Molluscan Shellfish Safety

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FOREWORD

Micheál O'Cinneide
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On behalf of the ICMSS 2004 Organising Committee, we are pleased to present this Book of Proceedings from the Conference, which was held in the National University of Ireland, Galway, Ireland in June 2004.

The aim of the global series of ICMSS Conferences has been well defined by our colleagues in Santiago de Compostela (Spain) who hosted the previous Conference in June 2002:

> **" To establish a forum where useful, enriching debate and interchange of knowledge flow easily on a broad spectrum in the area of Shellfish Safety "**

The ICMSS 04 Programme followed on the tradition and patterns which were set in Santiago de Compostela (2002); Southampton, New York, USA (2000) and in The Philippines (1998) of thematic sessions on a multi-disciplinary basis**.**
Our session topics included:

- **Microbiological Status of Shellfish**
- **Shellfish Viruses and Pathogens**
- **Harmful Algal Blooms (HAB) and Biotoxin Contamination**
- **HAB Mitigation and Depuration**
- **Toxicology of Shellfish Toxins**
- **Current and Emerging Analytical Methods**
- **Quality Assurance and Consumer Safety**
- **Regulation and Management of Shellfish Safety**
- **Role of Industry in Risk Management and Innovation**

We had 58 oral presentations and 92 posters in a 5-day programme and are delighted that the great majority of the contributors also submitted papers for these proceedings.

We wish to express our sincere thanks to the authors of these papers for their diligence and patience in working through the review process; to my colleagues on the ICMSS 04 Editorial Group, especially Dr. Terry McMahon, Dr. Kathy Henshilwood, Dr. Philipp Hess and our Design Editor, Bryan Deegan. Our thanks also to the many organisations that worked effectively together to co-host the ICMSS 04 Conference, including:

- Marine Institute, Galway, Ireland
- National University of Ireland, Galway
- Food Safety Authority of Ireland
- Irish Sea Fisheries Board (BIM), Dublin, Ireland
- European Commission (DG Sanco), Brussels
- ION Communications Ltd., Dublin, Ireland

The International Advisory Board provided valuable advice and leadership to our Committee during the Conference and were the decision-making group for the selection of the next country to host the ICMSS Conference.

We are delighted to welcome the initiative of New Zealand in taking on the challenge of hosting the next ICMSS in Blenheim in March 2007. We wish their Organising Committee, especially Helen Smale and Phil Busby, great success in their preparations.

It is fitting that New Zealand is the venue for ICMSS 2007, as they represent the ethos and spirit of the ICMSS Conferences very clearly in several respects, notably:

International Co-operation in tackling the challenges of managing Shellfish Safety

Industry Leadership in the effective Risk Management, Mitigation and Monitoring of the Biotoxin and Microbiological challenges in Molluscan Shellfish.

New Zealand has developed a competitive advantage for its Shellfish sector through investments in Quality Assurance programmes since the early 1990's and has been generous in promoting these standards with industry colleagues from around the globe. We in Ireland have greatly valued our interactions and exchange with regulators, scientists and industry representatives in countries such as New Zealand in developing our own Shellfish Safety programme in the past few years and we wish the ICMSS 2007 hosts every success for the next Conference.

CURRENT AND EMERGING ANALYTICAL METHODS

DEVELOPMENT OF A PCR-BASED METHOD FOR THE DETECTION OF THE DINOFLAGELLATE *ALEXANDRIUM MINUTUM* IN CONTAMINATED *MYTILUS GALLOPROVINCIALIS* MUSSELS

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Abstract
Paralytic Shellfish Poisoning (PSP) is a syndrome caused by the consumption of shellfish contaminated with neurotoxins produced by the marine dinoflagellate genus *Alexandrium*. *Alexandrium minutum* is one of the toxic species most widespread in the Mediterranean basin. The standard monitoring of shellfish farms for the presence of harmful algae and related toxins usually requires the microscopic examination of phytoplankton, bioassays and toxin determination by HPLC. The microscopy procedures are time-consuming and require taxonomic expertise and experience, thus limiting the number of specimens that can be analysed. Molecular biology techniques have great potential in the detection of target microrganisms in field samples. In this study, a PCR-based assay was developed for the detection of *A. minutum* in seawater samples and in contaminated mussels, using primers designed based on the ITS1-5.8S-ITS2 rDNA regions. Two primers specific for the genus *Alexandrium* were designed based on the conserved 5.8S rDNA region, while a primer specific for *A. minutum* species was designed based on the variable ITS1 region. The results showed that PCR is a valid, rapid alternative procedure for the detection of target phytoplankton species either in seawater or directly in mussels, where accumulation of PSP toxins may cause serious problems for human health.

Introduction

The toxic algae responsible for HAB events in Mediterranean Sea belong to the genera *Alexandrium*, *Pseudo-nitzschia* and *Dinophysis*. In particular, the toxins produced by the genus *Alexandrium*, when accumulated in mussels, may cause paralytic shellfish poisoning (PSP) in humans (Harvell *et al.*, 1999). The shellfish production areas along temperate coasts throughout the world are regularly affected by toxic phytoplankton that even when present in low cell concentration can contaminate shellfish. The dinoflagellate *A. minutum* is the most widespread toxic species in the Western Mediterranean basin (Vila *et al.*, 2001). Once a shellfish product is contaminated, mitigation strategies are relatively limited. For this reason most countries have implemented monitoring programmes to protect public health and reduce economic impacts. The standard monitoring of shellfish farms for the presence of toxic microalgal species and related toxins usually requires accurate morphological identification and the enumeration of phytoplankton by microscopy

(Steidinger *et al.*, 1997), bioassays and toxin determination by HPLC analysis. These procedures are time-consuming and demand taxonomic and technical experience. Molecular methods are attractive as diagnostic tools because they can rapidly detect limited numbers of specific organisms in mixed ecological scenarios. Although the first review detailing the use of molecular probes for the detection of HAB species was presented in Anderson (1995), the application of these methods are still not commonly used in the routine monitoring programmes for screening natural samples at present. Different regions of the ribosomal DNA (rDNA) cluster have been selected as targets for PCR amplification in microalgae. The PCR amplification technique of target rDNA regions has been successfully employed for the detection of various toxic dinoflagellates in seawater samples. In particular, Godhe *et al*. (2001) described a PCR-based method for the detection of *A. minutum* in field samples using primers designed based on the small subunit (SSU) rDNA region.

Methods

In this case, the primers were not species-specific since the PCR also yielded a product from two closely related species. We tried to improve the specificity and sensitivity of the assay by designing new primers based on the ITS1-5.8S rDNA regions of *A. minutum*. In fact, the internal transcribed spacers (ITS1 and ITS2) of the rDNA have been used as molecular targets for the identification of microbial species because they are usually variable enough to be used as species-specific target regions (Litaker *et al*. 2003). We designed the PCR primers using an alignment of all available rDNA *Alexandrium* sequences from GenBank database. Primers 5.8S-5' (5'-GCAADGAATGTCTTAGCTCAA-3') and 5.8S-3' (5'-GCAMACCTTCAAGMATATCCC-3') were designed to target consensus sequences specific for the genus *Alexandrium*. Primer ITS1m (5'-CATGCTGCTGTGTTGATGACC-3') was designed to a target sequence specific to the *A. minutum* species and was used along with the reverse primer 5.8S-3' in the PCR assays.

PCR reaction conditions were previously described by Galluzzi *et al*. (2005). Selected primers for the conserved 5.8S rDNA region of Alexandrium genus and primers specific for A. minutum species gave PCR products of 135 and 212 bp, respectively. The primers' specificity for the genus Alexandrium and for the species A. minutum was confirmed first in silico using BLAST, then by amplification of a representative sample of the genomic DNA of other dinoflagellates and by amplification of ITS1-5.8S rDNA-cloned sequences from A. minutum, A. catenella, A. tamarense, A. taylori and A. andersoni. PCR sensitivity was tested using a plasmid containing the ITS1-5.8S rDNA sequence of A. minutum.

Results

The assays were sensitive enough to detect 10 copies of ITS1-5.8S-ITS2 rDNA sequences, with or without 100 ng of background DNA purified from uncontaminated mussels, whether using genus-specific or species-specific primers. In the attempt to validate the PCR assay on seawater, 12 natural samples fixed with Lugol's solution were analysed by PCR. In four of these samples *A. minutum* was the dominant species while other species were not significantly represented. In five samples *A. minutum* was not the dominant species and it was present as small percentages of the total phytoplankton. Two samples did not contain *A. minutum* cells, instead these samples were made up of either very high densities of *A. taylori* or the dinoflagellate *Dinophysis sacculus* in which *Alexandrium* cells were not detected using LM techniques. The purified genomic DNA was amplified either with the *Alexandrium* genus- or *A. minutum* species-specific primers and the results showed that the PCR assays were able to detect *Alexandrium* cells in all seawater samples in which these cells were detected using LM techniques.

After validation using seawater samples, this assay was used to detect *A. minutum* cells in contaminated mussels, in order to provide a rapid method for monitoring the presence of toxin-producing algal species in mussel tissues. Cultures of *Mytilus galloprovincialis* acclimatised to laboratory conditions were spiked with *A. minutum* cells (Figure 1). Two mussels were removed at day 0 (7 hours after the addition of the *Alexandrium* cells), at day 3 and day 7. To confirm that cells were filtered by mussels, algal cell concentration was determined each time the mussels were removed. The hepatopancreas of the mussel was separated from the rest of the tissues to evaluate if any similarities in the distribution of microalgal DNA occurred in the digestive or non-digestive tissues. The gills, which harvest food particles toward the mouth, were always included in DNA extractions. Mussel genomic DNA was extracted using phenol-chloroform method as described by Rawson *et al.* (1999). Using 1000 or 100 ng of mussel DNA as template in the PCR assays, the amplified products were clearly visible until day 7, when algal cell concentration in the water was 2.5×10^3 cells·L^{-1} (Table 1). Positive controls spiked with 10^4 copies of ITS1-5.8S-ITS2 *A. minutum* cloned sequence were included in each PCR reaction to check DNA amplifiability.

The results obtained in this study demonstrate that *Alexandrium* cell detection by PCR in natural samples using ITS1-5.8S rDNA regions as targets is feasible, sensitive and specific. The species-specificity of the PCR assay was improved compared to that obtained in the study carried out by Godhe *et al.* (2001) because we have designed the *A. minutum*-specific primer (ITS1m) in the high variable ITS1 rDNA region. Moreover, we demonstrate that this PCR assay could be used for the detection of *Alexandrium* cells directly in mussels, where accumulation of PSP toxins may cause serious problems for human health. Further experiments will be needed to assess the effective toxicity of mussels in which *A. minutum* cells were detected by PCR. The assessment of *A. minutum* cell detectability in mussels containing PSP toxins will also be of importance in the future. In fact, in some cases it is possible to detect PSP toxicity in mussels even when cell concentrations in the water column are very low or cells are absent.

