

4th Irish Marine Science Biotoxin Workshop

Renvyle, County Galway, November 7th 2003

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

CONTENTS

	Page
Objectives of the 4th Irish Marine Science Biotoxin Workshop Micheal Ó Cinnéide, MEFS Director, Marine Institute	1
Session 1 Chair: Dr Peter Heffernan, CEO, Marine Institute	
Review of Irish Phytoplankton / Environmental Monitoring, 2003 Joe Silke / Caroline Cusack Marine Institute	3
Review of Shellfish Toxicity Monitoring in Ireland, 2003 Dave Clarke, Marine Institute	8
Review of Management Cell Operations, 2003 David Lyons, FSAI and Tim Coakley, ISA	16
Session 2: Irish Biotoxin Research (Chair Dr. Terry Mc Mahon, MI)	
Isolation and Purification of Azaspiracids from Naturally Contaminated Materials and Evaluation of Their Toxicological Effects. (ASTOX) Dr. Philipp Hess Marine Institute & Dr Michael Ryan/Gavin Ryan, UCD	18
Rapid Azaspiracid Shellfish Toxin Analysis: Culturing of <i>Protoperidinium spp.</i> Siobhán Moran, Marine Institute & Dr. Chris Elliot, DARD Northern Ireland	21
Morphology, Molecular Taxonomy and Toxin Composition of <i>Alexandrium spp.</i> Isolated from Irish Coastal Waters. Dr. Robin Raine & Nicolas Touzet, NUI Galway	24
Development of Molecular Probes for Toxigenic Phytoplankton Dr. Sinead Keaveney, NUI Galway	29
Phytoplankton Studies by DARD in Northern Ireland Dr. Richard Gowen, DARD, Northern Ireland	33
Session 3: Focus on Killary Harbour Research (Chair - Dr. Cilian Roden)	
Update on the Biological Oceanography of Harmful Algal Blooms (BOHAB) Project Dr. Glenn Nolan Martin Ryan Institute, NUIG & Joe Silke Marine Institute	38
Investigation into Toxic Algal Events in Killary Harbour by the Killary CLAMS Group Dr. Cillian Roden and Danny McNulty, Managing Director Atlantic Blackshells Ltd	43
Session 4 International Developments/ Microbiology (Chair: Alan Reilly, Chairman, MSSC & Deputy Chief Executive, FSAI)	
Role of Thin Layers in Controlling the Dynamics & Impact of HABs Dr. Percy L. Donaghay, University of Rhode Island, USA	44
The Classification of Shellfish Production Waters Andrew Kineen, Dept of Communications, Marine & Natural Resources	54
Conclusion Alan Reilly, Food Safety Authority of Ireland	57
List of Attendees	58

OBJECTIVES OF THE 4TH IRISH MARINE SCIENCE BIOTOXIN WORKSHOP

Michéal Ó Cinneide

Director, Marine Environment & Food Safety Services, Marine Institute

On behalf of the Marine Institute and our co-sponsors, BIM, the Food Safety Authority of Ireland (FSAI) and the Irish Shellfish Association, I would like to welcome the participants to this, our 4th annual Biotoxin Workshop.

The **Marine Institute's objectives** for the Irish Biotoxin programme since the reforms in 2000 have been to:

- **Support the continued development of the Irish Shellfish industry**
- **Promote food safety**
- **Build the best Biotoxin management system in the Northern hemisphere**

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

Our specific objectives for the 2003 Workshop are:

- Review the Irish Biotoxin Monitoring system and to assess the trends in toxicity during 2003
- Summarise current Irish research work in Harmful Algal Blooms and Phytoplankton
- Focus on research work in Killary Harbour under the BOHAB project
- Take stock of developments and provide a forum for debate/feedback.

Communications with Stakeholders.

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

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

Key Irish Developments in 2003

- Evolution of the **Management Cell**, to enable rapid decision making, according to protocols which were drafted by the Molluscan Shellfish Safety Committee (MSSC)
- 5 meetings were held by the Molluscan Shellfish Safety Committee, chaired by the FSAI and a strong ethos of partnership has been forged
- We have made great progress in Lab accreditation and quality systems in the areas of bioassay and Chemical testing (LC-MS and HPLC)
- Toxicity has again been low in 2003 (**3.6%** of shellfish samples were positive)
- Major **research** projects are underway in the areas of Biotoxins and Harmful Algal Events (HAE's)
- There has been an increasing focus on issues of microbiology and the classification of shellfish waters

Irish and International Research in Harmful Algal Events (HAE's)

The Workshop will give us an update on some key research projects, many of which are funded by the Marine Research Measure (2000- 2006). These include:

- Research work by Marine Institute staff on the Chemistry of Azaspiracids, Plankton and Oceanography
- Collaborative research with our neighbours NUI Galway on biological oceanography and plankton
- BioResearch Ireland, Galway are continuing to develop immuno assays
- Work on the toxicology of AZA with staff from the Conway Institute, University College Dublin
- Project on Azaspiracid assays with DARDNI and Queens University, Belfast.

Biotoxins and HAB's are a global issue. The scale of the processes underlying marine toxins means that international co-operation is essential. In 2003 the Marine Institute worked closely with partners from Europe, the USA, New Zealand and Japan and we look forward to building on these links in the years ahead. Key partners of MI include:

- The network of National Reference laboratories for Biotoxins in the EU
- Cawthron Institute, Nelson, New Zealand in the validation work on the LCMS tests for a range of biotoxins and in phytoplankton
- Woods Hole Oceanographic Institution, USA which is a world leader in oceanography and HAB research

Irish Molluscan Shellfish Exports, 1999 – 2002

I am delighted to report that the Irish shellfish industry has shown its resilience and potential in the past three years. As the Marine Institute and other agencies have invested in rebuilding our shellfish safety programme, the Irish industry has been able to win back and to develop new exports markets such as the USA.

BIM exports data show

Year	Tonnage	Value (€ million)
1999	14,000	32.8
2000	17,500	39.3
2001	22,300	43.5
2002	24,560	52.5

Ireland's investment in Shellfish Safety has supported a 60% resurgence in Irish exports of mussels, oysters and scallops since 1999.

REVIEW OF PHYTOPLANKTON AND ENVIRONMENTAL MONITORING 2003.

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This review provides a summary of the occurrence of potentially toxic and harmful phytoplankton found in Irish coastal and shelf waters in 2003 (January to November). The results below are derived from data collected by the Marine Institute as part of the National Phytoplankton and Biotoxin Monitoring Programme. In 2003 the number of phytoplankton samples analysed by the Marine Institute was approximately 1,800 of which ~1,700 were directly related to the National Monitoring programme.

Water samples were collected at shellfish and finfish production sites by the aquaculture industry. Where depth allowed, samples were collected using a Lund tube (5-15 m), otherwise surface samples were collected. Staff of the Department of Communications, Marine and Natural Resources (DoCMNR) were responsible for the management of this sampling programme. In 2003, the sampling programme was expanded to include offshore shelf sites sampled by the Irish Naval Service. At aquaculture production sites sample frequency was weekly during "high toxicity risk" periods (Spring to Autumn) and was reduced to monthly in winter when little or no phytoplankton growth occurs. Several sites, called "sentinel sites", were selected and were sampled on a weekly basis all year round. This provided information on the phytoplankton community structure and species succession throughout the year. Results were reported on a daily basis with over 250 reports being sent to the aquaculture industry and regulators by fax and e-mail in 2003. All results were also posted on the Marine Institute's Web site at www.marine.ie/habsdatabase.

Closures of shellfish growing areas as a result of biotoxin contamination in shellfish are common in the summer and autumn, when toxic algae are present. The duration of these closures varies from year to year. In 2003, there were relatively few closures compared to other years and most closures resulted from elevated levels of the biotoxins, Okadaic Acid and its derivatives, particularly DTX2, also known as Diarrhetic Shellfish Poisoning (DSP) toxins (Figure 1). Details of closures and toxin concentrations are provided in the paper by Clarke et al. in these proceedings. These toxins are produced by the dinoflagellates *Dinophysis spp.* and *Prorocentrum lima*. *Dinophysis spp.* were present at very low cell concentrations (0.04 cells.mL⁻¹) from January to May 2003. Elevated cell concentrations were detected in June with highest cell densities of 2.08 cells.mL⁻¹ recorded in Bantry Bay (southwest coast) on the 15th July. After this date, cell numbers declined and in November *Dinophysis spp.* were not detected in water samples collected (Figure 1). Diarrhetic Shellfish Poisoning toxins were present in shellfish in January, 2003. This is most likely the result of toxin carry over from the previous year. At the beginning of June, the first shellfish samples to contain DSP toxins at levels above the regulatory limit (0.16 µg.g⁻¹) were reported from sites off the northwest and southwest coasts (McSwynes Bay and Kenmare Bay). These toxins persisted in samples analysed in July and continued to increase in concentration with maximum levels of 0.57 µg.g⁻¹ recorded in shellfish from the southwest coast (Castletownbere, Bantry Bay) on the 27th August (Figure 1). The detection of *Dinophysis spp.* in water samples earlier in the summer, however, allowed time for the shellfish industry to make management decisions on harvesting. The availability of data on the occurrence of toxic phytoplankton also contributed to the Management Cell decisions detailed in the paper by Lyons and Coakley in these proceedings.

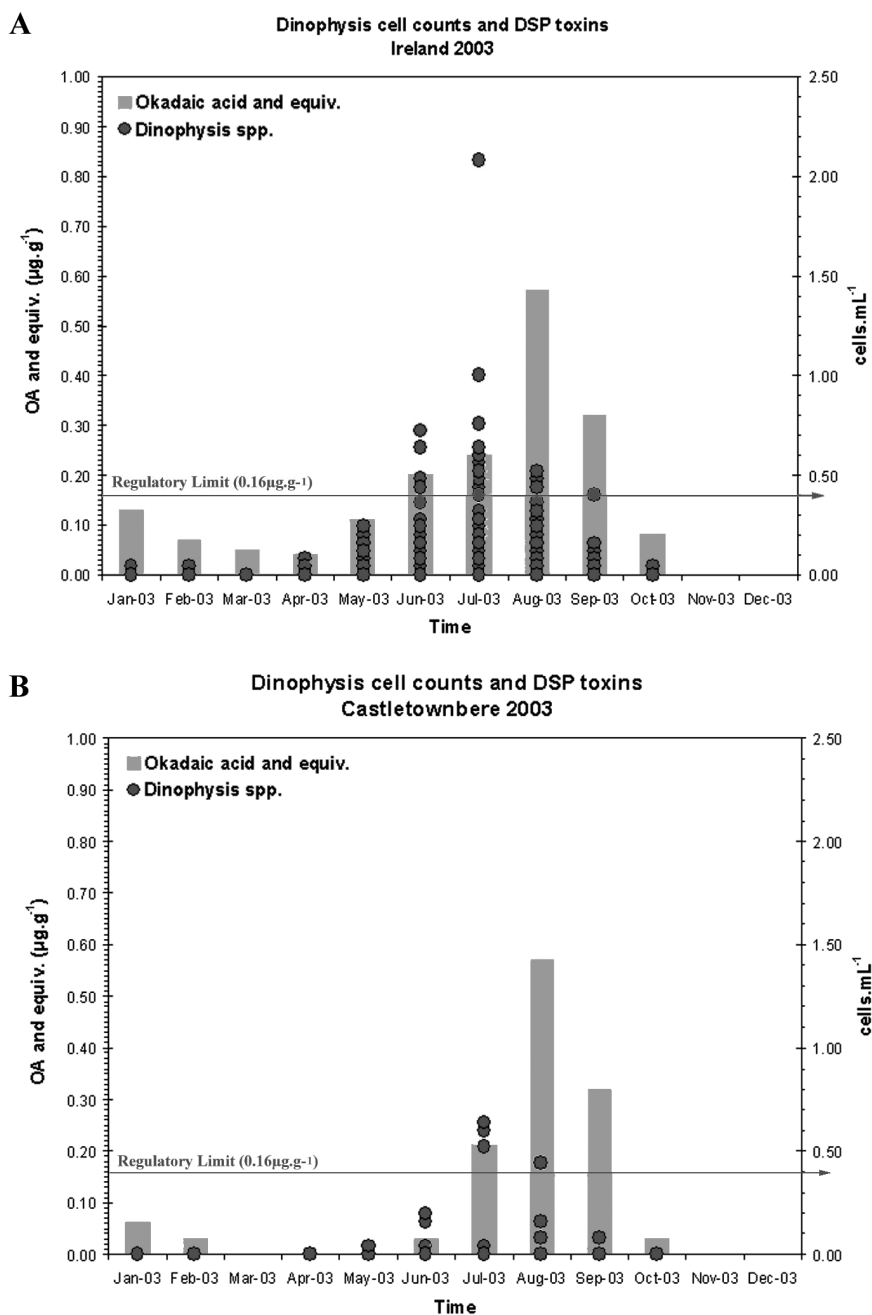


Figure 1. *Dinophysis* cell numbers and DSP toxins plotted against time. Data derived from samples collected at Aquaculture Production sites off the Irish coast (A) and in Castletownbere, Bantry Bay (B) in 2003.

Alexandrium spp. are also potentially toxic dinoflagellates, producing Paralytic Shellfish Poisoning (PSP) toxins and are frequently recorded in the phytoplankton populations in Irish waters (Figure 2). The presence of these dinoflagellates triggers the testing of shellfish samples for PSP toxins. The PSP mouse bioassay (AOAC Official Methods of Analysis, 1995) is used to detect these toxins and to date the only aquaculture area that has experienced closures as a result of PSP toxins is Cork Harbour (south coast) (Figure 2). In mid September 2003, shellfish sites in this area were closed as a result of a small bloom of *Alexandrium spp.* with highest cell concentrations in the order of 15 cells.mL⁻¹.

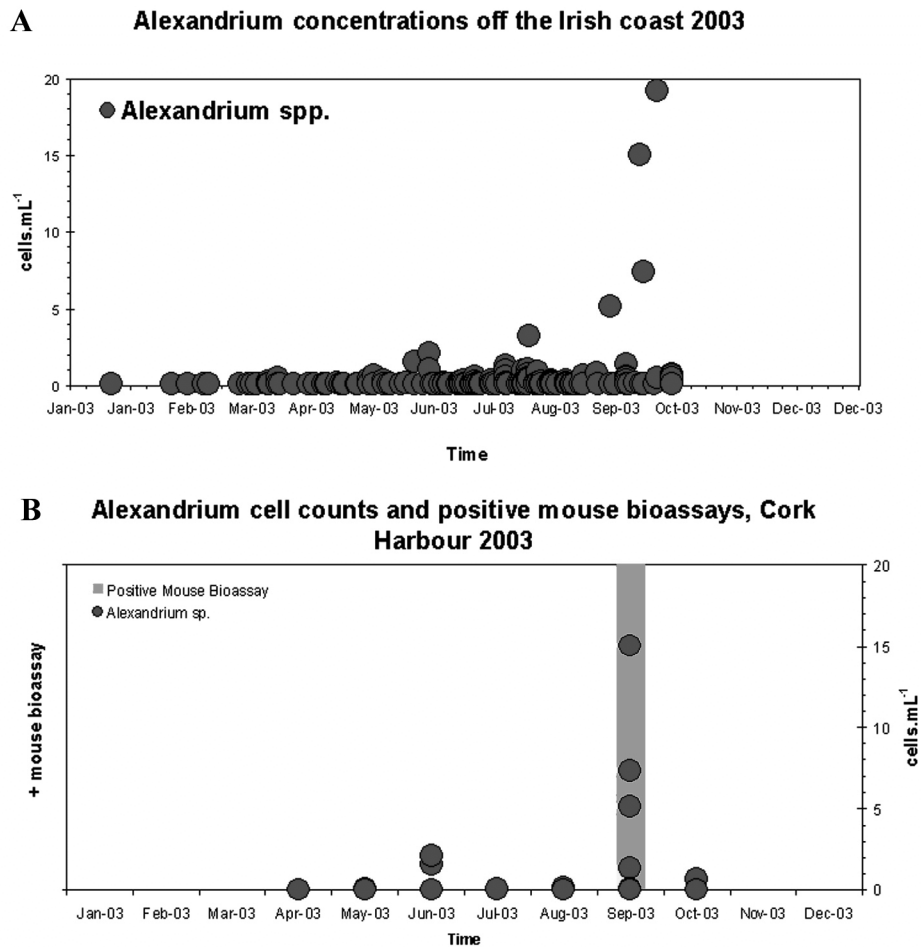
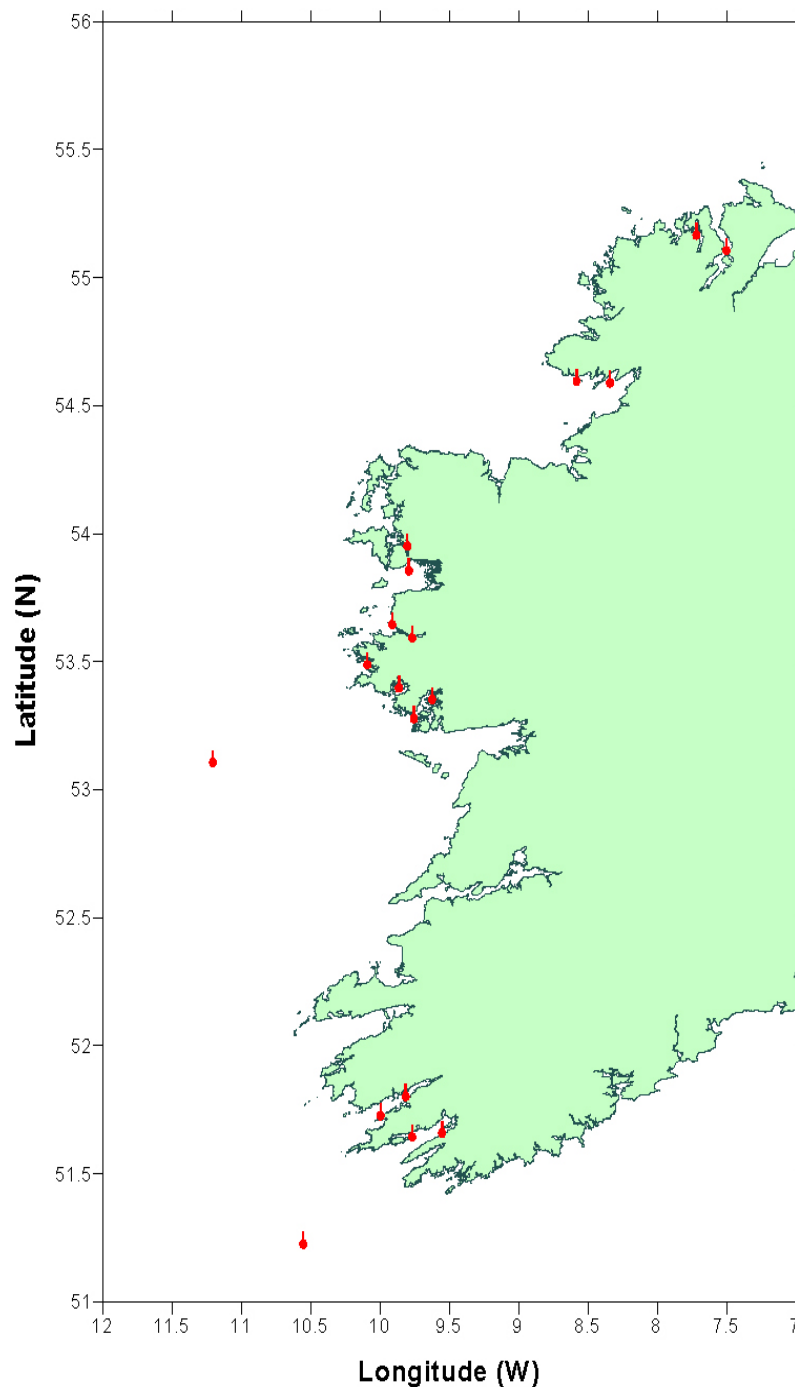


Figure 2. Scatter plot of cell concentrations of *Alexandrium spp.* recorded off the Irish coast plotted against time (A). Cell densities of *Alexandrium spp.* and positive PSP mouse bioassays recorded in Cork Harbour in 2003 plotted against time. Positive mouse bioassays were recorded on the 17th and 21st September (B).

Several phytoplankton species that are harmful to finfish were recorded in Irish waters in 2003. These included the heterotrophic dinoflagellate *Notilluca scintillans* which regularly blooms off the east coast in summer, *Karenia mikimotoi* and *Prorocentrum cf. balticum*. A bloom of *Notilluca scintillans* was observed from August to October, off the southeast to the southwest coasts. *Karenia mikimotoi* was observed at most stations off the Irish coast in April through to September, peaking in early autumn with maximum concentrations in the order of 56 cells.mL⁻¹ recorded off the west coast. A bloom of the dinoflagellate *Prorocentrum cf. balticum* (300 – 4,000 cells.mL⁻¹) occurred in the subsurface (4 m) brackish waters of Lough Furnace, Clew Bay (west coast) in October and Lough Swilly (north coast) in September.

Over the last few years the Marine Institute has deployed a number of temperature sensors (stowaway Tidbits) at numerous sites around the country (Figure 3). The time series generated from these sensors, that take measurements on an hourly basis, have provided useful information on the thermal structure of the water column at these sites. For example the temperature data collected off the northwest coast in Inver Bay in 2003 shows that there was a sudden drop in temperature (~3 °C) at the beginning of July. The dinoflagellate, *Oxytoxum caudatum*, generally associated with shelf waters, was observed in water samples collected in the bay at this time. The presence of this organism indicates that it was



transported from further offshore (Figure 4). Temperature data collected by the Marine Institute M1 Data Buoy, situated at 53°07.6' N, 11°12' W, shows that water temperatures off the west coast were up to 1°C higher in late summer 2003 than recorded in previous years. This compliments temperature records collected by Met Eireann at Malin Head (north coast) with highest temperatures above 16 °C in August. *Amphidoma caudata*, a dinoflagellate associated with thermally well stratified water (McDermott, 2002) was present in samples collected at the end of July / beginning of August when thermal stratification of the water column was well established in the area (Figure 4). Similar observations were also noted in the neighbouring McSwynes Bay (Figure 4). This highlights the fact that certain phytoplankton species can be indicative of particular water bodies and shows the importance of coupling oceanographic data such as temperature with records on the general trend of the phytoplankton composition.

Figure 3. Map showing the locations (mussel lines, salmon farms and oceanographic buoys) of internal logging temperature sensors around the Irish coast.

