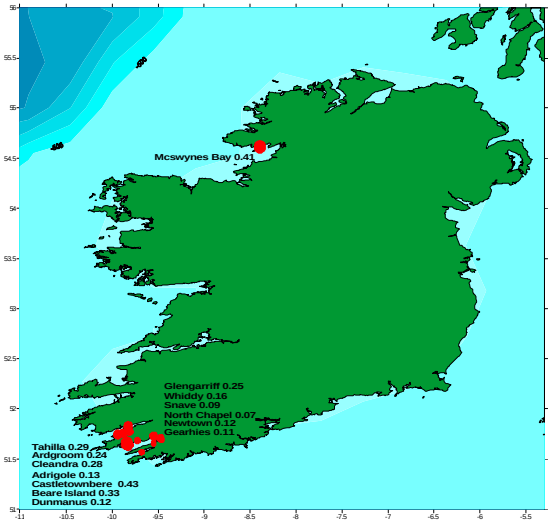


Figure 9.6 ***OA levels*** *OCTOBER*

Figures. 9.1 – 9.6 • Unhydrolysed Total Okadaic Acid / DTX-1 / DTX-2 in µg/g TT May – October 2005

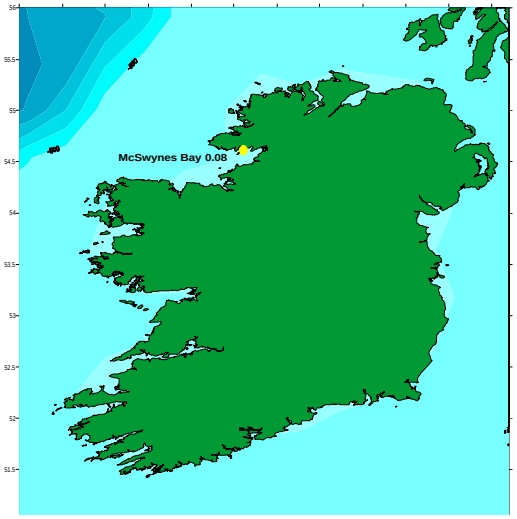


Figure 10.1 AZA levels JUNE

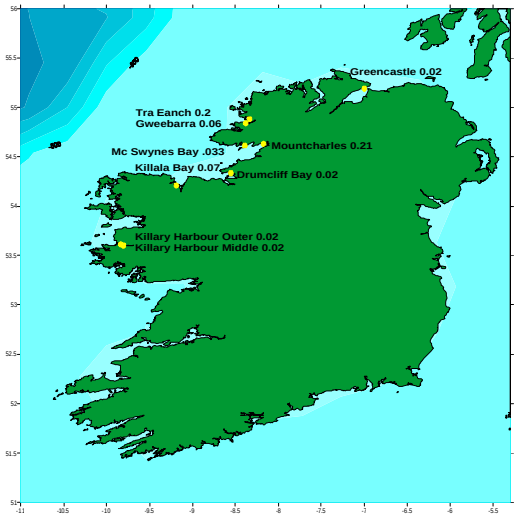


Figure 10.2 AZA levels JULY

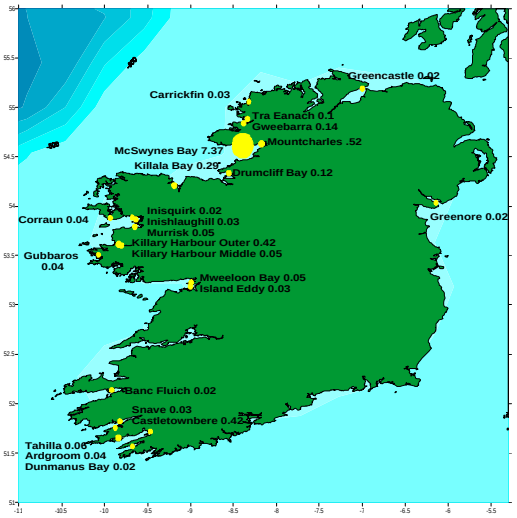


Figure 10.3 AZA levels AUGUST

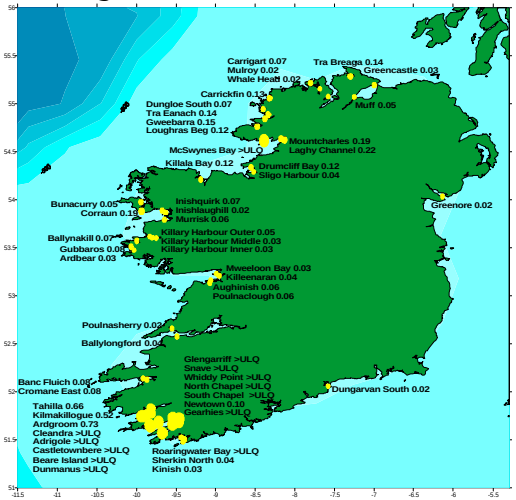


Figure 10.4 AZA levels SEPTEMBER

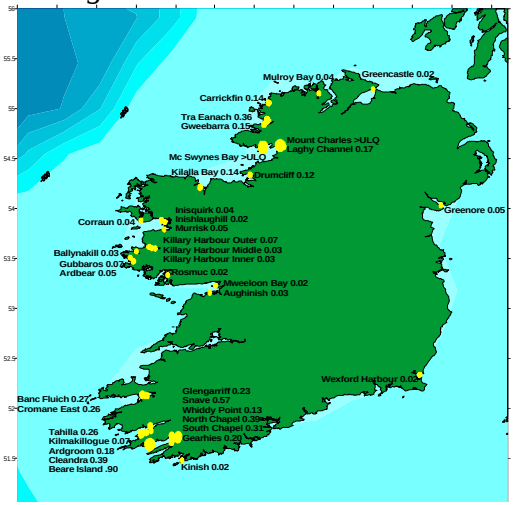
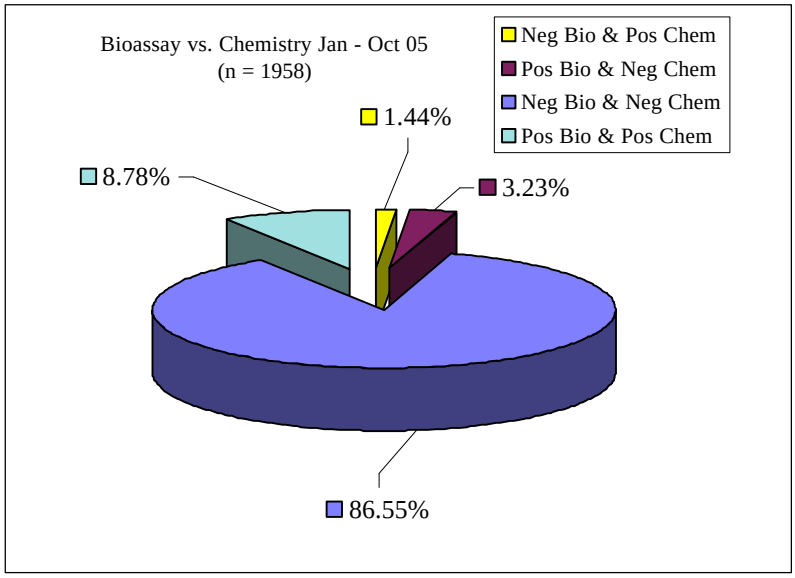


Figure 10.5 AZA levels OCTOBER

Figures. 10.1. to 10.6. AZA Results June – October 2005 • Total Azaspiracids AZA's 1, 2 & 3 in µg/g TT

Comparison: Bioassay & Chemical Analysis

The bioassay results were compared to the chemical results for OA equivalents (including Post Hydrolysis) & AZP (n= 1958 samples Jan – End of Oct 05), and are illustrated in figure 11. Overall, a 95.2% comparison was obtained between the two methods, the slightly lower value obtained for 2005 is primarily due to the increase in the no. of samples with toxicity present at the regulatory limit, where over the last 4 years on approximately 9000 samples, a 97.6% correlation was obtained between the two methods.



Year	Positive MBA & Positive Chemistry (%)	Negative MBA and Negative Chemistry (%)	Total Correlation (%)
2002	2.5	96.3	98.8
2003	1.3	96.2	97.5
2004	3.3	95.9	99.2
2005 (Oct 05)	8.8	86.2	95.0

Figure 11. Comparison of Bioassay vs. Chemical Analysis (n=1958)

Paralytic Shellfish Poisoning (PSP)

During January to October, 227 samples were submitted for PSP analysis. All samples were negative via Jellet PSP Rapid Test Kit, apart from two *M.edulis* samples observed in early to mid June from Cork Harbour. These positive samples were re-analysed via AOAC PSP bioassay, where the maximum level observed was **66.12** µg STXeq100g^{-1}. Since the introduction of the Jellett PSP Rapid Test last year, the number of bioassays performed has been reduced by 98%.

Levels of *Alexandrium sp.* were generally observed in low levels all around the Irish Coast throughout May to October (when compared to 2004), with the highest levels observed in North Channel, Kinsale, Oysterhaven, Loughras Beg & Greenore. The levels and distribution of *Alexandrium sp.* were observed to further decrease in September, and were observed to be mainly confined to the south and south west at low levels in October. Samples of shellfish from these areas (except those from North Channel in June) tested negative for the presence of PSP toxins.

Quality System

The full suite of Biotoxin & Phytoplankton Test Methods conducted within MI laboratories, are now all accredited by the Irish National Accreditation Board to ISO 17025;

Okadaic acid, Dinophysistoxins (DTX-1 & DTX-2) by LC-MS in Dublin.
Domoic acid by HPLC via DAD Galway
DSP Mouse bioassay in Galway
PSP by AOAC Mouse Bioassay in Galway
PSP by Jellett Biotek Rapid Test in Galway
Phytoplankton analysis in Galway & Bantry
Azaspiracids (AZA's 1, 2 & 3) via LC-MS in Dublin

Review of Management Cell Decisions for 2005

The Management Cell consists of a group of representatives from DCMNR, FSAI (Chair), ISA & MI and is called when the following situation occurs:

- Borderline or out of character biotoxin results, where results maybe inconsistent with local/national trends i.e. a single, unexpected negative/positive result occurs.
- Discrepancy between Bioassay & Chemistry Results
- Prolonged borderline toxicity. Borderline biotoxin results need consideration.
- Sampling continuity has been interrupted.
- LCMS breakdown.

In order to proactively manage a risk situation, the Management Cell considers the following factors when assigning a status to an area;

- Species
- Bioassay Results (no. dead, time of death)
- Chemical Results (OA, DTX-2, AZA's, Okadaic Acid Esters)
- Time of the year
- Results of adjacent areas
- Phytoplankton Results (numbers of associated toxic species present)
- Previous history of results from the area in question
- Any other associated data

Where the following options are available;

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

For 2005 (from January to early December), a total of 89 Management Cell Decisions were taken into account. This is an increase in the number of decisions than observed in previous years, primarily due to the increase in the toxicity periods and type of events observed in 2005. The following (Table 1) shows a breakdown on Management Cells taken in 2005.

Table 1. Breakdown of Management Cell decisions

Original Decision	**MC Decision**	**Frequency**
Open	Closed	3
Open	Closed Pending	4
Closed	Open	14
Closed	Closed Pending	5
Closed Pending	Open	15
Closed Pending	Closed	15
No Change in status		31
Precautionary advice		0
Issuing status advice prior to chem		1
Change in sampling frequency		1
Total Management cells		**89**

2005 PROGRESS REPORT ON ASTOX – RESEARCH ON AZASPIRACID STANDARDS AND TOXICOLOGY

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Introduction

The ASTOX project is a 3-year NDP-funded project that began in 2003 and is due to finish in mid 2006. Irish partners are MI and University College Dublin, outside partners (not Irish funded) are official control laboratories for marine biotoxins and university collaborators in Japan as well as the Center for Coastal Environmental Health & Biomolecular Research (NOAA/NOS/NCCOS),US. Additional collaborations were also sought during the project and the outcome of those is also described in this manuscript.

The structure of this outline follows the project modules, which were designed to fulfil the major objectives:

- Obtain pure standards of AZAs + DTX-2 for instrument calibration and method validation
- Obtain pure standards of AZAs for toxicological studies confirming and extending previous work
- Conducting studies into the functional toxicity of azaspiracids at the cellular level
- Conducting studies into the effects of azaspiracids on gene expression of cellular models

The first 2 objectives are supported by WP1 (retrieval of contaminated shellfish) to obtain material for purification and preparation of reference materials. The objectives 3 and 4 will eventually be used in a WP6 on the establishment of safe levels of azaspiracids in shellfish for human consumption.

WP 1: Retrieval of contaminated shellfish materials

Several bulk shellfish tissue materials were obtained during the course of the ASTOX project. These materials served both for the isolation and purification of the toxins from shellfish and for the preparation of shellfish tissue reference materials that can be used in method development and validation.

Shellfish initially harvested in 2001 have been obtained, stored and used by the project for a number of studies until 2005. Also, three additional bulk lots of shellfish have been made available to this project, originating from 1999 and 2000. The year 2005 has been particularly toxic and 3 further bulk materials have been obtained, processed and are available now to the project.

WP 2: Preparation of stable and homogenous reference materials for AZA- and DTX- contaminated shellfish

Previous reports in this forum have primarily focussed on the study of parameters affecting homogeneity of shellfish tissue reference materials. Here, we report on studies of the factors influencing stability of shellfish tissue reference materials and how these can be optimised. Apart from slow oxygenation of the analyte in the matrix over a long time, the degradation of shellfish tissues can be accelerated by microbiological processes, in particular at temperatures above 0 °C. We have studied two separate processes that potentially are capable of preventing this microbial degradation, through suppression of microbial activity:

a) Heat treatments
b) Gamma-irradiation

While heat treatments had been previously studied at conditions below 100 °C (Hess *et al*., 2005), where no degradations were observed, the total removal of microbial activity typically requires autoclaving of tissues at circa 120 C. Therefore, we tested this condition for shellfish tissues contaminated with AZAs (Figure 1). This harsher treatment at 120 °C leads to destruction of more than 50 % of the total AZA-content in the shellfish tissue. Therefore, heat treatment was not considered a practicable approach for the stabilisation of shellfish tissue reference materials for AZAs.

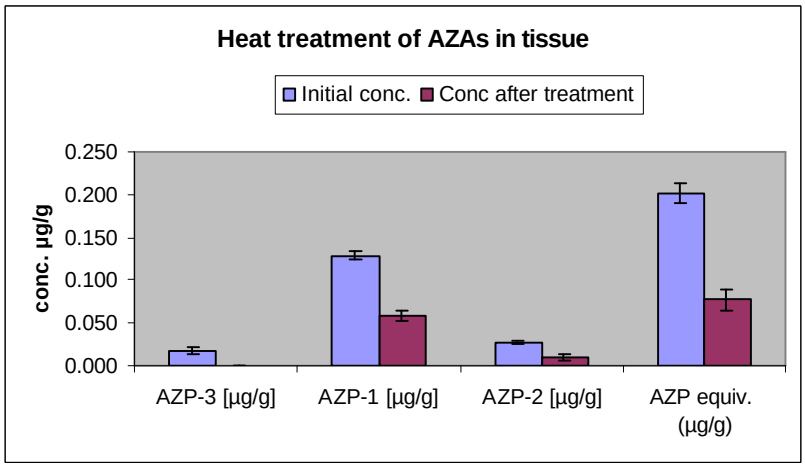

Figure 1. Effect of heat treatment (120 °C) of tissue on AZA-concentration

Subsequently, we tested gamma-irradiation as a technique and several doses from 5 to 20 kGy did not show any significant effect on the concentration of AZAs in shellfish tissues (Figure 2). Also, gamma-irradiation has a less detrimental effect on the other, major constituents of the matrix and therefore, we recommend to further investigate

the use of irradiation as a stabilisation technique of shellfish tissue materials as reference materials for AZAs.

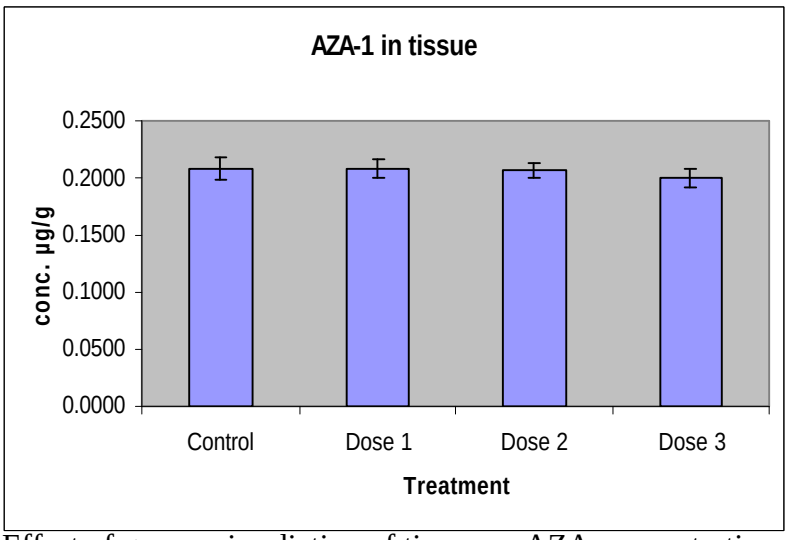

Figure 2. Effect of gamma-irradiation of tissue on AZA-concentration

WP 3: Isolation of Azaspiracids and Dinophysistoxin-2

In the first part of the study we implemented the procedure for isolation of AZAs as developed initially. Subsequently, we clarified and optimised this procedure to obtain the best possible procedure adapted to our laboratory environment. Over the past year, isolation of approximately 1 mg of AZA-1 was achieved. A spectrum acquired using nuclear magnetic resonance was used to determine the purity (Figure 3), and purity was confirmed to be in excess of 97 %. This purity is sufficient to use the compound either as calibrant or in toxicology studies. Current studies make use of the higher contaminated shellfish obtained in 2005 and are investigating the efficacy of the procedure by comparison with a procedure developed at the National Research Council, Canada.

Also, we have isolated in this project approximately 1 mg of DTX-2, which was characterised for its purity by NMR and found to be of sufficient purity for toxicology studies.

Both AZAs and DTX-2 have been made available to the European Community and National Reference Laboratories and to other research projects funded by the EU (see BIOTOX).

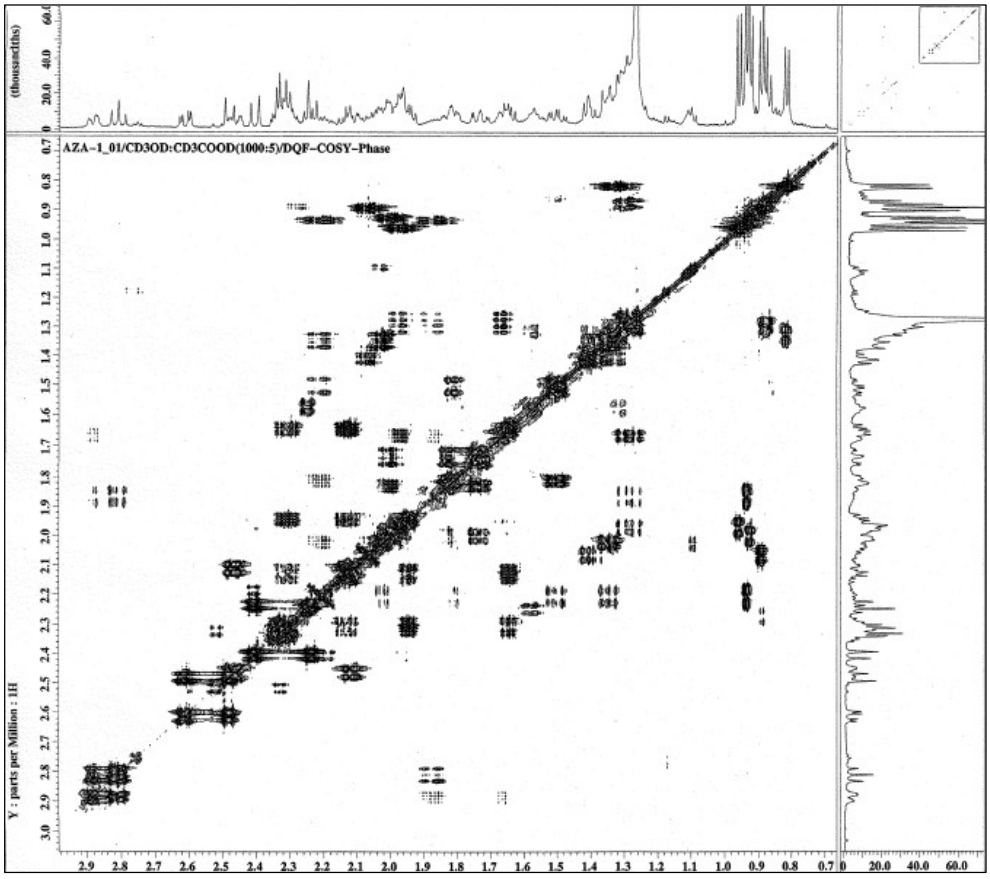

Figure 3. Purity of AZA-1 isolated at MI as characterised by NMR spectrum (Yasumoto and Naoki, 2005).