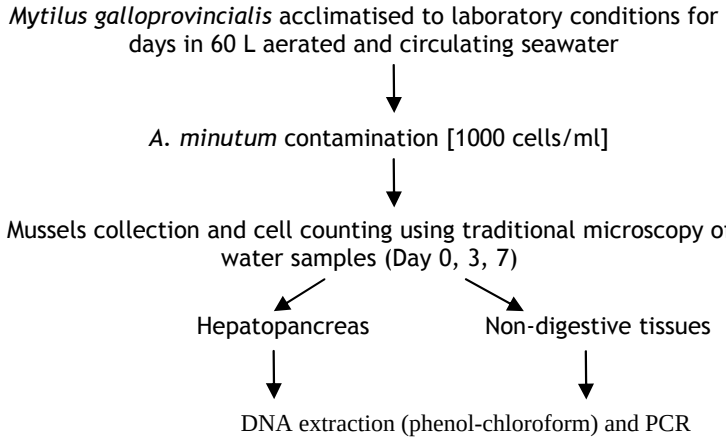

Figure 1: Flow chart of the procedures carried out during the mussel contamination experiment

Table 1: Results of PCR-analysis for the detection of *Alexandrium* cells in contaminated mussels. + indicates a detectable PCR product after agarose gel electrophoresis.

	Alexandrium genus-specific PCR		*A. minutum* species-specific PCR		*A. minutum* concentration (cells/ ml)
	Hepatopancreas	Other tissues	Hepatopancreas	Other tissues	
Day 0	+	+	+	+	100
Day 3	+	+	+	+	14
Day 7	+	+	+	+	2.5

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CHARACTERISTICS AND APPLICATIONS OF THE JELLETT RAPID TESTS

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Abstract
The Jellett Rapid Tests for PSP has been accepted for use in the USFDA's National Shellfish Sanitation Program of the USA as a screening tool. The ASP test detects domoic acid well below the regulatory limit, at around 5 µg/g, but with dilution, can be used with a higher detection limit. It is being used in processing plants to manage incoming shellfish and in field applications for both shellfish and phytoplankton monitoring. At one test site in Washington State, the Rapid Test for ASP used to monitor phytoplankton gave several days warning of impending shellfish toxicity compared to shellfish monitoring. These projects show that both phytoplankton and shellfish monitoring based on rapid testing could enhance public safety, especially in remote areas, and that phytoplankton monitoring could provide early warnings of shellfish toxicity. The Rapid Tests for PSP and ASP are being tested in a number of isolated communities, by shellfish farmers and by processors, and for phytoplankton monitoring.

Introduction
The mouse bioassay (Adams *et al.*, 2000) has successfully been used to monitor for shellfish toxicity for more than 50 years. Ethical and other issues have led toward replacement, such as the more sophisticated liquid chromatography with mass spectrometric or fluorescence detectors. They not only provide a higher degree of accuracy and sensitivity, but individual toxins can be identified in complex mixtures. Ongoing problems with sample clean up, the high capital cost of the instruments and their maintenance, and requirement for a well-equipped laboratory and trained staff has limited their use to sophisticated and well-equipped laboratories. The ELISA methods, based on antibodies, require little sample preparation and relatively inexpensive equipment. However, ELISA methods take several hours, and are difficult to use outside the laboratory or in unskilled hands. Lateral flow immunochromatography (LFI) is an alternative format for antibody detection of shellfish toxins and is widely used in medical and veterinary diagnostics, the best known being the home pregnancy test. This method is self-contained, simple enough to be used by anyone, and reliable. They are essentially yes/no tests engineered to indicate a specific analyte concentration. We have developed LFI tests for PSP and ASP, and one for DSP is being developed. The absence of a coloured test line on the strip indicates that the sample contained the toxin at a concentration around half the regulatory limit. The LFI format can be used to screen a large number of samples quickly and only those with toxin concentrations above or approaching regulatory limits need to be tested using confirmatory and quantitative methodology; thereby speeding throughput, and

reducing costs and the number of mice used in bioassays. The Jellett Rapid Test for PSP (formerly, MIST Alert™) is based on antibodies that recognise all of the saxitoxin (Stx) and neosaxitoxin (Neo) analogues, but not equally. Our first publication (AOACI, 1999) describing the characteristics of the PSP test showed relative sensitivities to a range of purified PSP toxins. All fell within the regulatory limit. Sensitivities to Neo and its 11-sulphated analogues (Gtx1/4) were about five fold less than to Stx and its analogues. Detection levels for the sulfamate analogues of Stx (C1/2 and B1) fell between the two (Gtx2/3 and Gtx1/4) extremes. The PSP test has been subjected to extensive field trials (Jellett *et al.*, 2002, Laycock *et al.*, 2001), which showed no false negatives in over 2000 samples. Extracts containing only Gtx1/4 or Neo are rare but if encountered at concentrations close to the regulatory limit, they would fall within the detection limit of the test (Laycock *et al.,* 2004). Independent studies have said that the test is "rugged and reliable" (Mackintosh *et al.,* 2002a). The ASP test has also been subjected to independent testing and shown to be easy to use and reliable (Mackintosh *et al.,* 2002a, Mackintosh *et al.,* 2002b). The detection limits of the ASP test were examined using spiked negative extract and shown to be about 5 µg/g (Laycock *et al.,* 2004). The Rapid Tests have also been shown to be effective for the detection of cultured and naturally occurring phytoplankton in seawater (Rafuse *et al.*, 2002, Silva *et al.*, 2001).

Results and Discussion

In January 2004, the USFDA approved the recommendation of the Interstate Shellfish Sanitation Committee to include the Jellett Rapid Test for PSP into the National Shellfish Sanitation Program. Its use as a screening tool to remove negative samples from the predominantly negative sample stream of most regulatory laboratories has the potential to reduce the use of mice by as much as 50 % (Mackintosh *et al.*, 2002a). A number of countries are testing the use of the Rapid Test for PSP in their regular monitoring systems, and some have already incorporated it into their programs. The Rapid Test for ASP and the novel Rapid (or "cold") Extraction Method are both in trials now that are underway in many countries, and the new DSP test is expected to be ready for trials soon. Preliminary work with the Rapid Tests for PSP was done using extracts from cultures. Domoic acid was determined in several samples of cultured *Pseudonitzschia pungens/multiseries* from New Brunswick, Canada, using the FMOC method. These extracts were tested in the Rapid Test by serially diluting them with Rapid Test phytoplankton buffer to determine the sensitivity of the tests. Seawater samples containing *P. pungens/multiseries* for La Push, Washington, and *P. delicatissima* from La Push and New Horizon, Washington, were similarly tested using HPLC and/or the receptor bioassay. Table 1 shows the results of these dilutions. The Rapid Tests for ASP were capable of detecting between 2-20 ng/ ml of DA in the phytoplankton extracts, with no apparent inhibition of co-occurring species in the naturally occurring samples.

TABLE 1. Preliminary Detection of Domoic Acid in Extracts from Cultured and Naturally Occurring *Pseudonitzschia* spp. with the Rapid Test for ASP

Sample	HPLC ng/ ml	Detection in 100 uL sample applied to the Rapid Test			
		1/5	1/25	1/125	1/625
Moncton					
1	578	+	+	-	-
2	622	+	+	-	-
3	5798	+	+	+	-
4	4928	+	+	+	-
5	8.7	-	-	-	-
6	4.7	-	-	-	-
7	control SW	-	-	-	-
La Push, WA					
1	0	-	-	-	
2	20	-	-	-	
3	225	+	-	-	
4	1026	+	+	-	
5	control SW	-	-	-	
La Push					
New Horizon					
1	300	+	+	+	-
2	458	+	+	+	-
3	170	+	+	-	-
4	80	-	-	-	-
5	100	+	-	-	-
6	75	-	-	-	-
7	control SW	-	-	-	-

The Rapid Tests for PSP and ASP have been used in a pilot project on the west coast of Washington State for both shellfish and phytoplankton monitoring and are being done with a great deal of success. One example of such success was seen at Kalaloch Beach, on the coast of Washington State, where razor clams are a tremendous resource both commercially and for ongoing subsistence use by native people (Smith *et al.*, 2002). Phytoplankton monitoring was already being performed using traditional microscopic methods (Trainer, 2003), and the Rapid Test for ASP was implemented in the summer of 2003. Toxic forms of *Pseudonitzschia* spp cannot be identified without electron microscopy, but it was possible to identify the main groups by light microscope. The different groups of species and relative percentages of each of the main *Pseudo-nitzschia* spp. were determined using microscopy (Figure 1). The main species fell into three groups: 1) the small cells of *P. delicatissima* and *P. pseudodelicatisima*, 2) the long and narrow *P. pungens* and *P. multiseries*, and 3) the fat cells of *P. fraudulenta*, *P. australis* and *P. heimii*. Rapid testing was initiated when the fat cells exceeded 4×10^4 cells/L or when the smaller cells exceeded 1×10^6 cells/L. There was only one occasion during the

summer/fall of 2003 when the cells counted reached the trigger level for a Rapid Test, with the smaller cells approaching 1 x 10^6 cells/L on August 28 (Figure 1, marked as 1). A Rapid Test was performed on the same sample as that counted by collecting the cells from 1L of unfixed seawater by filtration, then incubating the filters in 2.5 ml of 0.1 N acetic acid for one hour. A 100 µL aliquot of sample was diluted in Rapid Test buffer, then applied to the Rapid Test strip. The test strip produced a positive result (T line was absent), with the trigger level of the procedure having been determined to be about 300 ng particulate DA/L seawater filtered. Dilutions were performed with the extract to detect 600 ng/L and the test result was pos/neg (a pale T line result indicates that the toxin in the sample is near the detection limit of the test), and 1200 ng/L, which was negative (full T line present). This showed semi-quantitatively that the sample contained more than 300, approximately 600 ng/L, and less than 1200 of particulate DA/L in the seawater. Aliquots of the same seawater samples were also processed for determinations in the receptor binding assay, but the results are still being analysed. Figure 2 shows the test results on the test units.