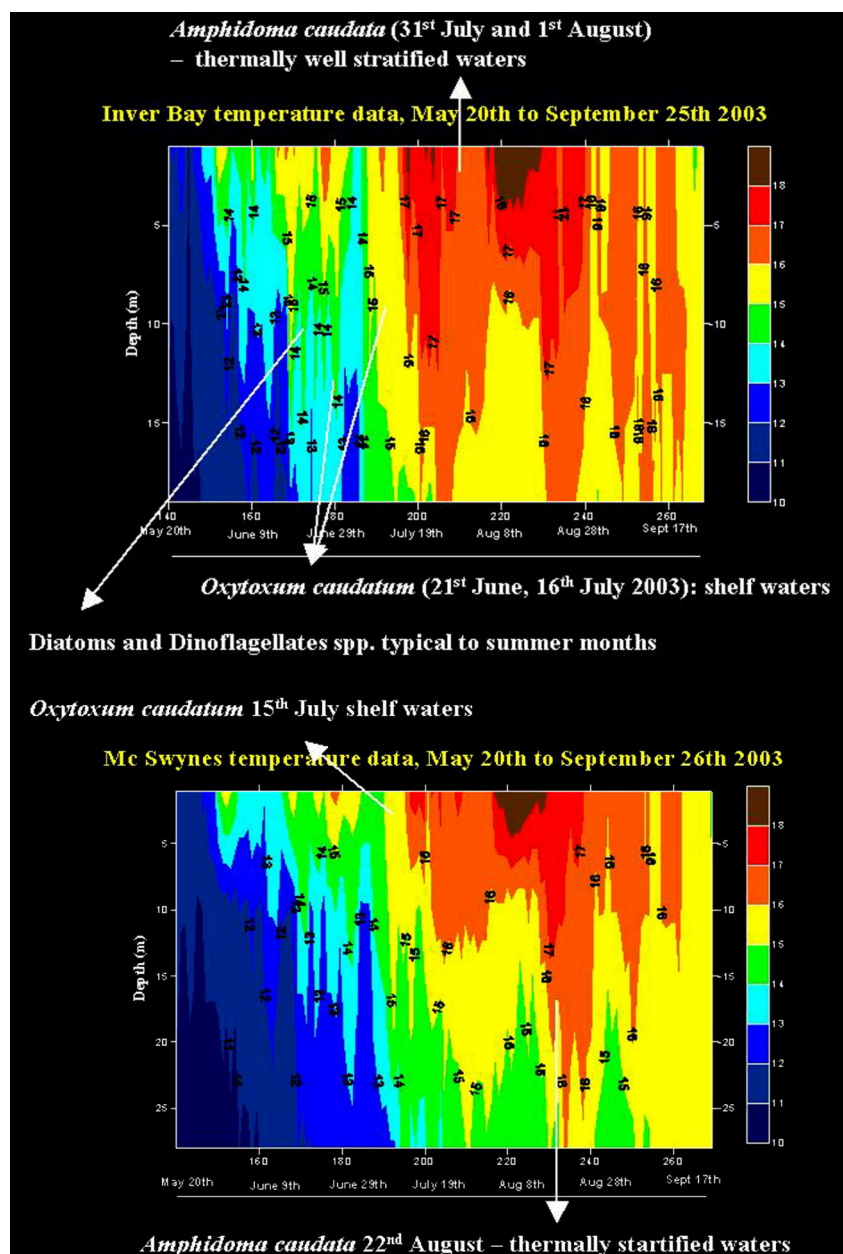


Figure 4. Water temperature plotted against time (x-axis) and depth (y-axis) at Inver Bay and McSwynes Bay in 2003. Temperature sensors were deployed at 1 m, 10 m and 19 m at the Inver Bay site and at 1 m, 10 m, 20 m and 28 m at the McSwynes Bay site.

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- McDermott. 2002. The Distribution of Net Armoured Dinoflagellates in the Continental Shelf Waters the North Eastern Atlantic. Ph.D. Dissertation. National University of Ireland, Galway. pp. 265.

A REVIEW OF SHELLFISH TOXICITY MONITORING IN IRELAND FOR 2003

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

Diarrhetic Shellfish Poisoning (DSP)

During 2003 (to end of October 2003) 2299 samples (2760 samples projected year end 2003 compared to 2854 for 2002) were submitted for DSP Bioassay analysis and chemical confirmatory analysis for the presence of Okadaic Acid equivalents (OA + DTX 2) and Azaspiracids (AZA 1, 2, 3). Mussel (*Mytilus edulis*) samples were submitted on a weekly basis while oysters (*Crassostrea gigas* and *Ostrea edulis*) were submitted on a monthly basis during winter months and a fortnightly basis during the summer months. A monthly testing frequency will be introduced for Razors (*Ensis ensis* and *Ensis siliqua*) from 1st December 2003, reverting to fortnightly during the summer periods.

Overall during 2003, (up to end October 2003) 3.6% of samples tested positive under DSP Mouse Bioassay (2299 samples analysed) compared to 3.4% over the same time period for 2002 & 17% for 2001. All the positive bioassay results were obtained in mussel samples and no oyster, cockle or clam samples submitted and analysed were positive for DSP/AZA during the same time period.

Figure 1 illustrates the percentage of positive samples analysed using DSP Bioassay during 2003. During January 1.8 % of samples analysed tested positive, probably due to carryover of toxicity from 2002. During February – May, no samples tested positive for the presence of DSP/AZP toxins. Towards the end of June the presence of DSP toxins was observed (1.02% of samples analysed were positive). DSP toxicity was observed to increase throughout July – September with the highest number of positives observed in September (9.68% of samples) falling to 3.61% in October. *Dinophysis* species were also observed to be present in the effected areas during this period.

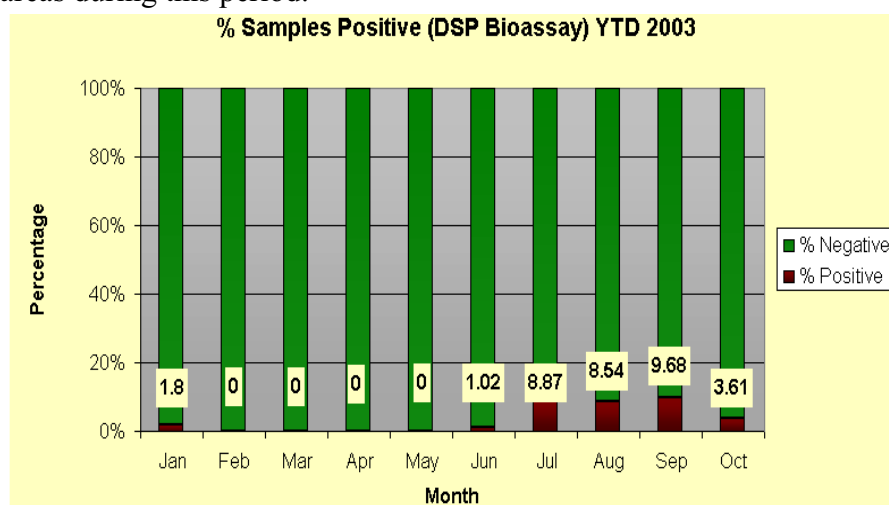


Figure 1. Percentage of Samples Positive for 2003.

Figures 2.1 – 2.4 illustrate the highest levels of OA equivalents observed in samples of mussels from June – September 2003, $>0.03\mu\text{g/g}$ total tissue. All other samples analysed had levels $<0.03\mu\text{g/g}$ total tissue. Highest levels of OA equivalents were observed predominantly in the south west.

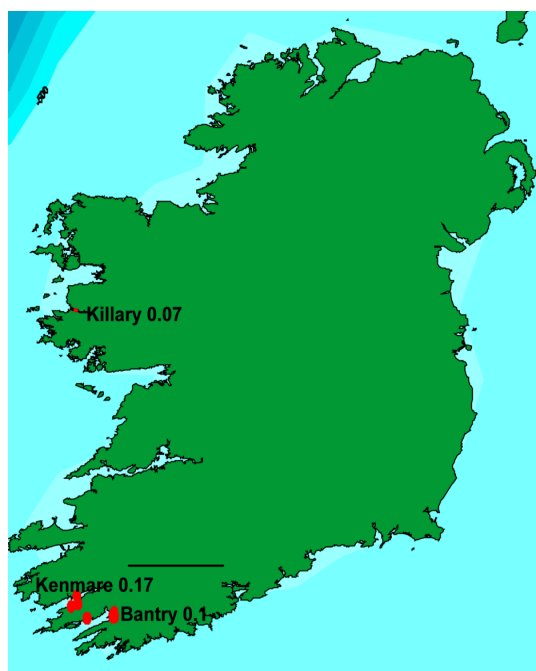


Figure 2.1 OA levels for June 2003

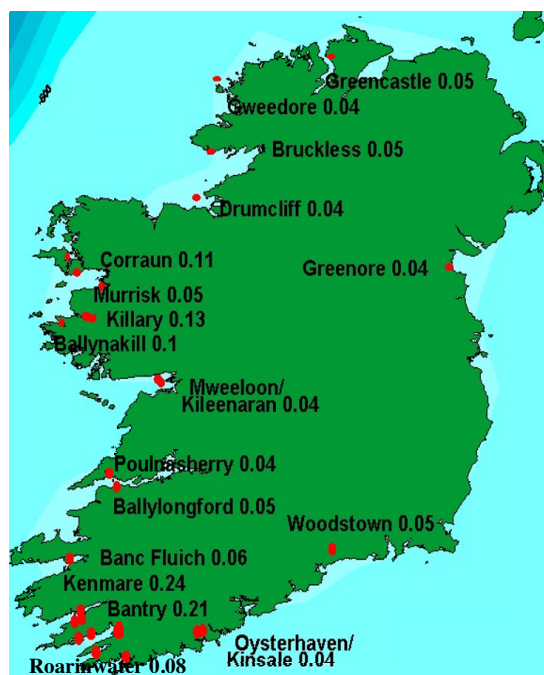


Figure 2.2 OA levels for July 2003

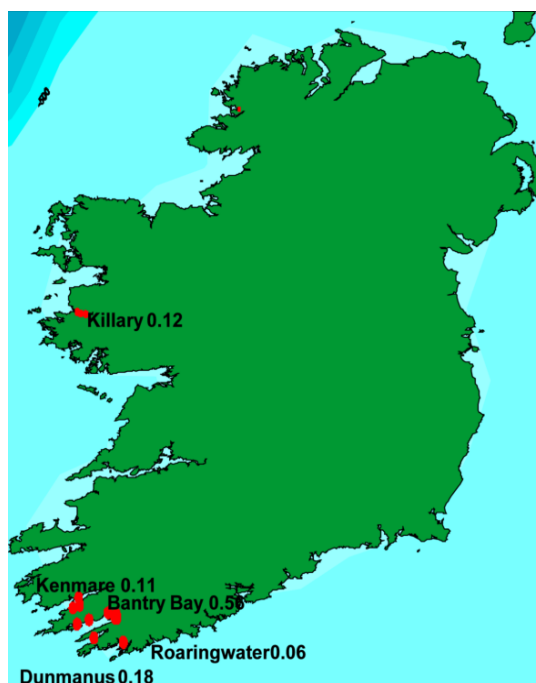


Figure 2.3 OA levels for August 2003

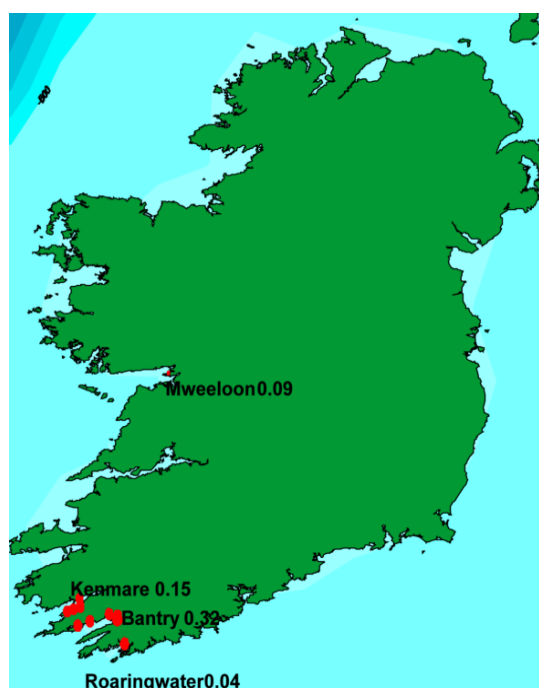


Figure 2.4 OA levels for September 2003

Paralytic Shellfish Poisoning (PSP)

During 2003 (up to end October 03), 112 samples (125 projected end of year) of shellfish were analysed for the presence of PSP toxicity from over 26 locations. Samples of shellfish species were requested from areas where *Alexandrium sp.* were observed in the water column. Figures 3.1 – 3.2, illustrate the locations where *Alexandrium sp.* were observed in 2003.

Two samples (*M. edulis* & *C. gigas*) from Cork Harbour in mid-September tested positive for the presence of PSP toxicity above the regulatory level. High concentrations of *Alexandrium sp.* (>15,000 cells/litre) were also observed at this time in the area. All other samples analysed tested negative.

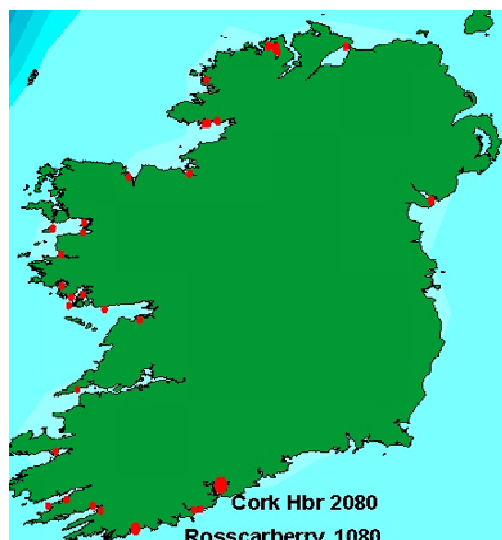


Figure 3.1 *Alexandrium sp.* Jan - Jun

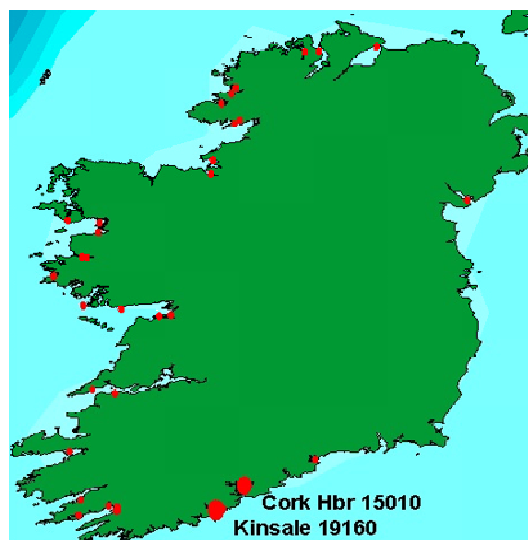


Figure 3.2 *Alexandrium sp.* Jul - Oct

Azaspiracid Shellfish Poisoning (AZP)

From January to October 2003 only two areas were closed due to the presence of Azaspiracids above the regulatory limit (>0.16 $\mu\text{g/g}$), with positive bioassays. These were Bruckless (September 2003) and Inverin (October 2003). Figs. 4.1 – 4.2 illustrate AZA levels >0.03 $\mu\text{g/g}$ total tissue observed nationally between September & October 2003. All other samples analysed during this time period were <0.03 $\mu\text{g/g}$ total tissue.

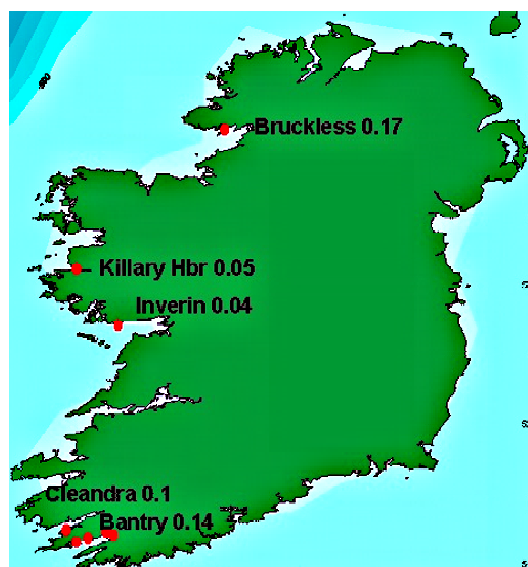


Figure 4.1 Azaspiracid levels for September

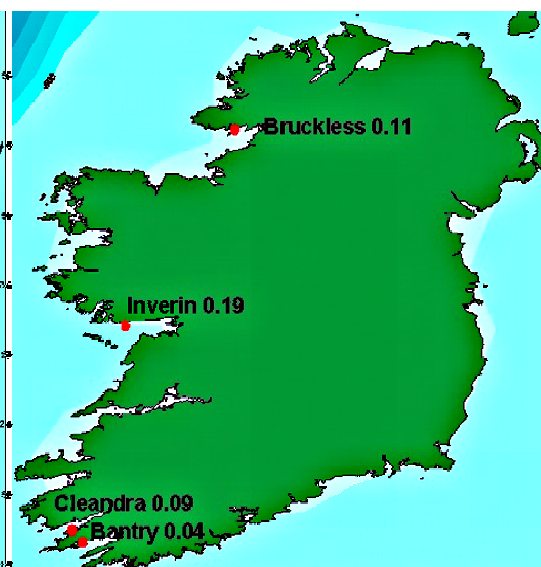


Figure 4.2 Azaspiracid levels for October

Amnesic Shellfish Poisoning (ASP)

In 2003 (up to end August 2003), 492 analyses of scallops had been conducted for the presence of Domoic Acid (DA) & Epi-Domoic Acid.

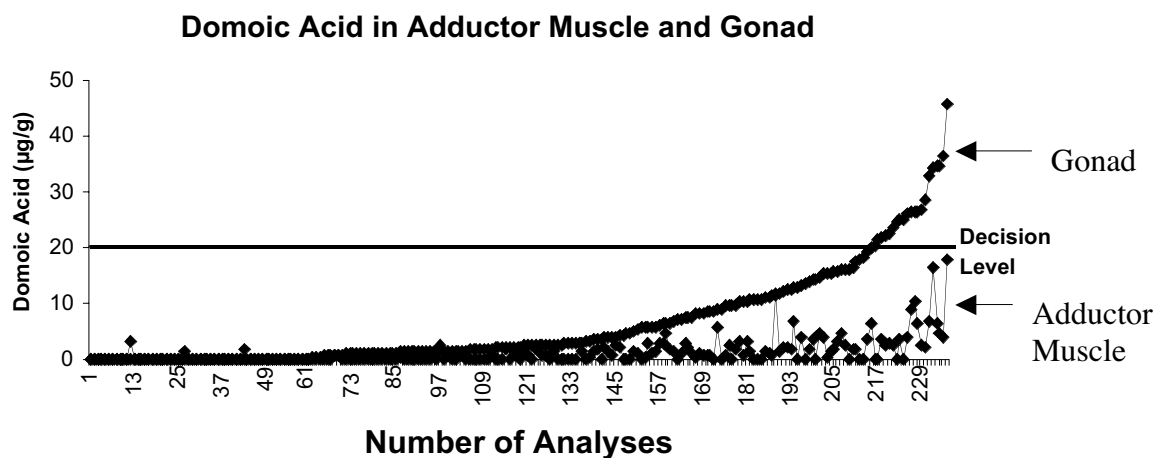


Figure 5. Comparison of DA in Adductor Muscle and Gonad tissues in scallops for 2003

Figure 5 illustrates the comparison of DA levels in Gonad and Adductor Muscle tissues. Approximately 8.8% of gonad tissues analysed (compared to 10% for the same time period in 2002) had levels of DA greater than the regulatory limit ($>20 \mu\text{g/g}$), with the maximum level observed $45.5 \mu\text{g/g}$.

For the adductor muscles analysed, no samples were observed to have levels of DA above the regulatory limit (compared to 2% for the same time period in 2002), with the maximum level observed $16.5 \mu\text{g/g}$. For the “Remainder” tissues, 79% analysed were observed to have levels above the regulatory limit (compared to 31.7% for the same time period in 2002), with the maximum level observed $300 \mu\text{g/g}$. Up to the end of August 2003, 21 *M. edulis* samples were analysed for DA presence, and were all below $20 \mu\text{g/g}$ Total Tissue, with the maximum level observed $7.5 \mu\text{g/g}$.

Report Turnaround

Of the 2115 samples analysed (up to September 2003) for DSP/AZP & PSP, 91.4% of samples were reported within less than / equal to 3 days (illustrated in Figure 6) from initial lab receipt in bioassay laboratories (74% reported within 2 days).

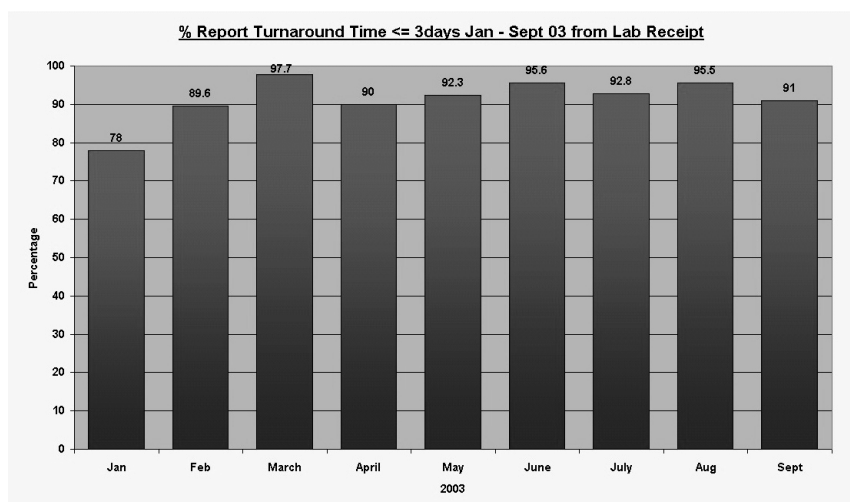


Figure 6. Percentage Total Report Turnaround for DSP/AZP/PSP for 2003

A total of 84.1% of these samples analysed chemically for OA equivalents & AZA's had a report turnaround time of 2 days or less. (illustrated in Figure 7).

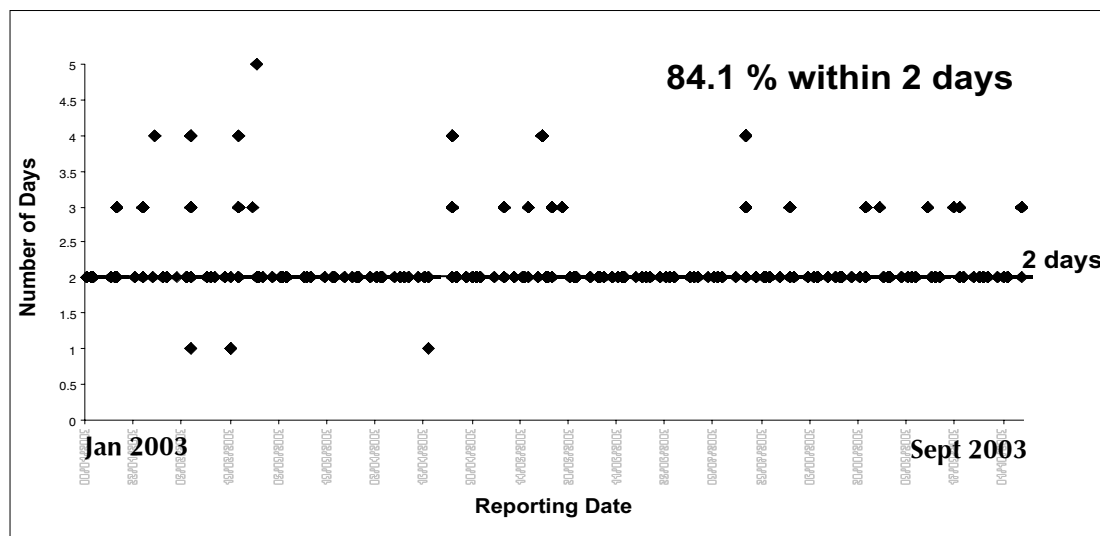


Figure 7. Percentage of Report Turnaround for OA & AZP chemical analysis

To ensure a high level of report turnaround, it is essential, where possible, that all samples arrive into the bioassay laboratories before or on Wednesday of each production week. It was observed that for 2003 on average 16% of samples received each production week arrived after Wednesday.