WP 4: Establishment of a functional in vitro assay for AZA-1

The aims of this study were to examine the effect that AZA-1 had on gastrointestinal cells and to determine potential mechanisms of action with a view to establishing an *in vitro* functional assay for AZA-1 detection. The human colon cell line - Caco-2 cells - were selected for these studies due to their ability to form tight junctions and allow for measurements of transepithelial electrical resistance (TEER). Transepithelial electrical resistance was measured using an electrical resistance measurement device across a monolayer of Caco-2 cells adhered to a semi-permeable membrane. When confluent monolayers of Caco-2 cells were exposed to increasing concentrations of AZA-1 there was a dose- and time-dependent decrease in TEER. The robustness of our model was tested using AZA-1 of lower purity. No alteration in sensitivity was observed compared to AZA-1 of higher purity.

In order to address the potential problems of matrix interference in our model a range of experiments examining the effect of uncooked mussel extract (UME) on Caco-2 cells was carried out. No effect on TEER was observed with a 10% concentration of UME or lower. The effect of UME in combination with a range of toxins was also examined. Uncooked mussel extract did not appear to have any significant effect on the toxicity of AZA-1, OA or PTX-2. The effect of UME on DTX-2 needs to be clarified. The data to date suggests that UME will not interfere with the sensitivity of our model.

Additional marine biotoxins were examined in our model; okadaic acid (OA), DTX-2 and PTX-2. OA (100 nM) and DTX-2 (100 nM) both reduced TEER significantly at 24 h, with OA (500 nM) reducing TEER to basal levels. DTX-2 (500 nM) did not reduce TER to basal levels until 48 h, this was significantly less than the equivalent concentration of OA ($p = 0.05$). PTX-2 (100 nM) reduced TEER to basal levels at 24 h, while a significant decrease was observed as early as 4 h.

WP 5: Analysis of AZA-1 induced gene alterations by genechip microarray

Gene microarray experiments using the Affymetrix human genome array U133A 2.0 have been carried out in Dublin and bioinformatic analysis of gene expression alteration by AZA-1 is under way (Figure 4). This data is enabling us to examine changes in gene expression after exposure to AZA-1. A concentration of 10 nM AZA-1 was selected with 24 and 48 h time points. Our current analysis has identified 132 genes significantly upregulated and 15 downregulated at 24 h and 209 genes upregulated and 18 downregulated at 48 h. Gene microarray experiments with the Jurkat - lymphocyte T cell line have also been carried out. We are exchanging data in an attempt to identify common mechanisms of action of AZA-1 in our different models.

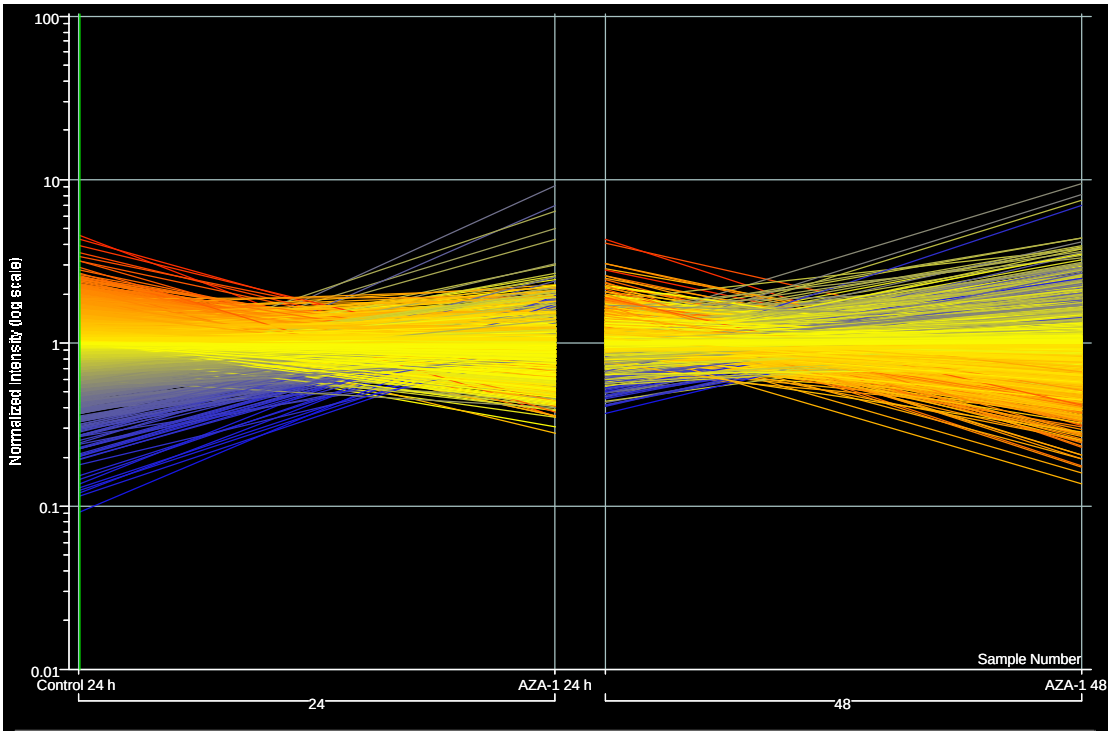

Figure 4. Representative display of gene changes using Affymetrix gene microarrays at 24 and 48 hours after exposure to 10 nM AZA-1.

Bioinformatic analysis has highlighted many biological pathways from our gene microarray data. Some of those identified are reported to be involved in response to stress, growth, differentiation, metabolism and structural integrity. Further analysis of these genes in combination with existing literature is necessary to attribute specific mechanisms to AZA-1.

By highlighting the modes of action of AZA-1 these studies will aid in the replacement of the *in vivo* mouse bioassay with the development of alternative *in vitro* test systems.

WP 6: Establishment of a NOAEL

This work-package is the last in the ASTOX project and is to be completed at UCD. Results of this work-package will be reported in the final report.

Additional collaborations

An additional collaboration was possible during 2005 with a group in Norway. The toxicity of DTX-2 had previously been questioned, since mouse bioassays in Ireland and Norway were negative even though doses of DTX-2 were comparable to the lethal dose of okadaic acid. The toxicity of DTX-2 was investigated using intraperitoneal injection into mice and a relative toxicity factor of circa 0.6 compared to OA was established.

Conclusions

A number of bulk samples of toxic shellfish have been gathered, processed and stored by the ASTOX project. These raw materials have been used successfully in the isolation of both AZA-1 and DTX-2, and have allowed us to study the parameters affecting the preparation of stable shellfish tissue reference materials. The isolated toxins and homogenous and stable reference materials are essential requisites in the development and validation of non-animal test methods for lipophilic shellfish toxins, and these materials will be used in further projects. The isolated DTX-2 was used in the study of its intraperitoneal toxicity in mice and a relative toxicity factor of DTX-2 versus OA was established (0.6). I*n-vitro* toxicology studies using transepithelial electrical resistance suggest that a functional assay may be developed for azaspiracids using this technique. Such a functional assay may be helpful in replacing the current mouse bioassay, either directly, or by cross-validation of other more rapid and cost-effective techniques. Bioinformatic analysis of changes in gene expression induced by AZA-1 using micro-array gene-chips have aided in determining possible mechanisms of action. In the future, this may allow an evaluation of some of the earlier indications of the carcinogenicity of azaspiracids. Work package 6 will focus on establishing a safe level of azaspiracids in shellfish destined for human consumption.

PRESENTATION OF THE BIOTOX PROJECT ON THE DEVELOPMENT OF COST-EFFECTIVE TOOLS FOR RISK MANAGEMENT AND TRACEABILITY SYSTEMS FOR MARINE BIOTOXINS

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Rationale for the BIOTOX project

Certain phytoplanctonic species are capable of producing biologically active compounds known as phycotoxins or marine biotoxins. The filter-feeding bivalves that prey on these toxigenic species become unsafe for human consumption and they can be responsible for various syndromes: diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), azaspiracids poisoning (AZP), paralytic shellfish poisoning (PSP), and neurological shellfish poisoning (NSP). In order to protect human consumers against the presence of marine biotoxins in shellfish, regulatory limits have been determined at the EU level; these are quantitative thresholds above which the shellfish are considered as unsafe and so far, 13 lipophilic marine biotoxins have been included in the EU regulation (Anonymous, 2002). A mouse bioassay (MBA) is considered as the reference method for the detection of these marine biotoxins (Anonymous, 2002 and Anonymous, 1991) and insures a good level of protection as this assay determines the overall toxicity of the tested samples. However, it raises other issues such as the controversial use of animals; the MBA is not very sensitive nor specific, not designed for multi-toxin detection and is not validated. Therefore DG SANCO identified the need for alternative and validated methods to replace the MBA. One of the aims of the EU-funded BIOTOX project is to address this issue by developing an LC-MS based reference method that will be used for the cross-validation of cost-effective tools such as biochemical (*e.g.* enzyme-linked immunoassays for YTX) and functional assays (*e.g.* Trans-epithelial electrical resistance assay for OA and AZAs).

The process of method development requires the use of toxin standards and test materials to (1) optimise the characteristics and (2) assess the performance of the developed method. However, these materials are not always commercially available and this is an important limitation factor for method development. To address this issue, a feasibility study on the production of certified reference materials (CRM) has been included in the BIOTOX project.

Being able to detect the presence of marine biotoxins in the contaminated shellfish is obviously a major aspect but, the ambition of the BIOTOX project goes even further as the project also focuses on “preventative” and “remediation” measures. Thus the development of early warning systems would enable the detection of toxic algal blooms and toxins in the seawater prior to their accumulation in shellfish. Once the shellfish is contaminated it is essential for the shellfish industry to be able to implement the appropriate measures to depurate the shellfish and this requires the acquisition of knowledge on the contamination and depuration rates of the shellfish. Although MI has not been contracted to work on depuration as part of BIOTOX, it is natural that the MI offers its scientific and analytical support to the partners dealing

with this topic and particularly to Oyster Creek Limited (Maree, Co. Galway), as the Irish shellfish industry would benefit from the progress on depuration.

Overview of the BIOTOX project

The BIOTOX project runs from January 2005 to December 2007 and involves 12 partners from 6 countries: Ireland, Norway, France, Belgium, Italy, and the Netherlands.

The project is divided into 10 different workpackages (WPs) presented in the Figure 1. The MI is involved in the 4 WPs highlighted in yellow:

- Development of an LC-MS based reference method (WP2)
- Method validation (WP5)
- Feasibility study on the production of CRMs (WP6)
- Early warning systems (WP7)

Three other Irish partners are involved in the project:

- The University College of Dublin (WP4 on the development of functional assays)
- The Food Safety Authority of Ireland (WP8 on the risk management and HACCP)
- Oyster Creek Ltd. (WP9 on the depuration).

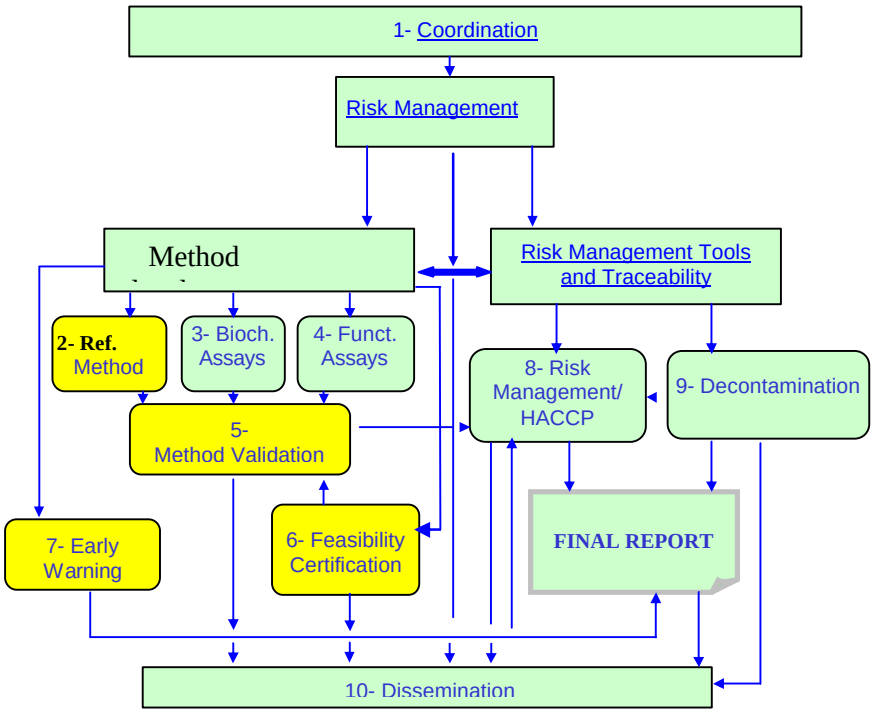

Figure 1: Presentation of the workpackages of the BIOTOX project

Analysis of the lipophilic biotoxins by LC-MS

Development of a LC-MS reference method

Each partner of WP2 (the Netherlands Institute for Fisheries Research [RIVO], Institute for Food Safety [RIKILT], the Norwegian School of Veterinary Science [NSVS]) started developing a liquid chromatography – mass spectrometry (LC-MS) method for specific toxins as shown in Table 1. Subsequently, the optimum LC conditions were chosen from the range of conditions assessed by the WP2 partners and the separation of the different toxins achieved in these conditions is shown in Figure 2.

In order to be recognised as a reference method, the LC-MS method must meet different criteria and be validated according to international standards such as those of the Association of Official Analytical Chemists (AOAC). With that prospect, the performance of the method will be assessed as part of the validation process that will start in the second quarter of 2006.

Table 1: Distribution of the different toxin groups between the WP2 partners.

Toxin group	Partner in charge
OA + DTX	RIVO [1]
AZA + DTX esters	MI [2]
PTX	RIKILT [3]
YTX	NSVS [4]

Analysis of the lipophilic biotoxins by UPLC-MS

Waters Micromass developed a new liquid chromatography system called ultra performance liquid chromatography (UPLC). The use of UPLC over the standard high performance LC enables:

- A shorter time of analysis due to higher flow rates, which means a better sample turnaround
- An increased sensitivity, as the smaller particles of the UPLC columns (1.7µm instead of 5-3µm) give sharper chromatographic peaks with a higher signal to noise ratio
- A better resolution of the chromatographic peaks due to the smaller particles size.

Because of the potential of UPLC-MS for the analysis of the lipophilic marine biotoxins, a collaboration was initiated with Waters Micromass (Manchester) to develop a method on this system. The initial results obtained are promising, as they show that it is possible to decrease the analysis time by a factor 3 with an acceptable resolution. These conditions have yet to be improved to make full use of the potential of UPLC-MS.

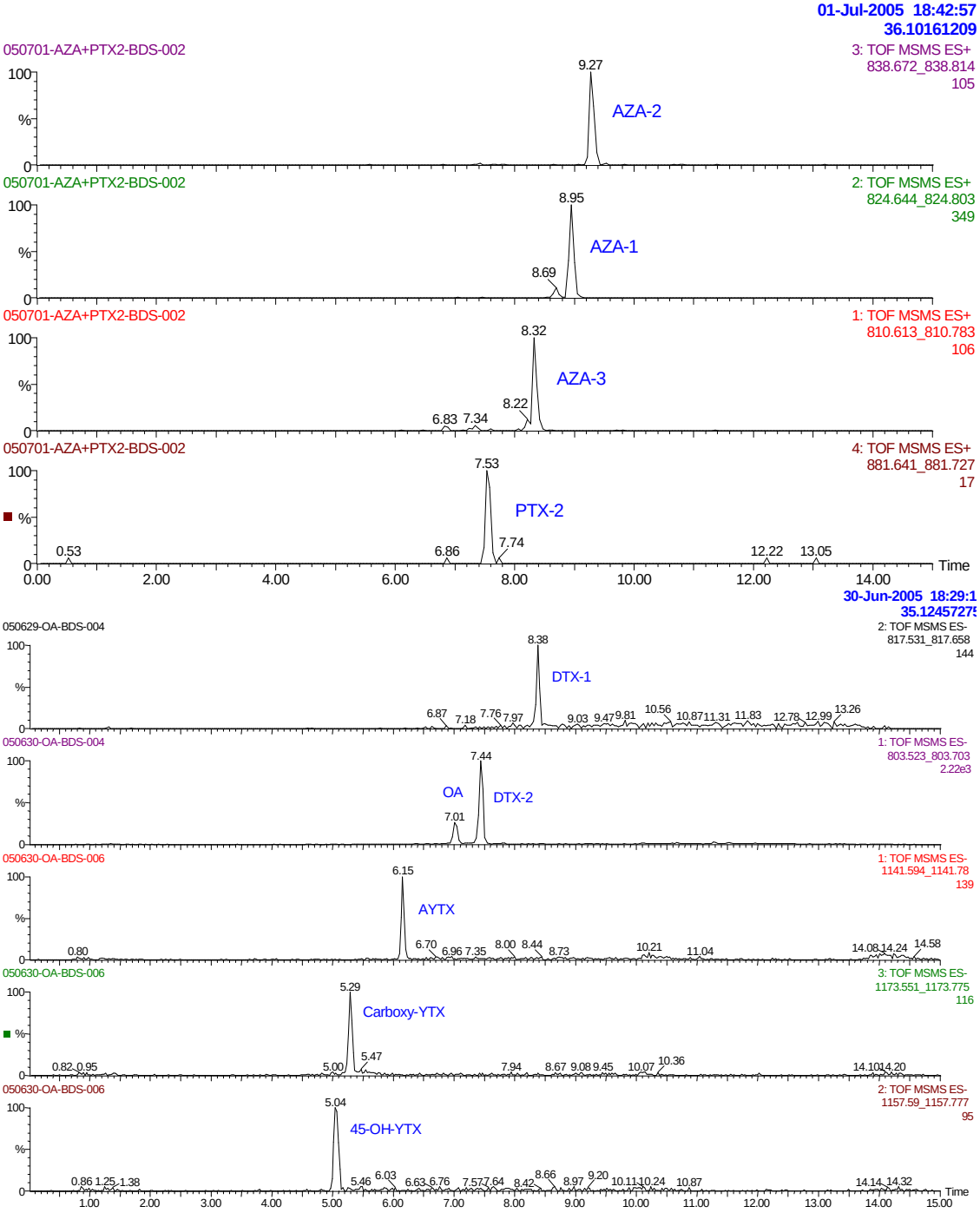

Figure 2. Separation of the lipophilic toxins on the BDS Hypersil C8 column (50×2mm, 3µm) in gradient conditions, at 0.2mL/min.