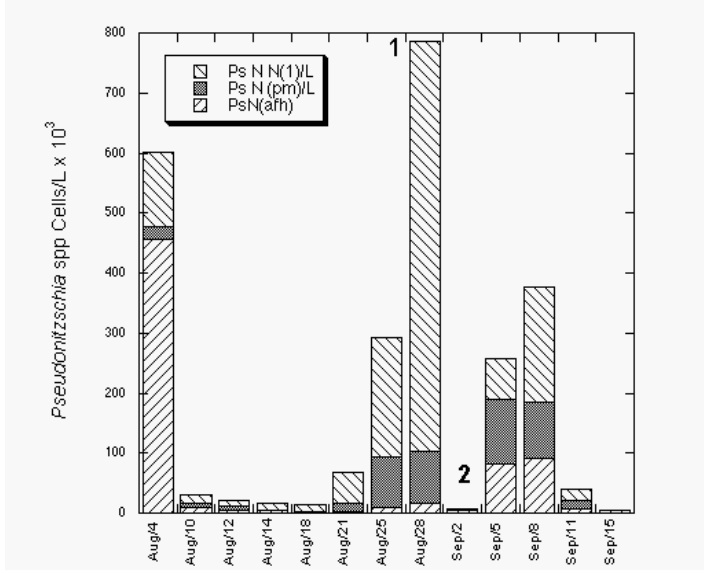


Figure 1. Counts and relative percentages of three main species of *Pseudonitzschia* spp.
Ps N (N)1: *P. pseudodelicatissima* and *P delicatissima* (smaller cells)
Ps N (pm): *P. pungens* and *P. multiseries* (larger cells)
Ps N (afh): *P. australis, P. fraudulenta*, and *P. heimii*
1: Positive Rapid Test
2. Positive shellfish tests occurred here

Shellfish testing was also being performed on shellfish from Kalaloch Beach by Washington Department of Health as usual at the north and south ends of the beach. The shellfish extracts were analyzed by HPLC. Shellfish samples taken on August 28 at the Department of Health north and south beach sites contained 8 and 15 µg/g by HPLC (Figure 3, marked "1"), respectively, although the seawater samples indicated high levels of toxin in the phytoplankton. The next razor clam samples were collected on September 2 at the north beach where they were found to contain 24

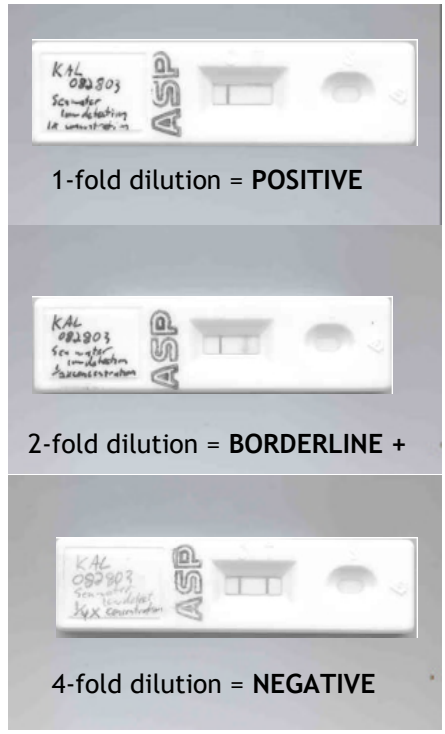
µg/g, which exceeds the regulatory limit of 20 µg/g. Another sample was taken on September 3 for Department of Health analysis and was found to contain 40 µg/g. Both of the September shellfish test results are shown in Figure 3 marked as "2."

Kalaloch Beach phytoplankton sample collected 8/28/03

1L seawater filtered then filters diluted in 2.5 ml 0.1 N acetic acid Sample then diluted 5x in phytoplankton buffer (= 1-fold dilution)

Detection limit is ~300 ng particulate domoic acid per L seawater filtered

Three dilutions were tested to determine approximate concentration



First line = control to make sure the test is working
If second line is absent, sample is STRONG POSITIVE

If second line is less than control, sample is BORDERLINE POSITIVE

If second line intensity equals the control, sample is NEGATIVE

RESULT: Sample domoic acid level is ~500-600 ng/L or a dangerous level for shellfish. This will be confirmed by receptor binding assay

Figure 2 Seawater testing by Jellett Rapid Tests

This showed that the phytoplankton test result gave 5-6d warning of impending toxicity in the razor clams, and is a clear indication that phytoplankton monitoring can provide early warnings. Since there are many pennate diatoms that resemble *Pseudonitzschia* and are common in coastal waters, only trained personnel can easily perform monitoring that would differentiate different species. Field-friendly Rapid Tests could allow voluntary monitoring by the general public using simple field microscopes to look for substantial numbers of pennate diatoms, and a Rapid Test to determine the presence of toxins. This approach to coastal monitoring for these harmful toxins could enhance public health safety, in addition to providing extra sampling for the regulatory agencies to give advanced warning of impending shellfish toxicity. The triage of shellfish samples using the Rapid Test in advance of quantitative analysis can reduce costs, reduce animal use, as well as speeding up throughput during critical toxic bloom periods.

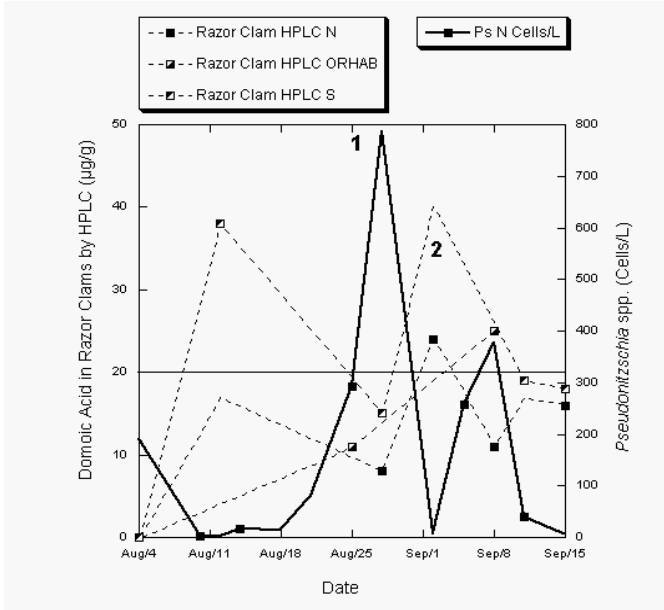


Figure 3. Total cells per Liter of *Pseudonitzschia* spp. in the seawater at Kalaloch Beach between August 3 and Sept 15, 2003, and µg of domoic acid (DA) per gram of shellfish tissue by HPLC from razor clams samples from three collection sites on the beach.
Razor Clam N: Department of Health North Beach collection site
Razor Clam S: Department of Health South Beach collection site
Razor Clam ORHAB: ORHAB Project collection site
1: Positive Rapid Test results in phytoplankton samples (but all shellfish results are below the regulatory limit (marked at 20 µg/g)
2: HPLC results from shellfish samples show that razor clams have exceeded the marked regulatory limit

Acknowledgements
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DEVELOPMENT OF REAL-TIME PCR ASSAYS FOR THE DETECTION OF *Alexandrium tamarense* AND *Alexandrium minutum*

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Abstract
It is well documented that species of the paralytic shellfish poisoning (PSP) marine dinoflagellate genus *Alexandrium,* present difficulties in accurate identification and differentiation from each other. In Ireland, the main *Alexandrium* species identified are *A. tamarense* and *A. minutum*. These species are difficult to distinguish reliably under conventional microscopy without the use of electron microscopy, which allows for the examination of detailed morphological features unique to a particular species. Species-specific molecular probes involving real-time PCR have the potential to provide accurate and rapid identification and enumeration of such species. Unialgal cultures of *A. tamarense* and *A. minutum* were cultured from cysts extracted from sediment samples from a number of locations around the Irish coastline. The D1-D2 region of the large ribosomal subunit (LSU) of *A. tamarense* and *A. minutum* isolates were PCR amplified and sequenced. Sequence alignments were performed against other *Alexandrium* species in the GenBank database using ClustalW. The *A. tamarense* isolates from Ireland showed distinct differences with *A. tamarense* of the North American ribotype (sequence identity of 93 %). The *A. minutum* isolates demonstrated high sequence similarity (95-100 %) to other *A. minutum* isolates. Based on the comparative sequence data generated, real-time PCR assays using FRET hybridisation probes were designed for *A. tamarense* (Western European ribotype) and *A. minutum*. The use of hybridisation probes allowed for the simultaneous identification of a number of closely related species based on sequence polymorphisms in the probe region among these species. The assay designed for the detection of *A. tamarense* (W.E.) allowed for simultaneous identification of the North American ribotype of *A. tamarense, A. fundyense* and *A. catenella*. Similarly the assay designed for the detection of *A. minutum* allowed for the simultaneous detection of *A. ostenfeldii*. The detection limit for both assays is approximately less than one cell. The application to the detection and identification of *A. tamarense* and *A. minutum* in environmental samples has been demonstrated.

Introduction
The Irish shellfish industry is vital to coastal communities and represents an important component of Irish food exports. In order to satisfy EU legislation pertaining to the production and export of shellfish (Council Directive 91/492), the Irish Marine Institute have implemented a national biotoxin monitoring programme that combines extensive phytoplankton monitoring from production sites as well as biotoxin testing of shellfish. Currently, the identification of phytoplankton species relies on the use of light microscopy, which is based on unique morphological features of the individual species. This method allows for a comprehensive overview of the phytoplankton population present in the water column and can precisely identify selected toxic species such as *Dinophysis* spp. In some cases it is difficult to discriminate between species of the same genera (e.g. *Alexandrium* spp. and *Pseudo-nitzschia* spp.). The genus *Alexandrium* is responsible for paralytic shellfish poisoning (PSP), and in Ireland the main *Alexandrium* species identified are *A. tamarense* and *A. minutum*. For accurate identification of these species electron

microscopy is required which examines detailed morphological features unique to a particular species. Such fine levels of discrimination are often not feasible in monitoring programs or studies that involve large numbers of samples. Molecular probes have the potential to provide accurate and rapid identification and enumeration of toxigenic phytoplankton based on the genetic diversity of different genera and species (Medlin *et al.,* 1998; Higman *et al.,* 2001). DNA probes for identifying harmful species have been applied in different formats including the whole cell format (Adachi *et al.,* 1996; Miller and Scholin, 1996; Miller and Scholin, 1998), sandwich hybridisation assays (Scholin *et al.,* 1997; Scholin *et al.,* 1999) and more recently real-time PCR assays (Bowers *et al.,* 2000; Galluzzi *et al.,* 2004). The genomic target chosen for these assays are the ribosomal DNA (rDNA) genes because of the high copy number of these sequences in cells and the range of very conserved to highly variable sites within the rDNA sequences (Sogin *et al.,* 1986; Stryer, 1995). The eukaryotic ribosome comprises of several subunits including the small subunit (SSU), the large subunit (LSU), the 5.8 subunit and two internal transcribed spacer regions, the ITS 1 and ITS 2.