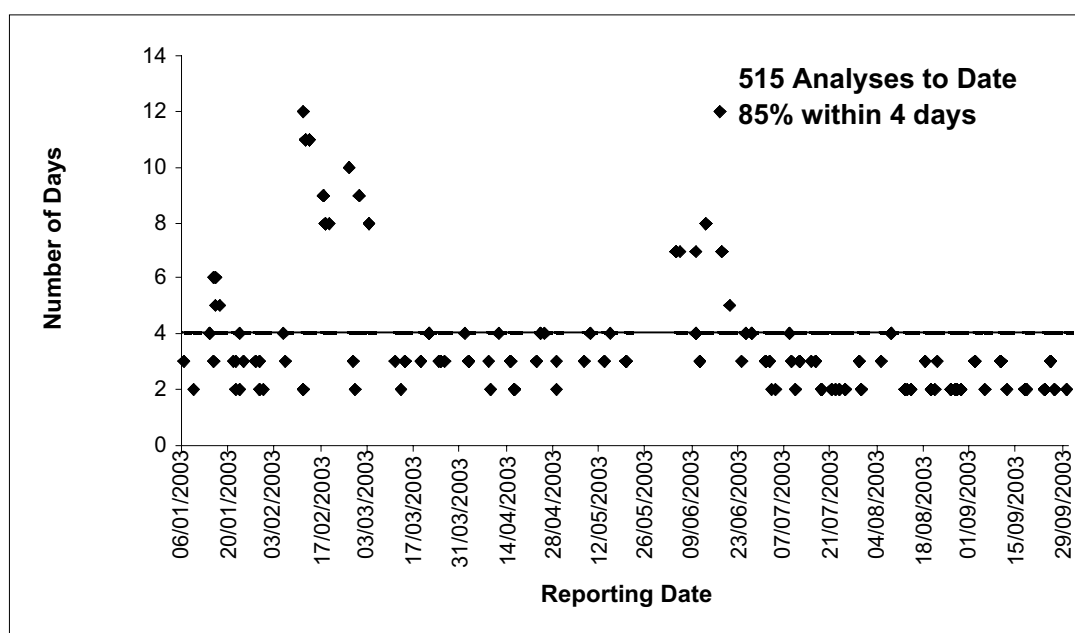


Figure 8. Report Turnaround

Figure 8. illustrates the report turnaround time for ASP analysis, with 85% of samples reported within 4 days. Following the transfer of all ASP analysis from the MI in Dublin to the MI Galway facility in July 2003, and the commissioning of a new HPLC, the turnaround improved, with the majority of samples reported within 2-3 days.

Comparison: Bioassay & Chemical Analysis

The bioassay results were compared to the chemical results for OA & AZP (n= 2299 samples Jan – Oct 2003), illustrated in Figure 9. Overall a 97.5% agreement was obtained between the two methods (compared to 98.8% for 2002).

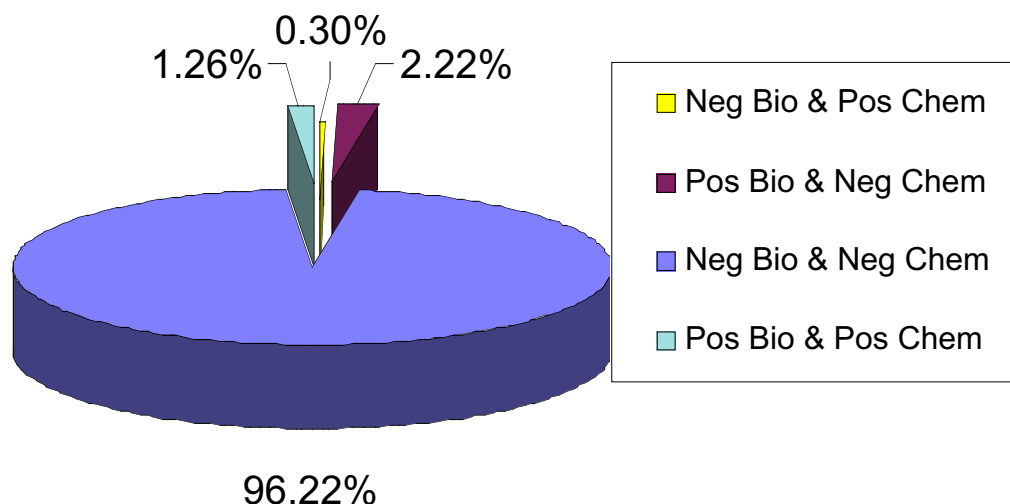


Figure 9. Comparison of Bioassay vs. Chemical Analysis (n=2299)

A total of 2.22% of samples (n= 51) were observed to have given positive bioassay results with okadaic equivalent (OA & DTX2) levels $<0.16 \mu\text{g/g}$ (compared to 0.96% in 2002). A total of 25 of these 51 samples were subsequently reanalysed for the presence of DTX3.

The analysis of DTX-3 requires an additional hydrolysis step and then re-analysis via LC-MS, which significantly lengthens the analytical procedure.

When these 25 samples were analysed, 22 were found to have total OA, DTX 2 & DTX 3 concentrations above the regulatory limit ($>0.16 \mu\text{g/g}$), therefore the presence of DTX-3 explains the conflict of results between bioassay and chemistry in 88% of the samples re-analysed.

Fig. 10 illustrates the percentage of correlations between the two methods pre and post DTX-3 analysis, where it is observed the original 2.22% non-correlation was reduced to 1.26%.

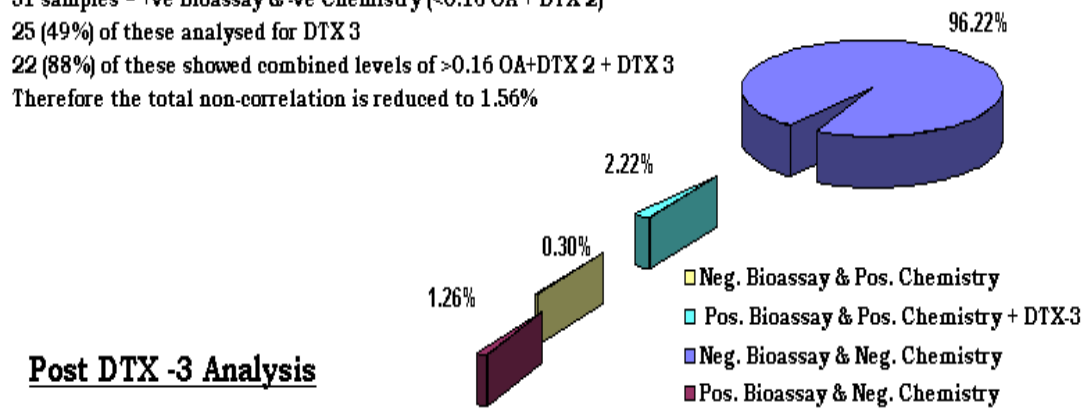
51 samples = +ve Bioassay & -ve Chemistry (<0.16 OA + DTX 2)

25 (49%) of these analysed for DTX 3

22 (88%) of these showed combined levels of >0.16 OA+DTX 2 + DTX 3

Therefore the total non-correlation is reduced to 1.56%

Post DTX -3 Analysis



Pre DTX -3 Analysis

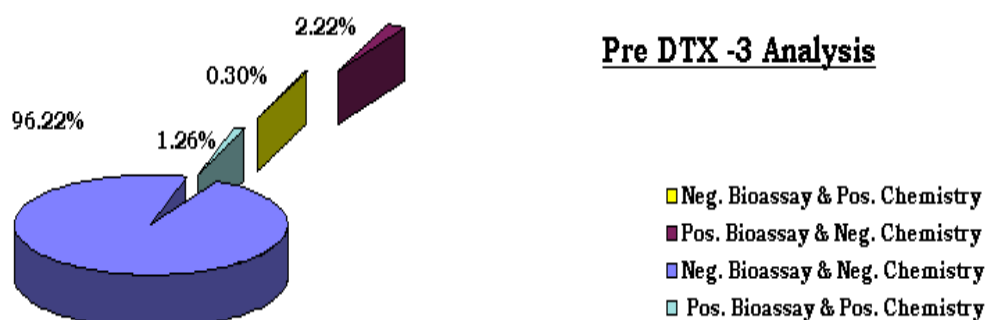


Figure 10. Comparison of Bioassay vs. Chemical Results Pre & Post DTX-3 Analysis

In total 111 analyses were performed for the presence of DTX-3. In those samples where DTX3 was detected it accounted for on average 38.7% of total DSP toxicity and ranged from 12% - 75%.

Communications

In 2003, MI staff actively participated with DCMNR, FSAI & ISA in Management Cell decisions (see paper by Lyons and Coakley in these proceedings) MI staff also worked closely with the DCMNR Shellfish Sample Co-ordinator and improvements were made in areas of sample labelling, sample rejections, samples not being taken and the timing of samples arriving into laboratories.

The Harmful Algal Blooms Database (HABS) was fully implemented in February 2003. HABS is a centralised database where regional laboratories input results of samples submitted for the National Biotoxin & Phytoplankton Monitoring Programmes and reports are generated and issued via fax, e-mail, SMS Text Message and on the web.

The online database, which is available at <http://www.marine.ie/habsdatabase> provides up to date information on the presence of toxic phytoplankton and biotoxins in shellfish growing areas around Ireland.

Results provide information on the production area, date, species of concern, bioassay results, Okadaic Acid equivalent and , AZA concentrations analysed by LCMS and PSP results as well as the operational status of the bay. Phytoplankton & ASP reports are also available. Results can be viewed by Report Name, or by Production Area. HABS also provides access to historical data on biotoxins and the operational status of bays around Ireland.

In 2004, it is proposed to provide a mapping service online, which will allow users to specifically query the HABS database for data of interest from the Biotoxin Monitoring Programme, and which can then be plotted in map and chart form. Temperature data from the TidBit Monitoring Programme will also be made available online.

The online availability of this information further improves the communication of results and ensures that up to date information is provided in an efficient and effective manner.

Quality System

In May 2003 the MI Biotoxin Chemistry Unit achieved accreditation for ASP analysis via HPLC in the Dublin laboratory from the Irish National Accreditation Board (INAB). In September 2003 applications were submitted for accreditation for ASP analysis in Galway and Okadaic Acid analysis by LC-MS in Dublin.

In January 2003 the MI Biotoxin Bioassay Unit in Galway participated in the EU- National Reference Laboratory Intercomparison exercise for PSP analysis and obtained satisfactory results. In May 2003, MI conducted 2 DSP Bioassay intercomparison exercises, with chemical confirmatory analysis. One exercise was held internally to assess individual staff performance, and the second exercise with the 2 regional Bioassay labs. In both exercises all results obtained were satisfactory and favourable.

In August 2003 the MI Biotoxin Bioassay Unit in Galway submitted an application for the accreditation of the DSP Bioassay Test Method to INAB. In October 2003, work commenced on the validation of the PSP Bioassay Test Method, with the aim of submitting an application in early 2004.

REVIEW OF MANAGEMENT CELL OPERATIONS, 2003

David Lyons¹ and Tim Coakley²

¹Food Safety Authority of Ireland

²Irish Shellfish Association

The statutory functions of the Food Safety Authority of Ireland are to:

- Co-ordinate the enforcement of food legislation at national level.
- Take all reasonable steps to ensure that food produced, distributed or marketed in the State meets the highest standards of food safety and hygiene, reasonably available.
- Ensure that food complies with legal requirements, or where appropriate with recognised codes of good practice.

Within this context and following on from the biotoxin taskforce, the Molluscan Shellfish Safety Committee (MSSC) was set up with the FSAI as chair. The aim of the MSSC is the protection of human health with the view to maintaining the excellent reputation of the shellfish industry.

As a national forum for all involved in the production and placing on the market of bivalve molluscs, it meets to discuss the safety of the product and the management of the industry from a consumer protection perspective. The main objective of the MSSC is the protection of consumer health in the areas of biotoxin, microbiological and virological contamination of shellfish.

Apart from the FSAI, membership of the MSSC includes the Department of Communications Marine & Natural Resources (DCMNR), the Marine Institute (MI), producers, processors, BIM, laboratories, and the Health Boards.

Some of the work to date of the committee has included mapping of production sites by the MI, improved communications (SMS, Fax, Website), refined methodologies, formalised sample management (by DCMNR), improved co-ordination between stakeholders, improved phytoplankton sampling, and improved risk management. It is under this last point that the work of the Management Cell (MC) falls.

The MC is a risk management tool, it is not a replacement for formal sampling nor is it a “court of appeal” or replacement for the MSSC. Its aim is to proactively manage the risk presented by marine biotoxins by facilitating rapid decision making in non-routine situations. Due their predominance and particular risk profile in relation to biotoxins, much of the work of the MSSC has focused on rope mussels, although some work has also been done in relation to oysters and razor clams.

The MC is generally asked to deal with such issues as borderline or out of character biotoxin results or prolonged borderline toxicity events. It comprises of one representative each from the FSAI, DCMNR, the MI and the producers.

In the risk management process, preliminary risk management activities are undertaken by the MI, DCMNR and the producers in the form of sampling, analysis, etc. In the event of the Cell being called upon, evaluation of options is carried out by the entire group. Any decision is implemented by the producers with necessary oversight provided by DCMNR, with the whole group and the MSSC, providing input into the monitoring and review of the system and the decisions it makes.

When considering its decisions the MC takes into account such factors as the species of bivalve mollusc, the details of the bioassay, any chemistry or phytoplankton result, the time of year or risk profile of the area, the status of adjacent areas, as well as any other relevant data or data analysis reports.

The outcome of the MC deliberations can range from no action or no change, through to changing an area's status. The MC may also recommend a voluntary closure or other voluntary actions to producers, or increase or reduce sampling frequency.

Decisions are arrived at, on a consensus basis, but where representatives feel they are unable to agree, the position of the FSAI is adopted in line with the Management Cell's brief as an instrument of consumer protection.

Between November 2002 and November 2003, 37 Management Cell Decisions were issued. There was on decision each where precautionary advice was issued, and the sampling frequency for oysters was changed. A total of 27 decisions dealt with situations where an assigned, or provisional status had been issued for a production area (see table).

Original Status	Decision	Frequency
Open	Closed	2
Open	Closed Pending	0
Closed	Open	6
Closed	Closed Pending	3
Closed Pending	Open	12
Closed Pending	Closed	0
No Change in Status		4

The remaining 8 decisions related to situations where a chemistry or bioassay result was available in isolation (typically because one or the other had been delayed) and a decision was made whether or not to allow harvesting to proceed.

For the industry it has meant that most of the decisions to date have facilitated early harvesting and an opportunity to correct genuine errors in the system. This allows producers to have a degree of control over the way their industry is managed.

To date the MC has been a qualified success, but some work remains. Before the next Workshop, a number of improvements are planned including improving the monitoring and review part of the process, better "housekeeping" to gather more information about decisions and better tracking of data. Efforts will also be made to plug gaps in the data (especially in the area of phytoplankton) and to improve access to non-ISA members.

ISOLATION AND PURIFICATION OF AZASPIRACIDS FROM NATURALLY CONTAMINATED MATERIALS AND EVALUATION OF THEIR TOXICOLOGICAL EFFECTS (ASTOX)

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Introduction

Azaspiracids (AZAs) were detected for the first time in mussels from Ireland in 1995. Since their initial detection, however, only very limited studies on the toxicity of (AZAs) have been carried out due to the limited supply of pure AZAs.

A three-year, collaborative research project, called ASTOX, involving the Biotoxin Unit of the Marine Institute and the Conway Institute, UCD began in January 2003 with the aim of isolating and purifying AZAs and evaluating their toxicological effects. The project also involves the isolation of DTX2, which can often be the dominant DSP toxin in shellfish in Ireland and for which few toxicological data are available. Progress to date on this project is outlined below and further information on the project is available at:

http://mdciis02/Marine+Institute/Funding/Marine+Institute+Funding/Marine+RTDI+Fund/Stategic+Marine+RTDI/ST_02_02.htm

Work carried out at the Marine Institute

Work at Marine Institute focused on the four work packages of project management, retrieval of contaminated materials, preparation of reference materials and preparative isolation of AZAs.

The objectives of these modules are:

- (i) Effective integration of work-packages and project progress.
- (ii) Retrieval of sufficiently naturally contaminated shellfish to prepare reference materials and isolate AZAs and DTX-2.
- (iii) Preparation of homogenous, stable reference materials for AZAs and DTX-2
- (iv) Isolation of AZAs and DTX-2 in sufficient quantities for toxicology studies and for calibration standards for method development and validation

A post-doctoral research scientist and 2 post-graduate students were recruited. The project partners met for their first steering committee meeting in May 2003. A first intermediate report has been prepared and the second steering committee meeting is scheduled for June 2004.

Contaminated shellfish have been obtained from the Southwest of Ireland and from Norway. While samples from Norway were contaminated with okadaic acid and azaspiracids, the levels in samples from Ireland were shown to be more appropriate for the preparation of reference materials and isolation studies. In total, ca. 2.4 tons of shellfish of various levels of contamination have been sourced as part of the project.

Reference materials contaminated with AZAs and DTX-2 have been prepared at pilot (0.5 – 1 kg) scale and medium (3 kg) scale. For five materials the homogeneity was assessed to yield coefficients of variance (CVs) between 5% and 35% depending on the analyte and concentration. It was noted that for concentrations between 0.01 and 1 µg/g CVs lay between 5 and 20 %, while for concentrations below 0.01 µg/g CVs typically exceed 20%.

The isolation of azaspiracids has yielded ca. 650 µg AZA-1 so far. This amount would suffice to conduct routine monitoring of azaspiracids by LC-MS in one laboratory for 80 years, or in eighty laboratories for 1 year. The purity of the AZA isolated is ca. 50 % and some additional purification may be necessary for this material to be used in toxicology studies.

Work carried out at the Conway Institute, UCD

Work in the Conway Institute, UCD focused on the mode of action and *in vitro* determination of AZA as part of Work Package 4:

The objectives are:

- (v) to establish the mode of action of AZA to provide an understanding of the basis of the toxicity and means to prevent it
- (vi) to establish an *in vitro* test as an alternative to the *in vivo* mouse bioassay

As the main symptoms of AZA toxicity in humans are gastrointestinal disturbances the use of human intestinal cells was chosen for toxicological studies rather than the mouse system as this may be much more relevant to the human toxicity induced by AZA.

The human colon cell line - Caco-2 cells - were selected for these studies due to their ability to form tight junctions and generate a transepithelial electrical resistance (TEER). When Caco-2 cells are grown on microporous membranes they form an intact monolayer similar to the *in-vivo* gastrointestinal tract. The intactness of the monolayer can be measured as TEER. The TEER reflects the barrier function of the gastrointestinal cells. The TEER measurement is therefore a useful index of the function of these cells in maintaining the transport of solutes and water.

The Caco-2 cells were cultured on microporous filters under standard conditions in the laboratory. TEER was measured using an electrical resistance measurement device. TEER was normalised to the area of the filter after removal of background resistance of a blank filter on which cells were not seeded and which contained only medium. TEER was thus measured as ohms x cm² (Ω.cm²). Varying concentrations (0 –100 nM) of AZA were added for different time-points of 24, 48 and 72 hours.

The results were expressed as the change in TEER with respect to time matched controls TER (Ω.cm²). No significant change in TEER was observed at concentrations below 10 nM. However, significant reductions in TEER were detected at 10 and 100 nM. These results are shown in Figure 1.

This assay has proved to be sensitive for detection of AZA. The level of 10 nM would be analogous to a concentration of 0.0084 µg/g. However, it must be stressed that we are using pure AZA whereas in the real life situation dilution factors and possible breakdown in the gastrointestinal tract would have to be taken into account.

Additional studies ongoing in the laboratory include:

- (i) precise determination of no observed adverse effect levels (NOAEL) and EC₅₀ values
- (ii) determination of mechanism of action of AZA
- (iii) comparison of effects in the presence and absence of other marine toxins

In the future, we intend to carry out gene microarray experiments to detect alterations in gene expression and address the issue of possible carcinogenicity. In the longer term, it is intended to carry out a comparison of the in vitro TEER assay with the existing mouse bioassay.

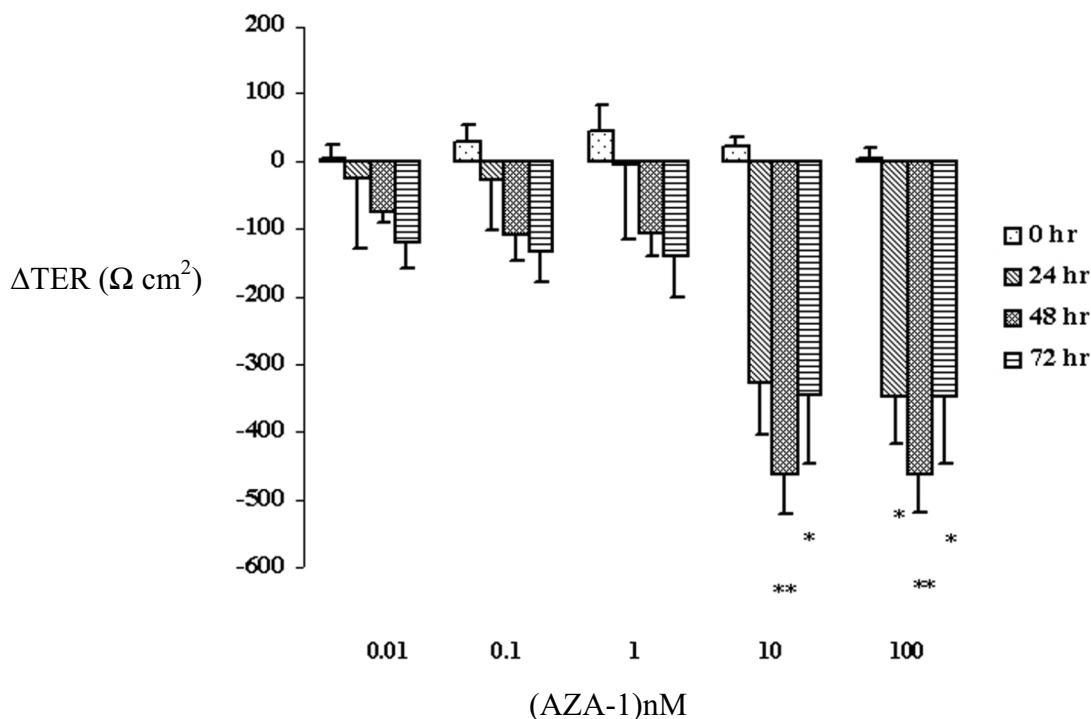


Figure 1. Effect of azaspiracid-1 on the transepithelial electrical resistance across caco-2 cell monolayers using the Endohm-6 apparatus. Cells were grown to confluency on Costar Transwell filters and treated with increasing concentrations of azaspiracid-1 for periods up to 72 hours.