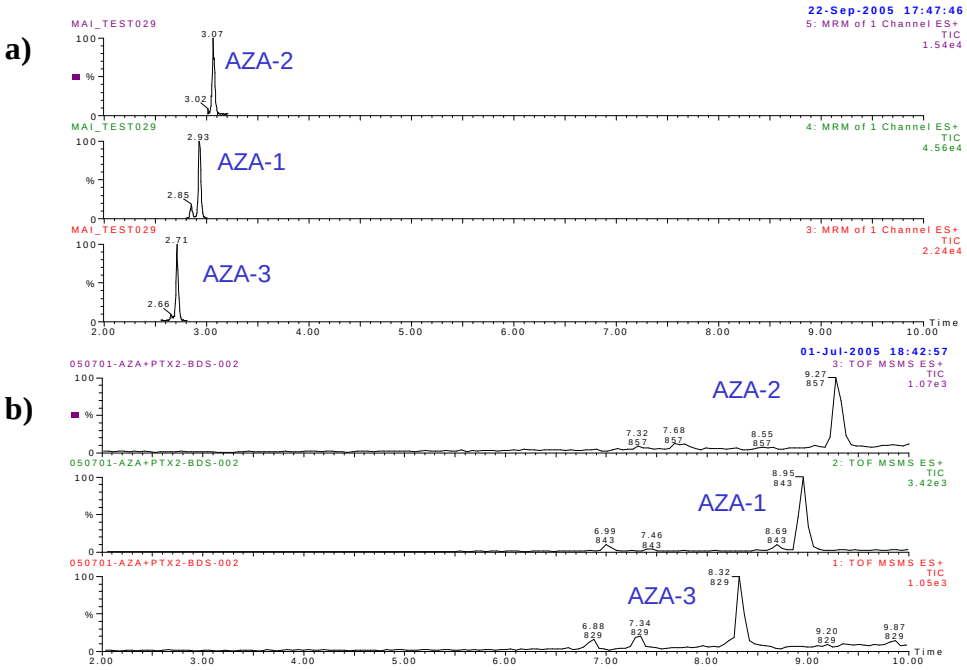

Figure 3: Separation of the AZAs using (a) the HPLC-Qtof Ultima (column BDS Hypersil C8, 50×2mm, 3µm; gradient of mobile phases; 0.2mL/min) and (b) the UPLC-Quattro Ultima (column Acquity C8, 50×2mm, 1.7µm; gradient of mobile phases; 0.4mL/min).

Early warning

The objective of the early warning is to forecast the accumulation of marine biotoxins in the shellfish. One way of meeting this objective is to detect the presence of the marine biotoxins in the seawater using a technique developed by Lincoln MacKenzie and colleagues (Mackenzie *et al.*, 2004), called solid phase adsorption toxin tracking (SPATT). This technique relies on the affinity of the lipophilic biotoxins for the hydrophobic resin that is used in the SPATT bags also called “Lincoln’s tea bags”.

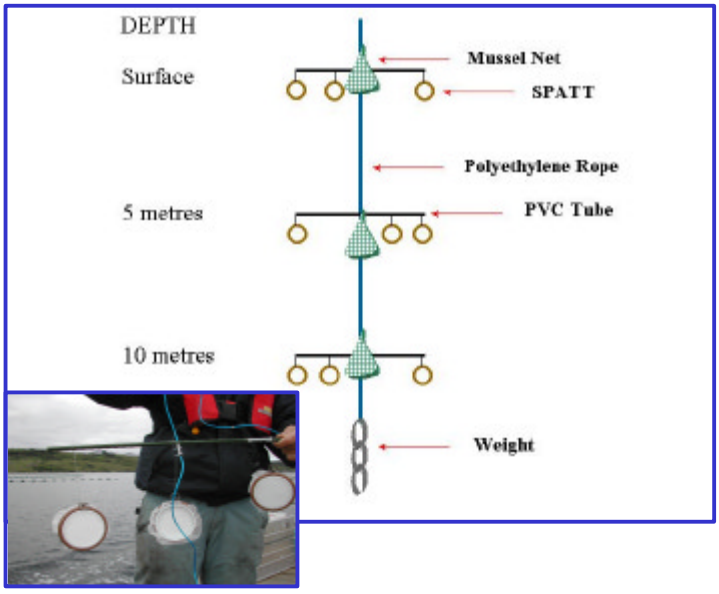

Figure 4. Sampling system used for the SPATT bags and the relocated mussels

Over the summer 2005, a field trial was carried out in 5 sites located on the west coast of Ireland (1 site in Bruckless and in Bantry and 3 in Killary). SPATT bags and relocated mussels were placed weekly at 3 different depths (surface, 5m and 10m) using the sampling system presented in Figure 4. Over the summer, 90 mussel samples and 420 SPATT bags were collected.

The LC-MS determination of the toxin content of indigenous mussels analysed as part of the routine monitoring and relocated mussels placed weekly on the same site (Killary outer), showed that within a week the relocated mussels can accumulate detectable levels of AZAs and OAs (Figure 5).

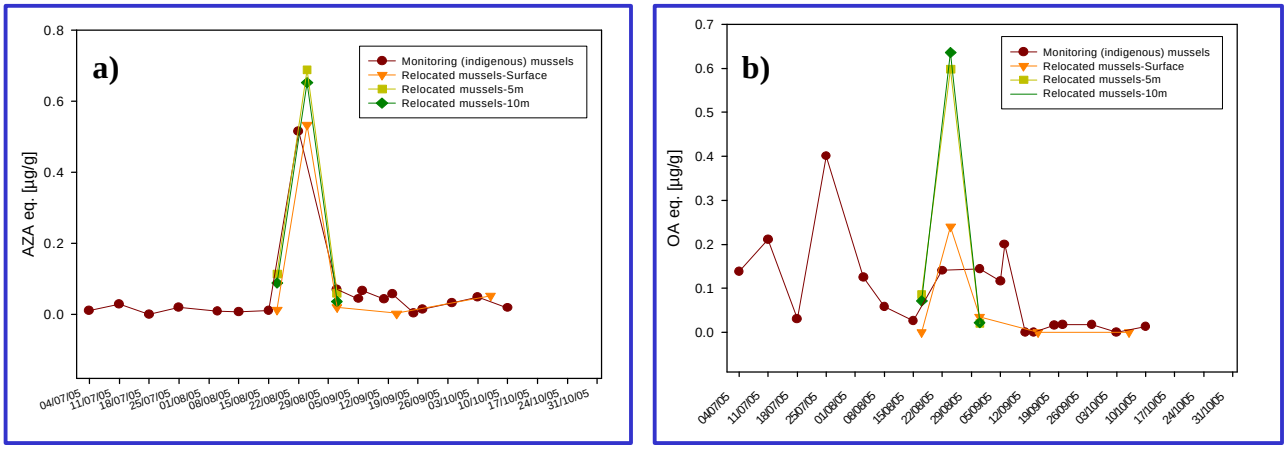

Figure 5: AZA (a) and OA (b) content of relocated and indigenous mussels from Killary outer.

The analysis of the first SPATT bags confirmed their ability to trap the marine biotoxins, as detectable levels of AZAs, pectenotoxins 2, OA and dinophysistoxin 2 were found in one of the sampling sites (Figure 6).

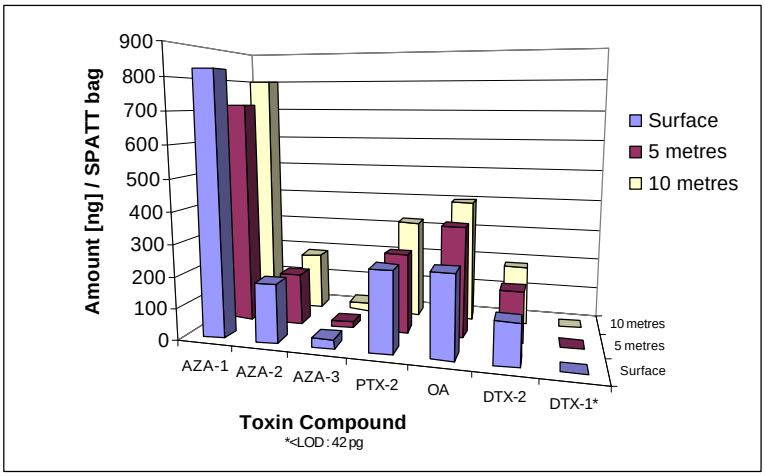

Figure 6 Toxin content of SPATT bags placed in Bruckless, determined by LC-MS.

Other examples of alternatives methods not included in the BIOTOX project

The DSP Jellett kit as an alternative method for the detection of the DSP toxins

The Rapid Jellett Testing Company developed immunochromatographic tests, based on the principle of the home pregnancy test. These semi-quantitave tests are commercially available for the detection of ASP and PSP toxins. The PSP Jellett kit is used for screening purposes as part of the routine monitoring carried out by the Biotoxin team of the MI.

The Rapid Jellett Testing is developing a DSP kit and the Biotoxin Chemistry team volunteered to assess the performance of the existing version of the kit. The tests performed showed that the strength of the DSP kit, similarly to the ASP and PSP kits, lies in its simplicity, its rapidity, and the fact that is does not require the use of extensive laboratory equipment; these characteristics make it a potentially interesting tool for the shellfish industry. However, the interpretation of the results in the "borderline" area (around 100µg/kg) is delicate with the tested version of the kit (Figure 7). This is an important drawback, since the regulatory limit for the OA and DTXs is at 160µg/kg.

A new version of the kit is already available, with an improved contrast of the coloured bands that should help the interpretation of the results in the "sensitive" borderline area. However, this new version has not been tested yet.

Negative "Borderline" (100µg/kg) Positive

Figure 7. Examples of negative, borderline and positive results obtained with the DSP Jellett kit still under development.

Alternative methods for the detection of the PSP toxins

A MBA is used as the reference method for the detection of PSP toxins in shellfish. This bioassay enables the determination of the overall toxicity of the tested samples but it is not possible to determine the toxin profile. This can only be achieved using complementary analytical methods such as LC with fluorescence detection (LC-FLD) or LC-MS.

Because the PSP toxins are not naturally fluorescent they must undergo a derivatisation process that will make them fluorescent and therefore detectable. This derivatisation process can be done pre (Lawrence and Niedzwiadek, 2001) or post-column (Oshima, 1995) depending on the method used. The Lawrence method (Lawrence and Niedzwiadek, 2001) using a pre-column derivatisation has gone through a validation process meeting the requirements of AOAC and has now been accepted as the AOAC official method 2005.06 (Anonymous, 2005).

Because there is now an internationally validated method for the detection of PSP toxins, DG SANCO wants to get away from the MBA and asked the Spanish Community Reference Laboratory to organise an intercomparison exercise for PSP toxins by LC-FLD in order to evaluate the method among the European Laboratories. The Marine Institute as being the Irish NRL participates in this intercomparison exercise.

LC-MS methods for PSP toxins have been developed as they offer an extra confirmatory power compared to LC-FLD methods, (Lagos *et al.*, 1999) (Pleasance *et al*., 1992) but they have a poor ionisation due to the aqueous mobile phase and they are subject to ion suppression because of the presence of salts to improve the separation of the toxins. In 2005 Dell'Aversano *et al.* (2005) reported the

development of a hydrophilic interaction LC-MS method that is more sensitive than the previous LC-MS methods, and collaboration has been initiated with Waters to work on improving this method.

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BIOTOX – Workpackage 8
David Lyons, Food Safety Authority of Ireland.

The purpose of the BIOTOX project is the development, validation, and standardisation of reference methods (LC-MS) and cost-effective assays for the identification and quantification of lipophilic marine biotoxins, the developments of early warning systems and the improvement of decontamination techniques and the implementation of traceability systems.

Among the potential deliverables from the project are:

- Assays for all lipophilic toxins included in European Directives
- An early warning system based on gene expression and/or passive sampling
- Recommendations on the certification of reference materials
- Improved decontamination procedures
- A report on the harmonisation of monitoring, control and handling contaminated shellfish
- A workshop
- Training
- Newsletters, website and scientific publications

Workpackage 8 of the project focuses on describing the potential for the integration of these new methodologies into the risk management regimes of member states and into the Hazard Analysis and Critical Control Point plans of individual producers and processors.

The workpackage will also aim to review and describe potential improvements and possibilities for the harmonisation of risk management practices across the EU and consider how the methodologies being developed as part of other workpackages might enhance the recall and traceability systems of stakeholders in the seafood supply chain.

Among the deliverables anticipated from this workpackage are a “harmonisation report,” recommended HACCP Model Plans as well as a work shop for risk managers.

ALEXANDRIUM IN CORK HARBOUR: MORPHOGENETICS, PSP TOXIN COMPOSITION AND SPECIES DISCRIMINATION BY WHOLE-CELL FISH.

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Martin Ryan Institute, National University of Ireland, Galway

Introduction

Dinoflagellates are single celled microalgae belonging to the class Dinophyceae and constitute a significant component of phytoplankton assemblages of coastal areas. The dinoflagellate genus *Alexandrium* is composed of about 28 morphologically similar species whose accurate identification relies on the microscopical examination of fine details of the theca, the external cell structure that covers the cell body (Balech, 1995). *Alexandrium* blooms have caused concerns in coastal areas around the world as some species produce potent neurotoxins that accumulate in filter-feeding organisms and can trigger serious neurological disorders in mammals that consume them. This syndrome, known as Paralytic Shellfish Poisoning (PSP), has mainly been attributed to *Alexandrium minutum* and varieties of the *A. tamarense* 'species complex' (Cembella, 1998). The compounds involved are saxitoxin derivatives that can cause muscular paralysis, neurological symptoms and in extreme cases death in humans (Kao, 1993). In Ireland, the only location where PSP events have been regularly recorded is a retentive inlet located on the south coast (Boelens *et al*., 1999).

Investigations into the ecology, phylogeny and evolutionary patterns of *Alexandrium* have attempted to elucidate questions related to its current and apparently expanding biogeography (Vila *et al*., 2001, Hansen *et al*., 2003 and John *et al*., 2003). These rely however on accurate identification of the species which is unreliable using ordinary light microscopy. Whole-cell Fluorescent *In Situ* Hybridization (FISH) is one of various molecular biology techniques which have been adapted to the study of harmful algal blooms (HABs) and *Alexandrium* in particular, which can alleviate this problem (Miller and Scholin, 1998 and Hosoi-Tanabe and Sako, 2004). The method relies on the use of epi-fluorescence microscopy to examine species-specific oligonucleotide probes bound to their rDNA targets in the ribosomes present in the cells. This study is an update of what is currently known of *Alexandrium* spp. in Cork Harbour and describes the successful adaptation of a FISH assay for studying the *Alexandrium* population dynamics in the area.

Methodology

Mono-specific cultures of *Alexandrium* spp. were derived from the isolation and germination of resting cysts taken from the surface of sediment samples in Cork Harbour. Cultures were maintained in 100 ml conical flasks in f/2 medium minus silicates (Guillard, 1975) at 15°C, with a 14:10 hour (light:dark) cycle and a photon flux density of 150 µmol m^{-2} s^{-1}. Species identification was performed using epi-fluorescence microscopy with Calcofluor White stain as described in Fritz and Triemer (1985). The phylogeny of *Alexandrium* spp. was assessed after sequencing of the D1-D2 domain of the LSU rDNA. Sequences were then compared with those of other *Alexandrium* spp. obtained from GenBank, and processed with the software PAUP version 4.0b10 (Swofford, 2003) to determine the phylogenetic relationships. The determination of the PSP toxin composition of cultures was carried out using the HPLC-FD method described by Franco and Fernandez-Vila (1993). Sequence alignments performed with Genedoc were used to design *A. tamarense* and *A. minutum* species-specific oligonucleotide probes which were labelled with the fluorophores FITC and CY.3. The procedure used for whole-cell FISH was derived from that described in Miller and Scholin (1998).

Results and discussion

Morphology

Alexandrium tamarense and *A. minutum* were both present in samples taken from the North Channel area of Cork Harbour. Their morphology largely conformed to Balech's (1995) emended descriptions regarding the shape of the thecal plates of the vegetative cells, the key plates for accurate species identification being the posterior sulcal plate, the first apical plate and the 6'' apical plate (Figure 1).

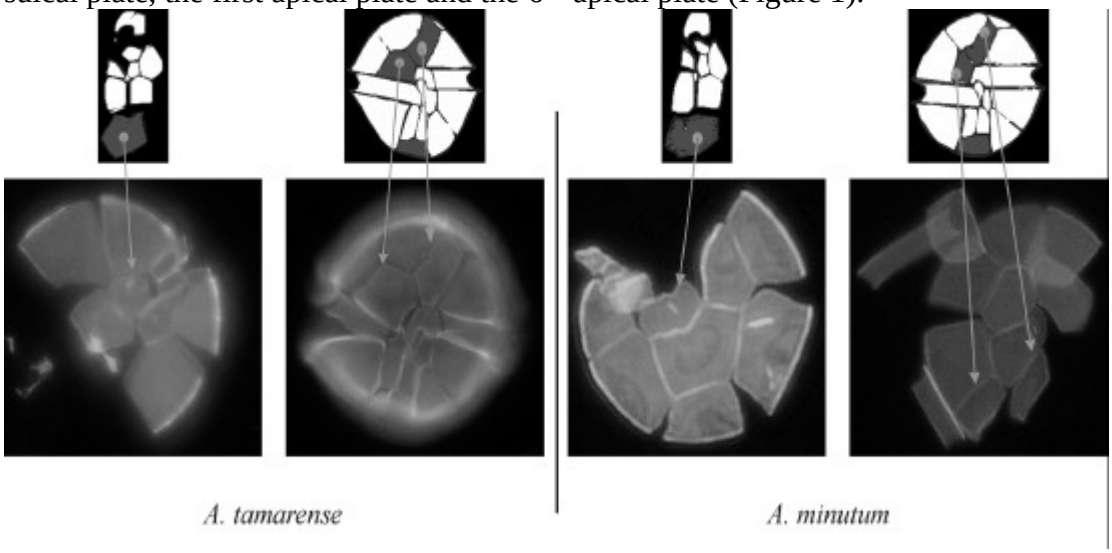
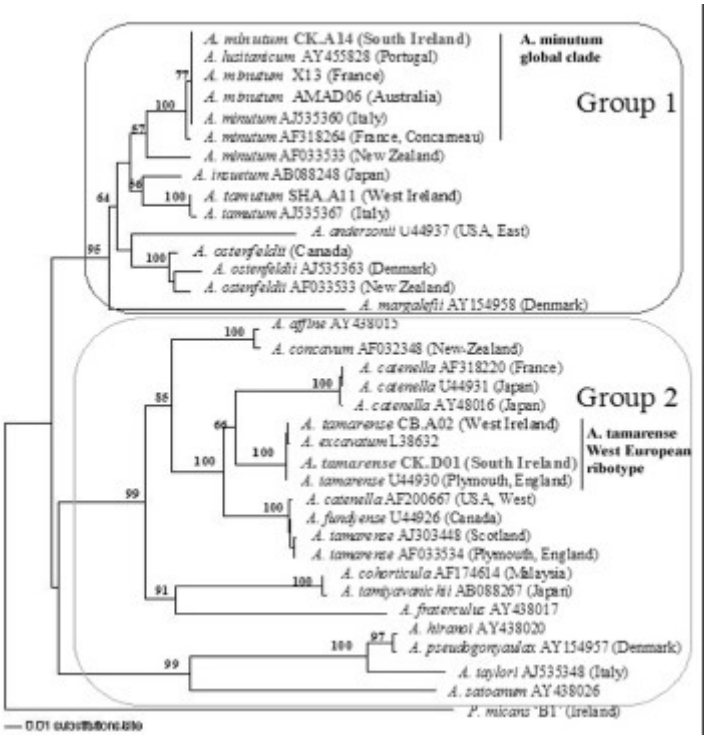


Figure 1. Fluorescence microscopy of *Alexandrium* spp. vegetative cells derived from Cork Harbour samples and whose theca have been stained with Calcofluor White. Key morphological thecal plates identifying *A. tamarense* and *A. minutum* are indicated with arrows.



Phylogeny

The phylogenetic analysis grouped *Alexandrium* species into two major groups or clades (Figure 2), a result similar to previous studies (Scholin *et al.*, 1995, Hansen *et al.*, 2003). *Alexandrium* strains from Cork were genetically similar to other strains commonly found in western Europe. *A. minutum* clustered into group 1 in an assemblage supporting *A. minutum* strains from all over the world. *A. tamarense* grouped with species in group 2 with other strains from the non-toxic western European rybotype (John *et al.*, 2003).

Figure 2. Phylogeny of *Alexandrium* (D1-D2 domain of LSU rDNA). The tree was constructed using the neighbour-joining method from a matrix of Logdet distances. Numbers on the branches are bootstrap values derived from 1000 replicates.

Toxin composition

Several *A. minutum* cultures isolated from the south coast of Ireland were analysed for the presence of PSP toxins. The chromatograms confirmed the presence of gonyautoxins 2 and 3 (Figure 3). As expected, (because clustering into the non-toxic western European ribotype) all *A. tamarense* strains analysed for PSP toxins proved negative. Culture experiments carried out with *A. minutum* while varying the initial nitrate and phosphate concentrations in the culture medium showed a great enhancement of the PSP toxin production in phosphate limiting conditions (Data not shown). The strain toxicity varied from 1.1 to 12.5 pg STX eq.cell^{-1}, a toxicity similar to that obtained from naturally occurring toxic populations from New Zealand (Chang *et al*., 1997).