In this study, we report on the development of two real-time PCR based assays for the identification of *A. tamarense* and *A. minutum* in Irish coastal waters. The whole cell format has been applied for the identification of *A. tamarense* of the North American ribotype (Miller and Scholin, 1998) and there are a number of PCR (Godhe *et al.,* 2001) and real-time PCR assays (Galluzzi *et al.,* 2004) described for *A. minutum*. PCR and particularly real-time PCR offer advantages over the whole cell assay format in terms of sensitivity and high-throughput of samples. In this study molecular probes for real-time PCR assays were designed following DNA sequencing of the D1-D2 region of the LSU from a range of Irish *A. tamarense* and *A. minutum* isolates. Real-time PCR assays were developed using the LightCycler™ and FRET hybridisation probe technology. The FRET hybridisation probe format involves the application of two specially designed sequence specific probes labeled with fluorescent dyes. The sequences of the two probes are selected such that they hybridise to the amplified DNA fragment in a head to tail arrangement. When the probes hybridise in this orientation, the two fluorescence dyes are positioned in close proximity to each other. The first dye (fluorescein) is excited by the LightCycler's LED (Light Emitting Diode) filtered light source, and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity the emitted energy excites the LC Red 640 dye attached to the second hybridisation probe that subsequently emits red fluorescent light at an even longer wavelength. This energy transfer, referred to as FRET (Fluorescence Resonance Energy Transfer) is highly dependent on the spacing between the two dye molecules. The probes need to be in close proximity (a distance between 1–5 nucleotides) for this energy to be transferred at high efficiency. The increasing amount of measured fluorescence is proportional to the increasing amount of PCR product generated. The real-time PCR assays developed in this study were evaluated for specificity and sensitivity, and their application for the detection of *A. tamarense* of the western European (W.E.) ribotype and *A. minutum* in field samples was assessed.

Materials and Methods

Algal cultures

A. tamarense and *A. minutum* cysts were isolated from sediment samples collected from the Irish coastline. Several unialgal cultures of both species were maintained for several transfers in mid to late exponential growth in modified f/2 media (Guillard and Ryther, 1962) made with 0.2 *µ*m filtered seawater. Cultures were grown at 15 ^{o}C on a 12:12 h LD cycle (ca. 100 µmol photons $m^{-2}sec^{-1}$ irradiance provided by cool white fluorescent bulbs). The precise identification of the species required the use of calcofluor white staining with epifluorescent microscopy which allowed the discrimination between *A. tamarense* and *A. minutum* based on the unique features of their thecal plates. Other cultures of *Alexandrium* including *A. tamarense* (ATIR/01, PW06 and SP3B8-3), *A. minutum* (AM1 and AMBOP006), *A. catenella* (ATTL01), *A. ostenfeldii* (K-0287) and *A. fundyense* (GTCA29) were grown under the same conditions.

DNA extraction from unialgal cultures

Unialgal cultures (15 ml) were centrifuged at 4000 g for 10 minutes. Genomic DNA was extracted from the cell pellets using the DNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions. DNA preparations were stored at –20 ^{o}C.

Amplification of D1-D2 region of LSU rDNA

PCR amplification was performed in a final volume of 100 µl containing the following: 100 ng genomic DNA, 0.1 µM of the forward (D1R) and reverse (D2C) primers (Table 1) (Lenaers *et al.*, 1989), 2 mM $MgCl_2$ (Promega, Wisconsin, USA), 1X PCR buffer (Promega, Wisconsin, USA), 200 µM of dNTPs (dUTP) (Roche Diagnostics, Ltd, UK) and 2 U Taq polymerase (Promega, Wisconsin, USA), and made up to 100 µl with PCR grade water (Sigma-Aldrich, UK). Thermocycling was performed on a Primus HT (MWG Biotech, UK) using the following conditions: an initial denaturation at 95 ^{o}C for 5 minutes, followed by 35 cycles of 94 ^{o}C for 1 minute, 50 ^{o}C for 1 minute and 74 ^{o}C for 1 minute, and an extension at 72^{o}C for 10 minutes. The resulting PCR products were analysed on a 1.5 % agarose gel and visualised under UV light source using the Bio Profil Imaging system and analysed using BIO ID V 6.11 software (Vilber Lourmat, France) in conjunction with a video copy processor (Mitsubishi Electric Corporation, Tokoyo, Japan).

DNA sequence analysis

The D1-D2 region LSU rDNA PCR products were purified using the PCR product Pre-sequencing kit (USB Corporation, USA) according to the manufacturer's instructions. Purified PCR products were sent to Sequiserve (Berlin, Germany) for sequencing. The D1-D2 region of LSU rDNA sequences for isolates of *A. tamarense* and *A. minutum* from the Irish coastline were compared to reported *Alexandrium* sequences available in GenBank using a Clustal W sequence alignment program.

Design of primers and probes for real-time PCR assays

Following analysis of the DNA sequence alignments PCR primers (AXLSU_F1 and AXLSU_R1) and DNA hybridisation probes were designed for the real-time PCR assays for *A. tamarense* and *A. minutum* on the LightCycler™. For the *A. tamarense* (W.E.) real-time PCR assay, the FRET hybridisation probes (AXTAM_FL and AXTAM_LC) were designed to be 100 % similar for *A. tamarense* (W.E.) based on the D1-D2 region of the 28S rDNA. The hybridisation detection probe (AXTAM_LC) was designed to have mismatches to *A. tamarense* (N.A.) (1 mismatch), *A. fundyense* (2 mismatches) and *A. catenella* (4 mismatches). A mismatch between the detection probe and a target will cause the probe to melt from these sequences approximately 4 °C lower than the primary target, *A. tamarense* (W.E.). Because the anchor probe (AXTAM_FL) has a T_m of approximately 5–10 °C higher than the detection probe it will not denature from the template until after the detection probe. Therefore, the melt and subsequent loss of fluorescence will be due to the detection probe and the melting of the anchor probe will not be detected. The FRET hybridisation probes for *A. minutum* (AXMIN_FL and AXMIN_LC) were designed using the same criteria as for *A. tamarense*. The hybridisation probe region was 100 % similar to *A. minutum*, with 2 mismatches with *A. ostenfeldii*. Therefore the detection probe was designed to melt from the *A. ostenfeldii* target sequence approximately 8 °C lower than from the *A. minutum* target sequence. The sequences of the primers and probes are detailed in Table 1. All primers and probes were synthesised by TIB Molbiol (Germany).

Table 1. Primers and probes used in this study

Name	Sequence (5' - 3')	T_m (°C)
Primers used for amplification of D1 - D2 region of LSU rDNA		
D1R (forward)	ACCCGCTGAATTTAAGCATA	59.2
D2C (reverse)	CCTTGGTCCGTGTTTCAAGA	62.1
Real-time PCR assay primers and probes		
AXLSU_F1	CATGAGGGAAATATGAAAAGG	56.2
AXLSU_R1	CCTTGGTCCGTGTTTC	55.8
AXTAM_FL	GGGTGGATTGCATGTGCATGTAATGAT--Fluorescein	67.8
AXTAM_LC	LC Red640-TGTGTTTTGATGAATGTGTCTGGTGTA--PH	65.5
AXMIN_FL	GTGCGATGGTTCTTACCT--Fluorescein	58.3
AXMIN_LC	LC Red640-GAATGTCAGCTTCTATTTCTGCAAAT--PH	62.6

Real-time PCR assays

Real-time PCR amplification was performed on the LightCycler™ (Roche Diagnostics, Germany). The thermocycling conditions for the *A. tamarense* (W.E.) and *A. minutum* real-time assays were identical and included an initial hot start step at 95 °C for 10 minutes followed by 45 cycles of denaturation at 95 °C for 15 seconds, annealing at 50 °C for 15 seconds and extension at 72 °C for 15 seconds. The temperature transition rate was 20°C/second. The fluorescence was measured at

the end of each annealing step and during the melting curve analysis, which was performed at the end of the PCR amplification programme by heating the PCR product at 20 °C/second to 95 °C for 1 minute and cooling to 40 °C at 20 °C/second and then slowly heating at 0.1 °C/second to 80 °C. PCR was performed in a final volume of 20 µl which contained 2 µl of 10X LightCycler FastStart DNA Master Hybridisation Probe mix (Roche Molecular Biochemicals), 4 mM $MgCl_2$, 0.5 µM of each primer (AXLSU_F1 and AXLSU_R1), 0.2 µM of each hybridisation probe (AXTAM_FL and AXTAM_LC) and 2 µl of template DNA for the *A. tamarense* assay. The real-time PCR amplification mixture for the *A. minutum* assay contained 2 µl of 10X LightCycler FastStart DNA Master Hybridisation Probe mix (Roche Molecular Biochemicals), 2 mM $MgCl_2$, 0.5 µM of each primer (AXLSU_F1 and AXLSU_R1), 0.2 µM of each hybridisation probe (AXMIN_FL and AXMIN_LC) and 2 µl of template DNA in a 20 µl reaction.

Results

DNA sequence analysis and real-time PCR assay design

The D1-D2 region of the LSU of five isolates of *A. tamarense* and two isolates of *A. minutum* from the Irish coastline were sequenced. All *A. tamarense* isolates were 100 % identical and there was also 100 % sequence agreement for the *A. minutum* isolates. Extensive sequence comparison with data available in the GenBank database revealed that the *A. tamarense* isolates were of the western European ribotype, sharing almost 100 % sequence similarity with other western European strains. The *A. tamarense* isolates from the Irish coastline shared a sequence identity of 93 % with north American strains of *A. tamarense*. The *A. minutum* isolates demonstrated a high sequence similarity (95-100 %) to other *A. minutum* strains from France (Guillou *et al.,* 2002) and New Zealand (Accession number AF033532). Sequence alignments were generated using the new sequence information and sequence data available from GenBank, which provided the basis for designing *A. tamarense* and *A. minutum* specific probes and PCR primers for the real-time PCR assays. Because of the sequence differences between the Western European (W.E.) ribotype and the north American ribotype of *A. tamarense*, it was not possible to design a probe that would detect both ribotypes without cross-reaction with closely related strains especially *A. fundyense*. The use of hybridisation probes allowed for the simultaneous identification of a number of closely related species based sequence polymorphisms in the probe region among these species. The hybridisation probes designed for the detection of *A. tamarense* (W.E.) allowed for simultaneous identification of the north American ribotype of *A. tamarense, A. fundyense* and *A. catenella*. Similarly the assay designed for the detection of *A. minutum* allowed for the simultaneous detection and discrimination of *A. ostenfeldii*.