Acknowledgements



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Marine Institute
Foras na Mara

RAPID AZASPIRACID SHELLFISH TOXIN ANALYSIS (R.A.S.T.A.) CULTURING OF PROTOPERIDINIUM SPP.

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Marine Institute, Parkmore, Galway

The shellfish toxin Azaspiracid (AZA) was first detected as the cause of poisoning that occurred in the Netherlands in 1995, after the consumption of mussels that were harvested in Ireland. From a phytoplankton sample taken in coastal waters off SW Ireland in 1999, the armoured heterotrophic dinoflagellate *Protoperidinium crassipes* was identified as being the source of AZA. (Yasumoto et al, 2002). This toxin is now known to be present in several Irish and other EU locations.

In September 2002, a research programme ‘**Rapid Azaspiracid Shellfish Toxin Analysis** (RASTA) was established, the main aim of which is the development of a rapid assay for the detection of AZA in shellfish. The project is a collaboration between the Marine Institute and Veterinary Sciences Division (VSD) of the Department of Agriculture and Rural Development (DARD) in Northern Ireland, and is funded by Food Safety Promotion Board. There is also collaboration between MI and Woods Hole Oceanographic Institute in Cape Cod.

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

1. Harvesting samples of bulk phytoplankton and extracting AZA toxins present.

This method can be time consuming and costly. Also if toxins are detected the source and the quantitative yield is not known. Chemical analysis was carried out on phytoplankton samples collected along the Irish West Coast, onboard the R.V Celtic Voyager in July / August 2001 and showed the presence of AZA at varying levels at all stations (Hess et al, 2002). However this method may not always supply a yield of toxins, as AZA was not detected in any samples taken during similar surveys in 2002 and 2003.

2. Collection and culturing of *Protoperidinium* species producing AZA toxins.

The culturing aspect of RASTA is concentrating on this method. As more than one species from a genus can produce different toxins of human health significance, it may be the case therefore that more than one *Protoperidinium* species may also produce AZA. Culturing under lab conditions allows us to be species specific and will help us to understand the role of food sources and culture conditions in the toxin production process. It also provides a continuous and reliable source of the toxin, if detected.

Culturing Heterotrophs

Some *Protoperidinium* species need an external food source for nutrition, and use various methods for consuming their prey. The capture method used by the *Protoperidinium*, involves circling around the potential prey and connecting to it by a filament. The *Protoperidinium* then pulls the prey toward itself, and using what is called a pallium or feeding veil, engulf their prey and extract the nutrients externally. The veil is then retracted into the cell.

Protoperidinium's own mechanism of swimming allows certain limited movement. In order to help them capture their prey more easily a slowly rotating phytoplankton wheel was designed and developed. A second more user-friendly wheel was adapted in Sept 2002 giving increased capacity. These wheels have proved invaluable not only in the lab, but also especially on the research vessels, where samples were collected and where initial treatment of cells is very important. Cultures of potential food sources were also established prior to the collection of *Protoperidinium*.

Collection of Samples

Due to the low level of AZA and *Protoperidinium* in Irish Coastal Waters since the commencement of RASTA, the only successful method of collecting *Protoperidinium* cells has been the use of multiple vertical plankton net hauls at off-shore stations, in shelf waters west of Ireland. This method was used during the R.V.Celtic Voyager cruise of 2002 during which 87 samples were collected from 21 stations (Figure 1) on the West Coast of Ireland. In order to maintain cell viability on board, samples were continuously rotated on the plankton wheel in the incubator. In 2003, 62 stations were sampled and replicate samples were taken at each station. *Protoperidinium* was present in sufficiently high numbers in samples from 9 of these stations (Figure 2) for isolation studies. Again cell viability was maintained by continuously rotating samples in the incubator.

Following these sampling cruises, all samples were returned to the Marine Institute laboratory in Galway where isolation work immediately commenced. Cells of all *Protoperidinium* species present were isolated using a micropipette and cultures. Initial cell numbers were very low. Further re-isolations of cultures were carried out using micro-pipetting or a pouring technique.

A network of contacts has also been established around the coast to respond, if necessary, to increases in AZA levels. Samples can be requested from Sea Fishery Officers from DCMNR, Marine Institute Sea lice Samplers, MI staff in Bantry and the Irish Naval Service.

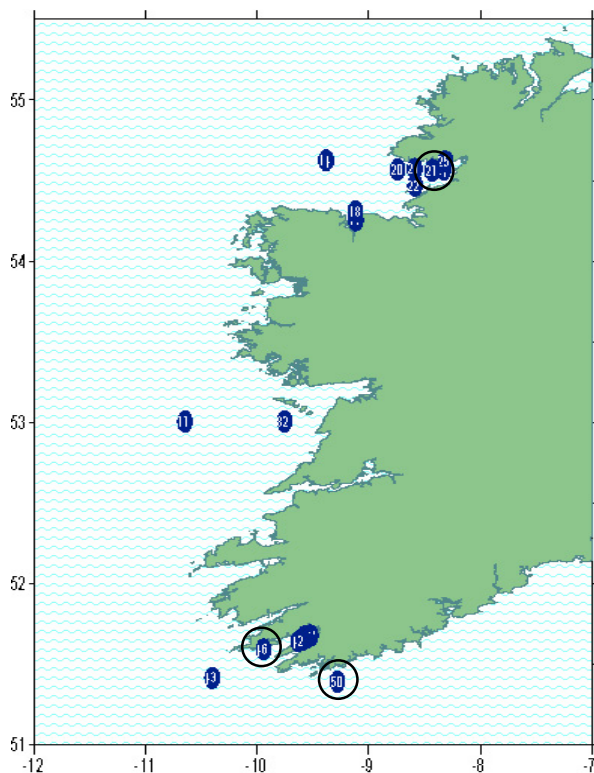


Figure 1
Phytoplankton Sampling Stations
July/August 2002 (Successful Culturing
from Stations 27, 46 and 50 (circled))
In 2002, 87 samples taken from 21 stations
during Phytoplankton Cruise

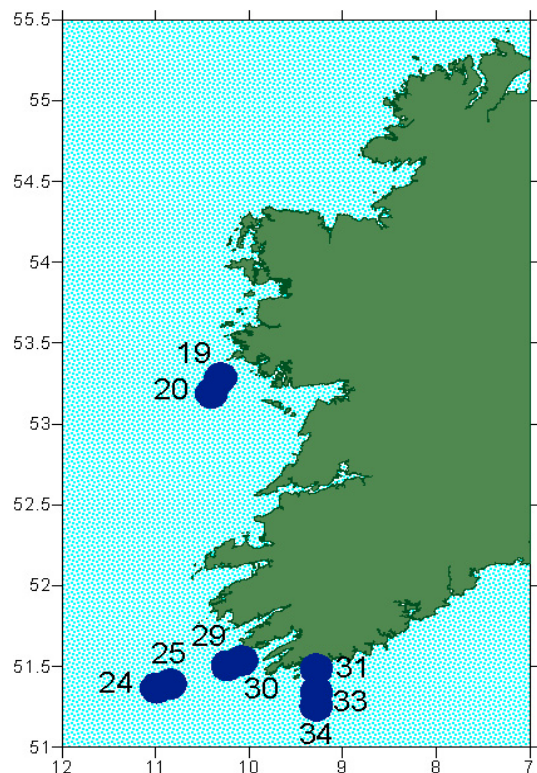
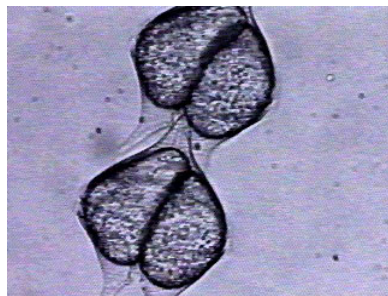


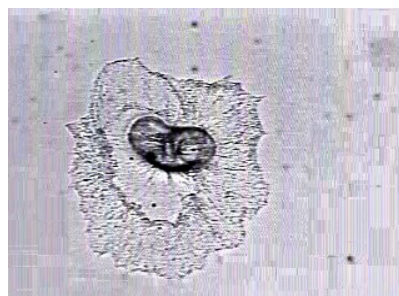
Figure 2
Phytoplankton Sampling Stations July
2003.(Successful Culturing from
Stations 24, 29 and 30)
In 2003, replicate samples from 62
stations during BOHAB cruise

Results

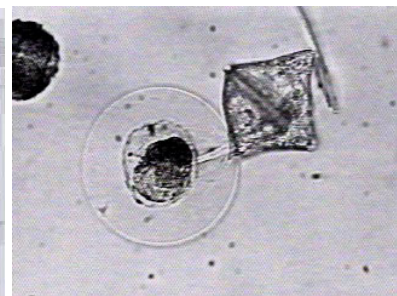
A number of different phytoplankton species have been tested as food sources for *Protoperidinium*. Initial experiments in 2002 tested various diatoms as a food source, and *D. brightwellii* proved most successful, for the species of *Protoperidinium* in culture at the time. After the summer sampling cruise of 2003, it was decided to concentrate only on cells resembling *P. crassipes*. The food source *L. polyedrum* was used exclusively. This is a documented preferred food source for a number of *Protoperidinium*, including *P. crassipes* (Jeong & Latz 1994) The *L. polyedrum* inoculum was sourced from the Instituto Español de Oceanografía, Vigo.



Cell division within
Protoperidinium



Retracting pallium (feeding
veil) of *P. depressum*



Pallium of *P. leonis* type cell,
containing *L. polyedrum* food
source

In 2002 *Protoperidinium depressum* was successfully maintained and densities increased.

In Dec 2002 / Jan 2003, 3709 cells were individually extracted and washed one to two times to eliminate food cells. These cells were tested for the presence of AZA by LCMS analysis and gave a negative result.

In 2003 techniques and procedures learned during the previous years culturing work proved invaluable in establishing cultures. Despite this years targetted cells being more delicate than *P. depressum*, cultures have been successfully established with overall numbers higher than this time last year. It is anticipated that in the near future there will be sufficient numbers of cells for toxin analysis, which will be carried out at the Marine Institute Laboratories in Galway.

Preliminary observations indicate that there may be more than one species of *Protoperidinium* in culture. As it is not possible to identify these cells to species level using light microscopy, fluorescent microscopy will be carried out using the calcofluor staining technique.

A colour difference in *Protoperidinium*, depending on the food source used, was noted. This is an important observation as colour difference is the main way of distinguishing *P. crassipes* from *P. curtipes* using light microscopy.

Still micrographs and video footage of actively dividing cells and the pallium (feeding veil) were prepared and some examples given in the plates below.

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MORPHOLOGY, MOLECULAR TAXONOMY AND TOXIN COMPOSITION OF *ALEXANDRIUM* SPP. ISOLATED FROM IRISH COASTAL WATERS.

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Introduction

This Higher Education Authority (HEA) sponsored project is about the development of molecular probes for the identification of toxigenic phytoplankton species in Irish waters and the development of rapid methods for toxin detection.

The work presented below has been carried out in the Martin Ryan Institute since September 2002. Research initially focused on *Alexandrium* due to an ongoing PhD program sponsored by the Marine Institute that has the objective of developing a bio-physical coupled model for the occurrence of *Alexandrium* in Cork Harbour. *Alexandrium* is known to be involved in toxic events and has been responsible for the closure of fishery activities in the south of Ireland; our latest results regarding this harmful micro-algae are presented.

Background and Context

Alexandrium is a photosynthetic dinoflagellate, with a size range between 20 and 40 µm. It possesses two locomotor flagella and is armoured with a thick cellulose theca made up of several plates that cover the cell body. The genus *Alexandrium* has a world-wide distribution and consists of several species, some are localised to particular regions while others have a cosmopolitan distribution (Balech, 1995). Their identification, from a morphological point of view, is very complex. Characteristics used to identify *Alexandrium* include the size, shape and the ability to form chains. To identify to species level, techniques such as Scanning Electron Microscopy or Calcofluor White stain are required to reveal fine details on the thecal plates. Size, shape and the presence of pores on these plates are the criteria used for identification (Yoshida, 2001).

The *Alexandrium* life cycle includes motile and non-motile stages. Under specific environmental conditions (physical and biological factors), *Alexandrium* populations can actively grow and reach high cell densities that can lead to discoloured waters (red tides). *Alexandrium* blooms can at times cause a toxic event when shellfish such as clams or mussels accumulate Paralytic Shellfish Poisoning (PSP) toxins in their flesh. This results in the temporary closure of aquaculture production areas.

Many species within the genus *Alexandrium* produce toxins that cause Paralytic Shellfish Poisoning (PSP) and can cause severe human intoxication after the consumption of shellfish contaminated by Paralytic Shellfish Toxins (PST) (Kao, 1993). Paralytic Shellfish Toxins are very potent neurotoxins that are not only produced by marine phytoplankton but also by cyanobacteria and bacteria (Negri and Jones, 1995; Gallacher *et al.*, 1997). These molecules are soluble in water and can pass through biological membranes. About 20 different forms have so far been isolated and classified in three groups: a) Saxitoxin (STX) and Neosaxitoxin are not sulphated, b) Gonyautoxins (GTX) are mono-sulphated and c) Carbamoyltoxins (C-toxins) are bi-sulfated (Oshima *et al.*, 1993). These toxins inhibit the transmission of nerve influx by blocking voltage-dependant Na⁺ channels and thus, prevent the depolarisation of the axonal membrane. Toxicity leads to muscular paralysis, mainly the respiratory muscles, and death occurs by asphyxia.

Alexandrium blooms have been recorded around Ireland. Some of these have been toxic, notably along the south coast of Ireland, and other blooms have apparently been non-toxic. For example, a bloom of *Alexandrium minutum* observed along the coast of Mayo in 2001 (Hansen et al., 2003) was not associated with contamination of shellfish, despite increased monitoring (Irish Marine Institute, unpublished records). This highlights the fact that problems linked to this genus must not be ignored, and further research is required to fully understand the situation.

Objectives

One of the objectives of the research carried out at the MRI is to determine which species of *Alexandrium* are present around Ireland and whether they are toxic or not. If different species are found, fluorescent molecular probes will be developed to ease identification and monitoring. To understand the physiology of the toxic strains, growth experiments will be carried out to investigate what physiological conditions enhance toxin production.

Preliminary Results

Alexandrium cysts have been extracted from sediments collected at several locations off the Irish coast. Stations sampled were situated along the south and west coasts. Additional samples will be collected along the north and east coasts. Two *Alexandrium*-type cysts have been identified from the south coast. This includes an *A. tamarense* ‘like’ cyst (an ellipsoidal shape) and an *A. minutum* ‘like’ cyst (a spherical shape) (Figure 1). These cysts have no ornamentation on the cyst wall and their granular cytoplasm contains lipid globules and a red accumulation body. The size range for the *A. tamarense* ‘like’ cyst is 40-50 µm and the size range of the *A. minutum* ‘like’ cysts is between 25-30 µm.

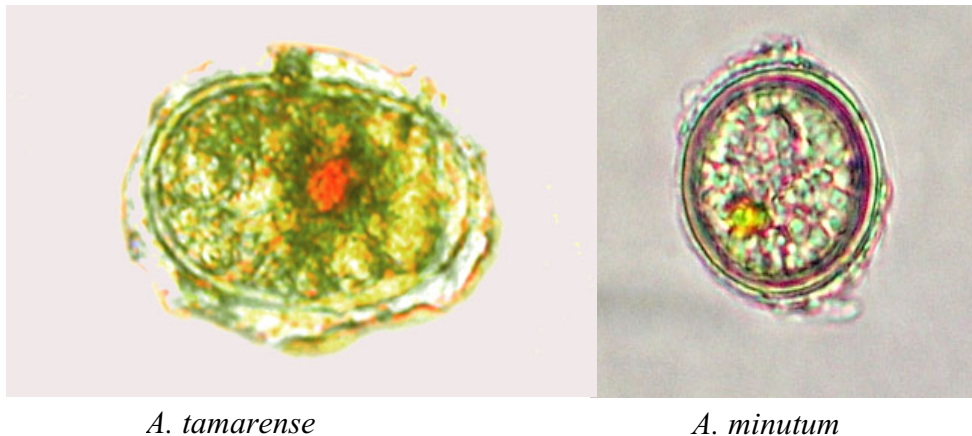


Figure.1 Photomicrographs of *A. tamarense* and *A. minutum* resting stages (cysts) isolated off the south of Ireland (not to scale).

Several cysts of both species have successfully been germinated and cultures of vegetative cells established. Batch cultures are being maintained in illuminated incubators under controlled conditions. The precise identification of vegetative cells required the use of Calcofluor to distinguish the important features on the thecal plates using fluorescent microscopy. This method has allowed the positive identification of *A. tamarense* and *A. minutum* from samples collected off the south coast. The *A. tamarense* cells had a posterior sulcal plate that is longer than broad, and the *A. minutum* cells had a posterior sulcal plate that was broader than long (Figure 2).

DNA has been extracted from the *Alexandrium* cultures and the LSU rDNA (large sub-unit ribosomal DNA) has been sequenced. Confirmed identification of the *Alexandrium spp.* in culture was carried out by comparing the sequence information obtained with other LSU rDNA sequences of *Alexandrium spp.* from other countries (downloaded from internet databases).

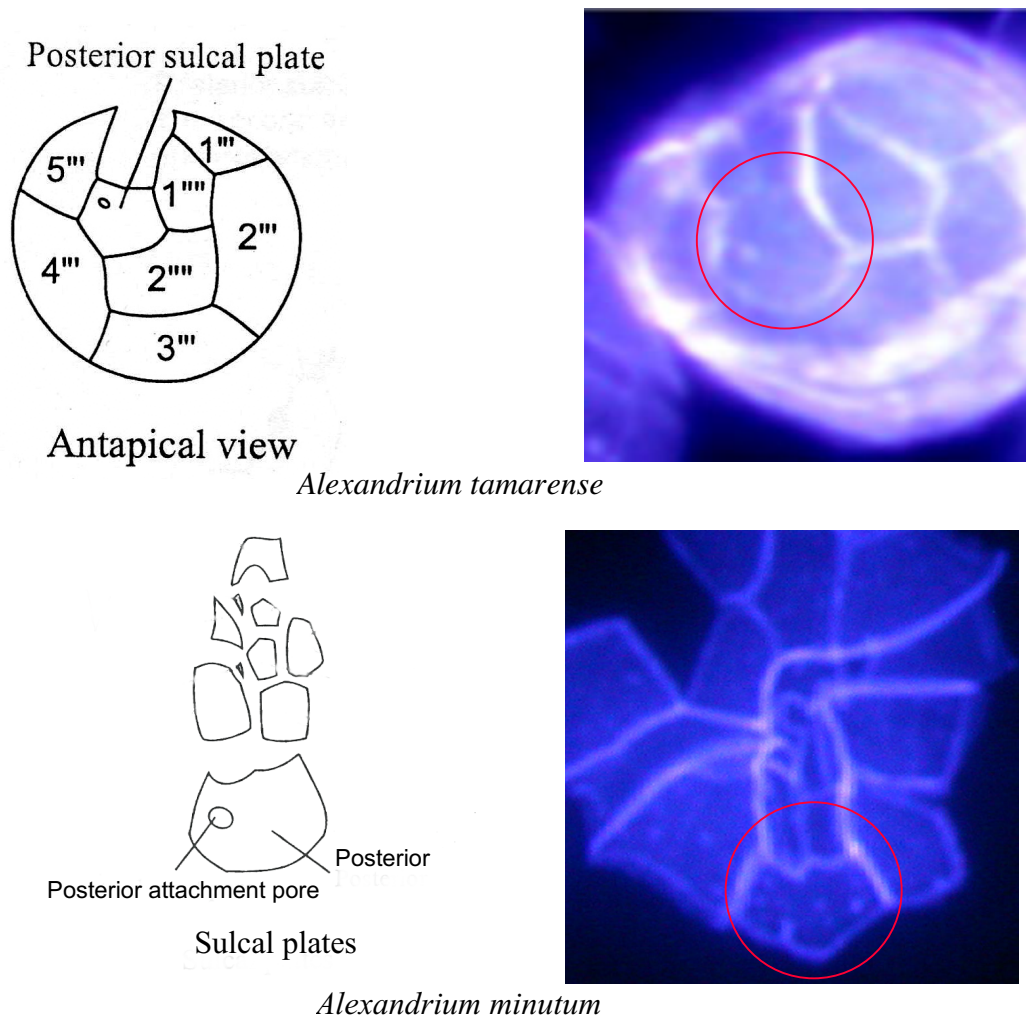


Figure.2. Details and shape of the Posterior Sulcal Plate of *A. tamarense* and *A. minutum* vegetative cells after treatment with Calcofluor White stain.

Two *A. minutum* cultures isolated from the south coast of Ireland were analysed for the presence of PSP toxins using HPLC-FD (J. M. Franco from Vigo in Spain). The chromatograms confirmed the presence of gonyautoxins 2 and 3 (Figure 3). Standard reference material of Saxitoxin was used in the analysis and was not detected in the *A. minutum* cultures (Figure 4). Carbamoyl-toxins were not detected and this is unusual for northwestern European strains of *A. minutum* (Hansen *et al.*, 2003). In order to investigate if the culture conditions affected the production of these missing toxins, a French strain (courtesy of Patrick Gentien, IFREMER, La Rochelle, France) will be grown under similar growth conditions and tested for PSP toxins.

Alexandrium tamarense cultures established from cysts collected off the south coast are currently being checked for Paralytic Shellfish Toxins. These cultures may not be toxic due to the fact that their DNA sequence information is homologous with other strains from western European populations that do not produce any PST (Higman *et al.*, 2001).

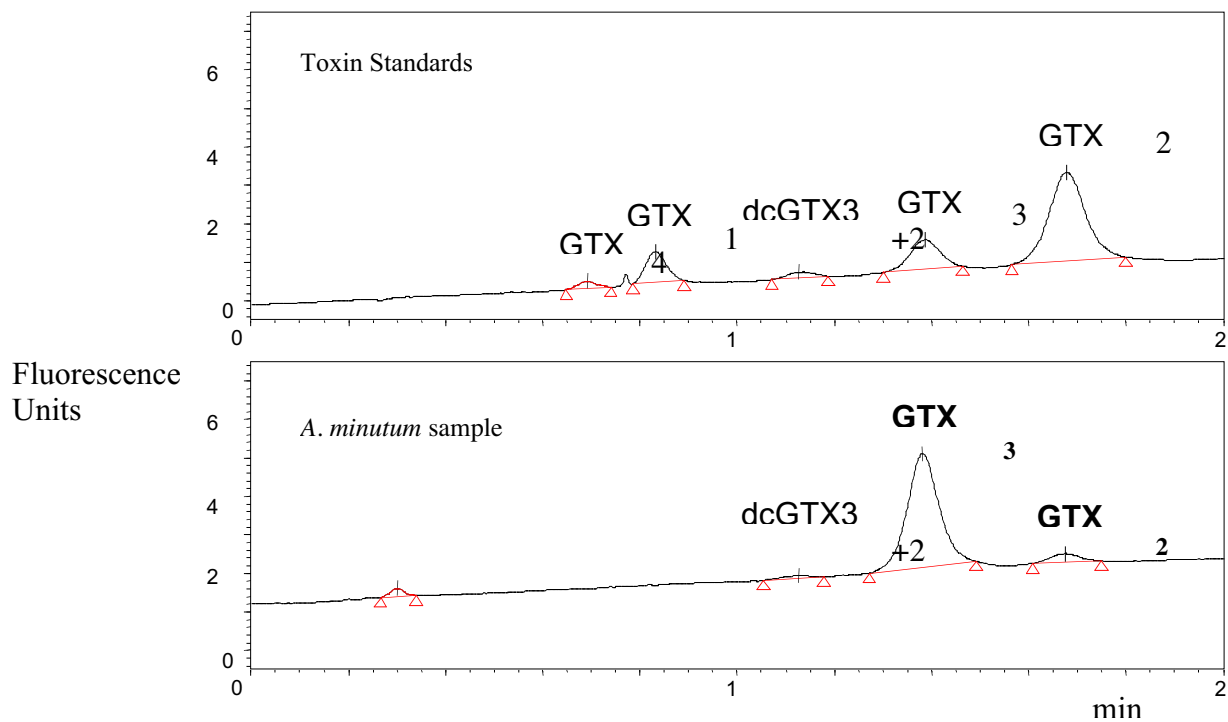


Figure 3. Chromatograms of an *A. minutum* extract (south coast of Ireland) showing the presence of GTX-2 and GTX-3.