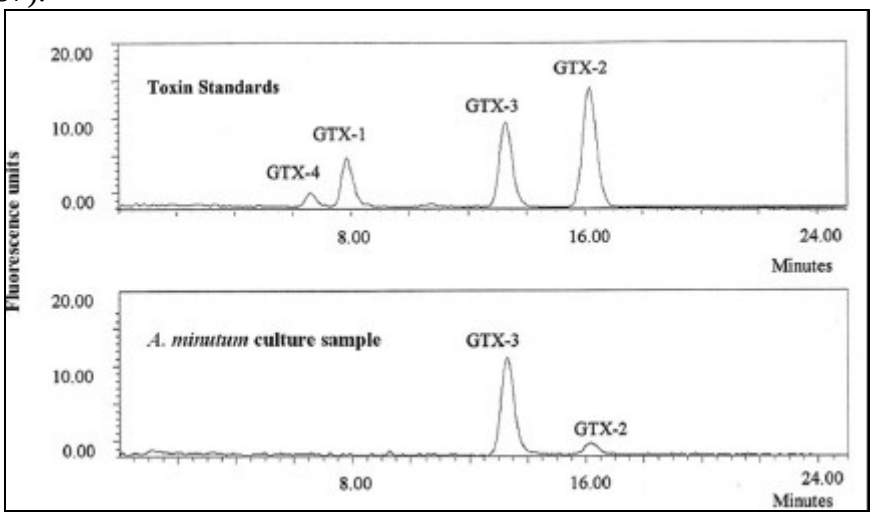


Figure 3. HPLC-FD chromatograms of PSP toxins of *A. minutum* from Cork Harbour. The top profile shows standards of gonyautoxins. The bottom profile shows the presence of GTX2 and GTX3 in an extract of A. minutum.

Whole-cell FISH. The CY.3 and FITC labelled oligonucleotide probes for *A. minutum* and *A. tamarense* showed a high specificity towards their target species. Cells were clearly recognizable and their enumeration straightforward at ×200 and ×400 magnification under epi-fluorescence microscopy (Fig. 4). In culture, the probes did not react with any other *Alexandrium* species tested or with any dinoflagellate species found in Irish coastal waters which usually co-occur with *Alexandrium* (*Scripsiella* spp., *Gymnodinium* spp., *Gonyaulax* spp. ...). All control assays were negative and only showed weak green or orange residual pigment autofluorescence.

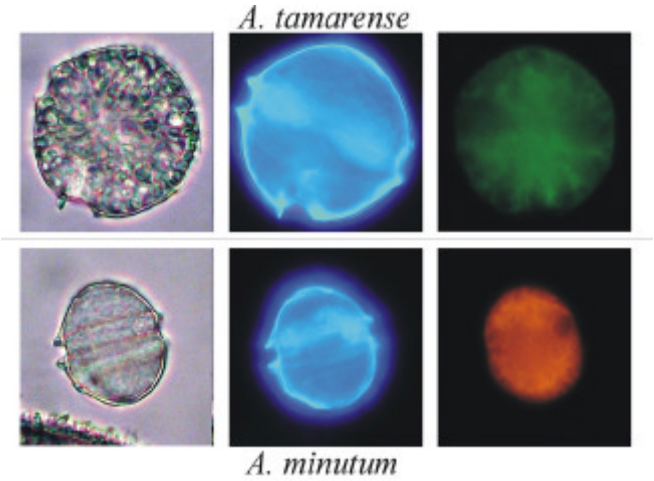


Figure 4. Discrimination of *A. tamarense* and *A. minutum* vegetative cells using light microscopy, calcofluor and fluorescent probes. *A. tamarense* cells tagged with TamA glow green while *A. minutum* cells labelled with MinA glow orange.

No matrix effect or apparent cross-reactivity with other phytoplankton species were observed during tests performed with field samples taken in summer 2005 from the North Channel area of Cork Harbour. The simultaneous use of DAPI and calcofluor proved particularly useful to confirm the specificity of the probes and to discriminate fluorescent detritus present in the field material from target cells (Figure 5). Some preliminary results derived through the use of the FISH probes showed that the composition of the *Alexandrium* community in the North Channel was variable. In July, the community was dominated by *A. tamarense* whereas two months later *A. minutum* was prevailing (Data not shown).

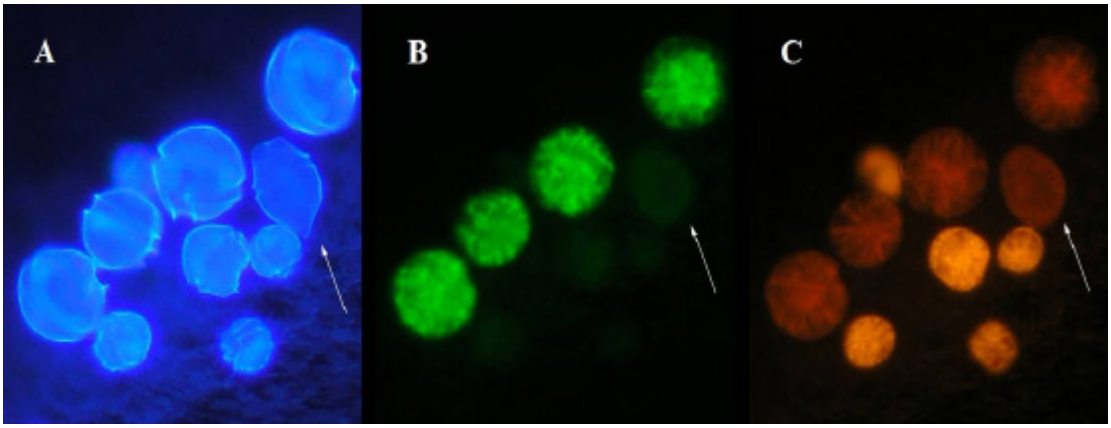


Figure 5. Epifluorescence photomicrographs showing the discrimination of FITC and CY.3 labelled *A. tamarense* (green) and *A. minutum* (orange) vegetative cells in a Cork Harbour field sample. Arrows show a vegetative cell of a non-target species (*Scripsiella* sp.).

Conclusions

There is a history of PSP contamination of shellfish in Cork Harbour. It is now well established that the *Alexandrium* community is mixed in the area, with the presence of the non-toxic *A. tamarense* and the PSP toxin-producing *A. minutum*. The latter species has been identified as the organism responsible for the PSP events recorded in this region as the toxin profiles obtained from cultures derived from locally *A. minutum* isolates coincided with those obtained from contaminated shellfish samples taken in 1996.

The tools for the quick and reliable discrimination between *Alexandrium* species in Cork Harbour are now available. FISH probes will prove invaluable to study the *Alexandrium* population dynamics at the species level in the area, and to implement bio-physical coupled models under development for the occurrence of *Alexandrium* in Cork Harbour.

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THE BIOLOGICAL OCEANOGRAPHY OF HARMFUL ALGAL BLOOMS (BOHAB) PROGRAMME: SPECIAL EMPHASIS ON THE DINOFLAGELLATE GENUS DINOPHYSIS

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Harmful algal blooms (HABs) are complex biological phenomena and the mechanisms involved in their development, proliferation and demise are not yet fully understood. It is however, possible to use observational data (physical, chemical and biological) to develop conceptual models in order to try to identify the underlying mechanisms responsible for these events.

The BOHAB (Biological Oceanography of Harmful Algal Blooms) project is a 3 year project, running from January 2003 to December 2005, whose primary objective was to research the means by which physical and biological processes interact to generate HABs in Irish waters. Observational data were collected throughout the project to meet this objective. The data collected were then used to develop a conceptual model for the west coast of Ireland, Bantry Bay and Killary Harbour (Figure 1).

In the first year of the project intensive fieldwork was carried out to study the spatial and temporal distributions of HABs in Irish waters. This included seasonal sampling in Killary Harbour and Bantry Bay, current meter deployments in Killary Harbour, a cyst survey along the west coast, as well as high frequency sampling and a preliminary mussel gut content study in Killary Harbour. Sample processing, data sorting, database development, a study of the occurrence of phytoplankton in "thin layers" in Killary Harbour and a gut content study was carried out in the second year of the project. Data analysis, additional intensive field work in Killary Harbour, ADCP and data buoy deployments and synthesis of results were the main activities carried out in year three.

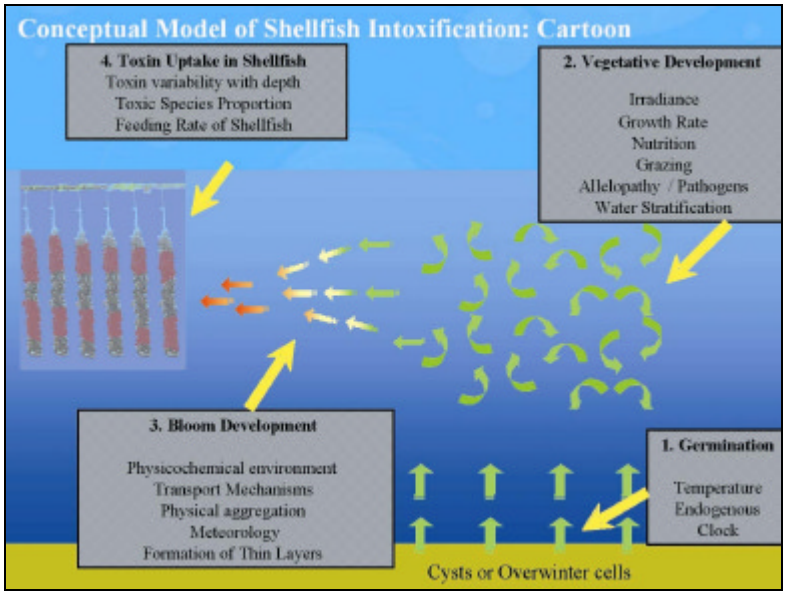

Figure 1. Example of a conceptual model developed during the BOHAB project.

In this paper data collected from fieldwork studies and information collected through literature reviews on the dinoflagellate genus *Dinophysis* (a DSP toxin producer) will be presented.

Germination (Life cycle)

It is well known that the genus *Dinophysis* has a complicated polymorphic life cycle and that its morphology is highly variable under different environmental conditions. Small and intermediate cells have been observed for some species. These small cells form when unfavourable environmental conditions arise. They can either play the role of anisogamous gametes (a gamete differing from the other conjugant in form or size) or if conditions improve, grow to become large cells again (Reguera and Gonzalez-Gil, 2001). Large-small cell couplets have been observed in *Dinophysis* species and are part of the sexual cycle and give rise to a planozygote (MacKenzie, 1991). It then settles out to form a hypnozygote. To date, little is known about the over winter populations of *Dinophysis.*

Vegetative Development / Seasonal Succession

A complex relationship exists between light availability, nutrients, physical mixing of the water column and the growth of *Dinophysis.* Some of the environmental parameters measured during BOHAB are discussed in more detail below. Sudden toxic events caused by *Dinophysis* are frequently experienced in the bays off the south west and west coasts. This suggests that the *Dinophysis* populations are being transported in currents from offshore.

In Ireland, a typical seasonal bloom of *Dinophysis* is represented by cell numbers from the mid-hundreds to about 5,000 cells per litre. Cell numbers recorded in other countries frequently reach numbers >100,000 cells per litre. In July 1992, an exceptional bloom of *Dinophysis* was recorded in thermally stratified waters of the Celtic Sea, due south of Cork, with cell concentrations in the order of 125,000 cells per litre (Raine and McMahon, 1998). *Dinophysis acuminata*, in this case was found in a thin layer adjacent to the pycnocline, at a depth of 25 m. During the BOHAB

project, examination of 15 years of phytoplankton data collected by the MI National Monitoring Programme showed that *Dinophysis* is typically present in the plankton from May through to November. Mean cell concentrations are, however, very low in February, October and November. The typical seasonal succession of *Dinophysis* in Irish waters can be characterised as follows: *D. acuminata* is most abundant in May, June and July and this is followed by *D. acuta* in August and September (Figure 2). Examination of the data shows that the monthly mean distribution of *Dinophysis* is more abundant off the south west coast. Off the west coast in Killary Harbour, *D. acuminata* is always the most abundant species present with highest monthly mean cell counts recorded in July and August. A seasonal succession is observed in Bantry Bay with higher mean monthly counts of *D. acuminata* recorded in early summer followed by *D. acuta* in August, September and October. *Dinophysis* spp. appear in the plankton when the day length is extended to >14 hours of light. Monthly mean cell concentrations are highest in August when day length is >15.5 hours. At this time of the year the water column is usually thermally stratified.

From 2003-2004, 472 water samples were collected during BOHAB surveys. These surveys were undertaken in March, May, July and August. *Dinophysis acuminata* was present in 220 of the samples while *D. acuta* was only present in 90 of the samples.

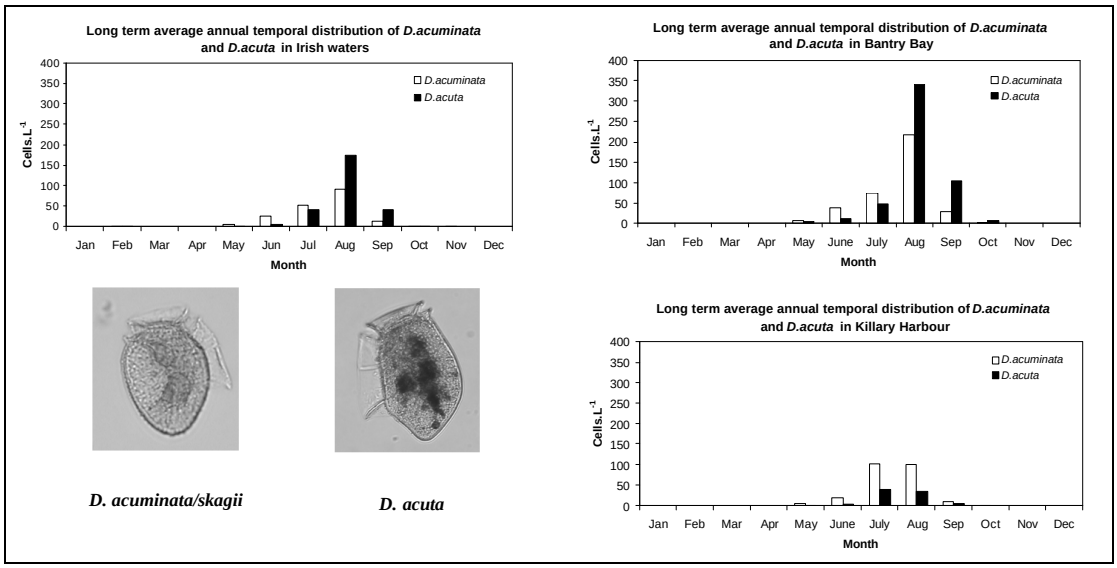

Figure 2. Monthly mean plots of *D. acuminata* and *D. acuta* in Irish waters, Bantry Bay and Killary Harbour. Fifteen years of data from 1990-2004 was used to generate these plots.

Daily division rates recorded in European and American waters show that *Dinophysis* grow faster at the pycnocline with a range between 0.16-0.97 divisions per day throughout the water column (Chang and Carpenter, 1991, Reguera *et al*., 1996, Graneli *et al*., 1997, Chang *et al*., 1991, Carpenter *et al*., 1995, Gisselson *et al*., 2002, and Reguera *et al*., 2002). *D*inophysis cells usually divide in the early hours of the morning just after sunrise. During a BOHAB survey off the southwest coast in July 2003, recently divided cells of *Dinophysis acuta* were observed in surface samples collected at about 8:30 am at the mouth of Bantry Bay (Sheep's Head).
Dinophysis species can be strictly heterotrophic (*D. rotundata*), some are photosynthetic while others are thought to be mixotrophic (cells that photosynthesis

and complements its nutrition by feeding on prey). Food vacuoles containing the remains of other organisms e.g. ciliates have been observed in cells of *D. acuminata* and *D. norvegica* (Jacobsen and Anderson, 1994, Koike *et al*., 2000). Hackett *et al*. (2003) observed *Dinophysis* cells that had engulfed other phytoplankton species and used the acquired chloroplasts (kleptoplasts) for photosynthesis. Graneli *et al*. (1997) found that net carbon uptake occurred during both light and dark periods in *D. acuta* and *D. acuminata*. The positive carbon uptake was suggested to be indicative of mixotrophic nutrition. Cells kept for prolonged periods in the dark (40hr) displayed the largest carbon uptake and it was suggested that heterotrophy in *Dinophysis* may be a mechanism to survive unfavourable conditions.

High densities of this dinoflagellate have been associated with ammonium rich waters close to the bottom of the photic zone in New Zealand (Chang, 1996). Other experiments with *D. acuta* and *D. acuminata* from natural populations have shown no difference in growth between N-rich, N-poor and phosphate enrichment (Johansson *et al*., 1996). This has also been observed with the addition of humic and fulvic acid (Maranda 1995). It has been reported that this genus is more competitive in low nutrient waters (Graneli *et al*., 1997). Results from such studies are however, difficult to compare since *Dinophysis* has not yet been successfully cultured in the laboratory where experiments can be careful controlled. *Dinophysis* was observed in both nutrient rich and nutrient poor waters during the BOHAB surveys.

Dinophysis spp. are not the only phytoplankton species to prey on other organisms for survival. In July 2003, during a BOHAB survey, empty *Dinophysis* thecae were observed packed inside *Noctiluca scintillans* cells in phytoplankton net samples collected in the Celtic Sea. The feeding on *Dinophysis* by heterotrophic organisms such as *Noctilluca* may be another way in which DSP toxins are concentrated and passed through the marine food web.

During the summer months in Irish waters, dinoflagellate distributions are normally associated with stratified waters. Water column stratification is influenced by a number of different factors including surface heating freshwater runoff, upwelling events, tidal mixing, wind mixing and the frequency of internal waves. In general, Dinophysis spp. were found in well stratified waters (Phi Φ ($J.m^{-3}$) >30) and temperature ranges from 10.0-17.5 °C (Table 1-2). Both *D. acuminata* and *D. acuta* were present in waters with a median salinity value of 34.77 (PSU) and 34.79 (PSU) respectively (Table 1-2).

Table 1. Summary the cell densities, depth cells recorded, temperature, salinity and the stratification index of *D. acuminata* during BOHAB surveys undertaken in 2003 and 2004. When Phi Φ = <10 $J.m^{-3}$ the water column is well mixed, Phi Φ = >10-30 $J.m^{-3}$ the water column is partially stratified (weak to moderate) and when Phi (Φ) = >30 $J.m^{-3}$ the water column is well stratified.

D. acuminata	cells.L^{-1}	Depth (m)	Temperature (°C)	Salinity (PSU)	Phi Φ ($J.m^{-3}$)
Number of samples	220	220	214	214	214
RANGE	1 to 7721	0 to 65	9.91 to 17.46	20.79 to 35.47	2 to 184
MEAN	263	9.5	15.00	33.79	46
MEDIAN	120	5.1	14.90	34.77	39
SD	614	10.3	0.98	2.45	35
MINIMUM	2	0.0	9.91	20.78	2
MAXIMUM	7721	65.0	17.46	35.47	184
max-min	7719	65.0	7.55	14.69	182

Table 2. Summary the cell densities, depth cells recorded, temperature, salinity and the stratification index of *D. acuta* during BOHAB surveys undertaken in 2003 and 2004. When Phi (Φ) = <10 $J.m^{-3}$ the water column is well mixed, Phi (Φ) = >10-30 $J.m^{-3}$ the water column is partially stratified (weak to moderate) and when Phi (Φ) = >30 $J.m^{-3}$ the water column is well stratified.