Specificity studies of real-time PCR assay for A. tamarense and A. minutum

PCR amplification of a 310 base pair amplicon for a range of *Alexandrium* spp. and other relevant phytoplankton species (Table 2) was performed on the LightCycler™ with PCR primers AXLSU_F1 and AXLSU_R1. Melting curve analysis of the *A. tamarense* (W.E.) specific hybridisation probe pair showed that the assay was specific for all of the *A. tamarense* (W.E.) isolates (n = 9) tested. The temperature peaks at which the probes dissociated from target sequences fell between 64.5 and 65.5 °C for all isolates of *A. tamarense* W.E. (Figure 1). This assay also allowed for simultaneous detection a number of species closely related to *A. tamarense* (W.E.) based on melting curve analysis including *A. tamarense* of the north American ribotype at 60 °C, *A. fundyense* at 61.3 °C and *A. catenella* at 50.6 °C. No other

Alexandrium spp. or phytoplankton species investigated in this specificity study were detected in this assay.

Initial specificity studies on the real-time PCR assay for *A. minutum* demonstrated that this assay was specific for all of the *A. minutum* isolates (n = 9) tested (Figure 2). This was confirmed by amplification curves and melting curve analysis. The temperature peaks at which the probes dissociated from target sequences fell between 55.5 and 56.5 ^{o}C for all isolates of *A. minutum* (Figure 2). The assay demonstrated the ability to discriminate between *A. minutum* and *A. ostenfeldii* as the probes dissociated from *A. ostenfeldii* at 45 ^{o}C. No other *Alexandrium* spp. or other phytoplankton species were detected in this assay.

Table 2 Specificity panel used in this study

	Species	Identification code	Location
1	*A. tamarense*	CKA07	Cork Harbour, Ireland
2	*A. tamarense*	CKB01	Cork Harbour, Ireland
3	*A. tamarense*	CKC01	Cork Harbour, Ireland
4	*A. tamarense*	CKD01	Cork Harbour, Ireland
5	*A. tamarense*	CKE03	Cork Harbour, Ireland
6	*A. tamarense*	CBA02	Clew Bay, Ireland
7	*A. tamarense*	CBB03	Clew Bay, Ireland
8	*A. tamarense*	ATIR/01	Cork Harbour, Ireland
9	*A. cf tamarense*	SP3B8-3	La Coruña Bay, Spain
10	*A. tamarense*	PW06	Port Benny, Prince William Sound, US
11	*A. minutum*	CKA02	Cork Harbour, Ireland
12	*A. minutum*	CKA17	Cork Harbour, Ireland
13	*A. minutum*	CKA20	Cork Harbour, Ireland
14	*A. minutum*	CKA23	Cork Harbour, Ireland
15	*A. minutum*	CKD04	Cork Harbour, Ireland
16	*A. minutum*	AM1	Morlaix Bay, France
17	*A. minutum*	FRA	France
18	*A. minutum*	AMBOP006	Western Bay of Plenty, New Zealand
19	*A. ostenfeldii*	K-0287	Limfjorden, Denmark
20	*A. fundyense*	GTCA20	Gulf of Maine, US
21	*A. catenella*	ATTL01	Mediterranean coast, France
22	*Glenodinium sp.*	CKA08	Cork Harbour, Ireland
23	*Gonyaulux sp.*	H10	Cork Harbour, Ireland
24	*Gymnodinium sp.*	CKA11	Cork Harbour, Ireland
25	*Prorocentrum lima*		
26	*Scrippsiella sp.*	KYB01	Killary Harbour, Ireland

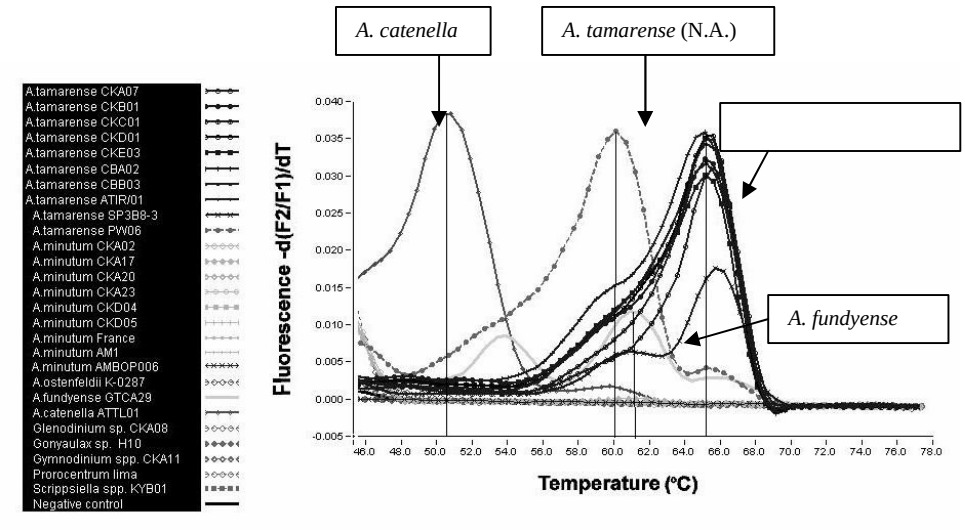

Figure 1. Melting curve analysis demonstrating specificity of *A. tamarense* assay

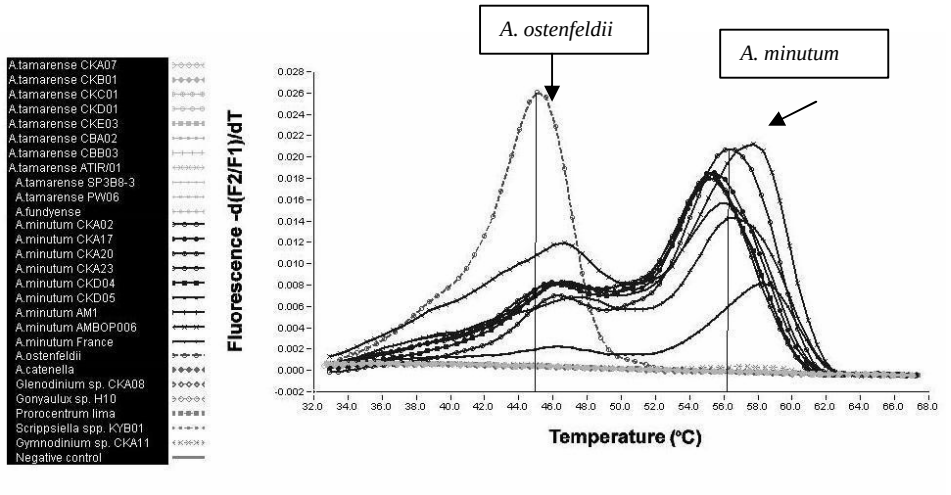

Figure 2. Melting curve analysis demonstrating specificity of *A. minutum* assay

Sensitivity of Real-time PCR assay for A.tamarense and A. minutum

To study the sensitivity of the assay DNA prepared from an *A. minutum* culture was serially diluted (10 ng to 1 fg) and tested in the assay. The detection limits for the real-time PCR assays for *A. tamarense* (Figure 3) and *A. minutum* (Figure 4) were 10 fg of DNA. Galluzzi *et al*. (2004) estimated that the DNA content per *A. minutum* cell was 11.4 ± 2.6 pg, therefore, in terms of *Alexandrium* cells, the sensitivity of the assays was less than 1 cell.

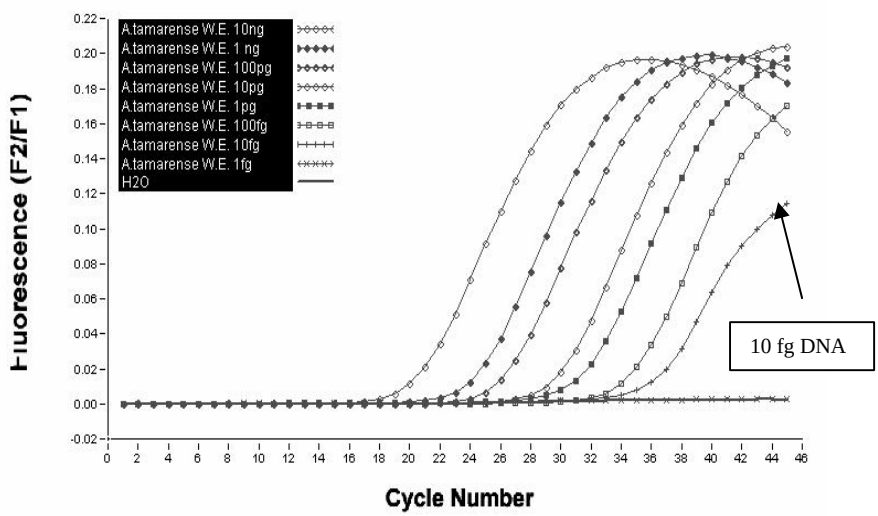

Figure 3. Amplification curves demonstrating *A. tamarense* assay sensitivity

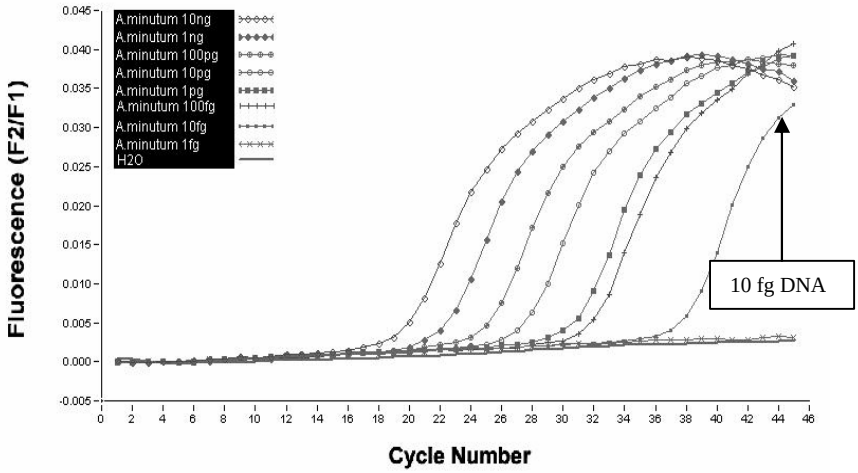

Figure 4. Amplification curves demonstrating *A. minutum* assay sensitivity

Detection of A. tamarense and A. minutum spiked seawater sample

Two separate seawater samples (25 ml) containing no *Alexandrium* cells were spiked with 5000 *A. tamarense* and 5000 *A. minutum* cells from a unialgal culture. DNA was extracted from the spiked samples and serial dilutions (down to 10^{-5}) were prepared (Figures 5 and 6). The neat and diluted samples were analysed in their respective real-time PCR assays. *A. tamarense* and *A. minutum* cells were detected in the diluted samples down to 10^{-3}. This dilution of the DNA is equivalent to approximately 0.1 cells (DNA from 100 cells in neat preparation, 2 µl, per LightCycler™ reaction).