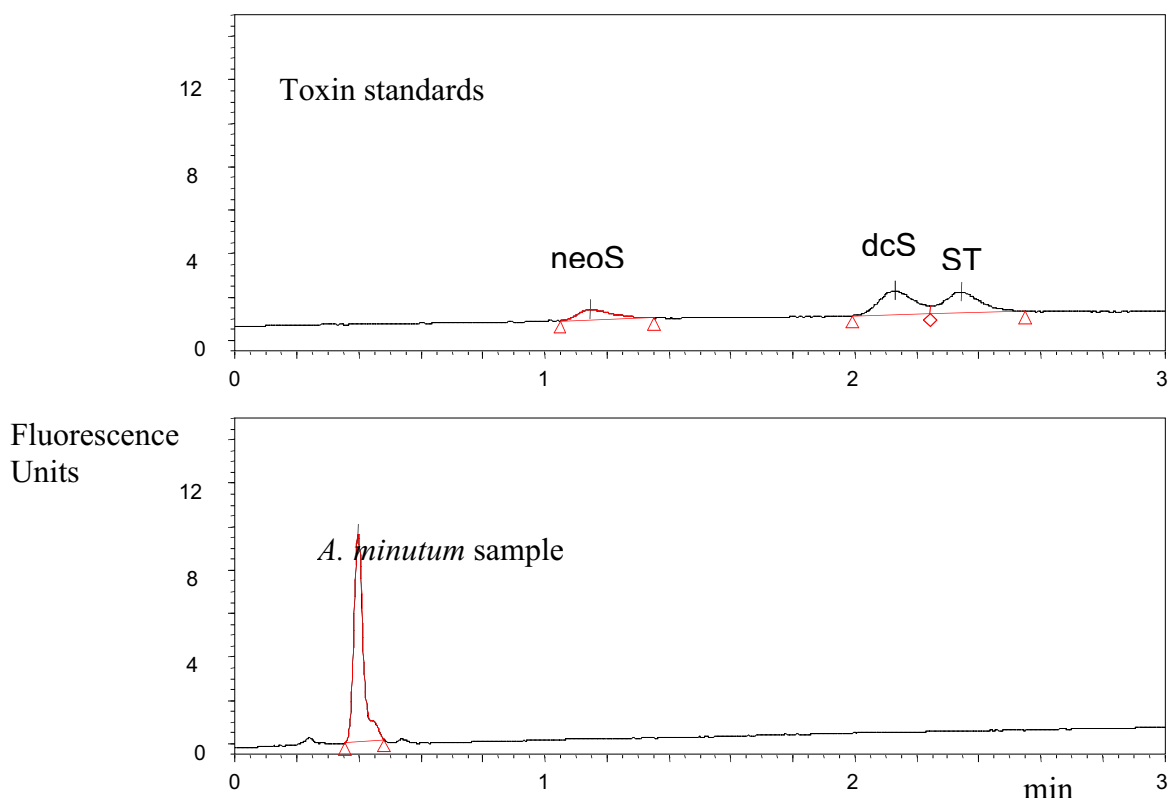


Figure 4. Chromatograms of an *A. minutum* extract (south coast of Ireland) showing the absence of saxitoxin and first derivatives.

Future Work

Future work will include the isolation of *Alexandrium* spp. from sediments collected off the west coast of Ireland. Dinoflagellate species isolated into cultures during a survey on the R.V. Celtic-Voyager in July 2003 also need to be identified and their toxicity profiles established. Finally, the development of a method to evaluate the specificity of the molecular probes designed for Irish *Alexandrium* species is under way.

Several other experiments are planned for 2004. The toxin profile of the toxic *A. minutum* strain isolated from Irish waters will be investigated further under different environmental conditions. Inter-specific interactions will be also investigated through competition and allelopathy experiments, the question being: "Can toxin producing *A. minutum* strains inhibit the development of other phytoplankton species or is *A. minutum* inhibited by competitive abilities of other species or molecules they release?" It is also considered important that the bacterial flora associated with toxic and non-toxic Irish dinoflagellates are investigated.

Conclusions

There is a history of PSP contamination of shellfish with PST derived from *Alexandrium* blooms in Irish coastal waters. It is likely that these harmful events have been due to the presence of *A. minutum*. Toxic *A. tamarense* populations have been found locally in Scottish coastal waters (Higman et al., 2001). It is possible that this population may expand in its distribution to Ireland. It is essential therefore that monitoring procedures are maintained and the life cycle of the organisms are studied to allow the development of predictive models.

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DEVELOPMENT OF MOLECULAR PROBES FOR TOXIGENIC PHYTOPLANKTON

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This project, funded by the PRLTI (Program for Research in Third Level Institutions) from the Higher Education Authority (HEA) and is a collaborative venture between the National Diagnostics Centre and the Martin Ryan Institute, both at NUI Galway. The overall aim of the project is to develop molecular probes for toxigenic phytoplankton that present problems for the Irish shellfish industry. A collection of DNA sequence data will be compiled for a collection of potentially toxic phytoplankton species, including *Alexandrium* spp., *Dinophysis* spp. and *Pseudo-nitzschia* spp. The sequence data generated will provide the information required to design molecular probes for the rapid detection and identification of toxigenic species. Moreover, sequence information may be used to investigate phylogenetic relationships of Irish isolates compared to other strains from different geographical locations.

The most common method for the identification of phytoplankton species involves the use of a light microscope. Identification is based on unique morphological features of the individual species. Several of the potentially toxic species, are however, morphologically very similar to non-toxic species. In some cases it is difficult to discriminate between species of the same genera (e.g. *Alexandrium* spp. and *Pseudo-nitzschia* spp.). For accurate identification of these species electron microscopy is required. However, this is a very laborious technique and is simply not very suitable for monitoring purposes.

Molecular probes have the potential to provide accurate and rapid identification and enumeration of toxigenic phytoplankton. They are targeted at the nucleic acids within cells. Nucleic acids (DNA and RNA) provide the unique genetic information required for protein synthesis in all organisms. Nucleic acids form a uniform stable chemical structure consisting of a linear sequence of bases (A, T, G and C) along the length of the molecule (Figure. 1.), which is common in prokaryotic and eukaryotic organisms. These bases provide complementarity between the two DNA strands (Figure. 1).

It is this complementarity that enables the hybridisation of short specific sequences of DNA, known as molecular probes, to their complementary DNA target (Figure. 2). This enables the detection and identification of a specific target sequence and consequently a specific organism (Smith *et al.*, 2000).

Molecular probes are short specific sequences of DNA usually 20 to 40 bases in length comprising of the four bases arranged in a sequence that is complimentary to the sequence of the target genomic DNA of the species of interest. Molecular probes can be commercially synthesised and labelled with a fluorescent, chemiluminescent or colourimetric tag, which facilitates the detection of the probe/target analyte in a sample.

Ribosomal DNA (rDNA)-targeted molecular probes are widely used for identification of a wide variety of microorganisms in environmental and clinical samples. The eukaryotic ribosome comprises of several subunits including the small subunit (SSU), the large subunit (LSU) the 5S and 5.8S subunit and two internal transcribed spacer regions, the ITS 1 and ITS 2 regions. This genetic target is chosen because of the large amount of rRNA present in cells and the range of very conserved to quite variable sites within the ribosomal subunits. The conserved sequences are useful in determining higher orders of taxa, and the less conserved regions for separating lower taxonomic orders, such as species. The closer the relationship between two species the less sequence variation there is between their ribosomal genes.

Ribosomal sequences offer ample opportunity to design molecular probes for genus or species specific targets.

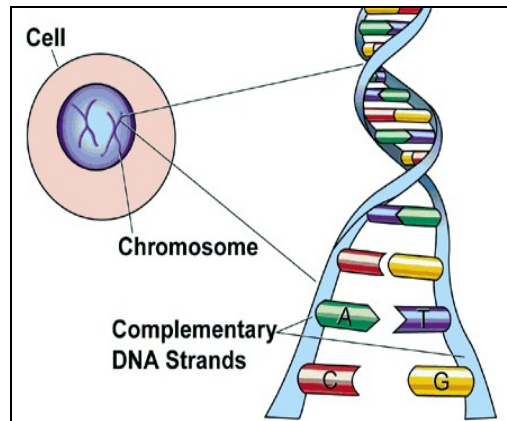


Figure 1. DNA molecule demonstrating complementarity between the bases (A, adenine; T, Thymine; G, Guanine and C, Cytosine)

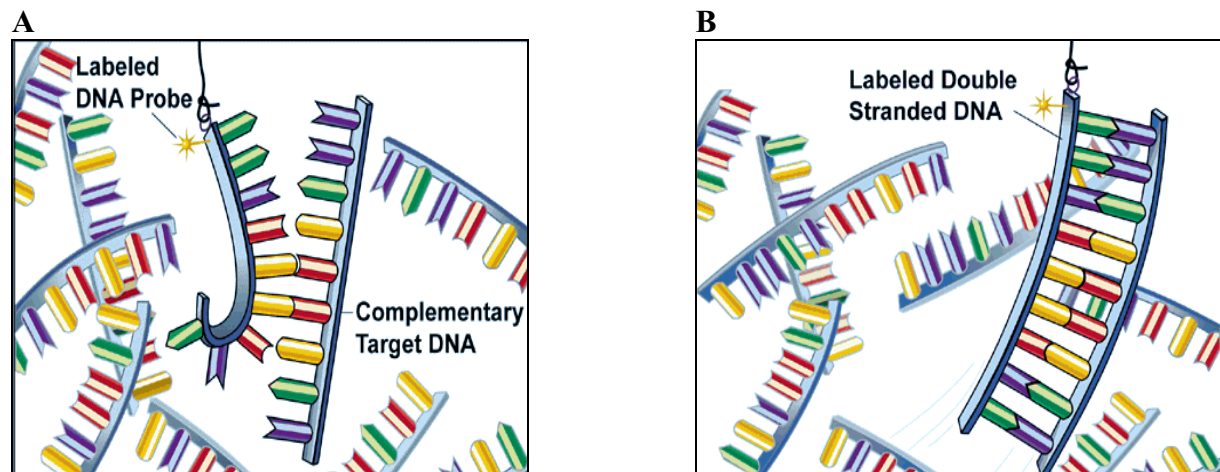


Figure 2. The labelled molecular probe binds specifically to DNA target (A) resulting in a labelled probe/target analyte (B)

The most common application of molecular probes for the identification of toxigenic phytoplankton includes the whole cell format and the cell homogenate format. The whole cell format relies on the 'labelling' of molecules on the cell surface or within the cell. This is most commonly carried out with fluorescently labelled probes. The cells are usually chemically preserved to retain the cellular integrity and also to render the cellular membrane permeable to a fluorescently labelled probe designed to detect a specific toxigenic species. The probe binds to the rDNA/rRNA targets and unbound probe is washed away. The probe/target DNA hybrid can be visualised using a fluorescent microscope. This methodology has been employed by a number of research groups for the identification of a range of toxigenic species (Miller and Scholin, 1998; Rhodes et al., 1998; Simon et al., 2000).

The cell homogenate format involves the disruption of the cells, usually by chemical lysis, which releases their contents. The molecular probes target the released molecules either by

directly hybridising to the target molecule (hybridisation assays) or the amplification of the target molecules is required followed by detection with the probe (amplification assays). Initial work carried out on the development of molecular probes for toxigenic phytoplankton species in Irish waters has been with cultures of a dinoflagellate called *Alexandrium* spp. It is very difficult to differentiate between species of this genus. Since *A. tamarensense* and *A. minutum* have been identified from samples collected in Irish waters, it is important that species-specific molecular probes are developed for these species. To date the LSU (690 base pairs) of four *A. tamarensense* isolates and two *A. minutum* isolates from Cork Harbour have been sequenced. The sequence information has confirmed the species identification of these *Alexandrium* isolates. The *A. tamarensense* isolates shared 100% sequence identity and demonstrated high sequence similarity to other *A. tamarensense* isolates of the western European ribotype. The *A. tamarensense* isolates from Cork Harbour showed distinct differences with *A. tamarensense* of the North American ribotype. The two *A. minutum* isolates from Cork Harbour shared 100% sequence identity and demonstrated high sequence similarity to other *A. minutum* isolates from France (Guillou *et al.* 2002) and New Zealand (Walsh *et al.* 1997; Accession number AF033532).

The sequence information generated for *A. tamarensense* and *A. minutum* isolates from Cork Harbour were compared to the LSU sequence information for a range of other *Alexandrium* spp. using the Clustal W sequencing alignment program. These sequence alignments provided the basis for designing *A. tamarensense* and *A. minutum* specific probes. Because of sequence differences between the western European ribotype and the north American ribotype of *A. tamarensense*, it was not possible to design a probe that would detect both ribotypes. Probes specific for *A. tamarensense* western European ribotype were therefore designed (Table 1). The sequence similarity between the *A. minutum* isolates allowed *A. minutum* specific probes to be designed (Table 2). The *A. tamarensense* and *A. minutum* probes were evaluated by Southern blot hybridisation analysis before they were applied to the whole cell assay. Southern blot analysis allows the specificity of each probe to be evaluated before the probe is applied to the whole cell hybridisation assay. The probes were evaluated in preliminary specificity studies against other *Alexandrium* spp. and other algal species. Don Anderson and Dave Kulis from Woods Hole Oceanographic Institute in the U.S. kindly provided cultures of other *Alexandrium* species for specificity studies.

Table 1. *Alexandrium tamarensense*-western European strain specific probes

<i>Name</i>	<i>Sequence (5' - 3')</i>	<i>A. tamarensense</i> <i>W.E. detection</i>	<i>Cross-reactivity to other</i> <i>Alexandrium spp.</i>
A.tam1	TCTGTTTTTGTTCATGTGT	Yes	No
A.tam2	GCTGTGGGTGAAATGATTC	Yes	Yes
A.tam3	TCTTGCATGCCAGGTTCTA	Yes	Yes

Table 2. *Alexandrium minutum* specific probes

<i>Name</i>	<i>Sequence (5' - 3')</i>	<i>A. minutum</i> <i>detection</i>	<i>Cross-reactivity to other</i> <i>Alexandrium spp.</i>
A.min1	GGGTGCGATGGTTCTTA	Yes	Yes
A.min2	GTTCTTACCTTGAATGTCAGCT	Yes	No
A.min3	CGCATGTGTTTGGTGAAATT	Yes	Yes
A.min4	GTAATTTGCCTGCGGGTATTGG	Yes	Yes

The results of the Southern blot analysis are summarised briefly in Tables 1 and 2. Each probe was tested on *A. tamarens* isolates from Ireland, Spain and North America, *A. minutum* isolates from Ireland, France and New Zealand, *A. fundyense* from North America, *A. catenella* from France, and *Scrippsiella* spp. from Ireland. The Southern blot analysis highlighted the cross-reactivity of some of the probes with other *Alexandrium* spp. and the information generated from these results allowed the selection of an *A. tamarens* W.E. (A.tam1) specific probe and an *A. minutum* specific probe (A.min2). These probes hybridised specifically to their target sequences and not to the LSU PCR (polymerase chain reaction) products from other *Alexandrium* spp. tested. Future work calls for these species-specific probes to be labelled with a fluorescent marker, fluorescein, and applied in the whole cell hybridisation assay.

In addition to developing and advancing the whole cell hybridisation assay, a real-time PCR based method will be established to identify and enumerate *A. tamarens* and *A. minutum* in the same sample collected from Irish waters. An advantage of real-time PCR based methods is that they are highly amenable to automation and a high throughput of samples is possible. It is important to establish both the whole cell hybridisation assay and the real-time PCR method for the identification of toxigenic phytoplankton, as they will play a crucial role in enhancing phytoplankton research and monitoring in Ireland.

Future work will involve the sequencing and design of molecular probes for the DSP toxin producing dinoflagellate, genus, *Dinophysis*.

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PHYTOPLANKTON STUDIES BY DARD IN NORTHERN IRELAND

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Introduction.

This short paper describes work carried out by DARD on routine monitoring for the presence of potentially toxic algae and biotoxins. Analysis of data, undertaken by the UK Co-ordinator of Fisheries Research and Development (CFRD) Working Group on Toxin Producing Algae (TPA), and long-term surveillance of key environmental variables (including phytoplankton) undertaken by DARD in the western Irish Sea are also described.

Monitoring for the presence of toxin producing algae.

To comply with the EU Shellfish Hygiene Directive, routine monitoring of phytoplankton in

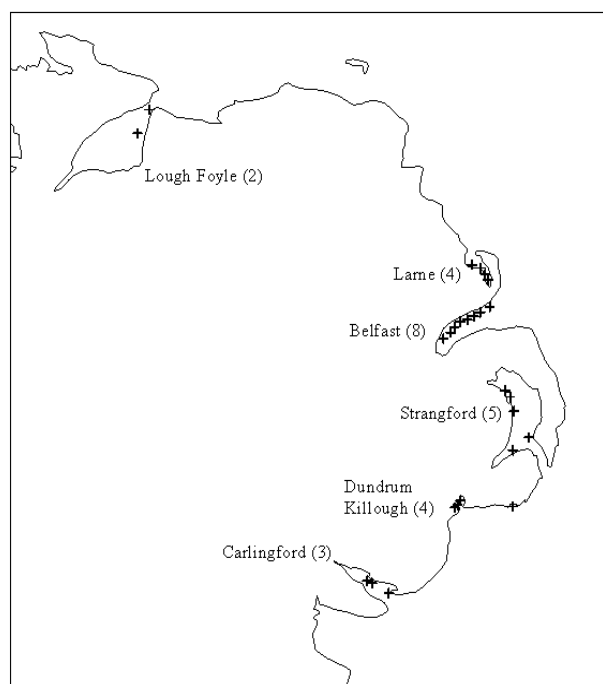


Figure 1. Sampling Sites

coastal waters of Northern Ireland began in 1993. Water samples were collected from 15 locations at fortnightly and monthly intervals during summer and winter respectively and analysed using light microscopy for the presence of toxin producing algae and other harmful/nuisance species. Currently, 26 sites (Fig. 1) are sampled fortnightly all year round. A standard near surface water sample is collected from each site and additional concentrated phytoplankton samples are collected using a small phytoplankton net at selected sites. Both types of sample are preserved with acidic Lugol's iodine at the time of collection. A standard sedimentation method is used to concentrate the phytoplankton (50 ml sub-samples of each standard sample) before

toxin producing and harmful species are identified and counted with the aid of an

inverted light microscope. Net samples are used to identify those algae present at numbers too low to be detected in the standard near surface samples. Toxin producing species which have been identified during the monitoring programme are listed in Table 1, although *Lingulodinium polyedra* and *Dinophysis rotundata* are considered rare, and only one major bloom of *Prorocentrum minimum* has been recorded. Biotoxin monitoring has revealed the presence of toxins in a variety of shellfish (Table 1) which has resulted in the periodic closure of shellfish beds. In addition, phytoplankton species known or considered to be toxic to fish *Dictyocha speculum*, *Gymnodinium* spp., *Heterosigma akashiwo*, *Karenia mikimotoi*, *Noctiluca scintillans* and those regarded as 'nuisance' species:- *Phaeocystis pouchetti* have been recorded.

The general impression gained from the monitoring data is that toxin producing phytoplankton species only occur at low levels in coastal waters of Northern Ireland. This is illustrated by the data in Fig. 2, which shows the percentage occurrence of *Alexandrium* and *Pseudo-nitzschia* spp. in samples analysed each year. The presence of *Alexandrium* in samples has not exceeded 20 % and in recent years is \approx 5 %. This reflects its short seasonal

occurrence and restricted distribution. In contrast, *Pseudonitzschia* spp. are generally more widespread and abundant, typically present in 40 to 60 % of samples. However, the threshold abundance for these species is high (150,000 cells l⁻¹) and has only been exceeded on 6 occasions since 1996.

Table 1. Toxin producing algae, their general distribution and associated toxicity in coastal waters of Northern Ireland.

Species	Distribution	Occurrence	Associated toxicity
<i>Alexandrium</i>	localised	rare	Mussels
<i>Prorocentrum lima</i>	localised	rare	Cockles (?)
<i>Dinophysis</i> spp. ¹	widespread	low	Mussels, Oysters
<i>Pseudonitzschia</i> spp.	widespread	common	Scallops
<i>Lingulodinium polyedra</i>	localised	rare	
<i>Prorocentrum minimum</i>	localised	rare	Mussels
<i>Protoperidinium</i> spp.	widespread	low	

¹includes *D. acuta*, *D. acuminata*, *D. norvegica* and *D. rotundata*

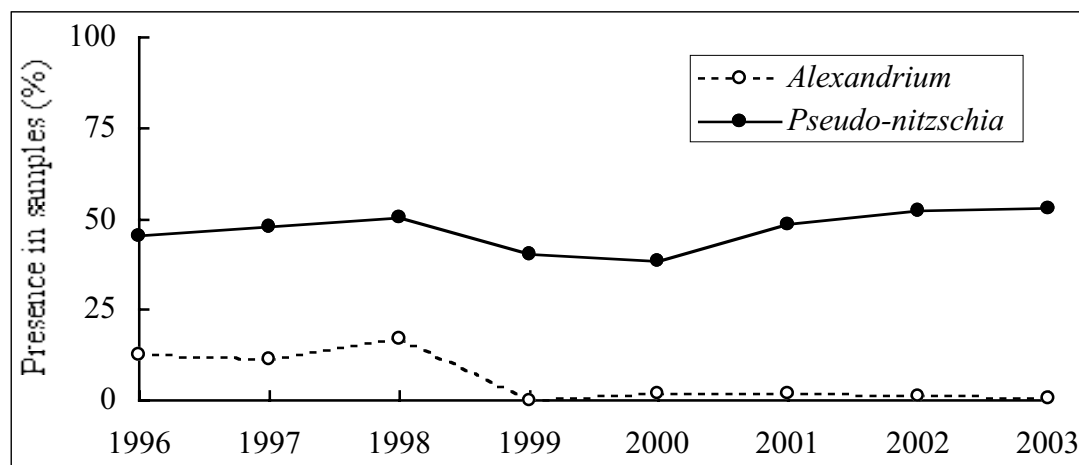


Figure 2. The occurrence of *Alexandrium* and *Pseudo-nitzschia* spp. (% presence in samples) in coastal waters of Northern Ireland.