D. acuta	cells.L^{-1}	Depth (m)	Temperature (°C)	Salinity (PSU)	Phi Φ ($J.m^{-3}$)
Number of samples	90	90	87	87	87
RANGE	1 to 1680	1 to 70	10.44 to 17.46	29.52 to 35.26	2 to 186
MEAN	290	11.1	14.95	34.64	60
MEDIAN	120	7.0	14.94	34.79	39
SD	377	11.8	2.12	3.77	51
MINIMUM	1	1	10.44	29.52	2
MAXIMUM	1680	70.0	17.46	35.26	186
max-min	1679	69.0	7.02	5.74	184

3. Bloom development

In order to develop future forecasting systems for HABs, a good understanding of the physical transport mechanisms (currents) that carry these toxic algae ashore is needed. Work carried out by Raine and McMahon (1998) off the southwest coast of Ireland has shown that a clockwise coastal current exists close to land. The position of a front (boundary between two different water body types) between the coastal and Atlantic ocean waters depends on local meteorological conditions (Figure 3.). A south westerly wind will restrict the position of the front to close to the coast while a north easterly or easterly wind will allow the front to be positioned further offshore. The position of the front will determine if phytoplankton populations in waters off the south coast can be transported further north.

In the summer subsurface baroclinic-like jets or bottom density fronts form at depth in shelf waters off the west coast of Ireland (Nolan, 2004). Currents generated by these bottom density fronts, characterised by a flow northward along the temperature front, are an ideal mechanism for transport of phytoplankton along the west coast.

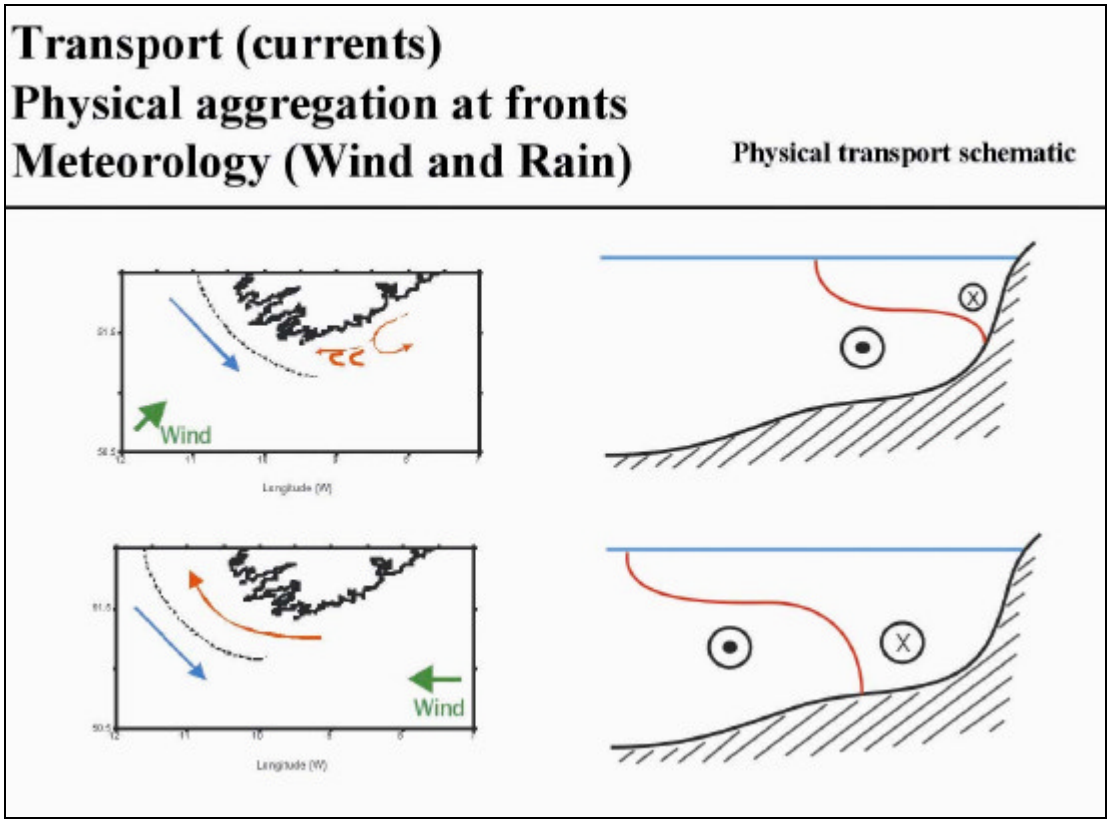

Figure 3. Schematic drawing showing the position of the frontal system off the south west coast of Ireland, in relation to local wind conditions.

The advection of offshore populations into bays off the south west coast is well understood. McDermott and Raine (2004) carried out a detailed study from 2001-2003 on predicting the transport of offshore *Dinophysis* populations into Bantry Bay. Under normal conditions south westerly winds prevail off the southwest coast. When the wind direction changes to north easterlies cold bottom water (and offshore phytoplankton) enters the bay under the pycnocline replacing much of the warmer water in the bay. This warmer water will then be carried northward with the coastal current. When the wind direction returns to normal conditions (i.e. south westerlies) warm offshore water re-enters the bay and with it If a *Dinophysis* population is present offshore at the time of the event, it is advected into the bay. (Figure 4).

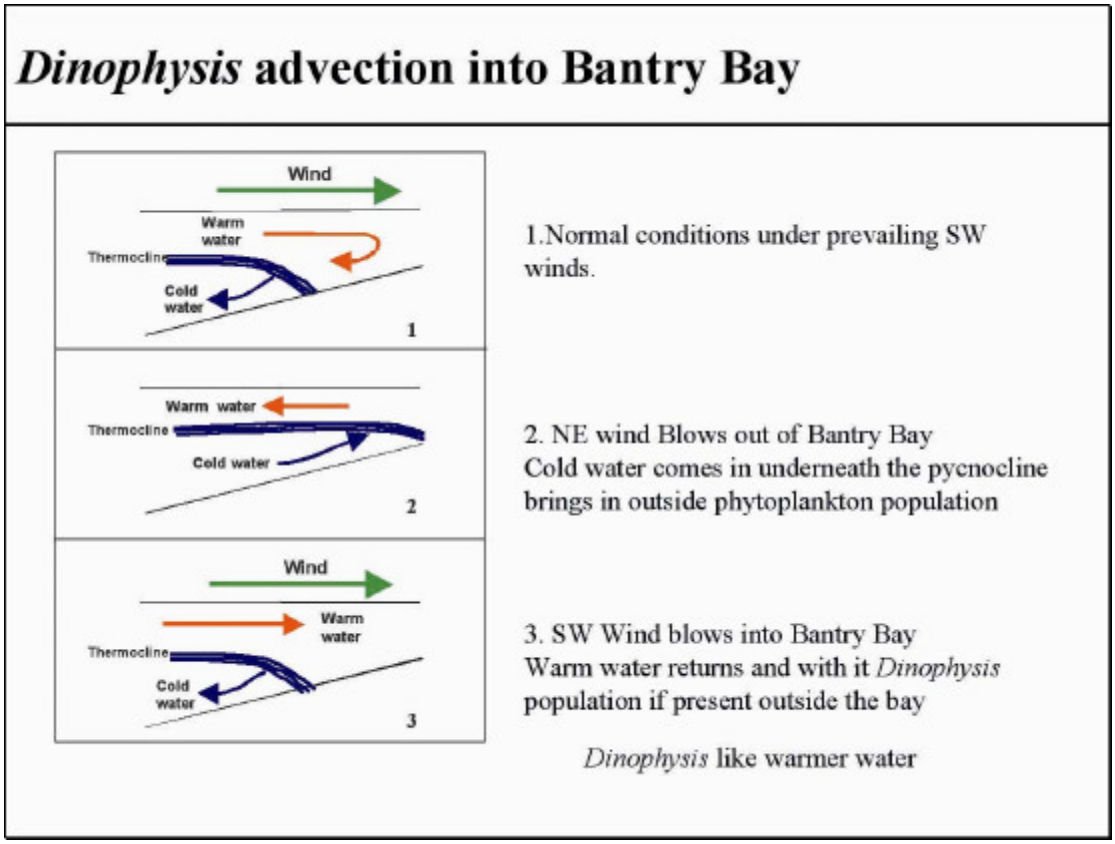

Figure 4. Schematic drawing showing a series of water exchange events related to meteorological conditions required to transport *Dinophysis* populations into Bantry Bay in summer.

4. Toxin uptake in shellfish

A study to investigate variations in toxicity of mussels with depth and the phytoplankton species composition in the gut contents of rope mussels was carried out in Killary Harbour in 2003 and 2004. Samples were collected from the top (1-2 m), middle (4-5 m) and bottom (9-10 m) of the long lines where the mussels were cultured. Results showed that toxicity patterns throughout Killary Harbour varied. In general, the surface and middle depths at the outer site exhibited similar patterns. At middle Killary all depths sampled showed similar patterns in toxicity and at the innermost site the surface differed in toxicity from the middle and bottom samples. *Dinophysis* was found in the gut contents during the toxic periods. The results from this study fit well with the current understanding of the hydrography of the area. Other work carried out has shown that toxic events caused by *Dinophysis* spp. can occur when this dinoflagellate is present at low abundances, < 25 % of the total phytoplankton community (>20 µm) (Figure 5). In other words, a monospecific bloom is not required for a DSP event to occur.

In summary, the new insights on the spatial and temporal distributions of *Dinophysis* and its interaction with different physical and chemical water properties have allowed the formulation of a conceptual model. This model brings us closer to developing early warning systems for the onset of DSP toxicity in Ireland.

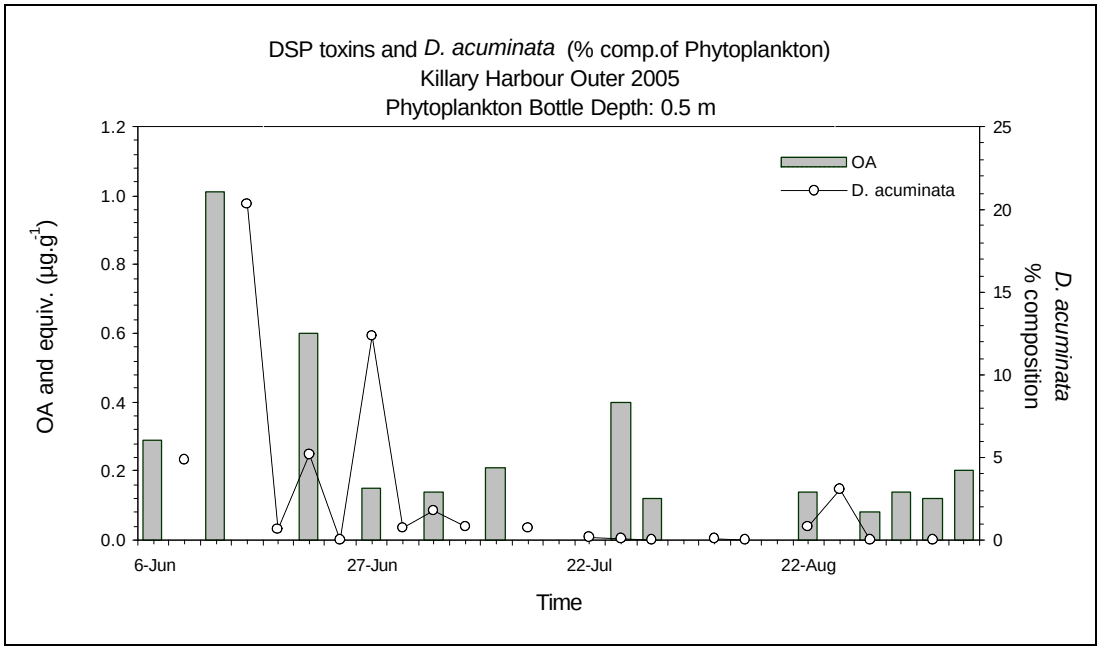

Figure 5. Plot of okadaic acid levels in mussels (primary Y-axis) and *Dinophysis acuminata* as a percentage of the total identifiable phytoplankton population (phytoplankton >10µm, secondary Y-axis) from June to August 2005 at the mouth of Killary Harbour. OA = shaded bars, *Dinophysis* = open circles.

Acknowledgements: Special thanks to the BOHAB Scientific Steering Group (Liz Abbot, Tim Coakley, Cilian Roden, Terry McMahon, Patrick Gentien, Dennis McGillicuddy, Daniel Lynch) and project partner (Don Anderson Woods Hole Oceanographic Institute). Thanks also to members of the Aquaculture Industry: Simon Kennedy, Abden Ryan, Anthony Murphy Danny McNulty and Kevin Lydon (Killary CLAMs group), Mike Salmon (Cuan Baoi Salmon Farm), Deirdre Collins (Bantry Bay Seafoods), Niall O'Boyle (Clew Bay Marine Forum), William Murphy, Dan O'Shea (Bantry Bay). Special thanks goes to all members of the Marine Environmental Food Safety Services group and the Oceanographic Science Services group at the Marine Institute. We are grateful the assistance of the sea fishery officers (Dept. of Marine, Communications and Natural Resources), Robert Boyne and Ian Richardson (Office of Public Works consultant, Eowen Associates), Hugh McGinely, Micheal MacCarthaigh and Shane O'Boyle (Evironmental Protection Agency), Tomas Burke (Bord Iascaigh Mhara) and John Wallace (Marine Informatics). Thanks to our colleagues at NUI, Galway (Hazel Farrell, Sandra Lyons, Aoife Ní Rathlaithe, Nicolas Touzet, Rachel Cave, Martin White, Christian Mohn and Robert Kennedy).

This project (Grant-aid Agreement No. ST/02/01) is carried out with the support of the Marine Institute and the Marine RTDI (Research, Technology, Development and Innovation) Measure, Productive Sector Operational Programme, National Development Plan 2000 – 2006. Part of the work carried out during this project was also financially supported by the EU funded projects NEMEDA and HABES.

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EU DEVELOPMENTS FOR SHELLFISH MICROBIOLOGY: FORTHCOMING REGULATION AND THE DEVELOPMENT OF AN EU GOOD PRACTICE GUIDE FOR MONITORING SHELLFISH HARVEST AREAS

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Introduction

EU directive 91/492/EEC lays down the health conditions for the production and the placing on the market of live bivalve molluscs and now has been in place for nearly 15 years. It provides a framework for food safety controls through all stages of bivalve production. It is just one of 17 vertical directives relating to food safety which have developed in the EU since 1964. Following the publication of a European white paper on food safety in 2000, a review these 17 directives was carried out by the European Commission. The review recognised that the high number of these directives and their individual complexity was unnecessary and needed replacing. As a result this has recently led to a recasting of these Directives into a simplified set of legislation. The package separates aspects of food hygiene from animal health and official control issues and is commonly referred to the "hygiene package". This came into force on 1st January 2006.

Current application of legislative controls across Europe is variable and open to different interpretation between member states. This potentially creates an "uneven playing field" for producers and a variable level of consumer protection throughout Europe. In recognition of this fact and seeing the introduction of the new legislation as an opportunity for standardisation of the application of controls throughout Europe DG Sanco have commissioned a working group to establish a good practice guide for the bacteriological monitoring of shellfish harvesting areas. This group aims to develop a best practice for carrying out classifications of shellfish harvesting areas, which is a key element of the controls for producing bivalve shellfish. It is the intention of the working group that the good practice guide will be finalised in early 2006, although it remains unclear at this stage as to exactly when it will be published. National reference laboratories have been shown copies of initial drafts of the good practice guide for comment on scientific aspects.

This paper highlights the contents of the new hygiene legislation with regard to changes from 91/492. It also highlights significant areas of the forthcoming good practice guide. The implications of two aspects of the good practice guide, namely the introduction of sanitary surveys and proposals fro data interpretation, are discussed. However, the comments in this paper should be seen in the context of assessing a draft document which may be changed before being published.

New Legislation –Hygiene Package

The new hygiene package consists of 5 pieces for legislation. These are EU regulations, which means that they must be applied within the member state and are legally binding. This contrasts to the previous directives that required implementation within each member state through national legislation and allowed for some interpretation of their requirements. Three regulations are of prime concern for bivalve mollusc production;

- Hygiene 1. Regulation EC No. 852/2004 on the hygiene of foodstuffs
- Hygiene 2. Regulation EC No 853/2004, lays down specific hygiene rules for food of animal origin
- Hygiene 3. Regulation EC No 854/2004, lays down specific rules for the organisation of official control on products of animal origin intended for human consumption

Hygiene 1 sets down the general requirements for food operators and establishes that the principle responsibility for food safety lies with the food business operator (FBO). The regulation introduces the requirements for application of hazard analysis critical control point (HACCP) principles. However, this does not currently apply to primary production. So in the context of bivalve mollusc production HACCP principles must be applied during treatment such as depuration or cooking, but not during harvesting. The regulation also establishes requirements for traceability of food products. The regulation further refers to the microbiological criteria for food stuff regulations which are being revised currently.

Hygiene 2 gives the requirements for foods of animal origin for industry. The specific rules for the production of live bivalve molluscs are given in section VII of the annex. There are no significant changes within the regulation from those in place in 91/492.

Hygiene 3 concerns the organisation and application of official controls for products of animal origin by competent authorities in member states. Specific details for live bivalve molluscs are given in annex II of the regulation. There are two significant changes within this legislation compared with 91/492.

Firstly the regulation makes provision for ensuring that where the competent authority decides in principle to classify a production or relay area, it must undertake a sanitary survey and that results for the sanitary survey must be used when establishing an ongoing sampling programme. Advice given to the community reference laboratory by the EU indicates that requirement for a sanitary survey legally only applies where new harvesting areas are identified after January 1st 2006.

Secondly within the legislation the requirement for category B classification areas has been changed. The new regulations do not allow for any tolerance within the number of results allowed above the upper limit of 4600 *E. coli* 100g^{-1}. Previously under 91/492 10% of samples from a harvest area were allowed to be above this upper limit and the area would still qualify for category B status. This equates to a tightening of the standard for category B classifications. This requirement presents difficulty in interpretation as no time limits are placed on compliance. If read literally, the requirement is for continuous compliance with 4600 *E. coli* 100g^{-1} for 100% of the time.

Good Practice Guide for monitoring of shellfish harvest areas.
An EU working group on the microbiological monitoring of bivalve mollusc shellfish harvesting areas was established in early 2004. The working group was made up or participants from Italy, Denmark, Ireland, France, Spain, The Netherlands and UK. DG Sanco from the European Commission was the overall coordinator and the community reference laboratory was given the role of scientific co-ordination.

Essentially the aim of the working group was to establish good practice procedures for undertaking classifications of shellfish harvest areas in member states.

The NRLs throughout Europe were given sight of an initial draft of the good practice guide and asked to comment on the content of the good practice guide from a scientific standpoint. As part if the NRL's role to disseminate information at national level, some details of the initial draft and potential implications are presented here. These have also been discussed previously at the Molluscan Shellfish Safety Committee. However, it must be remembered that the final document has not been produced and the contents may change.

Contents
The good practice guide will cover the following aspects of microbiological monitoring:

- Sanitary Surveys
- Sampling Plans
- Sampling and sample transport
- Microbiological testing
- Data handling and storage
- Interpretation of monitoring data

Within the guide a clear distinction is given on which elements is a legal requirement under the new legislative arrangements and what is recommended good practice. Two aspects with the good practice guide that merit further discussion in particular are recommendations for carrying out sanitary surveys and for interpreting data gathered during monitoring.

Sanitary Surveys
Within Hygiene 3. (Regulation EC No 854/2004) it is only a requirement to undertake a sanitary survey for new shellfish harvesting areas designated after January 1st 2006. However, the good practice guide recognises that this will create a two tier system and recognises that this is undesirable. Therefore it recommends that by January 1st 2009 member states should introduce a programme of work to complete sanitary surveys for all harvesting areas classified as at 21st December 2005 by 1st January 2011 at the latest.

The approach recommended by the good practice guide requires

- Characterisation of the shellfishery including location, extent and culture & harvest procedures.
- Identification of both animal and human pollution sources including continuous sewage discharges, rainfall dependent sewage discharges and diffuse pollution sources

Initially as much information as possible should be obtained from existing sources in order to minimise the resource implications. In addition however shoreline surveys should be undertaken where doubt exists as to whether all significant pollution sources have been revealed by the desk study. Use may also be made of hydrographic models. A report of the sanitary survey should be made and kept. Where possible the guide recommends that information should be store in a geographic information

system (GIS) format. The sanitary survey should be reviewed periodically on an ongoing basis. A complete review should be undertaken every three years.

The results of the sanitary survey should only be used to determine the location of microbiological sampling points which should be identified to determine the site for the "worst case scenario".