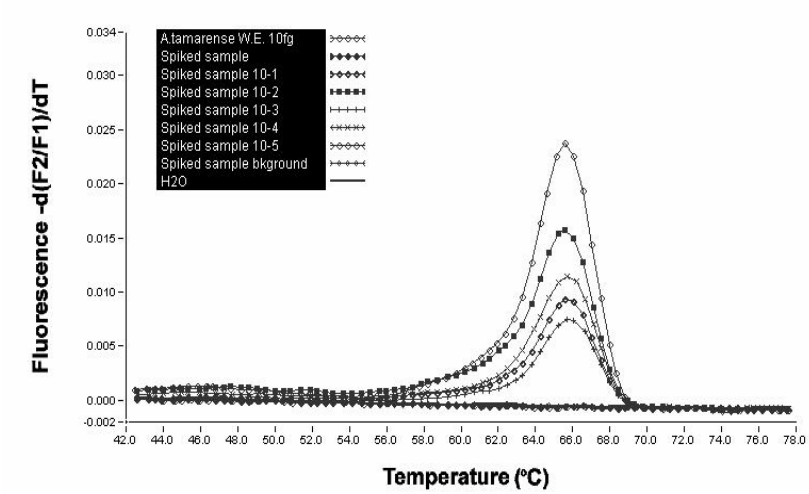

Figure 5. Detection of *A. tamarense* (W.E.) in spiked environmental sample

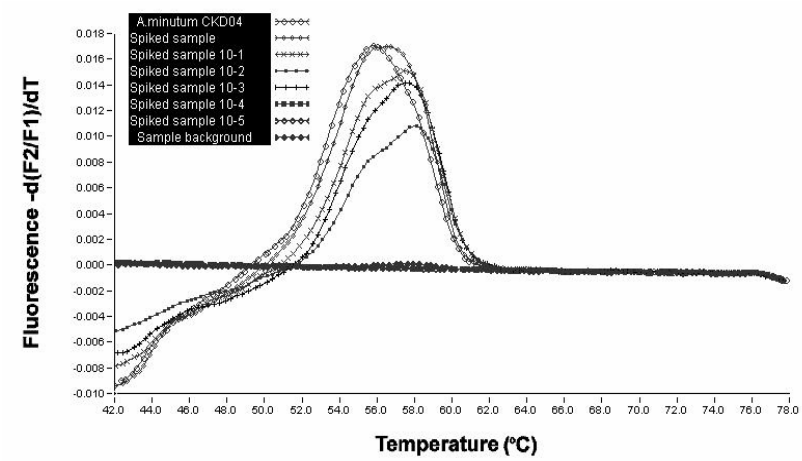

Figure 6. Detection of *A. minutum* in spiked environmental sample

Discussion

It has been well documented that species of the genus *Alexandrium* present difficulties in accurate identification and differentiation from each other. A number of PCR and real-time PCR assays have been described for *Alexandrium* spp. (Godhe *et al.*, 2001; Asai *et al.*, 2003; Galac *et al.*, 2003; Galluzzi *et al.*, 2004). Godhe *et al*. (2001) described a PCR assay for *A. minutum* using species-group-specific SSU rDNA primers which also amplified the closely related species *A. ostenfeldii* and *A. andersoni*. Galluzzi *et al*. (2004) developed a quantitative real-time PCR assay for *A. minutum* based on genus specific primers and the intercalating dye SYBR green as the detection system with a detection limit of 10 copies of 5.8S rDNA sequences. This assay however, also detected *A. catenella* and *A. taylori*, and was only used to quantify *A. minutum* cells from environmental blooms where these other species were not significantly represented.

In this study, two real-time PCR assays are under development for the detection of *A. tamarense* (W.E.) and *A. minutum*. Both assays have demonstrated good specificity and sensitivity for their target cells in culture and in spiked environmental samples. The added advantage of both assay systems, which employed FRET hybridisation probes, is that they allowed for the simultaneous identification of a number of closely related species based on sequence polymorphisms in the probe region among these species. The assay designed for the detection of *A. tamarense* (W.E.) allowed for simultaneous identification of the North American ribotype of *A. tamarense, A. fundyense* and *A. catenella*. The *A. tamarense* isolates from the Irish coastline have been tested for toxicity and were shown not to contain any PSP toxins (Touzet *et al.*, 2003). This complies with a study carried out by Higman *et al*. (2001), where the investigators demonstrated that *A. tamarense* isolates from the southern coasts of Ireland and the UK did not contain any PSP toxins, however isolates of the north American ribotype of *A. tamarense* did contain PSP toxins. The real-time PCR assay also allows for the differentiation between two closely related strains of *A. tamarense*, a non-toxic strain (*A. tamarense* of the W.E. ribotype) and a toxic strain (*A. tamarense* of the north American ribotype). In addition, the assay designed for the detection of *A. minutum* allowed for the simultaneous detection of *A. ostenfeldii*. The use of FRET hybridisation probes has not been described previously for the detection of these organisms.

Further evaluation of the simultaneous detection systems of both assays will be carried out on an extensive specificity panel currently being assembled, to include more isolates of *A. tamarense* of the north American ribotype, *A. fundyense*, *A. catenella* and *A. ostenfeldii*. In addition a range of field samples which have been analysed as part of the Irish Marine Institute's Phytoplankton Monitoring Programme are being evaluated in the real-time PCR assays for *A. tamarense* (W.E.) and *A. minutum* for the presence of these species in Irish seawater samples.

Acknowledgements

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EVALUATION OF BROADLY-REACTIVE REAL-TIME PCR (TAQMAN™) ASSAYS FOR THE DETECTION OF NOROVIRUSES IN ENVIRONMENTALLY CONTAMINATED BIVALVE SHELLFISH

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Abstract
The risk to human health associated with the consumption of molluscan shellfish grown in sewage-contaminated waters is well established. Noroviruses, are the principal agent of shellfish-related viral gastroenteritis. Several different methods using the polymerase chain reaction (PCR) for the detection of norovirus RNA in shellfish have been reported. However, these methods do not provide quantitative data and positive results require confirmation by, for example, probe hybridisation or DNA sequencing. The recent development of real-time PCR technologies offers potential improvements compared with conventional PCR for sensitivity, rapidity, quantification and ease of interpretation of test results. We describe the application of a norovirus genogroup I- and a norovirus genogroup II-specific real-time (TaqMan™) reverse transcription-PCR (RT-PCR) assay for detection of noroviruses to a panel of over 100 bivalve shellfish samples taken from commercial harvesting areas. The TaqMan™assays were found to be as or more sensitive than conventional nested RT-PCR, and to detect an equally broad range of norovirus strains. Our results indicated the presence of low levels of norovirus template in naturally contaminated shellfish, which were close to the limit of detection for the assay. In conclusion the TaqMan™ RT-PCR assay approach offers significant advantages for development of routine assays for the detection of noroviruses in bivalve shellfish.

Introduction
Filter-feeding bivalve molluscan shellfish such as oysters, mussels and clams can concentrate microorganisms, including pathogenic bacteria and viruses, present in their growing waters as a result of contamination with sewage. The risk to human health from consumption of such animals, especially when eaten raw or lightly cooked, is well established (Richards, 1985; Lowry *et al*. 1989; Halliday *et al*., 1991; Sobsey and Jaykus, 1991). Consequently, legal sanitary standards for the bacteriological quality of shellfish sold for consumption are established in many areas of the world. In the EU a maximum of <230 *E.coli* (or <300 faecal coliforms) per 100g of shellfish flesh is permitted. It is however well documented that outbreaks of viral illness can occur following the consumption of shellfish that meet such bacteriological standards (Gill *et al*., 1983; Chalmers and McMillan, 1995; Ang, 1998). In the UK, and elsewhere, the principal agents of shellfish-associated viral illness are noroviruses, previously called Norwalk-like viruses, which cause acute gastroenteritis (Lees, 2000). Norovirus strains can be classified into several

genogroups, of which genogroups I and II (GI, GII) are responsible for the majority of infections in humans.

No cell culture method exists for noroviruses and methods such as electron microscopy and enzyme-linked immunosorbent assay, which are used for detection of noroviruses in stool samples (Caul and Appleton, 1982; Richards *et al.*, 2003) lack the sensitivity required for detection of low levels of virus in shellfish. Consequently, molecular methods for the detection of noroviruses in shellfish utilising the polymerase chain reaction (PCR) have been developed (Atmar *et al.*, 1993, 1995; Lees *et al.*, 1995; Green *et al.*, 1998). These methods have predominantly been based on the use of conventional reverse transcription PCR (RT-PCR). However, conventional PCR does not provide quantitative data and in addition positive results require confirmation by, for example, probe hybridisation or DNA sequencing. The recent development of real-time PCR technologies, such as the SYBR® Green DNA-binding assay and the 5' fluorogenic nuclease (TaqMan™) assay, has offered significant potential improvements in terms of sensitivity, rapidity, quantification and ease of interpretation of test results. The use of real-time PCR methods offers significant advantages for a standardised, robust assay for detection of norovirus RNA in shellfish that could potentially be used in a legislative context. The high sequence variability between and within norovirus genogroups significantly complicates the design of real-time PCR primers. However, primer and probe sequences for a number of broadly-reactive GI- and GII-specific TaqMan™ assays based on the relatively conserved ORF1/ORF2 junction of the norovirus genome have recently been published (Kageyama *et al.*, 2003; Höhne and Schreier, 2004; Jothikumar *et al.*, 2005; Loisy *et al.*, 2005).

In this study we have evaluated the sensitivity and specificity of norovirus detection for broadly-reactive GI- (Jothikumar *et al.*, 2005) and GII-specific (Kageyama *et al.*, 2003) TaqMan™ real-time RT-PCR assays in comparison with a conventional nested RT-PCR method (Green *et al.*, 1998) in more than 100 environmentally contaminated bivalve shellfish samples. We have also analysed the quantitative aspects of the real-time PCR dataset.

Materials and Methods

Shellfish samples

Bivalve shellfish used in this study were collected from sanitary class A and class B commercial harvesting areas (European Union Council Directive 91/492/EEC) in the north Atlantic region during 2002-2004. The numbers of samples of each species used were as follows: Pacific oyster (*Crassostrea gigas*), 105 samples; native oyster (*Ostrea edulis*), 19 samples; common mussel (*Mytilus edulis*), 5 samples; Manila clam (*Tapes philippinarum*), 1 sample. Both unpurified and commercially depurated shellfish were analysed.