UK Co-ordinator of Fisheries Research and Development (CFRD) Working Group on Toxin Producing Algae (TPA)

Over the years two questions have frequently been asked about the presence of toxin producing algae and shellfish toxicity in coastal waters: “Has the distribution or abundance of toxin producing algae changed over time?” and “Has the incidence of toxicity in shellfish increased in recent years?” These questions have largely remained unanswered because of a lack of data collected systematically from the same locations over time. However, it may now be possible to address these and similar questions using information collected under the EU Shellfish Hygiene Directive. At some coastal monitoring sites, phytoplankton and related biotoxin data have been collected for up to 10 years and it is now possible to look for trends in the data. To undertake this work a UK, CFRD working group was established in 2003 to specify the statistical analysis required of data sets to identify trends and ‘hot spots’ in the occurrence of TPA and toxic shellfish events. Participation of the Marine Institute in the working group (Table 2) will provide additional data sets and broader geographical coverage of coastal waters.

Geographical trends and changes in the seasonal occurrence of toxin producing algae will be assessed using the dates of first and last occurrence in the year and the date of peak abundance. The frequency with which species exceed their threshold abundance will provide

an indication of whether the incidence of particular species has increased. Links between individual toxin producing species and toxicity in shellfish will be examined using data on *Alexandrium* and the occurrence of Paralytic Shellfish Poisoning (PSP) and *Dinophysis* and Diarrhetic Shellfish Poisoning (DSP). For *Dinophysis*, toxicity will be related to individual species and total *Dinophysis* abundance. Finally, changes in the severity of toxic events over time will be assessed on the basis of the duration of closures and number of closures per year.

Table 2. Members of the CFRD Working Group on Toxin producing Algae

David Maxwell, Steve Milligan, Linda Percy, Caroline Whalley (Secretary, 2003); Centre for Environment, Fisheries and Aquaculture Science
Richard Gowen (Chair), April McKinney ; Department of Agriculture and Rural Development
Eileen Bresnan, Shelia Fraser, Andrew Newton (Secretary, 2003/4) ; Fisheries Research Services
Joe Silke ; Marine Institute
Duncan Purdie ; Southampton Oceanography Centre
Jane Lewis ;University of Westminster

Long-term surveillance of environmental variables in the western Irish Sea

In 1990, DARD established a programme of biological oceanographic research in the western Irish Sea to gain a better understanding of how the Irish Sea functions as a semi-enclosed coastal marine ecosystem. Initially, a broad based study was undertaken to describe seasonal cycles of water column structure, concentrations of dissolved nutrients and phytoplankton production and standing stock. Building on this work, DARD has established two standard sampling stations in the western Irish Sea (Fig. 3) to undertake long-term surveillance of key environmental variables (e.g. temperature, salinity, dissolved nutrients, zooplankton and phytoplankton species abundance). The aim of this work is to describe present day conditions (particularly the scale of short-term variability) against which future change can be quantified. Sampling is undertaken by traditional shipboard methods (e.g. vertical haul with a 200 µm ring net for zooplankton abundance) and instrumented moorings. The offshore mooring (Fig. 3) supports near surface conductivity temperature and depth sensors (CTD) with a fluorometer for estimating phytoplankton biomass as chlorophyll and automated water samplers for dissolved inorganic nutrients (ammonium, nitrate, nitrite, phosphate and silicate) and phytoplankton species abundance. Thermistors located at selected depths provide details of the temperature structure of the water column and a bottom CTD is used to acquire data on seasonal changes in bottom water temperature and salinity.

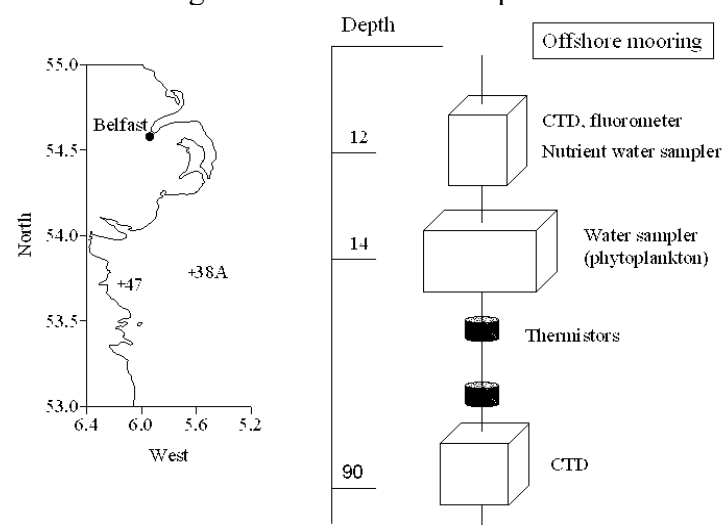


Figure 3. Offshore Mooring

High frequency measurements of water temperature have been made almost continuously since 1997 (Fig. 4). Of interest are short-term variability and any long-term trend in late winter temperature because of the role of winter temperature in the life cycle of commercially important species of fish such as cod. A similar time-series is being assembled for dissolved inorganic nutrients (Fig. 4) and again one of the purposes of this work is to identify any long-term trends. Quantifying the relative contribution of natural and anthropogenic nutrient sources to Irish Sea winter nutrients stocks is also an important element of this work.

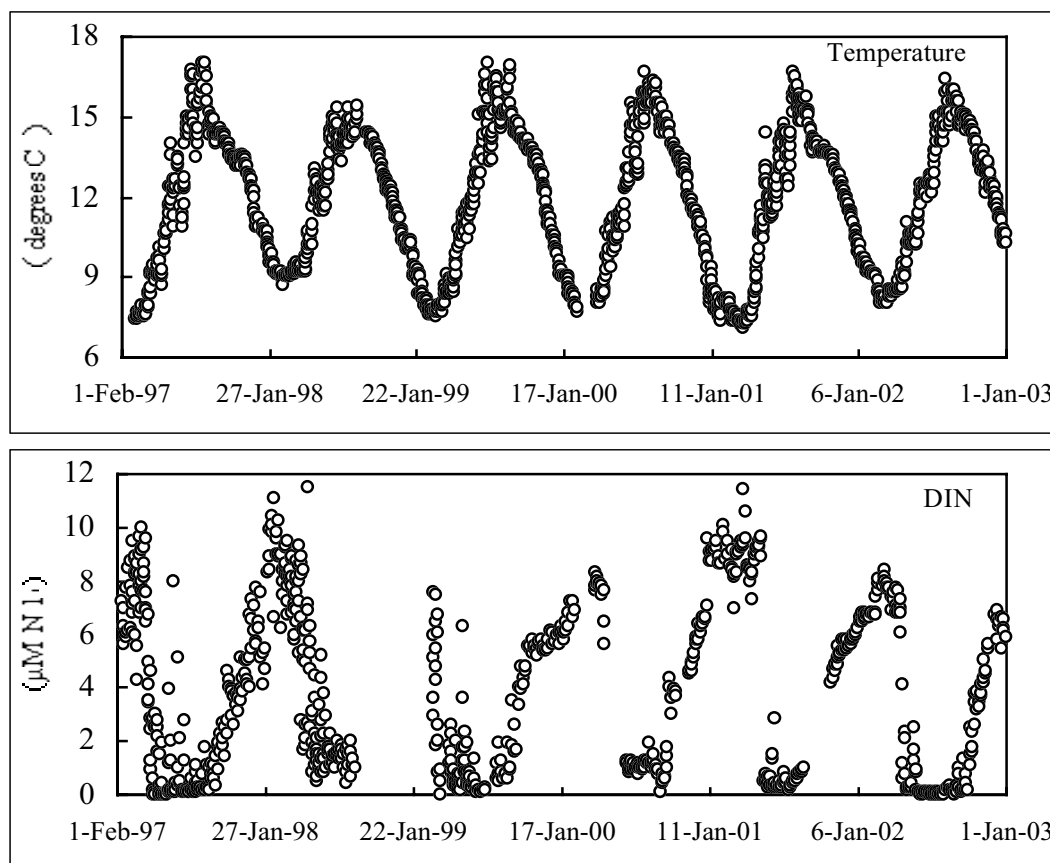


Figure 4. Time series of seasonal changes in near surface temperature and dissolve inorganic nitrogen (as nitrate + nitrite) at the DARD mooring station in the western Irish Sea.

The phytoplankton production season in the western Irish Sea begins with a spring bloom. The bloom is a recurrent annual event, which is of major importance to the over-all productivity of the region. The characteristics of the bloom (timing, duration, magnitude and composition) all influence the pathways by which energy is transferred through the food chain to higher trophic levels. A time-series of species abundance and composition during the spring bloom is being assembled for the offshore station 38A. These observations, supported with data on water column structure and nutrient concentrations, reveal considerable inter-annual variability in the characteristics of the spring bloom. The onset of the bloom (and hence the beginning of the production season) can vary by up to one month. In 2002 for example, the peak of the bloom occurred in early May but in 2003 the bloom was over by early April (Fig. 5). Although diatoms normally dominate the spring bloom, this is not always the case. In 1997 and 2001 the diatom crop failed and the spring bloom was dominated by autotrophic microflagellates. In 2000, 2002 and 2003, when diatoms dominated the bloom the predominant species differed each year. In 2000, small species of the diatoms, genus *Chaetoceros* represented 48 % of the peak diatom biomass. In 2002 and 2003, the species contributing most to the total diatom biomass were *Guinardia delicatula* (28 %) and

Thalassiosira nordenskiöldii (44 %) respectively. Quantifying the type of short-term inter annual variability illustrated by the data in Fig. 5 and understanding what controls bloom characteristics is an essential part of gauging how climate change and long-term changes in anthropogenic nutrient inputs will impact ecosystem structure and functioning in the Irish Sea.

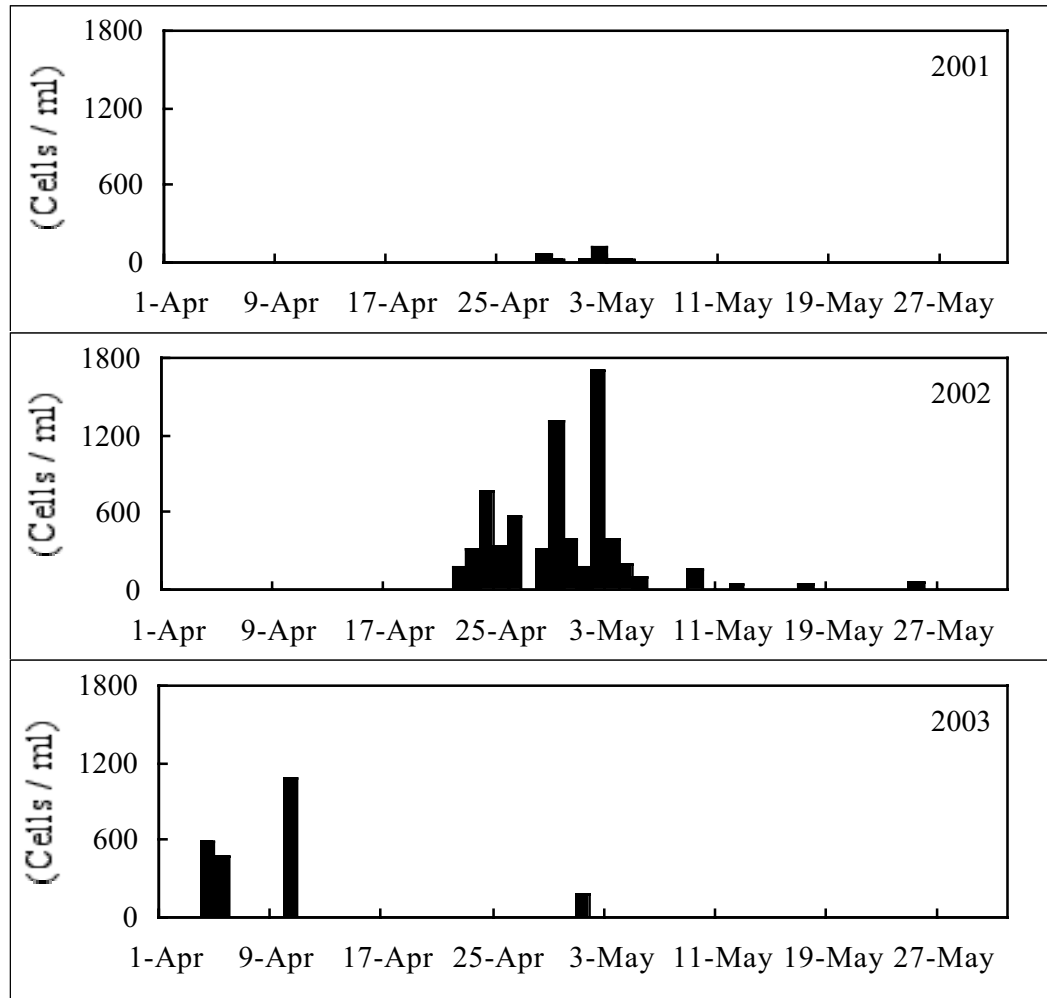


Figure 5. A preliminary assessment of diatom abundance during the spring bloom of phytoplankton at the DARD mooring station in the western Irish Sea.

Acknowledgements. I would like to acknowledge the support of colleagues who are involved in the DARD biological oceanographic programme. April McKinney undertakes the analysis of phytoplankton samples for the EU Shellfish Directive. Brian Stewart, Clare Smyth, Anne-Marie Coile and William Clarke contribute to the long-term surveillance programme in the western Irish Sea.

UPDATE ON THE BIOLOGICAL OCEANOGRAPHY OF HARMFUL ALGAL BLOOMS (BOHAB) PROJECT

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Introduction

The BOHAB project began in January 2003 to investigate the link between physical processes (currents and water masses) and the occurrence of harmful algal blooms (HABs). These HABs can cause fish mortalities and toxicity in some shellfish species and cost the Irish economy dearly in years when HAB events are prevalent. The project brings together scientists from the Martin Ryan Institute at NUI, Galway, the Woods Hole Oceanographic Institution (USA) and the Irish Marine Institute to tackle the complex HAB problem. The end goal of the project is to be closer to a HAB predictive capacity by developing a conceptual model of the sequence of events leading to the establishment, maintenance and dissipation of HABs in Irish coastal waters.

BOHAB Progress by workpackage.

The BOHAB project is divided into 10 distinctive workpackages (shown in figure 1). This paper focuses primarily on progress with distribution studies (Workpackage 1), intoxication studies (Workpackage 2) and bio-physical interactions (Workpackage 6). Most of the remaining workpackages are either desk studies to be conducted in 2004 or related to data and overall management of the project.

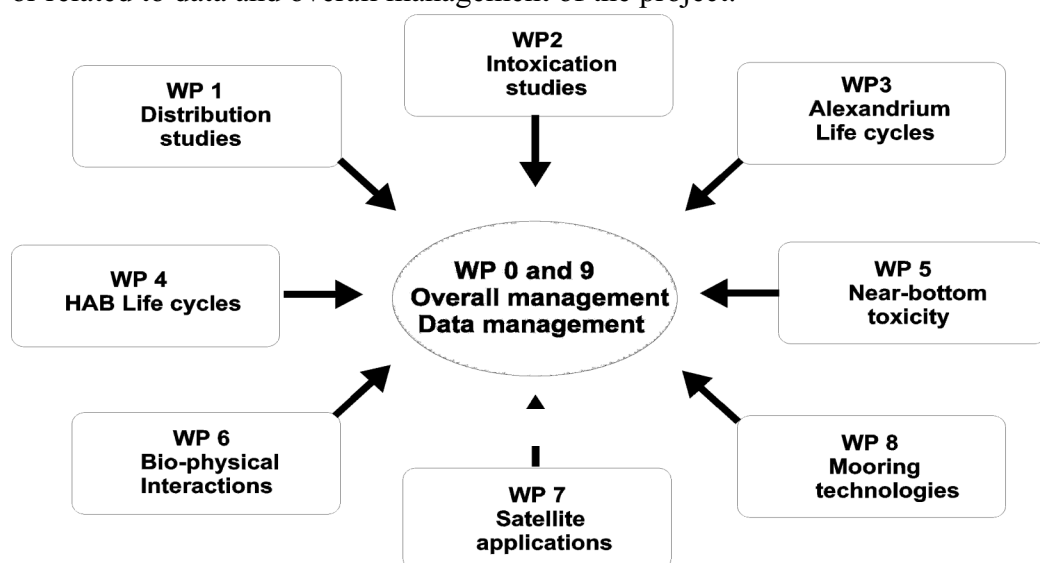


Figure 1. Schematic overview of the workpackages in the Biological Oceanography of Harmful Algal Blooms (BOHAB) project

Six research cruises were carried out in total in 2003 in the two fieldwork focal bays of Bantry and Killary Harbour. Extensive measurements of temperature, salinity, currents, phytoplankton distribution, nutrients and sediments were made on each programme to establish the seasonal variability in HAB species and the relationship with key oceanographic features such as fronts and coastal jets. The stations occupied are shown in figure 2. Much of the data is still being analysed but an interesting picture is emerging already from the Killary Harbour oceanographic data from cruise

BHO503 on the RV Celtic Voyager. Thin layers of fluorescent material have been observed in the vicinity of Killary in the past such as that observed by Silke et al in summer 2002. The layer at that time was 2-5 m thick and is shown in detail in figure 3 of this paper. Microscope analysis of water samples taken from within this layer revealed that the phytoplankton comprised a community dominated by a *Hyalochaete Chaetoceros* species with cell densities between 100,000-200,000 cells per litre. High resolution work in 2004 will focus on establishing the persistence of the layer and the phytoplankton species therein.

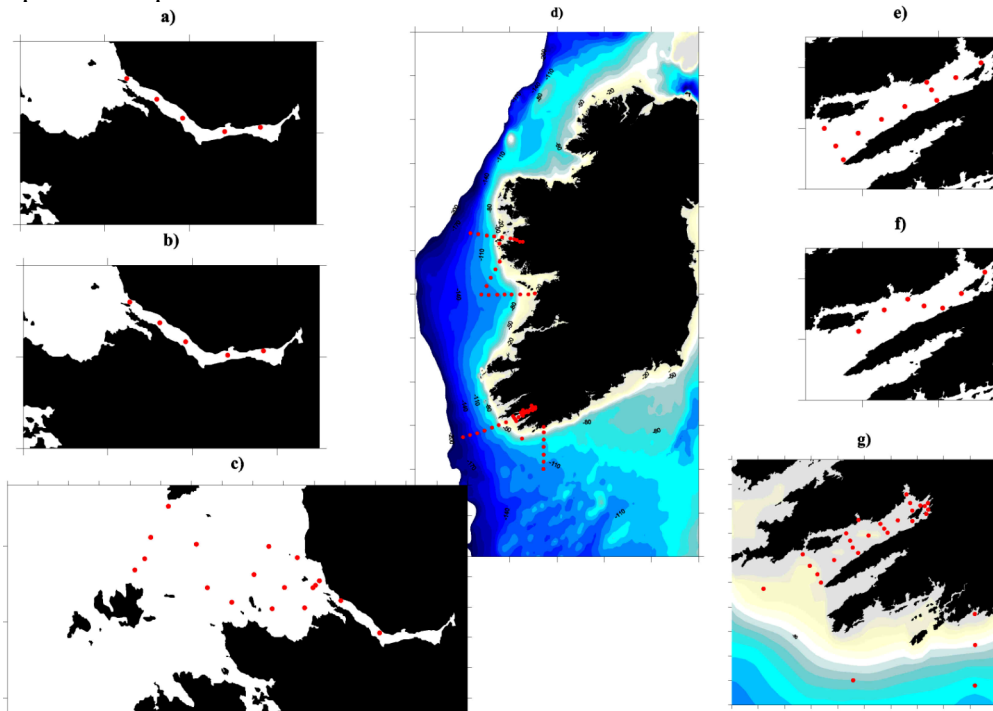


Figure 2. Research cruises carried out for BOHAB in 2003, a) Killary, March, b) Killary, May, c) Killary, August, d) Celtic Voyager shelf cruise, July, e) Bantry, March, f) Bantry, May and g) Bantry, July (as part of Voyager cruise)

Some progress has been made in intoxication studies examining susceptibility of shellfish to toxic phytoplankton. Samples of mussels were taken from 3 sites in Killary Harbour at 3 depths (surface, mid-water and near bottom) over a nine week period in summer 2003. The samples were analysed for the presence of Okadaic acid toxin and a sub-sample was analysed for gut content to assess the relationship between number of problematic phytoplankton in the mussel gut and the presence/absence of toxin in the mussel flesh. A small number of *Dinophysis* spp. were found in gut samples in week six of the study concurrent with an increase in the levels of toxin in the mussel flesh but the numbers of phytoplankton or toxin level never reached the regulatory limit of 0.16 $\mu\text{g/g}$ that would close a mussel production area. The relationship between increased toxin production and the presence of the toxin producing species is shown in figure 4. The method is however viable and will be repeated in the context of higher toxicity where possible in the future.

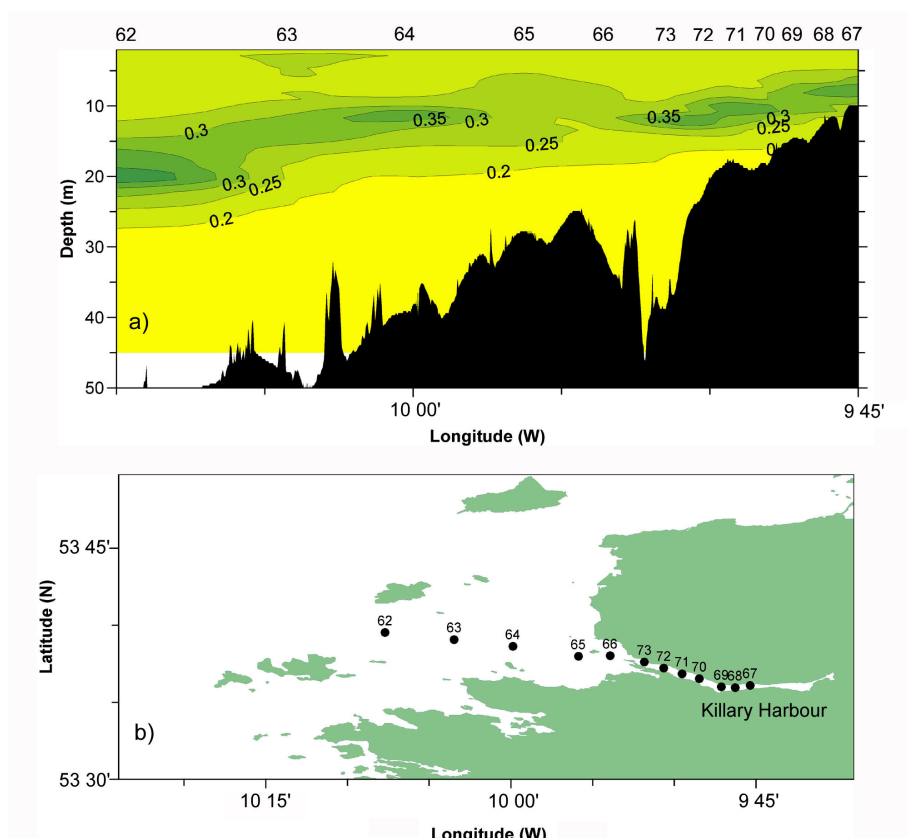


Figure 3. a) Distribution of *in situ* chlorophyll fluorescence (relative units) along Killary Harbour, 4th August 2002. Station numbers are indicated along the top axis. Locations of the sampling stations are shown in b).