There are a number of implications that arise if the good practice guide recommendations for introducing sanitary surveys are adopted. Firstly the proposal to carry out sanitary surveys in all harvesting areas has clear resource implications for the competent authority which need to be considered in the light of competing calls for resources. A further consideration is that if the results from the sanitary surveys result in modifications to the sampling point location this could lead to changes in the classification status in a particular harvest area. Where changes to classification do occur these are likely to be deteriorations in the status as the major objective of the sanitary survey is to identify "worst case" sampling points. In the long-term, the introduction of sanitary surveys in harvesting areas has a potential beneficial effect, as an up to date inventory of pollution sources in a harvesting area is a significant step in producing management plans for improving water quality in shellfish harvesting areas.

Interpretation of monitoring data

The good practice guide recommends that classifications should be made on the basis of time series data collected on a monthly frequency. A final decision on the period of data to be reviewed is not given in the draft good practice guide however, examples of data interpretation given in the guide point to a likelihood of the last three years data being reviewed on an annual frequency. Hygiene 3. Regulation No. 854/2004 requires 100% of samples from an area to comply with the ascribed classification. However the guide recognises that continuous 100% compliance in any harvesting area is an unrealistic aspiration. The draft good practice guide therefore makes a recommendation that a tolerance allowing 5% of samples to be above the upper limit of a particular classification category i.e. 95% compliance. Currently within Ireland classification of shellfish harvesting areas is made by reviewing data every six months requiring 90% of samples to comply with the upper limit for a particular category. This 90% compliance level has been adopted by several countries including France and the UK.

Both the new regulations (100% compliance) and the likely recommended approach in the good practice guide (95%) represent a significant increase in the classification standard compared with the current approach in Ireland (90% compliance). If increased compliance levels as suggested in the good practice guide were adopted this would mean that if 2 samples in a three year review period (assuming 36 monthly samples) were above the upper limit for the classification category the area must be classified in the higher category. An initial review of three years data from classified oyster sites in Ireland indicates a significant number of harvesting areas would be downgraded under the new system (Table 1.).

Table 1. Numbers of classified oyster sites in Ireland currently classified in each category and if the requirement for 95% of samples to be below the upper limit for the classification category was applied.

Category	Number of areas currently classified in each category	Number of areas classified in each category with 95% compliance.
A	21 (37.5%)	4 (7.2%)
B	35 (62.5%)	46 (82.2%)
C	0	6 (10.7%)

Conclusions

The implementation of both the new EU hygiene regulations and the recommendations contained in the draft good practice guide have major implications for the competent authorities and the industry in Ireland. Recommendations contained in the good practice guide on sanitary surveys will have resource implications if adopted. Rearranging the sampling programmes in harvesting areas as a result of the sanitary surveys may influence the classification of harvesting areas. The requirement to undertake sanitary surveys in all harvesting areas would identify sources of pollution in harvesting areas which is a major step in developing water quality improvement plans which could have a beneficial effect on the classification status of same shellfish harvesting area. If the 95% compliance level for shellfish sample results contained in the good practice guide is adopted, it will have a significant impact on harvest area classifications in Ireland. Whilst the introduction of sanitary surveys and changes in sample result compliance levels are the major issues within the good practice guide there are several additional recommendations which may have implications if adopted in Ireland. Implementation of the new EU hygiene regulations and adoption of the recommendations contained in the forthcoming good practice guide in a way that balances the potential impact on the shellfish industry while considering the desire for improved food safety requirements at the EU level represents a significant challenge for all involved.

DETECTION OF HUMAN VIRUSES IN SHELLFISH AND UPDATE ON REDRISK RESEARCH PROJECT, CLEW BAY, CO. MAYO.

Sinead Keaveney, Fergal Guilfoyle, John Flannery and Bill Dore, Marine Institute

Introduction

Bivalve shellfish are filter feeders and can accumulate human pathogens when grown in sewage-contaminated waters. This, allied to the fact that they may be consumed raw, means that they can present a public health risk. Current control measures rely on classification of harvesting areas based on their sanitary quality using *E. coli* as an indicator of sewage contamination. However, *E. coli* may underestimate the risk of viral contamination and outbreaks of viral illness can still occur.

In northern Europe gastroenteritis caused by norovirus (NV) is the most prevalent viral infection associated with shellfish consumption. As NV cannot be cultured, detection methods in shellfish have relied on the use of molecular techniques. In particular the use of the polymerase chain reaction (PCR) is common. Conventional PCR methods for detection of viruses in shellfish have a number of technical limitations, which have acted as a barrier to their application in monitoring shellfisheries for viral quality. However, more recently, the development of real-time PCR methods, has addressed some of these technical deficiencies and provides a more robust and reliable assay for detection of noroviruses in shellfish.

Given the continued public health risks associated with shellfish consumption, improved risk management procedures for controlling viral hazards are required. To address this, the Irish National Reference Laboratory (NRL) is involved in a European-wide project called Reduction of risk in shellfish harvesting areas (REDRISK). The REDRISK project aims to identify the main environmental risk factors causing viral contamination of shellfish to allow the development of improved risk management approaches during primary production of shellfish. The ultimate aim is to identify environmental conditions that can be monitored in real-time to determine when viral contamination may occur.

This paper describe the progress in norovirus detection methods and initial results from the REDRISK study.

Detection of norovirus in shellfish

Detection of NV in shellfish using molecular techniques is complicated compared with detection in clinical samples. This is because of the complex non-homogenous nature of the sample matrix which contains PCR inhibitors and the low levels of virus present. In addition, the existence of two virus genogroups further complicates the situation. A number of strategies have been developed to overcome these problems.

Virus and RNA extraction

All human viruses present in shellfish are restricted to the digestive tract, primarily the hepataopancreas, of the animal. In the procedure used the hepatopancreas of at least 6 animals (minimum weight 3 g) are isolated, finely chopped and treated with the enzyme proteinase K. The procedure allows extraction of the virus from the hepatopancreas, without homogenisation of the animal tissue and substantially reduces the amount of inhibitory material introduced into the assay.

The nucleic acid material in NV is RNA. Unlike DNA, RNA is not stable as it is prone to digestion by cellular enzymes. Therefore careful RNA isolation is a must for the preparation of template for the PCR assays. The application of commercially available kits used with clinical samples is limited due to the shellfish matrix. The method used by the Irish NRL is commonly referred to as the Boom method (Boom *et al.,* 1990). This facilitates extraction and purification of nucleic acid from a variety of sample types using guanidine isothiocyanate to denature cells releasing the nucleic acid. The RNA released from the virus particles is adsorbed onto silica particles to facilitate purification through several washing steps. RNA itself cannot be used as a template in PCR assays and therefore must be converted back or "reverse transcribed" to its complementary DNA (cDNA) copy using the enzyme reverse transcriptase.

DNA amplification and Real-time PCR

Once the cDNA is isolated it is necessary to amplify the DNA before it can be detected. The PCR amplifies a specific sequence of DNA using two short DNA sequences (primers) each of which is complementary to either end of the DNA target sequence. Figure 1 demonstrates the principle of PCR.

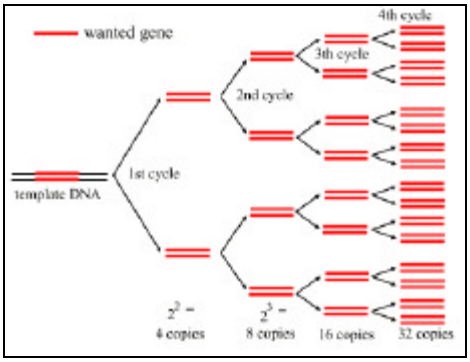


Figure 1. The polymerase chain reaction (PCR). This diagram represents the first four cycles of a PCR.

Real-time PCR makes use of sophisticated instrumentation to detect amplified PCR product in real time. In the TaqMan® PCR format an additional short DNA sequence (the probe) binds internally of the two primers. Two fluorescent labels are attached to either end of the probe sequence. The chemistry of the primer/probe arrangement is such that as the quantity of amplified product increases, the fluorescent signal also increases proportionately, therefore allowing the early stages of the PCR reaction to be detected. This allows the quantification of the initial starting material i.e. virus level. Separate assays have been developed for genogroup I and II NVs. The use of NV GI or GII specific probes allows for "in built" confirmation of a NV positive result without the need to sequence the PCR product.

The units of quantification in the real-time PCR are known as cycle threshold (Ct) values. The Ct value is the cycle number at which the fluorescence generated from the amplification of the target sequence crosses the threshold (Figure 2). **The lower the Ct value the more virus is present in the sample.** The quantity of target sequence i.e. virus copies, in the samples can be determined by extrapolation from the standard curve. Appropriate standards for the absolute quantification of NV are not readily available. However, close observation of the Ct values allows relative

quantification of the virus copy number. Although absolute quantification is not currently possible, results from trials within the Irish NRL indicate that an increase in the Ct value of 3 approximately equates to 10 fold increase in virus levels (Table 1). To date experience both in Ireland and in other European countries indicates that in general the level of virus detected in category B shellfish is at the limit of detection of the assay (Ct values of approximately 35-37). Ct values higher than this often results in NV being detected in only 1 or 2 of the 3 replicates as observed in Figure 3. It remains unclear as to whether these low levels of virus present a public health problem.

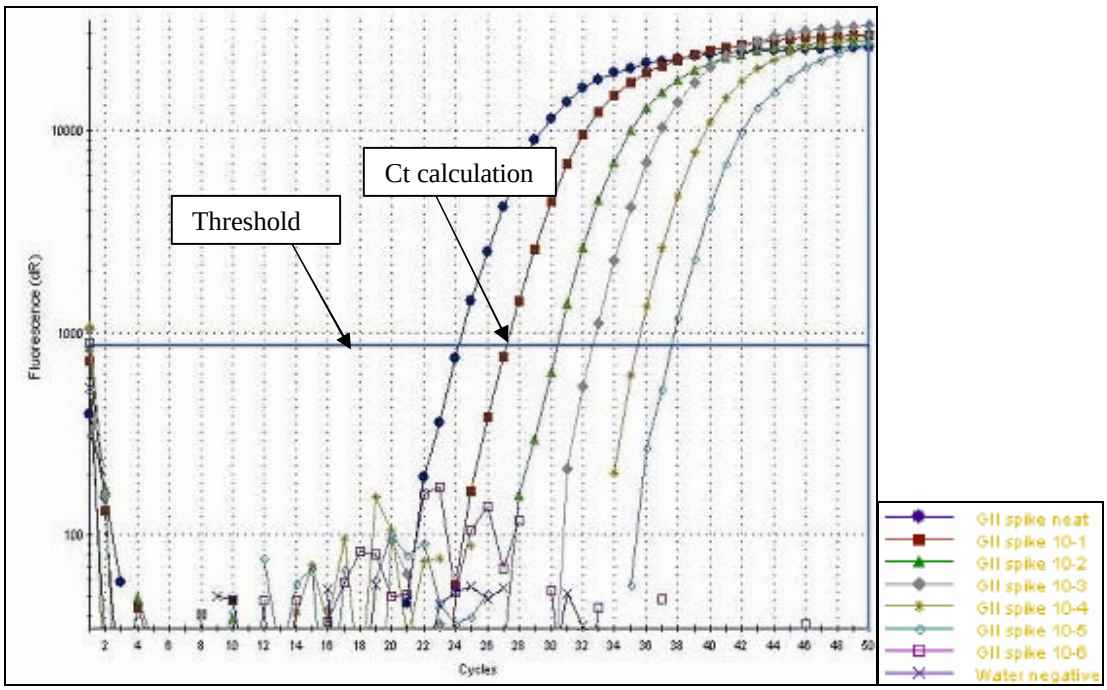


Figure 2. Amplification plots for NV GII assay demonstrating the threshold and calculation of the cycle threshold (Ct)

Table 1. Ct values for NV GI and GII detected in an oyster sample spiked with GI and GII positive stool sample

Spiked oyster sample	**Ct NV GI assay**	**Ct NV GII assay (Fig 4)**
Neat	23.7	24.2
10^{-1}	26.7	27.2
10^{-2}	29.3	30.3
10^{-3}	33.3	32.6
10^{-4}	35.8	35.3
10^{-5}	44.3	37.5
10^{-6}	No Ct	No Ct

NV negative oyster samples were spiked separately with NV GI and GII positive faecal material. Viral RNA was isolated and reverse transcribed to cDNA. The cDNA was serially diluted to 10-6 and all dilutions were run on the respective assays

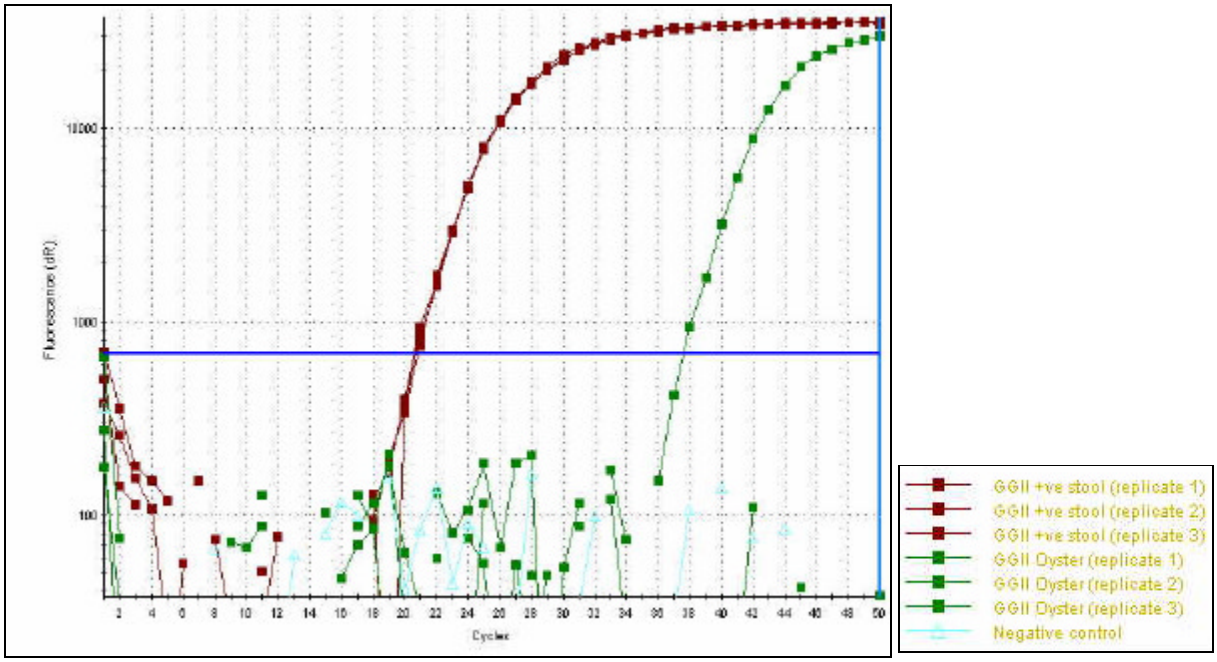


Figure 3. Amplification plots for NV GII identified in a stool sample (Ct average of 20.7) and an oyster sample. Note only 1 of the 3 replicates tested for the oyster sample was positive for NV GII with a Ct value of 37.5.

Real-time PCR Controls

The quantitative nature of the real-time PCR assay allows the use of accurate controls within the assay allowing greater standardisation and reliability. This is a major improvement over conventional PCR systems. A number of internal process controls have been introduced into the real time PCR assay.

Controls for the RNA extraction step ensure the successful isolation of viral RNA from the shellfish matrix and its subsequent reverse transcription into cDNA. The control used is another virus of the calicivirus family called feline calicivirus (FCV). A known amount of FCV is spiked into the sample homogenate. If the extraction step is successful, the level of FCV detected (as judged by the Ct value) is consistent with the previously determined Ct value for the amount of FCV added. If Ct values are higher than expected this indicates poor extraction of the RNA and requires repeat testing of the sample.

To control for inhibitors of the real-time PCR reaction an "internal exogenous control (IPC)" is used. This IPC kit contains a piece of DNA that is totally unrelated to the target DNA i.e. NV or FCV, and primers and probe designed to amplify this DNA only. The reagents for this assay are included when each sample is analysed for NV. If this IPC DNA target is not amplified in the reaction, this indicates the presence of inhibitors from the shellfish extract and if the IPC reaction has been inhibited it can be concluded that the NV reaction is inhibited also. These control measures provide confidence in the accurate detection of NV GI and GII from shellfish samples.

Norovirus negative and positive controls are also included in each assay run.

REDRISK project

The REDRISK project is part of the wider SEAFOODplus EU Framework six integrated research project. It is being carried out in four European countries, England, Spain, France and Ireland. In Ireland Clew Bay was selected as the study site because it has generally good water quality but within the bay there are also a variety of pollution sources, which may impact on its quality. There are areas classified as both Category A and B for shellfish production. The Clew Bay Marine Forum provides logistical support and local advice on the REDRISK project. Essentially the project is divided into two parts:

- A data collection programme to investigate possible sources of pollution and collect environmental data
- A sampling programme collecting shellfish for microbiological analysis

Environmental data collection and site selection:

Clew Bay is a westerly-facing bay made up of a complex series of islands and interlocking bays. The two main towns are Westport and Newport (Figure 4). Westport has a population of 6000 serviced by a new wastewater treatment plant (WWTP). Secondary treatment involving aeration and settlement is provided for a population equivalent of 15,000. Newport has a population of 600 but has only a very basic wastewater collection and treatment system and septic tanks.

Background data were collected to characterise the bay. This data comprised of human and animal population numbers (Central statistics office, CSO), *E. coli* results both historical and from ongoing monitoring (DCMNR), wastewater treatment details and licensed discharges (Mayo County Council), integrated pollution control licenses and river flow volumes (EPA), aquaculture production (BIM), hydrographic model details (MI) and health data from pharmacies. From a survey of this background data four sites were selected for further investigation. The characteristics of each of these sites are outlined in Table 2.

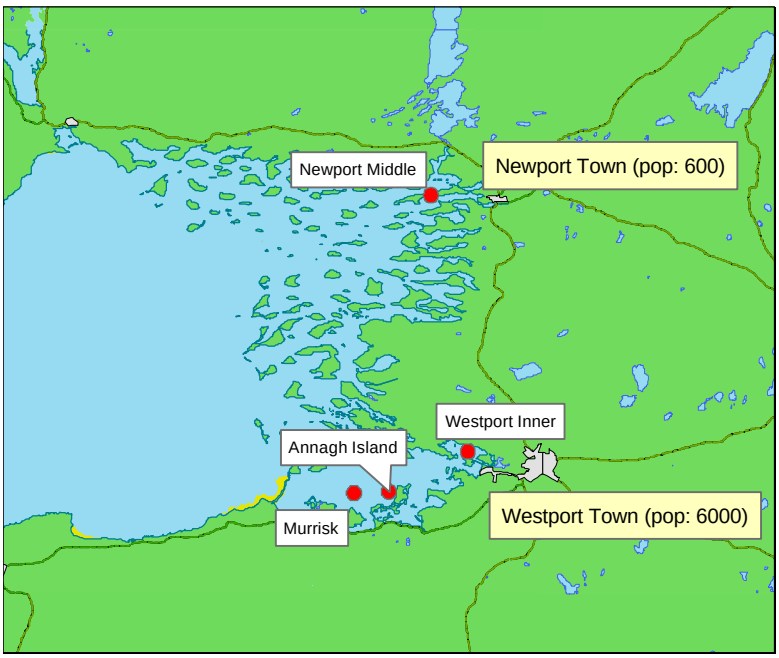


Figure 4. Clew Bay with major towns impacting the bay and sampling REDRISK points .

Table 2. Comparison of REDRISK sampling sites.

Westport Inner	**Annagh Island**	**Murrisk**	**Newport Middle**
Native and Pacific oysters	Native and Pacific oysters	Pacific oysters	Pacific oysters
Class B area	Class B area	Class A area	Class B area
300 m from WWTP outfall	3500 m from Westport town	4500 m from Westport town	1600 m from Newport sewage outfall (minimum treatment)
Population 6000	Local population 0	Local population low (some septic tanks)	Population approx. 600
Close to mouth of Carrowbeg river (average flow 1 m^3/s)	Some distance from two rivers of influence	Very little freshwater input	High freshwater input, Newport river (average flow 6 m^3/s)
Some agricultural input	Some animal farming nearby	High numbers of animals	Some agricultural input

WWTP = Waste water treatment plant

Oysters were placed at each site in conditions as close to natural conditions as possible.Pacific oysters (*Crassostrea gigas*) were placed at all four sites in growing bags, on tressles (Figures 5 and 6). At 2 sites, Westport and Annagh Island native oysters (*Ostrea edulis*) were placed in boxes on the bottom.