Shellfish homogenate
For each sample a minimum of 6 shellfish were opened and the animals removed from their shells. The peripheral flesh and organs of each animal were then cut away from the hepatopancreas and discarded. The hepatopancreases were finely chopped using a razor blade before being added to an equal volume of 100 µg/ml Proteinase K solution (Promega). The sample was incubated at 37°C with shaking at 320 rpm for 1 hr, and subsequently incubated at 65°C for 15 min. in order to inactivate the proteinase. Finally, the sample was centrifuged at 2000 x g for 5 min., and the supernatant (homogenate) retained.

Purification of viral RNA and generation of complementary DNA
For each sample, one aliquot each of 400 µl and 133 µl shellfish homogenate for nested RT-PCR, and 1-3 aliquots of 133 µl shellfish homogenate for real-time RT-PCR, were added to separate 1.5 ml microcentrifuge tubes containing 10 µl of silica bead suspension ('glassmilk'; Anachem). Phosphate buffered saline buffer (PBSa; Oxoid) was added where necessary to make a final volume of 410 µl per tube and viral nucleic acid was subsequently purified as described previously (Lees *et al.*, 1994). Nucleic acids were precipitated in ethanol and sodium acetate, before complementary DNA (cDNA) was synthesised in a reverse transcription (RT) step as described previously (Green *et al.*, 1998).

Nested PCR
Nested PCR assays were carried out as described previously (Green *et al.*, 1998).. PCR products were then analysed using gel electrophoresis. The unambiguous presence of a band of the correct size (GI and GII primers amplify 116bp and 114bp products respectively) was counted as a presumptive norovirus positive for that sample.

5' fluorogenic nuclease assay (TaqMan™) analysis
For each sample all cDNA replicate tubes were pooled together prior to real-time analysis. For each primer/probe set (Table 1), duplicate 2 µl aliquots of cDNA were added to adjacent wells of a 96-well optical reaction plate
made up to 25 µl with TaqMan™ reaction and subjected to the following amplification cycle; 50°C for 2 min., then 95°C for 10 min., followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.using an Applied Biosystems GeneAmp SDS 5700 real-time PCR machine Threshold cycles (C_t values) were determined using the GeneAmp system software. Samples giving a C_t value of <50 in either replicate were counted as positive.

Table 1. Sequences of primers and probes used in this study

Primer/probe name	Sequence	Reference
First round primers for nested PCR		
G1	TCN GAA ATG GAT GTT GG[a]	Green *et al.*, 1998
G2	AGC CNT NGA AAT NAT GGT[a]	Green *et al.*, 1998
SM31	CGA TTT CAT CAT CAC CAT A	Norcott *et al.*, 1994
Second round primers for nested PCR		
Ando[b]	GTG AAC AGY ATA AAY CAN TGG[a]	Ando *et al.*, 1995
NI	GAA TTC CAT CGC CCA CTG GCT	Green *et al.*, 1995
E3	ATC TCA TCA TCA CCA TA	Green *et al.*, 1995
GI real-time assay primers and probes		
JJVIF	GCC ATG TTC CGI TGG ATG[a]	Jothikumar *et al.*, 2005
JJVIR	TCC TTA GAC GCC ATC ATC AT	Jothikumar *et al.*, 2005
JJVIP	TGT GGA CAG GAG ATC GCA ATC TC[c]	Jothikumar *et al.*, 2005
GII real-time assay primers and probes		
COG2F	CAR GAR BCN ATG TTY AGR TGG ATG AG[a]	Kageyama *et al.*, 2003
COG2R	TCG ACG CCA TCT TCA TTC ACA	Kageyama *et al.*, 2003
RING2	TGG GAG GGC GAT CGC AAT CT[c]	Kageyama *et al.*, 2003
PROBE		

[a] N=A/T/C/G, R=A/G, B=T/C/G, Y=C/T, I=Inosine.

[b] Ando primer is a degenerate combination of the published sequences SR48, SR50 and SR52.

[c] Probes were labelled 5' 6-carboxyfluorescein (FAM), 3' 6-carboxy-tetramethylrhodamine (TAMRA).

Discrepancy testing

Any cDNA giving a discrepant real-time RT-PCR result compared with the corresponding nested RT-PCR result for the same genogroup was subject to further testing. Duplicate 2 µl aliquots of the same cDNA pool used for the real-time PCR analysis was tested in the nested PCR by adding 48 µl first-round reaction mix containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 20 pmol each of G1, G2 and SM31 primers and 1 unit AmpliTaq Gold enzyme/reaction, and performing PCR as before. Second round nested PCR was then performed for the relevant genogroup followed by agarose gel electrophoresis. Samples giving a band of the correct size in either replicate were counted as a presumptive positive.

Generation of sequence data

Positive nested PCR amplicons were cloned and sequenced using previously described methods (Henshilwood *et al.*, 1998). Up to three clones per genogroup for each sample were sequenced. Phylogenetic analysis was performed using Lasergene software (DNAStar).

Results

Comparative analysis of conventional nested RT-PCR and real-time RT-PCR assays

Parallel detection assays using conventional nested RT-PCR and real-time RT-PCR were carried out on subsets of 80, 27 and 23 of the 130 shellfish samples collected for this study for both norovirus GI and GII, GI only and GII only respectively (giving totals of 107 samples tested for GI and 103 samples for GII). Norovirus GI and GII RNA was detected in 68 of 107 (64 %) and 72 of 103 samples (70 %) respectively using the real-time assays compared with 65 of 107 (61 %) and 67 of 103 samples (65 %) using conventional nested RT-PCR (Figure 1A and 1C). Prior to discrepancy testing the nested and real-time assays gave the same results in 72 of 107 samples (67 %) tested for norovirus GI and 72 of 103 (70 %) tested for norovirus GII. Following discrepancy testing the nested and real-time assays provided concordant results in 94 of 107 samples (88 %) tested for GI and 92 of 103 samples (89 %) tested for GII (Figure 1B and 1D).

A

Nested \ Real-time	+	-	Σ
+	49	16	65
-	19	23	42
Σ	68	39	

n=107

B

Nested \ Real-time	+	-	Σ
+	59	4	63
-	9[a]	35	44
Σ	68	39	

n=107

C

Nested \ Real-time	+	-	Σ
+	54	13	67
-	18	18	36
Σ	72	31	

n=103

D

Nested \ Real-time	+	-	Σ
+	66	5	71
-	6	26	32
Σ	72	31	

n=103

Figure 1. Two-row by two-column contingency tables for the comparison of conventional nested RT-PCR and real-time RT-PCR. Norovirus GI before (A), and after discrepancy testing (B). Norovirus GII before (C) and after discrepancy testing (D). Discrepancy testing was not carried out for 2 out of 9 of these samples due to insufficient cDNA template

Sequence analysis of nested PCR amplicons

Nested PCR amplicons from all samples that tested presumptive norovirus positive for the relevant genogroup with nested PCR in both initial and discrepancy testing phases but negative with real-time PCR (4 samples for GI, 5 samples for GII) were cloned and sequenced, as described in materials and methods, in the polymerase region of the genome. In addition, amplicons from a subset of samples testing positive by both assays (22 samples for GI, 20 samples for GII) were also cloned and sequenced. Sequence analysis for norovirus GI showed that 3 samples, including one that had tested negative by real-time PCR, gave sequence that was clearly non-noroviral in origin, suggesting a false positive nested PCR result in these cases. Norovirus GI sequence was identified in the remaining 23 samples. In total 30 different clones (more than one sequence per sample may be identified) yielded 17 distinct sequences, representing 6 different norovirus polymerase types (Luton/Valetta, Bkm658-12/99-QC, Peleliu, WUG1, Southampton, UK2/12121/89/UK; Figure 2A). Sequence analysis for GII showed that only a single

sample gave sequence that was non-noroviral in origin. This sample had also tested negative by real-time PCR. Norovirus GII sequence was identified in the remaining 24 samples. In total 34 different clones yielded 18 distinct sequences, representing 3 different norovirus polymerase types (Bad Berleburg, Grimsby/Lordsdale, Wak1B-1997-JP; Figure 2B).

The sequence identity of samples testing positive by nested PCR but negative by real-time PCR was examined to evaluate the broadness of reactivity of the real-time primers. In all cases other samples providing identical, or highly similar polymerase region sequence data had tested positive by real-time PCR (Figure 2).

Evaluation of norovirus template quantification

Of 68 samples that tested positive for norovirus GI using real-time PCR 34 (50 %) tested positive in one duplicate reaction only. This indicates that the quantity of GI virus in these samples was on the limit of sensitivity for the assay. This is supported by C_t values for these samples, which ranged from 37.6 - 44.2, indicating low norovirus template titre. For those samples testing positive for norovirus GI in both duplicates (50 %), again the large majority produced average C_t values in the range 37.6 - 43.7, indicating low titres of virus. A single sample produced a C_t of 33.2 (average of two values), corresponding to detectable viral RNA levels at least 10 times higher than in other samples (Figure 3A). Of the 72 samples that tested positive for norovirus GII using real-time PCR, 16 (22 %) tested positive in one duplicate reaction only. C_t values for these samples ranged from 37.6-45.5. Of those samples testing positive in both duplicates (78 %), average C_t values ranged from 34.9-44.1, again indicating low titres of virus template (Figure 3B). However, in comparison to the norovirus GI assay, a greater proportion of C_t values were found in the lower part of the range (higher viral template titres). In total 41/72 (57 %) samples positive by the norovirus GII assay tested positive in both duplicate reactions and produced an average C_t of less than 40, in comparison to 26/68 (38 %) of positive samples in the GI assay. This indicates that although titres of GII virus in shellfish samples are normally low, they are perhaps on average higher than GI titres. Examination of Figure 3 suggests that the occurrence of single replicate positive samples was associated with lower viral template copy numbers (higher C_t values) and is thus probably due to insufficient viral template being present to give a reliable positive result in both duplicates. The distributions of samples testing positive in either one or both replicates were compared using the Kolmogorov-Smirnoff test separately for norovirus GI and norovirus GII. The distributions were found to be very highly significantly different ($p<0.001$) for GI and significantly different ($p=0.045$) for GII. This supports the conclusion drawn from Figure 3 that single replicate positive assay results are associated with very low viral template titres. For both genogroup-specific assays, the total absence of positive reactions producing C_t values in the range 46-50 indicates that there is a genuine discontinuity between positive and negative reactions, and validates the use of 50 reaction cycles.