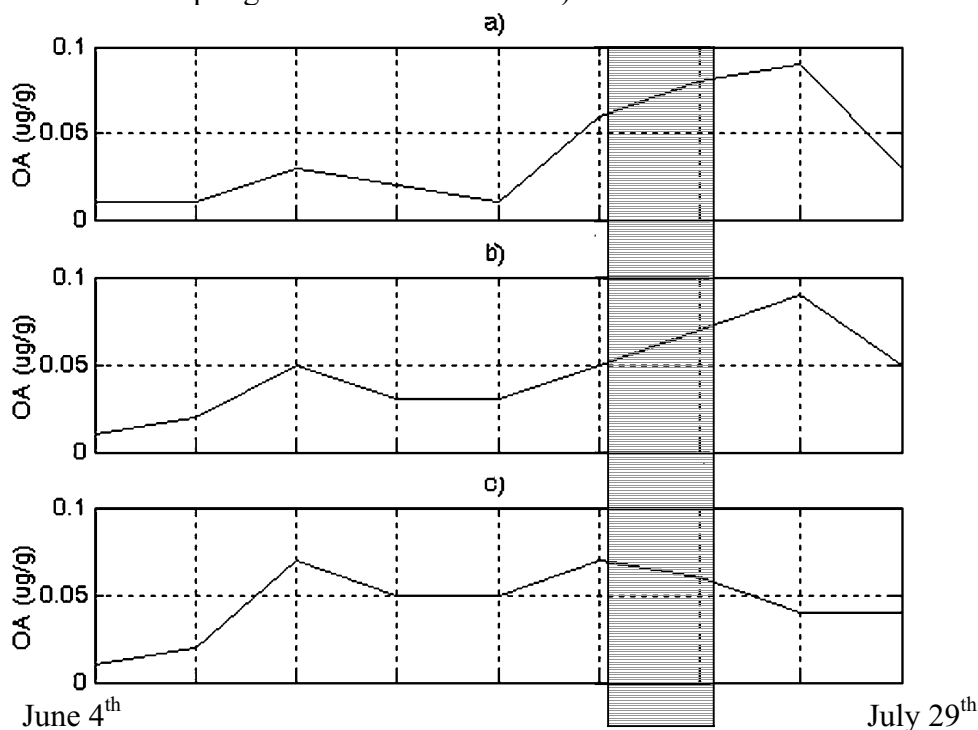


Figure 4. Plots of okadaic acid in mussel flesh samples over a nine week period in outer Killary Harbour during summer 2003, a) surface, b) mid-water and c) near bottom. Blue shaded area denotes the time during which *Dinophysis* spp. were found in the mussel intestine coincident with a rise in toxin level in the mussel flesh.

The Killary Harbour area was extensively instrumented in 2003 to examine the relationship between current patterns within Killary and on the adjacent shelf to the west. Two acoustic current profilers and five recording current meters were deployed as well as twenty temperature sensors and two multi-parameter probes. One of the two acoustic profilers has been recovered to date yielding an interesting picture of current patterns outside the entrance to Killary. Figure 5 shows the location of two profilers deployed west of Killary and a progressive vector plot of currents at three vertical levels in the water column at the inner site. The key feature of the currents at the inner (Carrickgaddy) site is that they are relatively slack at all depths ($\leq 1 \text{ cm s}^{-1}$, or overall movement of ca. 1 km per day).

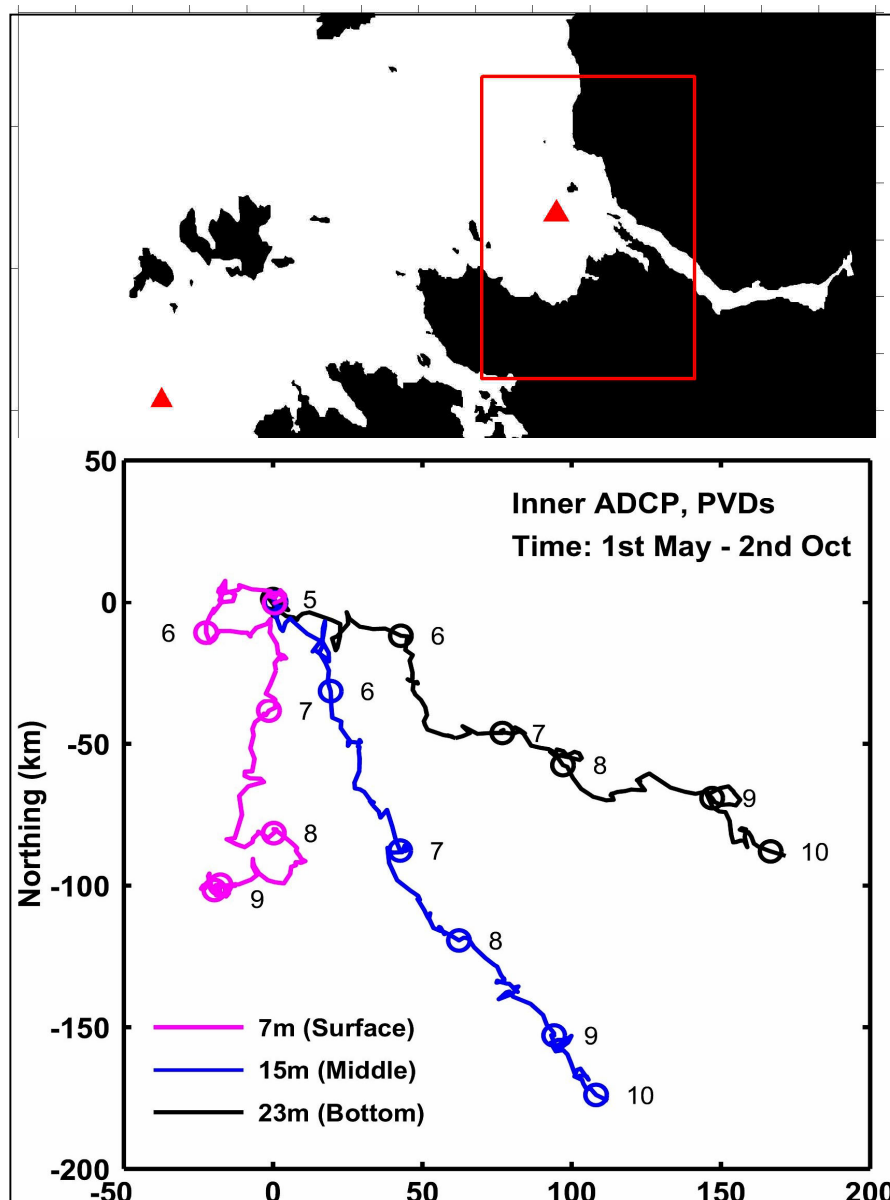


Figure 5. Progressive vector diagram of currents at the inner ADCP site at Carrickgaddy Rocks between May and October 2003. Numbers at circled points of diagram denote first day on each month eg. 5 = May 1st, 6 = June 1st. Location map is also shown in the insert panel with red triangles denoting the two mooring locations in 2003. Carrickgaddy is marked by the triangle in the upper right of the panel. The key

feature are the **very slack currents** at this location with typical speeds of 1-2 cm s⁻¹ amounting to a drift of 1-2 km per day suggesting that this is an **area where particles or phytoplankton could be retained** for an extended period.

This is about 5-10% of the current speed observed on the adjacent western Irish shelf and means that particles (phytoplankton etc) could easily be retained in this area for an extended period of time, perhaps acting as an incubation site for harmful phytoplankton in the vicinity of Killary Harbour. Another interesting aspect of the observed circulation pattern is the periodic easterly surface (7m depth) flow in figure 5 that is related to changes in the prevailing wind direction. This area of slack circulation may be retaining phytoplankton while the periodic easterly currents could then transport phytoplankton to the aquaculture sites within Killary Harbour.

A project examining near bottom and water column toxicity in scallops (*P. maximus*) has been initiated in Clew Bay in 2003 but is still in the early stages at present. The results will feed into the BOHAB project in due course. A comprehensive cyst survey of the focal bays and the adjacent coastal ocean will be conducted on vessels of opportunity in 2004 to examine distribution and substrate preference for certain cyst forming dinoflagellates. Extensive work is being undertaken at NUI, Galway and Woods Hole Oceanographic Institution (U.S. into life cycle strategies of the *Protoperinium* and *Alexandrium* HAB species) which feeds directly to the BOHAB project scientists. Desk studies are planned for 2004 in the areas of satellite remote sensing of HABs and the use of moored technologies to provide an early warning system for HABs in Irish waters.

Acknowledgements

The BOHAB project scientists would like to thank the Killary CLAMS group including Tomas Burke, Kevin Lydon, Danny Mc Nulty and Cilian Roden for their assistance in the project's first year. The assistance of Liz Abbott and Jimmy Burke at Bantry Bay Mussels is gratefully acknowledged for Bantry fieldwork. Mike Sammon and Abdon Ryan are thanked for locating instrumentation at their aquaculture sites while we thank the Irish Naval Service for providing offshore phytoplankton samples to support monitoring and the BOHAB Project. Finally, sincere thanks to Georgina Mc Dermott, Maeve Gilmartin, Mairead Murphy, Eileen Mongahan, Cian O'Hora and Niocolas Touzet on sampling, analysis and data management for BOHAB.

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INVESTIGATIONS INTO TOXIC ALGAL EVENTS IN KILLARY HARBOUR BY THE KILLARY CLAMS GROUP

Dr. Cillian Roden and Danny McNulty, Managing Director Atlantic Blackshells Ltd

Killary Harbour is an important aquaculture centre in the west of Ireland. Over 2500 tonnes of salmon and 1000 tonnes of mussels are produced in the harbour each year and up to one hundred people are employed. The aquaculture industry tackles common environmental problems by means of the Killary CLAMS (Co-ordinated Local Aquaculture Management System) group. Members meet monthly and undertake monitoring of toxic algae, spatfall, bacterial levels etc. The group is funded both by a levy on tonnage and grant aid from Bord Iascaigh Mhara. To date the most serious problem facing the group has been the frequent closure of mussel harvesting due to the presence of *Dinophysis sp* which produce toxins, thus contaminating shellfish.

Since 2001, the group has monitored harmful algal blooms in the harbour. After three years fieldwork and sampling it has been possible to draw preliminary conclusions about the *Dinophysis* problem. Our results can be summarised as follows.

- In 1981, 2001, 2002 and 2003 *Dinophysis* exceeded 40 cells per litre in June and July. Only in 2001 did cell numbers exceed these values in August and September. Significant concentrations of *Dinophysis sp.* have not been recorded in other months.
- The severity of closures could be matched with the diversity of species which appeared in June and July. Thus in 2001 a great diversity of dinoflagellates were noted in June/July including the commonly encountered *Dinophysis acuminata* and the rarer *D. acuta*. Closures in 2001 were prolonged. In the other years only *D. acuminata* was seen in a largely diatom assemblage and closures were less severe.
- *Dinophysis* is always most abundant in the outer harbour and at depth. Its presence decreases rapidly as one samples further into the harbour. We suggest that *Dinophysis* is carried into the harbour by a landward moving bottom current which underlies an outward flowing brackish layer. Detailed counts made at 1 metre vertical intervals show that *Dinophysis* moves upward and is probably then carried seawards by the surface brackish layer. This proposed mechanism would explain why closures are far commoner in the outer harbour and justifies splitting the harbour into three HAB monitoring zones.

Given this mechanism, the CLAMS group would challenge closures in spring and early summer in the absence of chemical and plankton evidence of toxins and harmful algae. The group also feels that offshore work is necessary to explain why plankton composition varies from year to year must be undertaken by the Marine Institute.

In future years the CLAMS group will continue to sample for HABs.

PROFILING SYSTEMS FOR UNDERSTANDING THE DYNAMICS AND IMPACTS OF THIN LAYERS OF HARMFUL ALGAE IN STRATIFIED COASTAL WATERS

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Introduction

Phytoplankton and zooplankton can form layers that range in thickness from 10 cm to a few meters, but persist for hours to days and stretch for hundreds of meters to kilometers (Donaghay and Osborn, 1997; Holliday, et al., 2003). Although such layers are too thin to be adequately sampled with standard methods using bottle and nets, the application of “smart” sampling methods has shown that these thin layers can contain high concentrations of harmful algae. This raises several issues: (1) what are the temporal and spatial scales of thin layers of harmful algae; (2) what are the mechanisms that control the formation, maintenance and dissipation of thin layers of harmful algae; and (3) what are the impacts of thin layers of harmful algae? Addressing these issues creates six sampling challenges: (1) sample at fine enough vertical scales to resolve the distribution of the harmful algae; (2) simultaneously sample biological, chemical and physical structures and processes, (3) avoid confounding of temporal and spatial variability, (4) sample over concentration ranges that control physical, chemical and biological responses, (5) sample over long enough time to detect patch formation, maintenance and dissipation responses, and (6) collect biological samples needed for identification and rate processes measurements. Herein, we will first consider the scales we need to make measurements, and then discuss how a series of new sensors and deployment techniques are allowing us to begin to address fundamental questions about the structure, dynamics and impacts of thin layers of harmful algae.

PART 1: Ship-Deployed High resolution profilers

Question 1: What are the critical sampling scales for assessing peak concentrations of harmful algae and the rates at which those concentrations change? Can sampling at multi-meter scales be used to adequately assess peak concentrations of harmful algae and the rates at which those concentrations change?

Approach: In order to address this question we developed a technique for simultaneously sampling the finescale vertical distribution of organisms of interest along with that of the potentially controlling physical, chemical and biological structures and processes (Donaghay et al., 1992). In this technique we use a high-resolution profiler (Figure 1) to first define the physical, optical and chemical structure of the water column at centimeter scales, and then use real-time data from this system to guide the collection with a siphon of discrete biological and chemical samples from features of interest such as thin optical layers (Figure 2), regions of intrusive flows (Figure 3), or strong chemical gradients (Donaghay et al., 1992; Sieburth and Donaghay, 1994; Hanson and Donaghay, 1998). The concept here is to over-sample the water column to identify the scales at which concentration and/or optical properties change and then use that information to select the depths and spacing for collection of discrete samples needed to identify organisms and measure rate processes. This technique has been used extensively to study the role of thin

layers in biogeochemical processes (Sieburth and Donaghay, 1992; Scranton et al., 1993, 1995; Mason et al., 1993; Hanson and Donaghay, 1998), finescale optical characteristics and dynamics of coastal waters (Twardowski, et al., 1999; Dekshenieks, et al., 2001; Twardowski and Donaghay, 2001; Alldredge et al., 2002; Twardowski and Donaghay, 2002), and the finescale structure and dynamics of a variety of organisms including bacteria (Bazalinski, et al., 1995), harmful algae (Rines et al., 2002; Sullivan et al., 2003), microzooplankton (Johnson, et al., 1995), larvae of benthic organisms (Donaghay, et al., 1992), and the coupling of phytoplankton and zooplankton (Johnson et al., 1995, Holliday, et al., 2003).

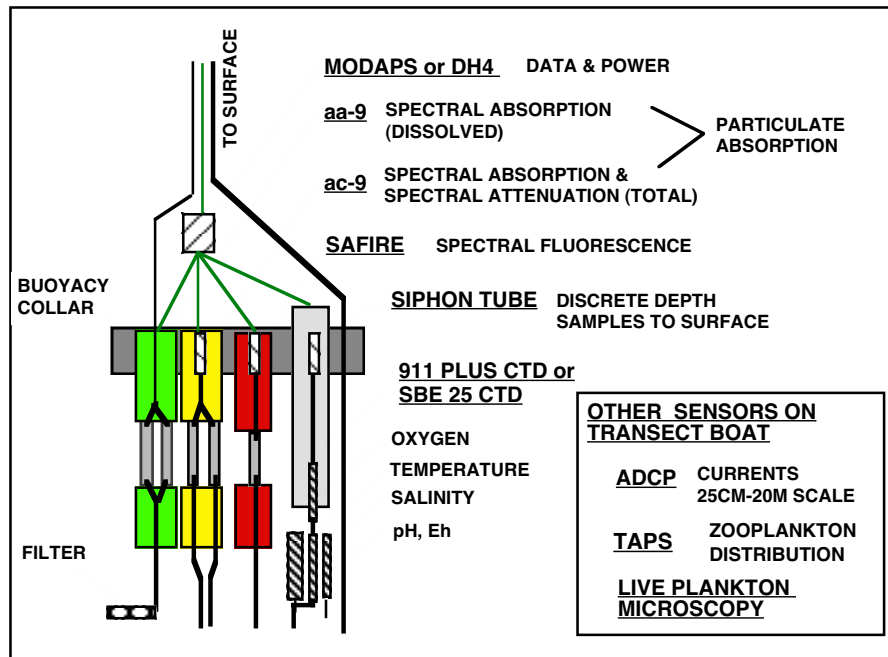


Figure 1. Schematic design of the ship-deployed high-resolution profiler and other sensors used to simultaneously measure the finescale physical, chemical, biological, optical, and acoustic structure of coastal waters. The system uses a suite of sensors designed to quantify and optically characterise the finescale vertical structure of dissolved and particulate material. The core optical sensors are the dual WET Labs ac-9s (one with a 0.2 micron pre-filter and the other with direct intakes) that allow us to measure separately the vertical structure of particulate material and coloured dissolved material (CDOM). This separation can be critical in estuarine waters where CDOM may dominate the absorption of light. Current versions of the system include additional sensors for measuring optical backscattering (WET Labs VSF or BB1) and chlorophyll a (WET Labs WetStar fluorometer). The profiler is buoyancy compensated so that it can slowly free-fall through the water column and can be decoupled from ship motions.

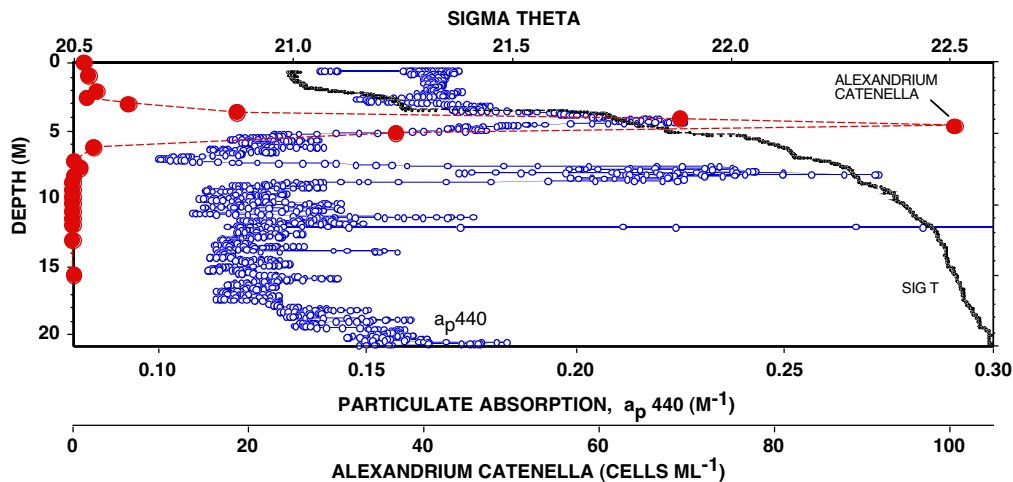


Figure 2. Finescale vertical structure of density (small black circles), particulate absorption at 440 nm (the wavelength for maximum absorption by chlorophyll a) (blue circles) and *Alexandrium catenella* (solid red circles) in 1997 in East Sound, WA. Samples for measuring species abundance were collected by siphoning samples from the depth of the thin layers seen in the absorption data.

Conclusion: Optical structure and concentrations of specific algae can change at sub-meter scales that cannot be resolved by standard bottle cast methods. However, changes in optical properties are highly correlated with changes in harmful algae (HA) and thus can be used to guide sampling of the HA.

Question 2: Do we need to sample physical and chemical structure at similar scales?

Approach: This question can be addressed by using data collected simultaneously by the high-resolution profiler and auxiliary sensors to examine the relationship between the position of the thin layer and the finescale vertical structure of potentially controlling factors such as current velocity (Figure 3). In this example, the harmful algae *Alexandrium catenella* is restricted to a thin layer of water that is flowing toward the upper end of East Sound where there are extensive aquaculture operations. Thus the simultaneous measurement of currents and organism distributions not only provides a key insight into the role of currents in controlling the dynamics of this species, but also insights into its potential impact.

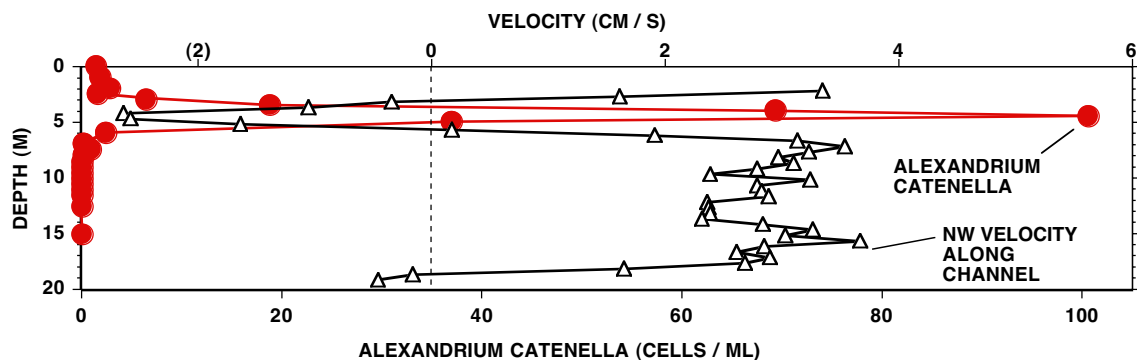


Figure 3. Overlay of the thin layer of *Alexandrium catenella* (from Figure 2) on the current velocity along the axis of East Sound, WA. Currents were measured simultaneously by the ship deployed ADCP. Figure adapted from Sullivan et al., 2003.

Conclusion: Lateral advection varies at similar vertical scales and thus must be simultaneously measured since it can be an important mechanism in controlling the thickness, intensity and depth of this harmful algal layer and its potential impact on aquaculture.

Question 3: Are these “critical scale” structures ephemeral or persistent? If persistent, how long do they last? What is their spatial scale?

Approach: A series of transects with the high-resolution profiler can be used to address this question in fjord systems where flows are topographically constrained and harmful algal blooms can persist for days and extend for kilometres along the axis of the system (Figure 4). This works particularly well if the bloom has a strong optical signal and the currents are slow relative to the rate at which the sampling boat can complete the transects. In this example we were able to map changes in the intensity and spatial extent of a thin layer bloom of the harmful algae *Pseudo-nitzschia spp.* for 9 days. The persistent association of this thin layer with water of 29.8 ppt salinity indicated that changes in depth of this species were not due to sinking, but instead were driven by advection of lighter water into the system that eventually pushed the layer downwards until it came in contact with the benthos (Figure 4c). Thus the simultaneous measurement of salinity and organism distributions not only provides a key insight into the role of sinking and buoyant plumes in controlling the dynamics of this species, but also into how to predict the impact of such blooms on benthic filter feeders.