Figure 5 and 6. Fergal Guilfoyle (MI) and Mike Struth (CBMF) sampling Pacific oysters from tressle and sampling native oysters from "Ortek" box. Photos courtesy of Niall O'Boyle, CBMF.

Microbiological monitoring

Weekly sampling of oysters from the four sites began in August 2005 and 15 samples have been collected in 2005. Each oyster sample was tested for *E. coli*, FRNA bacteriophage and norovirus.

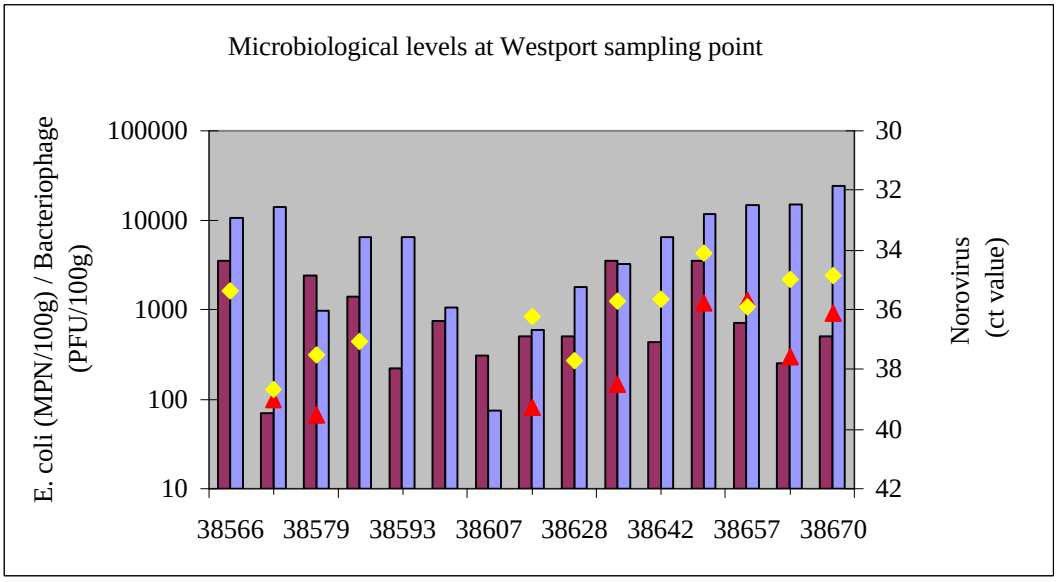
FRNA bacteriophage form a group of viruses which infect a group of bacteria and are found in high numbers in sewage, they are not pathogenic to humans and are considered to be a good indicator of viral contamination as they exhibits similar survival characteristics to pathogenic viruses i.e. norovirus.

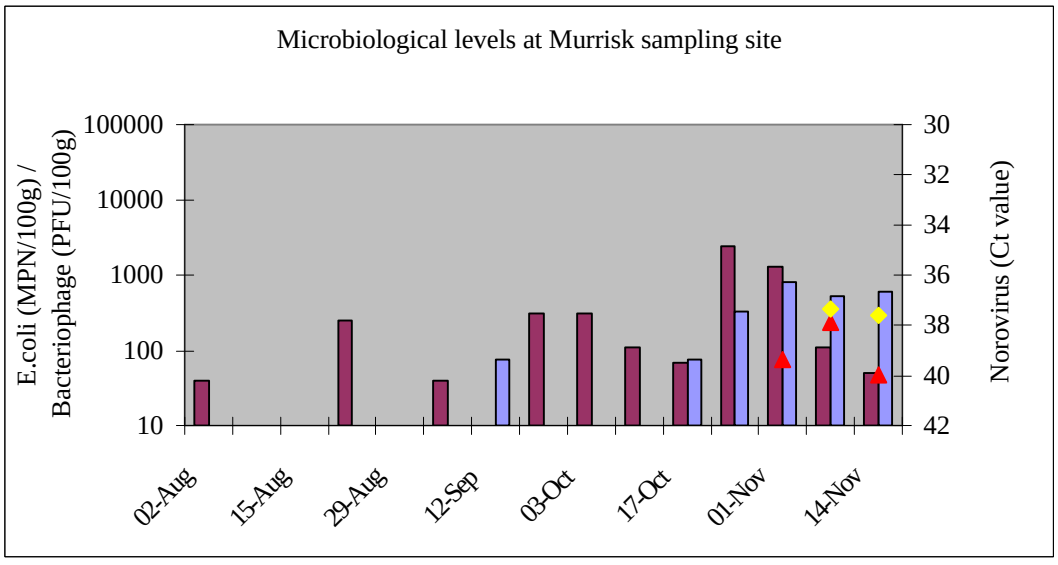
Environmental monitoring

Environmental data were also collected at each site, temperature is logged continuously at each site, salinity is collected at each site during sampling and turbidity is recorded during sampling. Riverflows are automatically recorded by the EPA, weather data was collected by MI/Met Eireann in Newport, wastewater treatment plant output volume is recorded by Mayo County Council and all the pharmacies in the area recorded sales of diarrhoea medicines.

REDRISK results (August – November, 2005)

The REDRISK project is ongoing and therefore only initial results are available. The preliminary results reported here are for two sampling sites (Murrisk and Westport) from August – November 2005. Figures 7 and 8 show levels of the different microbiological parameters from pacific oysters from these sites.





Figures 7 and 8. Levels of *E. coli*, (purple bar), FRNA bacteriophage (blue bar) and GI (red triangle) and GII (yellow diamond) norovirus in Pacific oysters (C. gigas) from Westport Inner and Murrisk. Ct values for norovirus have been inverted.

The initial results indicate;

1. Clear differences were observed in levels of both *E. coli* and FRNA bacteriophage in oysters collected from the Westport site and in oysters collected from the Murrisk site. This was consistent with the proximity of sources of sewage pollution to each site.
2. Norovirus (GI and GII) has been detected in oysters from the Westport Inner sampling site on a regular basis correlating with the levels of indicator organisms and demonstrating the potential risk associate with shellfish from this site.
3. The Ct values obtained for norovirus from August 2nd to October 17th indicates low level of virus contamination (average Ct value of 38). However, a decrease in Ct values (average Ct value of 35) from November 1st, may indicate a ten-fold increase in virus level. This is consistent with the known winter seasonality of norovirus infection in the community.
4. Samples collected from Murrisk contained low levels of *E. coli* and FRNA bacteriophage during the sampling period.
5. Norovirus was absent from shellfish in this area except for a contamination event in November. The Ct values at this time were high (near limit of detection) indicating a very low number of virus present.
6. Similar results have been observed by project partners in the contributing to the REDRISK project.

The next step is to further analyse the microbiological results with the environmental information gathered to establish if there is a relationship with viral contamination and environmental factors. Work to date has shown that there is possible link between river flow, wastewater treatment plant outfall volumes and levels of norovirus and microbiological indicators. However, further sampling and data analysis is required to establish this link. The REDRISK project will continue to collect data and test shellfish in 2006

Conclusions

The new real-time PCR method being deployed by the Irish NRL allows relative quantifcation of norovirus in shellfish samples for the first time. In addition, the introduction of quantitative controls within the assay allows better standardisation and increased degree of certainty of the result over conventional PCR procedures. The application of the real-time PCR procedure to shellfish samples is giving an increased understanding of the viral risk associated with shellfish.

Using the real-time PCR procedures to determine the relative levels of norovirus in shellfish during the REDRISK project will allow the possibility of identifying environmental factors responsible for viral contamination of shellfisheries. This may provide the framework for introducing improved risk-management procedures for controlling the risk associated with viral contamination in shellfisheries.

The authors would like to acknowledge the assistance of the following: Terence O'Carroll (BIM), Niall O'Boyle, Sean O'Grady, Mike Struth (CBMF), National Diagnostics Centre (NUIG), Hugh McGinley (EPA), Jimmy Carney (DCMNR) and Mayo County Council.

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DEVELOPMENTS IN RAPID TEST KITS FOR TOXIN TESTING (PSP, ASP AND DSP)

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Jellett has pioneered the application of lateral flow immunochromatography (LFI), which is more commonly known in use as a home pregnancy test, into the marine biotoxin field. In 1997, in Jellett Biotek Ltd., the first prototype LFI tests for PSP were developed with saxitoxin standard from Sherwood Hall and funding from the Alaska Science and Technology Foundation.

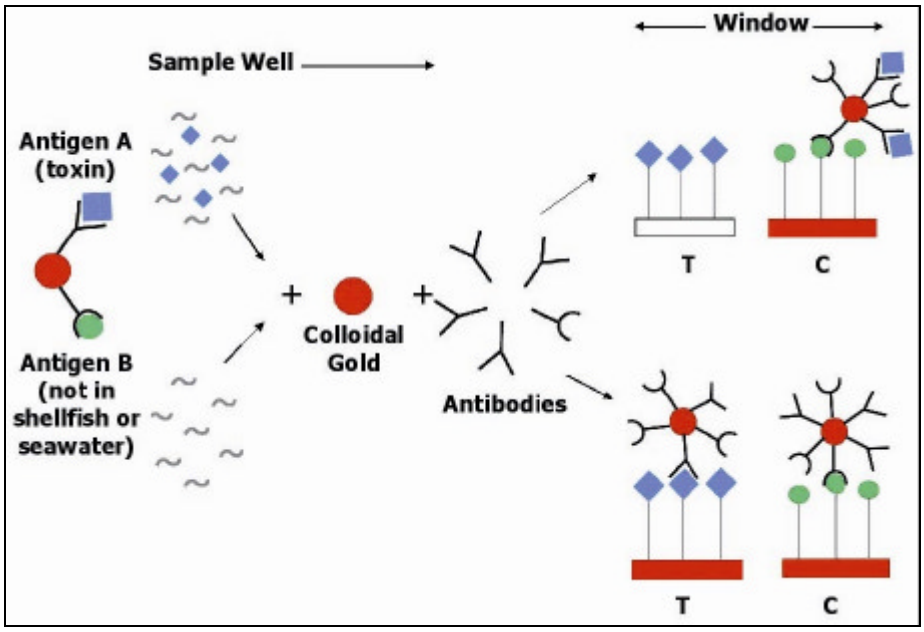

Figure 1. Lateral flow immunochromatography for marine biotoxins

All the LFI tests used for marine biotoxins operate similarly. Antibodies and colloidal gold (the red colour) are dried under the sample well, and antigen is tethered to the capture lines. When the sample is added, the antibodies and other reagents are mobilised, the antibodies bind to the gold and move forward to the capture lines. If biotoxins are present, at or above half the regulatory level in the sample, then the antibodies will not be able to bind at the toxin (T) capture line and only one line (C) will form. This indicates a positive response for toxin. If there is no toxin in the sample, then the antibodies will have lots of binding capacity to attach to the T and C lines, and two lines will form, indicating a negative response for the presence of the target toxin. Detection limits for the three tests are 40 µg/100g for PSP, 10 µg/g for ASP (although this can be easily modified to 15 µg/g with dilution, and tests as low as 1 µg/g have been developed for use with phytoplankton, or for shellfish monitoring in areas where ASP is unlikely to occur), and 0.1 µg/g for DSP.

The critical reagents, the capture antibodies, had been developed several years before in a project at the National Research Council of Canada's Institute for Marine Biosciences (NRC-IMB) and shelved, and these were licensed to Jellett Biotek Ltd.

Jellett then obtained training and expertise in GMP management and developed a comprehensive quality assurance program based on certified reference materials from NRC-IMB. Early work on the cross reactivities of the antibodies to the different pure toxin standards can be seen in Laycock *et al.* (2002). Extensive beta-level validation was performed in collaboration with NRC-IMB to prove the efficacy of the test using spiked and naturally contaminated shellfish tissue from many different countries using comparative HPLC testing. Validation then continued with comparative testing against the mouse bioassay performed in many countries and with many different shellfish types over time (Jellett *et al*., 2001). In 2001, Jellett submitted the comparative data to the Interstate Shellfish Sanitation Committee for inclusion into the National Shellfish Sanitation Plan (NSSP) of the USFDA.

In 2001, Jellett Biotek also made an agreement with AgResearch of New Zealand to use their anti-ASP antibody to develop and market an LFI test for ASP, and began the development of anti-DSP antibodies.

In 2002, Jellett continued the work toward regulatory approval of the PSP test, and the process of validation of the ASP test following the same basic procedure, from a new company called Jellett Rapid Testing. The names of the tests were changed from "MIST Alert" to "Rapid Test" to eliminate trademark costs and keep the costs of the tests as low as possible. In 2003, the PSP test was approved for screening use in the NSSP of the USA. Since then, many other countries have adopted the PSP test for regulatory screening (such as the UK, Ireland, Portugal, and Australia), but as yet not within any official regulatory framework. Several other countries are assessing the PSP test, such as Canada and Australia.

The ASP test has been used in an extensive community-based monitoring pilot project under ORHAB, where several west coast tribes used phytoplankton monitoring in conjunction with toxin testing with the ASP test to protect valuable razor clam beaches under native control. An extensive assessment was performed in a project with BIM and the Marine Institute which showed that the small scale field extract recommended with the test was as effective as the full scale extraction used for HPLC testing at MI (Bogan *et al*. in prep).

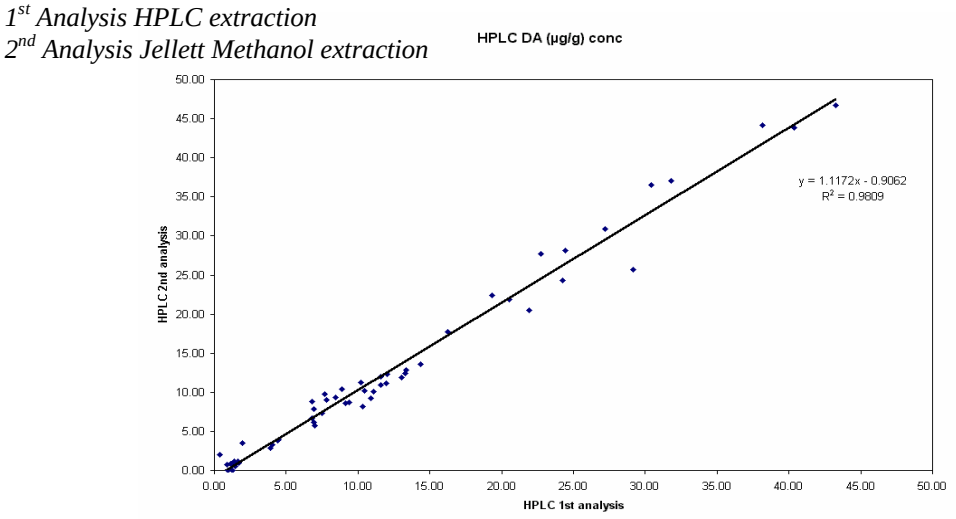

Figure 2a. Comparison of DA conc in whole scallop (µg/g) samples from 1st and 2nd HPLC analysis

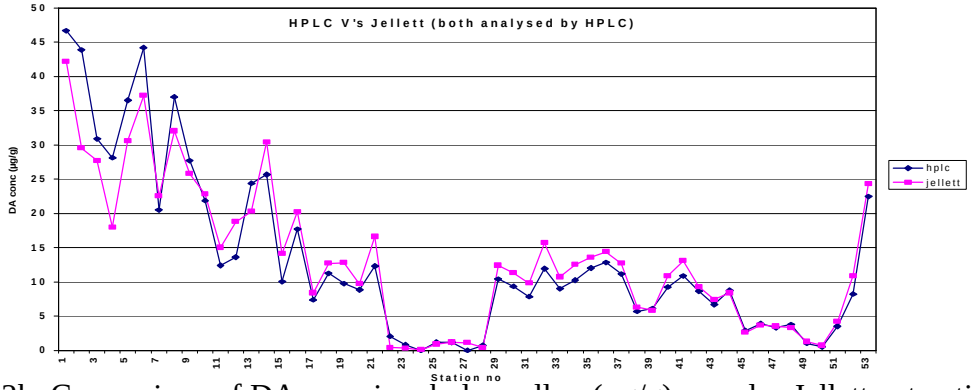

Figure 2b. Comparison of DA conc in whole scallop (µg/g) samples Jellett extractions vs HPLC extractions

Figure 2. Comparison of Regulatory (100g) and Jellett Extracts (10 g sub-sample) for scallop samples from 53 Stations Irish HPLC Data (from Bogan *et al*., 2005 In Prep)

Extracts from fifty-three positive and negative scallop samples were also correctly identified as positive or negative for toxin by 3 different analysts, although there was some variation at the detection limit (Figure 3). This is because the analysts must identify a decrease in the intensity of the T line (compared to the C line) to a specific level (50% or 75% depending on the test), which is subjective. However, all samples at or above the action level are easily identified by all analysts as positive, and all samples containing little or no toxin were identified as negative.

This project was designed to determine if the ASP test would be suitable for use by scallop fishers at sea to monitor for toxin in their catches. It was found that the test should (and could be) modified through dilution to 15 µg/g detection to eliminate many false positives. This is because the scallops in Ireland appear to carry toxin in their tissues for extended times after contamination, although at levels well below the action level of 20µg/g.

The new DSP test has been tested extensively in collaboration with the NRC-IMB using pure toxins, spiked tissue, and naturally contaminated tissue from Ireland, France and Holland (Laycock et al. 2005, 2006), and is now under assessment in many laboratories, including the Marine Institute, while it is perfected. A rapid sample preparation has also been developed for use with the test, which is 100% methanol extraction with a volume to weight ratio of 3:1. A rapid cleanup method has been developed to allow detection of very low levels of DSP toxins in shellfish. Both the sample preparation and the sample cleanup method have been investigated for efficacy using LCMS at NRC-IMB. Simple sample preparation is important for rapid screening use of the tests in the regulatory environment, but it is critical for use of the tests in the field, on ships, and at processing and aquaculture facilities. Jellett has focused on this aspect through the development of all of the biotoxin LFI tests over the years, and also previously with the cell bioassay. All rapid methods require rapid and simple sample preparation methods, which must be robust and reliable. Without the close association of Dr. Quilliam's group at the NRC-IMB and their provision of analytical standards and testing, the development and validation of the LFI tests and their associated sample preparation methods would not have been possible. Good

research and quality control produce tools like the LFI tests that make it possible for industry to take control of their own biotoxin testing.

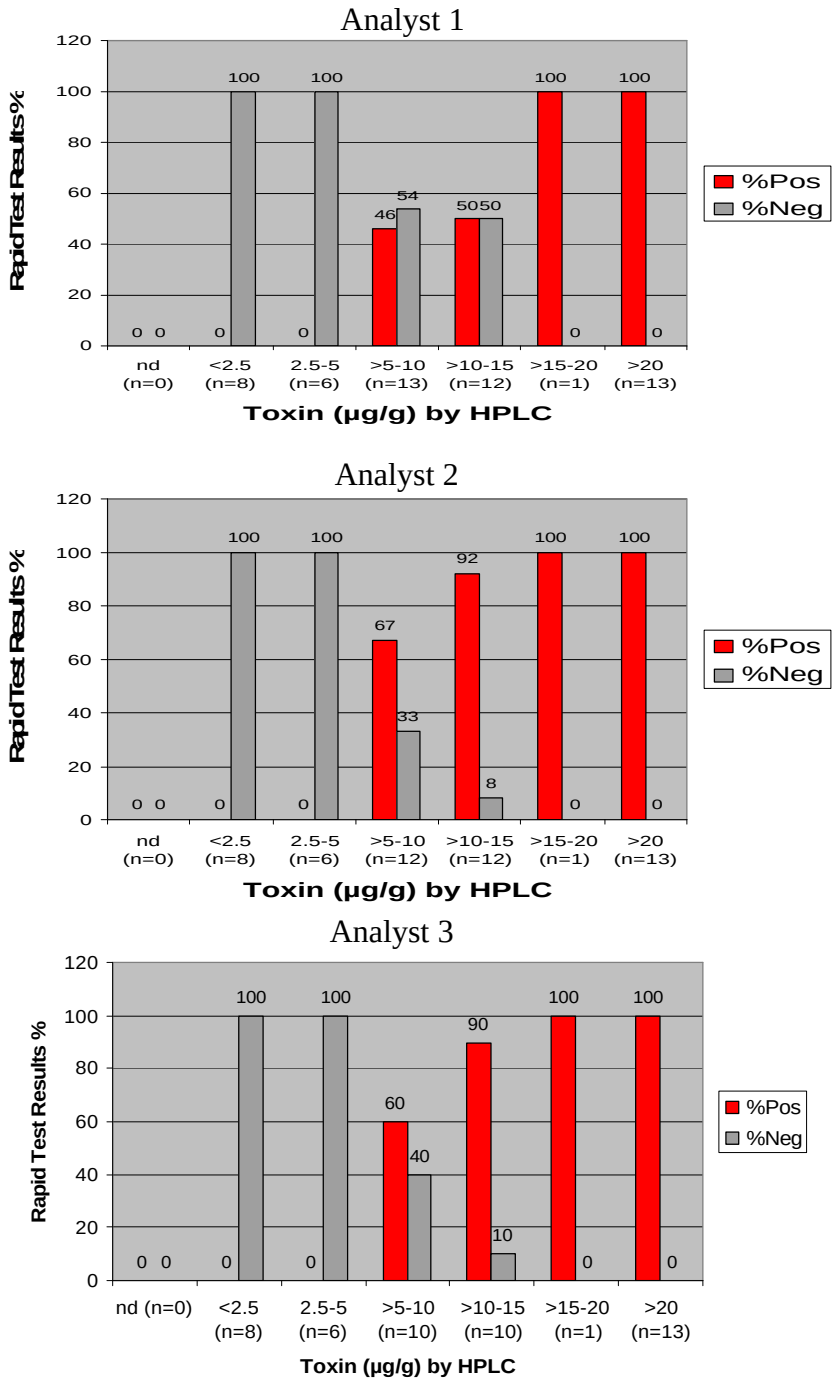

Figure 3. Extracts from fifty-three positive and negative scallop samples using 3 different analysts.

The USA is pioneering a new wave of testing called "distributed testing", whereby volunteers or paid people with lay backgrounds and minimal training perform field testing with rapid and simple tools like the LFI tests. These applications require simple sample preparation methods for field use. Although no food safety decisions are made in the field, the network of field tests provide data which is transmitted to the central regulatory laboratories, that can use it to accelerate sampling in high risk

areas. Distributed testing also provides a larger scale overview of toxic events, especially in areas like Alaska where monitoring is based on commercial activity almost exclusively and therefore can be oblivious to major toxic events in non-commercial areas that can then advect into shellfish beds quickly and unexpectedly. The LFI tests have proven very useful in distributed testing on the west coast of the USA when applied to phytoplankton monitoring as an early warning for shellfish contamination, but this application is dependent on the hydrography of each area and some areas are simply not suitable for phytoplankton monitoring for various reasons. Furthermore, critical levels of phytoplankton which cause shellfish contamination must be determined (and are different in each area) before the LFI tests can effectively be implemented for phytoplankton detection. Nevertheless, distributed testing, or community-based monitoring, can broaden the food safety net and eventually empower industry.

Although at present there are no rapid field tests for some of the regulated toxins, like azospiracid and pectenotoxin, these rapid tools continue to be developed in many laboratories. At present there are LFI tests for PSP, ASP and DSP, and for many bacterial food contaminants. There are rapid tests for coliforms in other formats, and rapid ELISA tests for ASP and yessotoxin. Hopefully the not too distant future will provide all the tools needed for the industry to ensure that what is harvested is safe for human consumption before it is sent to the regulatory laboratory.

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EVOLUTION OF IRISH PROGRAMME AND EU POLICIES ON BIOTOXIN MANAGEMENT

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Introduction

The Irish National Biotoxin Monitoring Programme is carried out under EU Directive 91/492/EEC and involves the analysis of shellfish samples for the presence of lipophilic (DSP, AZP, YTX, PTX) PSP and ASP toxins.

In this paper a series of actions to be implemented in 2006/2007 as part of the Irish Biotoxin Monitoring Programme based on agreement at the MSSC meeting on 22nd November 2005 is presented. These actions were agreed taking into account:

- Evolution of EU Commission Policy on the use of animal assays for biotoxin detection and monitoring
- Recent developments at Community Reference Laboratory / National Reference Laboratory (CRL/NRL) level on actions to validate alternative, non-animal based methods e.g. HPLC, LCMS, functional assays for PSP and lipophilic toxins
- Feedback and discussion at recent meetings with Industry

EU Legislation

The first EU wide legislation laying down the health conditions for the production and the placing on the market of live bivalve molluscs came in to force in 1991 (Council Directive 91/492/EEC). Under this Directive live bivalve molluscs intended for immediate human consumption must comply with the following requirements;

> The total Paralytic Shellfish Poison (PSP) content in the edible parts of molluscs (the whole body or any part edible separately) must not exceed 80 microgrammes per 100 g of mollusc flesh in accordance with the biological testing method - in association if necessary with a chemical method for detection of Saxitoxin - or any other method recognized in accordance with the procedure laid down in Article 12 of this Directive.
>
> If the results are challenged, the reference method shall be the biological method
>
> The customary biological testing methods must not give a positive result to the presence of Diarrhetic Shellfish Poison (DSP) in the edible parts of molluscs (the whole body or any part edible separately).

No detailed guidance on the testing methods to be used was provided but in most EU Member States mouse bioassays were used for both PSP and DSP toxin detection. However, in the case of DSP toxins, no clear definition of a positive result was provided which led to a lack of harmonisation between Member States.

During the 1990s the detection of new toxins e.g. azaspiracids, as well as new analogues of known toxins, developments in alternative, non-animal based, analytical methods of toxin detection and increasing ethical concerns regarding the use of animal assays led to the adoption in 2002 of Commission Decision 2002/225/EC

laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods.

The maximum level of okadaic acid, dinophysistoxins and pectenotoxins together (the whole body or any part edible separately) was set at 160 µg of okadaic acid equivalents/kg, for azaspiracids the maximum level was set at 160 µg of azaspiracid equivalents/kg while the maximum level for yessotoxins was set at 1 mg of yessotoxin equivalent/kg.

Guidance on the use of biological and alternative methods and the definition of a positive bioassay result are set out in the Annex to the Decision including:

A series of mouse bioassay procedures, differing in the test portion (hepatopancreas or whole body) and in the solvents used for the extraction and purification steps, can be used for detection of the toxins mentioned in Article 1. Sensitivity and selectivity depend on the choice of the solvents used for the extraction and purification steps and this should be taken into account when making a decision on the method to be used, in order to cover the full range of toxins.

Three mice should be used for each test. The death of two out of three mice within 24 hours after inoculation into each of them of an extract equivalent to 5 g of hepatopancreas or 25 g whole body should be considered as a positive result for the presence of one or more of the toxins mentioned in Article 1 at levels above those established in Article 2, 3 and 4.

A series of methods such as high performance liquid chromatography (HPLC) with fluorimetric detection, liquid chromatography (LC)-mass spectrometry (MS), inmunoassays and functional assays such as the phosphatase inhibition assay can be used as alternative or complementary methods to the biological testing methods, provided that either alone or combined they can detect at least the following analogues, that they are not less effective than the biological methods and that their implementation provides an equivalent level of public health protection:

- okadaic acid and dinophysistoxins: an hydrolysis step may be required in order to detect the presence of DTX3,
- pectenotoxins: PTX1 and PTX2,
- yessotoxins: YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX,
- azaspiracids: AZA1, AZA2 and AZA3.

If new analogues of public health significance are discovered they should be included in the analysis. Standards will have to be available before chemical analysis will be possible. Total toxicity will be calculated using conversion factors based on the toxicity data available for each toxin.

The performance characteristics of these methods should be defined after validation following an internationally agreed protocol.

When the results of the analyses performed demonstrate discrepancies between the different methods, the mouse bioassay should be considered as the reference method.

Use of alternative test methods for lipophilic & PSP toxins in Ireland

Since 2001, all samples received by the Marine Institute as part of the national biotoxin monitoring programme are tested using both

- Yasumoto 1984 mouse bioassay and
- LC-MS (OA, DTXs, AZA1,2,3).

The Marine Institute has been accredited to ISO 17025 standard by INAB for these methods. During this period more than 8,000 samples, including mussels, oysters, clams, razor fish and scallops have been analysed and a 95 -99% agreement between the results of both methods has been found and have been presented at previous workshops. The results for the period 2002 – mid Oct 2005 are summarised in Table 1 below. Based on the results from this dataset, it is clear that, at least in the case of Ireland, the LC-MS method provides an equivalent level of human health protection to that provided by the mouse bioassay.

Table 1. Comparison of results of lipophilic toxin analysis by mouse bioassay and LC_MS, 2002 – 2005.

Year	Positive MBA & Positive Chemistry	Negative MBA & Negative Chemistry	Positive MBA & Negative Chemistry	Negative MBA & Positive Chemistry
2002	2.5%	96.3%	0.9%	0.3%
2003	1.3%	96.2%	2.2%	0.3%
2004	3.3%	95.9%	0.11%	0.6%
2005 (to Oct)	8.8%	86.2%	3.2%	1.4%

The relationship between the results obtained using the mouse bioassay and AZA analysis by LC_MS during the period 1st October - 4th November are plotted in Figure 1 below. During this time period the levels of DSP toxins (Okadaic acid, DTX2) was minimal and thus any potential interferences, particularly with the results of the mouse bioassay, was likely to be minimised.

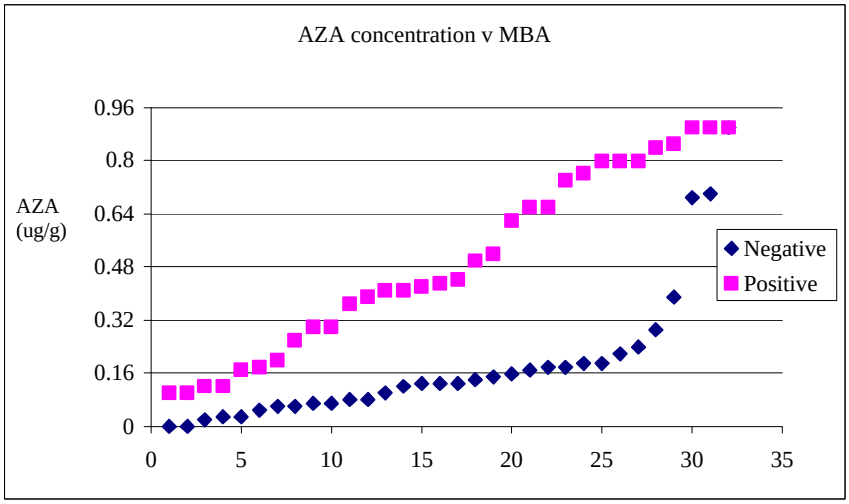

Figure 1. The relationship between the results of the mouse bioassay and AZA analysis by LC-MS during the period 1st October – 4th November 2005

The data show that in general there is a good relationship between the results obtained by both tests. Negative bioassay results were obtained when the AZA level was typically below 0.16 µg/g while positive bioassay results are obtained when the AZA levels exceed 0.16 µg/g. The results also show that on 4 occasions a negative mouse bioassay result was obtained when the measured level of AZA was significantly greater than 0.16 µg/g and on 4 occasions a positive mouse bioassay was obtained when the measured level of AZA was less than 0.16 µg/g. The uncertainty of measurement of both test methods may explain some of the apparent discrepancies at AZA levels close to the regulatory limit of 0.16 µg/g. The samples with clear discrepancies at high AZA levels cannot be easily explained.

The Marine Institute is of the view that the availability of LC-MS data has strengthened Ireland's ability to make regulatory decisions since 2001. If and when the EU Commission decides to permit the use of alternative methods in place of the mouse bioassay as reference method, the Marine Institute believes that the number of bioassays could be reduced, or phased out completely, and the LC-MS used without compromising human health.

Currently in Ireland, samples received for PSP testing are screened using the Jellet Rapid Test (JRT) for PSP toxins. Samples that give positive results using the JRT are retested using the mouse bioassay as the reference method. This has resulted in a considerable reduction in the numbers of mice used without compromising human heath. The JRT has also been accredited by INAB. It is proposed to continue with the use of the JRT as a screening tool as part of the National Biotoxin Monitoring Programme.

EU Commission Policy on alternative testing methods

The European Commission has a policy of complete removal of animal testing methods as soon as collaboratively validated methods are available. Commission Regulation (EC) 2074/2005 states that 'Provision should be made for the replacement of biological tests as soon as possible' and Council Directive 86/609/EEC requires that 'elements of replacement, refinement and reduction must be taken into account when biological methods are used'

In early 2005, the EU Commission tasked the Community Reference Laboratory (CRL) with introducing non-animal based toxin detection methods by 31st December 2005. At the VII meeting of the CRL and all National Reference Laboratories (NRL) in Cesenatico, Italy, 26th –28th November 2005, the DG-SANCO representative re-affirmed the Commission's policy of complete removal of animal testing methods as soon as collaboratively validated methods are available but recognised that the target date of 1st January 2006 was too ambitious.

The replacement of the bioassay as the reference method for detecting the Paralytic Shellfish Poisoning (PSP) group of toxins is well advanced. A HPLC based method for PSP toxin detection- the Lawrence Method - has been validated through the AOAC and is expected to be implemented at the end of March or April 2006. After this, the Commission will activate the procedure for an EU Decision on the adoption of this method as the reference method in official control. A favourable decision will result in the method becoming official and mandatory.

Steps towards the replacement of the mouse bioassay for lipophilic toxin detection with alternative methods were discussed and agreed at the CRL/NRL meeting in 2005. No NRL disagreed with the policy of replacement of the mouse bioassay over time and some e.g. Germany, Norway, The Netherlands, France, Denmark, Belgium and Ireland were in favour of replacement with "in-house" / single laboratory validated LC_MS methods. Single laboratory validation would be carried out using an internationally agreed protocol and to an internationally agreed standard as set out by e.g. European Centre for the Validation of Alternative Methods (ECVAM), Association of Official Analytical Chemists (AOAC). The use of single laboratory validated methods is already permitted in the residues monitoring programme as set out under Commission Decision 2002/657/EC implementing Council Directive 96/23/EC Concerning the performance of analytical methods and the interpretation of results. This Decision sets down performance criteria as well as validation procedures that need to be met before the test method can be used and it can be reasonably argued that a similar approach could be applied to the case of biotoxin monitoring in the EU.

The CRL / NRL group agreed to continue the activities of the LC-MS Working Group, including collaborative trials for the validation of a LC-MS method for the determination of lipophilic toxins covered in 2002/225 (including spirolides). It is proposed that this LC-MS method will include a screening and a confirmatory part**.** The CRL have been able to obtain toxin standards from a number of different sources, including the Marine Institute for Azaspiracids, to enable this work to take place. It is proposed that this CRL/NRL validation will be completed by end of 2006. Validation studies of alternative methods to be carried out as part of the EU funded Biotox project, in which the Marine Institute is a lead partner, will complement the CRL/NRL activity. The CRL will also conduct pre-validation trials using a functional assay, the Protein Phosphatase 2a (PP2a) assay for detection of the Okadaic acid group of toxins.

Revision of maximum allowable limits for biotoxins

In association with the CRL/NRL meeting in November 2005 the 1st meeting of an EU Working Group on Toxicology was held to discuss the toxicological data, analytical methods, legal status and regulatory limits for the okadaic acid group, the pectenotoxin group, the yessotoxin group, the azaspiracid group, the palytoxin group, the cyclic imine group and the ciguatoxin group.

The report of the Working Group will be sent to the European Food Safety Authority (EFSA) for consideration and review. On the basis of this report and other relevant documents EFSA will carry out a risk assessment and provide a report to the Commission. The Commission will then consider the EFSA report, propose risk management strategies and, if needed, new draft legislation which will then be put to Member States for a vote.

The Working Group recommended that:

- The regulatory limit for the Okadaic acid group be set at 80 µg Okadaic acid equivalents / kg of shellfish meat
- The regulatory limit for pectenotoxins be set at 720 µg pectenotoxins / kg of shellfish meat. Because of their comparatively low toxicity , PTX-8, PTX-9, PTX-10 and the pectenotoxin seco acid should be excluded from regulation.
- Yessotoxins should be de-regulated
- An interim limit for azaspiracids should be set at 32 µg / Kg shellfish flesh
- Gymnodimine should not be regulated
- The regulatory limit for spirolides should be set at 400 µg / Kg shellfish flesh

The Working Group also agreed that

Each toxin group should be considered as a distinct toxicological entity. Within a group of analogues, it should be assumed that each substance has the same toxic potential as the most harmful member of the group until data is available to set toxicity conversion factors

"*The mouse bioassay is no longer to be used as a reference method. From a toxicological standpoint, the mouse bioassay suffers the disadvantage of being non-specific, and with compounds that show high intraperitoneal toxicity, such as the pectenotoxins and spirolides, it will give false positives. Work is urgently required to develop alternative methods for toxin assay. It is recommended that the mouse bioassay be retained as a research tool. It should be employed periodically in order to detect new compounds or new toxin groups. In this situation, however, it will be used only for research purposes in order to detect new activities, not as an analytical method".*

Irish Monitoring Programme 2006/2007

Based on the above the following actions were discussed and agreed at the Molluscan Shellfish Safety Committee (MSSC) meeting on 22nd November 2005.

1. **LC-MS testing**

- The Marine Institute will continue to work towards international validation of LC_MS method and participate in LC-MS method validation exercise to be organized by CRL.
- Hydrolysis will be carried out only when requested by Management Cell in cases of positive mouse bioassay results or in the event of a rapid alert or product recall.

2. **Mouse Bioassay**

- Marine Institute will participate in the CRL/NRL Working Group on the development of a harmonized mouse bioassay protocol for lipophilic toxins
- Marine Institute will adopt harmonized protocol when finalized and implemented in all EU Member States. The MI is of the view that this work will improve the comparability of the mouse bioassay for as long as it remains the reference test within the EU. This will also introduce efficiencies in the test method and improved animal welfare.
- Marine Institute will only completely replace the mouse bioassay by LC-MS analysis when a validated method is finalized and accepted at EU level and

implemented in EU Member States. At that time it is envisaged that the use of the mouse bioassay will be prohibited under EU animal welfare legislation because of the availability of an alternative, collaboratively validated, method The validation will be carried out to an internationally accepted protocol as set out by e.g. ECVAM, AOAC

3. Phytoplankton Analysis

- Marine Institute will continue phytoplankton monitoring and reporting the results of analysis of approximately 1800 samples as heretofore and as set out in EU Directive 91/492 and CODEX ad-hoc Expert Consultation Group recommendations of 2004.
- Marine Institute recommends the increased use of phytoplankton data in the work of the Management Cell and in decisions to switch from low to high risk sampling and testing.
- Marine Institute will reallocate resources towards the production of synthesis reports of phytoplankton distribution and occurrence

4. Frequency of testing by Mouse bioassay

- Marine Institute will reduce the basic frequency of testing by mouse bioassay of "low risk" species, to include oysters (*C. gigas* and *O. edulis*), clams, razor fish and cockles, to monthly all year round. The frequency will be increased as appropriate when phytoplankton or other data indicates an increased risk.

5. PSP Testing:

- MSSC requests that the Irish Competent Authorities work with DG-SANCO to expedite approval of the Jellet Rapid Test (JRT) and other similar rapid screening assays
- Based on the low incidence of PSP in Ireland and experience to date on the use of the JRT, the MSSC approves the continued use of the JRT as a screening method
- Marine Institute to implement the HPLC based Lawrence method for PSP toxin detection. This test to be used only on those samples which test positive on the Jellet Rapid Test.

6. Management Cell

- Marine Institute will continue to provide data and record the MC decisions.
- The results of the mouse bioassay will be considered as the reference method as set out in Commission Decision 2002/225/EC.

7. Consultation

- Marine Institute in association with Irish Shellfish Association will prepare an information booklet, which will explain in easily understandable terms, issues related to the monitoring and regulation of biotoxins in Ireland.

8. Regulatory Levels of DSP, AZA etc

- FSAI to provide an update on the AZA Risk Assessment carried out in 2000 as well as provide an Irish position paper on the limits proposed by the Codex Ad Hoc Expert Consultation and the October meeting of the EU Toxicology Working Group.

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