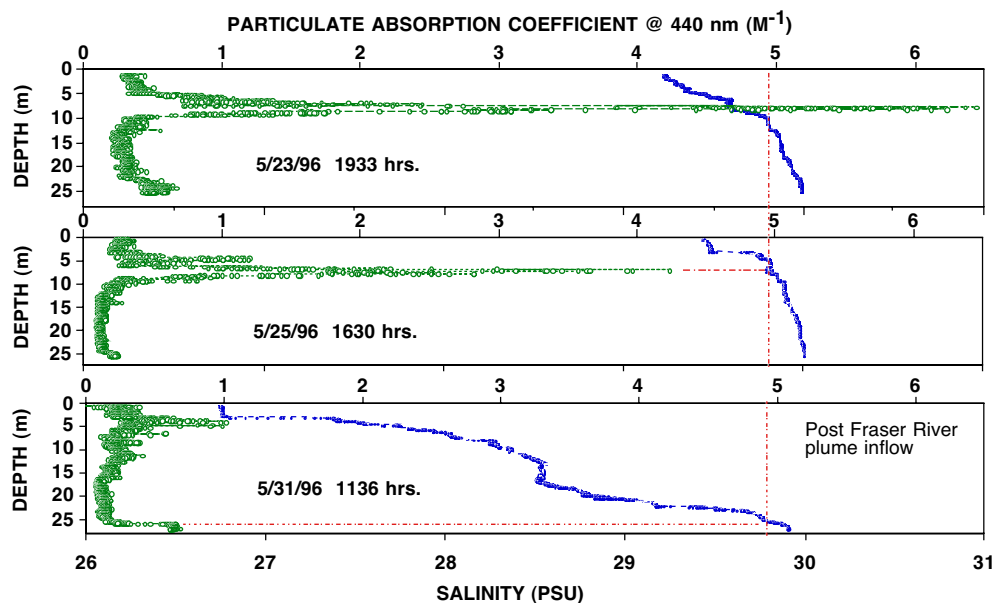


Figure 4. Persistent thin layer of phytoplankton (measured as particulate absorption at 440 nm, green circles) associated with 29.8 salinity water (blue circles) in East Sound, May 23-31, 1996. This layer was about 1 m thick at half peak height and dominated by *Pseudonitzschia spp.* (see Rines et al., 2002 for discussion). Profiles collected each day along the 12 km long axis of East Sound were used to identify the depth and spatial extent of the thin optical layer. Siphon samples collected from inside the thin optical layer were examined on the microscope to identify and confirm species composition of the layer.

Conclusion: Thin layers can extend for km, persist for days and stay closely associated with a particular water mass. However, the depth at which these layers occur (and thus their potential for interacting with the benthos) can change dramatically in response to large-scale advection of less dense waters into an area.

Part 2: Development of autonomous bottom-up profilers

Question 4: How thin can such layers be and still extend for hundreds of meters and persist for hours? How frequently do thin layers occur at a particular location (such as an aquaculture site) and how long do they persist there?

Approach: Although ship-deployed high-resolution profilers are powerful tools for characterising and tracking a thin layer once it is developed over a multi-kilometre spatial scale, they are really not suitable for addressing these questions. Given this, we have developed a semi-autonomous bottom-up profiler that can provide real-time data on thin layers and collect extended time series of centimetre-resolution profiles of physical, chemical and optical structure (Figure 5). These systems can be deployed in arrays so that we can identify those thin layers that exceed some minimal horizontal spatial scale and observe changes in the layer as it is advected through the array. For example, we used two semi-autonomous bottom-up profilers to simultaneously collect hourly profiles for two weeks at two locations 300 m apart in upper East Sound, WA. This approach demonstrated that layers as thin as 12 cm could be very intense (> 50 ug/l chlorophyll a), spatially coherent at scales in excess of 300 m, and persist for more than 18 hours (Figure 6). It also demonstrated that thin layers were not present during storms, but could rapidly reform following re-stratification.

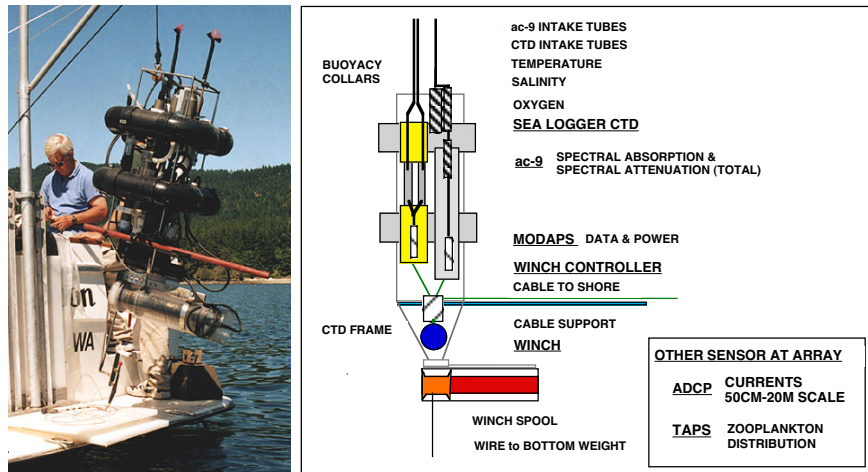


Figure 5. Photograph and schematic of semi-autonomous profiler deployed in East Sound, WA in 1998. The profiler uses a small underwater winch to collect centimetre-resolution profiles of physical, chemical, and optical structure. These profilers consist of a positively buoyant sensor package with attached winch, a bottom weight and a thin cable connecting the weight to the winch. Profiles are collected from the bottom-up as the winch slowly releases cable. Once the profiler reaches the surface, the winch pulls it back down to the bottom where it waits until the next cast. These profilers were connected to shore by a cable that provided power and 2-way real-time communications. Currents were measured nearby with a 300 kHz ADCP at 50 cm resolution and zooplankton distributions were measured with a TAPS once per minute with 12.5 cm resolution.

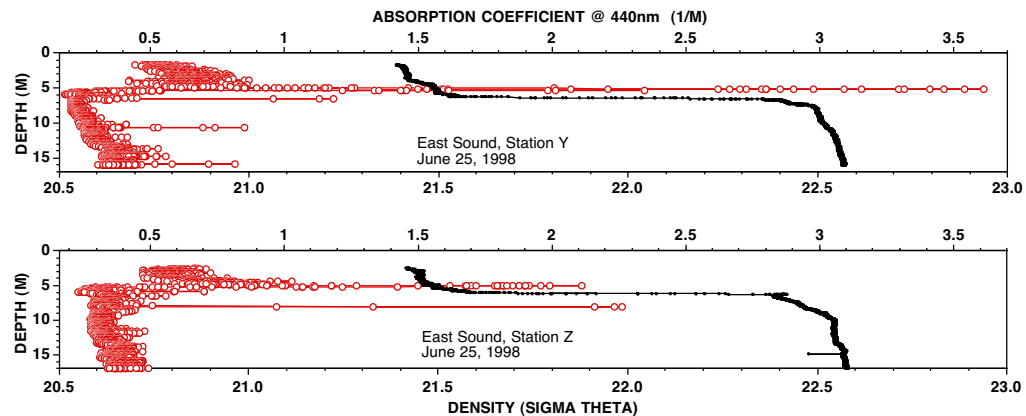


Figure 6. Finescale vertical structure of density (black dots) and absorption at 440 nm (the peak wavelength of absorption by chlorophyll a) measured simultaneously at 1600 hours at two stations located 300 m apart in upper East Sound, WA. Data were collected from the bottom-up by two semi-autonomous winch profilers. Note that the thin layer at about 6 m was 12 cm thick at half peak height. Although it was not possible to siphon sample the thin layer, phytoplankton samples collected above and below the thin layer were dominated by *Chaetoceros socialis*, but contained *Pseudo-nitzschia* spp. (see discussion in Rines et al, 2002).

Conclusion: Layers as thin as 10 cm can be spatially coherent at scales of 300 m and persist for 18 hours. Given the average current velocities at the site, this means this thin layer extended for 3.6 km.

Question 5: Can similar thin layers form and persist in more open coastal waters such as Monterey Bay? Can such layers be as intense and persistent as those seen in the more sheltered waters of coastal fjords?

Approach: Addressing this question represented a major challenge since it required developing totally autonomous, self-contained bottom-up profilers that can be used in the open coastal ocean to provide real-time data on thin layers and collect extended time series of centimetre-resolution profiles of physical, chemical and optical structure (Figure 7). As part of an effort to address these questions, we deployed one of the autonomous profilers several kilometres offshore in 20 m of water in northeastern Monterey Bay and used it to collect 178 sequential hourly centimetre-resolution profiles of physical, chemical and optical structure. The resulting data demonstrated not only that thin layers can occur in open coastal system (Figure 8), but also that they can be just as intense (Figure 8) and persistent as any seen in East Sound (Figure 9). Diver collected samples from within the layer indicated that it was dominated by *Pseudo-nitzschia* spp.

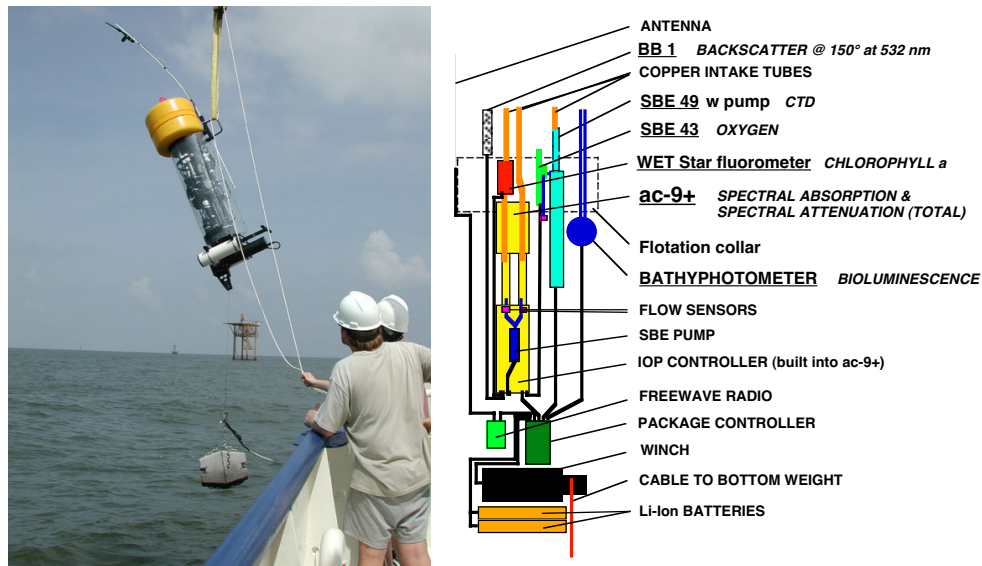


Figure 7. Picture and diagram of the autonomous bottom-up profiler used to collect a week-long time series of centimetre resolution profiles in northeastern Monterey Bay, CA. The profiler uses a positively buoyant sensor package and a small underwater winch to profile finescale physical, chemical and optical structure from the bottom up. These profilers are fully self-contained with onboard microprocessors, controllers, batteries and radio communication systems. These systems are designed to optically characterise vertical structure using sensors for spectral absorption, spectral attenuation, spectral scatter, chlorophyll a fluorescence, optical backscatter and mechanically stimulated bioluminescence. These profilers were co-developed with WET Labs.

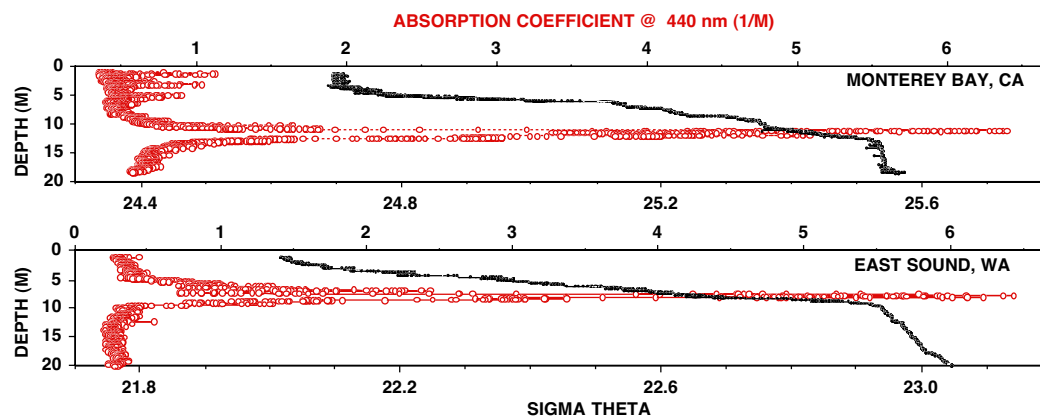


Figure 8. Comparison of finescale density (sigma theta, small black circles) and optical (absorption coefficient at 440 nm, the peak absorption of chlorophyll a, red circles) structure of the thin layer of *Pseudonitzschia* spp. in East Sound and Monterey Bay. Chlorophyll a concentrations at the peak were approximately 100 ug/l.

Conclusion: Thin layers of harmful algae in open coastal waters can be just as intense as those observed in the more sheltered waters of East Sound, WA.

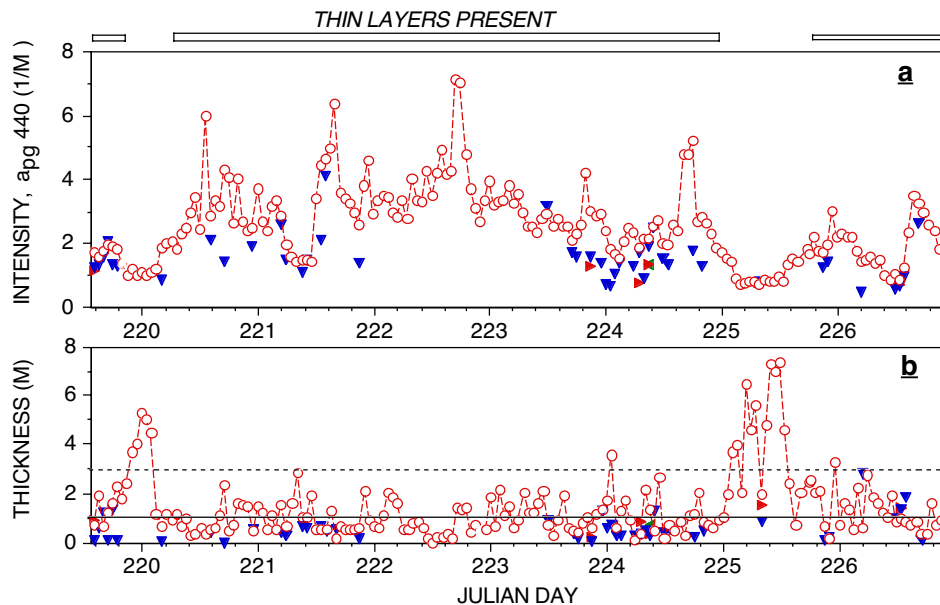


Figure 9. Temporal variation in intensity (a) and thickness (b) of optical layers in northeastern Monterey Bay, CA, August 2002. The open red circles are for the primary (*Pseudonitzschia* spp.) thin layer and the blue triangles are for thin layers that occurred elsewhere in the water column (such as the 3 thin layers that occur near the surface in Figure 8 a). The solid line and dashed lines in **b** are the modal and the 95% upper limit of the thickness of thin layers in East Sound reported by Dekshenieks, et al. (2001).

Conclusion: Thin optical layers of harmful algae in open coastal waters can have similar persistence and thickness to those in East Sound. However, biological sampling of such layers is greatly complicated by the intense internal wave fields.

Summary and Conclusions

(1) A variety of harmful algae can develop as thin layers in stratified coastal and estuarine waters. Such layers cannot be sampled with standard bottle cast methods. However, high-resolution profiling techniques can be used to detect persistent thin layers of harmful algae and increasing our understanding of their dynamics and impacts. This technology is commercially available and can be deployed from small boats as well as large ships.

(2) Cabled and fully autonomous bottom-up profilers have considerable potential for real-time assessment of the occurrence, intensity, thickness, dynamics and local persistence of thin layers. These techniques are not commercially available yet, but efforts are underway to transition them.

(3) The new generation of optical sensors on these profilers provides dramatic improvements in our ability to quantitatively assess vertical changes in the abundance and types of particles in the water column. Although techniques for extracting this information are still being developed, it is already clear that these techniques will be useful in understanding harmful algal blooms and evaluating the availability of the other types of algae needed to support aquaculture of shellfish.

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THE CLASSIFICATION OF SHELLFISH PRODUCTION WATERS

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Department of Communications, Marine and Natural Resources

This is a brief paper on the Classification of Shellfish Production Areas, a summary of particular aspects of the European Legislation, how this legislation is currently interpreted, what scope there is to redesign our system and what process will be used to develop an improved system.

In the recent past the focus of the collective energies of the MSSC has been primarily directed at issues associated with the monitoring of marine biotoxins in bivalve shellfish. There has been considerable progress in this area over the last two years and much has been learned as to the value of genuine consultation between the various parties involved in production, processing, analysis and regulation of shellfish.

Having made significant progress in the area of marine biotoxins it is now time to address a number of issues in the area of the monitoring of the microbiological quality of live bivalve molluscs. This is the fourth workshop on marine biotoxins, we may be now moving towards a situation where in the future these workshops may be expanded to give greater attention to issues related to the microbiology of shellfish.

The legal requirements

The legal framework for the classification of shellfish production areas is laid down in EU Directive 91/492/EC. This Directive focuses on the **health** conditions for the production and placing on the market of live bivalve shellfish. The Directive gives clear limits as to what the microbiological standards of a live bivalve shellfish must conform to prior to being placed on the market. In order to ensure that the shellfish will meet these market requirements it is necessary that the production areas be tested and be defined according to the categories described in the Directive.

Chapter 1 of the Annex (Council Directive 91/492/EEC) lays down the criteria that define whether a shellfish production area is classified as being A, B or C. These criteria are intended to ensure that whatever the levels of microbiological contamination, present in a production area, that the shellfish may be produced without risk to Human Health upon consumption, once subjected to the proscribed treatments.

A guideline to the DCMNR procedures for the classification of shellfish production waters has recently been presented to the MSSC. This document outlines in detail the legal basis for classification and the methods used by the DCMNR to implement the directive. There are copies of this document available to interested parties, please contact Jimmy Carney at the DCMNR, Michael Davitt House, Castlebar, Co. Mayo with your details.

The Community Reference Laboratory (CRL) CEFAS has produced guidelines that the DCMNR has adopted for the management and Classification of shellfish production areas. Details of the criteria used in the classification can be found page 12 of the document on the Classification of Shellfish Waters. The guidelines deal with issues as to the interpretation of results and the assigning of a classification category, in addition issues such as anomalous results.

Scope for modifications to the Classification System

EU Directive 91/492/EC does not prescribe in exact terms a number of parameters for of the system used to classify shellfish production waters. The list below gives some aspects of the shellfish production area classification process that are open to interpretation by the individual Member States. All the listed items above will be discussed within the MSSC with a view to improving the classification system overall. Whereas there is scope for discussion, the decisions to be made must be dictated by the imperative to protect public health and to provide a system of classification that meets the approval of DG SANCO.

- Sample frequency/Sample sites/Area Boundaries
- Classification periods/ Classification frequency/ Management of anomalous results

Other issues to be addressed

The list below represents some of the technical aspects of the classification system that require attention. The DCMNR and the MI, with the assistance of the FSAI will work to resolve these issues in a timely fashion.

- Laboratory inter-calibration
- Ireland's NRL for Micro and Virus
- A code of practice for Classification
- FVO approval
- Equality of management systems within the European Community

Partnership in the change process

The DCMNR is anxious to involve shellfish producers and processors in the process of review of the production area classification regime. There are a number of ways by which interested parties may keep themselves informed or contribute to the consideration of the issues as they arise.

How to contribute

- Communicate with the MSSC through the ISA, the local Sea Fishery Officer, Jimmy Carney, etc.
- Detailed information will always be the basis of being able to modify decisions.

Local producers should provide information on the characteristics of their productions areas, the tidal conditions, known sources of contamination, production patterns, etc. This detailed information is essential to be able to modify sampling points, sampling frequencies and area boundaries. In addition follow-up actions to resolve the reasons for anomalous results or to conduct shoreline surveys require direct co-operation with the Industry.

Immediate Actions already taken

- “Mini” Classification of a limited number of Production Areas
- Changing the time when classifications will be made.
- The MSSC will be informed of proposed Classifications in advance of the Order being made.

Following consultation with the ISA and local producer groups the DCMNR has modified the classification of five shellfish production areas. The next Classification will take place in March 2004 and will begin a cycle of classifications that will cover April to August and September to March each year. The DCMNR will present, for review, the proposed Schedule of the Order to the MSSC, in time for comment and examination.

We intend to use what we have learned in the work done to date in the development of a successful biotoxin management regime. Ultimately our decisions made must withstand external scrutiny (e.g. the FVO, trading partners, etc.)

Consumer Health is the key issue.

CONCLUDING REMARKS

Alan Reilly, Chairman, Molluscan Shellfish Safety Committee

The Marine Biotoxin Science Workshop is now an annual event where all stakeholders in the Irish shellfish industry come together to share information on the latest national and international developments in monitoring and surveillance of biotoxins. The organisers of this fourth national event, particularly the Marine Institute as the driving force behind the workshop, are to be warmly congratulated.

With regard to the toxicity of shellfish, 2003 has been a good year and toxicity was very low in comparison to other years. The introduction of the Management Cell involving all stakeholders has brought openness and transparency to decision making. It is seen by all as a major development in the risk management process.

Its success has been due to the provision of sound scientific data, which are essential for the development and expansion of the shellfish industry. The industry has seen continuous growth over the past 4 years to an estimated value of € 52.5 million in 2002. Sound science is the basis for risk management decisions that underpins the regulatory programme which demonstrates to customers and consumers alike that the highest standards of hygiene and safety apply in the production, processing and marketing of Irish shellfish.

The success of the management cell has also been due to the attitude, efforts and hard work of the representatives from the producers, the Marine Institute, the Department of Communications, Marine and Natural Resources and the Food Safety Authority of Ireland.

Over the past few years we have also seen a rapid development in the techniques used for screening biotoxins in shellfish with the introduction of chemical methods that reduce the need for over-reliance on the mouse bioassay. The introduction of LC-MS and HPLC as routine methods for the analysis of biotoxins is a major step forward in shedding light on biotoxin levels in shellfish. None of this would have been possible without an active research programme feeding results into the monitoring programme. These techniques are complemented by phytoplankton and environmental monitoring which adds to our understanding of the ecology of growing areas.

The highlights for me of this workshop were to hear about some of the cutting edge scientific research that is currently underway which is leading to the development of methodologies for future use in the monitoring and surveillance programmes. I look forward to a time when we have sufficient data to map out the toxicity profiles of bays where shellfish are cultivated, and to use this new data to proactively reduce risks to consumers and the industry.

Looking forward to next year we will need to use the data gathered to date to further improve management of biotoxins, and most importantly, to persuade legislators that the argument for moving away from the mouse bioassay is becoming stronger.

Finally, on a related note, the co-operation and communication that are now characteristics of the biotoxin monitoring need to be translated into a strengthened programme for the microbiological monitoring and classification of bays. Much work needs to be done to develop this programme along the same lines as the biotoxin monitoring programme.

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