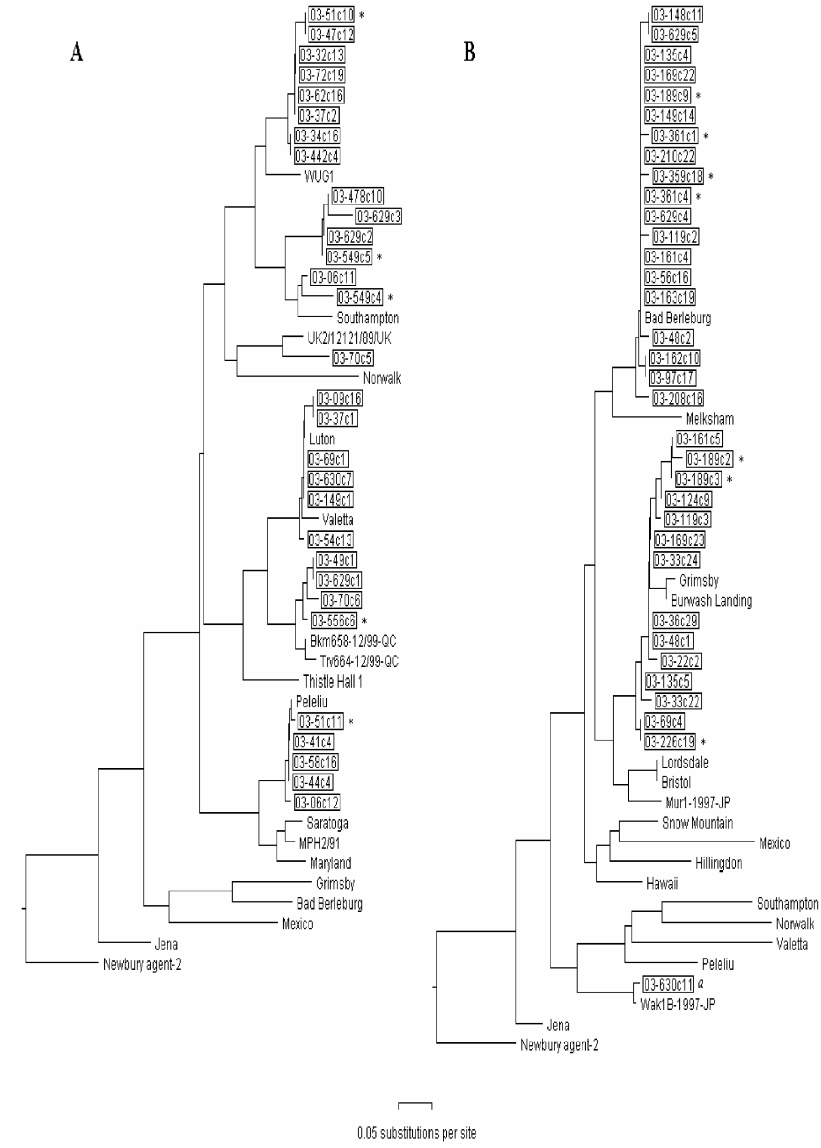

Figure 2. Phylogenetic trees of the polymerase region vRNA sequences of GI (A) and GII (B) noroviruses detected in shellfish samples during this study using nested PCR. The trees were generated using MegAlign software (DNAStar). The newly isolated sequences are represented boxed, previously published norovirus sequences are represented unboxed. Sequences marked with asterisks (*) were isolated from samples testing negative for the relevant genogroup using real-time RT-PCR. [a] Although sequence 03-630c11 is aligned more closely to GI strains in this tree, it was originally amplified using GII-specific nested PCR primers

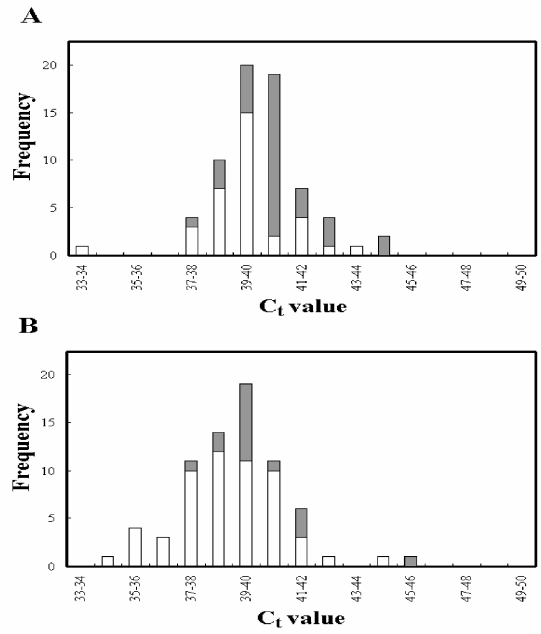

Figure 3. C_t value ranges produced by samples testing positive for GI (A) and GII (B) noroviruses using real-time RT-PCR. The white portion of each bar represents the number of samples testing positive in both duplicate reactions (average values are plotted). The grey portion represents the number of samples testing positive in one reaction only.

Discussion

Norovirus is one of the most significant of all food-borne pathogens, estimated to be responsible, for example, for two thirds of all food-related illnesses in the United States (Bresee *et al.*, 2002). Bivalve molluscan shellfish are established as one of the most frequently implicated food vehicles involved in such outbreaks (Gill *et al.*, 1983; Chalmers and McMillan, 1995; Ang, 1998; Parashar and Monroe, 2001). Improvement of shellfish hygiene controls through introduction of monitoring of commercially produced shellfish for norovirus has been suggested as a possible option for control of this problem. Conventional RT-PCR methods have been used for norovirus detection in shellfish for several years (Atmar *et al.*, 1993, 1995; Lees *et al.*, 1995; Green *et al.*, 1998). However, the qualitative nature of the data and the lengthy and expensive confirmatory steps (for example probe hybridisation (Legeay *et al.*, 2000), DNA sequencing of the RT-PCR products (Henshilwood *et al.*, 1998) or heteroduplex mobility assay (Mattick *et al.*, 2000)) required to eliminate false positives caused by non-specific amplification (this problem was, for example, noted in our study where 3/26 (12 %) and 1/25 (4 %) of conventional RT-PCR positive samples analysed for GI and GII respectively could not be confirmed as noroviral in origin by sequencing) have provided a barrier to the widespread implementation of such methods. The real-time PCR-based norovirus detection assays presented in this study offer an alternative to conventional PCR that is more rapid, equally or more sensitive, includes an in-built probe confirmation, and provides semi-quantitative data.

We and others have recently reported the development of TaqMan™-based real-time RT-PCR assays utilising carefully designed primer/probe sets, as well as rigorous validation to ensure both theoretical and practical cross-reactivity with a broad spectrum of genogroup I and II noroviruses in clinical as well as shellfish samples (Kageyama *et al.*, 2003; Nishida *et al.*, 2003; Jothikumar *et al.*, 2005). In our study we have confirmed the broad reactivity of the real-time RT-PCR assays with low template levels using a panel of naturally contaminated shellfish samples that contained an array of genetically diverse noroviruses (based on polymerase region sequences). For all samples testing positive by conventional PCR, but negative by real-time PCR, other samples with the same or genetically similar norovirus sequences had given positive real-time PCR results. It should be noted however, that the polymerase region of the norovirus genome used for strain-typing in this study is distinct from the ORF1/ORF2 junction region targeted by the real-time PCR, which complicates interpretation of our data. For example, the Bad Berleburg (genotype GIIb) polymerase type is associated with recombinant strains, and has been reported with at least three capsid types, Hawaii (genotype GII.1)(Gourdon78 isolate, GenBank accession number AY580335), Snow Mountain (GII.2)(Pont de Roide 673 isolate, AY682549) and Mexico (GII.3)(Paris Island isolate, AY652979). To date, no single conventional PCR primer pair for norovirus detection is capable of reacting with all strains with equal sensitivity (Vinjé *et al.*, 2003), indicating the challenges for detecting this group of viruses in environmental samples. Although most of the published TaqMan™ primers and probes for norovirus detection are targeted against the most conserved region of the genome (Kageyama *et al.*, 2003; Höhne and Schreier, 2004; Jothikumar *et al.*, 2005; Loisy *et al.*, 2005), careful design and validation are similarly essential for such assays to ensure continuing appropriate strain coverage and sensitivity. Subsequent to the completion of the practical element of this study several groups have modified original proposals for GII-specific primer and probe sequences in order to improve assay sensitivity and specificity (Kageyama *et al.*, 2004; Loisy *et al.*, 2005). Similarly, our group has successfully trialled another modified GII real-time primer/probe set, which showed improved performance with clinical samples, on a smaller collection of shellfish samples (Jothikumar *et al.*, 2005). Particular vigilance must also be paid to newly-emergent strains of noroviruses in order to ensure currently proposed primer/probe sets retain their usefulness into the future.

The potential of real-time PCR to provide quantitative data is often cited as a major advantage of this technology. For diagnostic testing of shellfish for noroviruses, quantitative data may enable correlations to be drawn between the level of viral template present in a sample and the public health risk posed by such shellfish. Ultimately addressing this question will require studies on virus infectivity, which are currently only possible using human volunteers. Several conventional PCR-based methods for quantification of noroviruses have been proposed utilising end-point dilution (Jiang *et al.*, 1992; Schwab *et al.*, 1998) or most probable number (Le Guyader *et al.*, 2003) approaches. However, the emergence of real-time RT-PCR assays for norovirus detection has greatly improved the quality of quantitative data available. Quantification using the SYBR® Green DNA-binding assay is complicated by the possibility of non-specific amplification but the TaqMan™ PCR is able to circumvent this problem through the use of a sequence-specific probe, and the development of assays using this technology should facilitate the development of a simple, standardised method for quantification. The results obtained in this study support this conclusion but also indicate that most contaminated shellfish contain levels of virus template close to the limit of detection of the assay used (this is not necessarily indicative of a low risk to health however - possibly as few as 10 virus

particles are required to effect an infection (Caul, 1996)). GII noroviruses were more prevalent than GI, both in terms of incidence and possibly also virus template titre. This probably reflects the lower frequency of norovirus outbreaks caused by GI as opposed to GII strains (Hale *et al.*, 2000; Fankhauser *et al.*, 2002; Lopman *et al.*, 2004) and the resultant lower levels in the environment. Whilst valuable, we experienced several technical difficulties in applying the real-time PCR approach to quantification of noroviruses in shellfish samples, as a result of the low virus template levels. In many cases amplification was noted in only a single replicate real-time PCR reaction. Additionally in those samples where both replicates were positive, variation between the individual C_t values was often significant. The complex multi-step RNA extraction procedures used and the possible presence in shellfish tissues of substances inhibitory to RT-PCR (Metcalf, 1978; Atmar *et al.*, 1993, 1995), also significantly complicates accurate quantification in individual samples. These factors combine to confound the quantification of noroviruses in shellfish and illustrate the need both to develop a rational system for quantifying low copy-number samples and to further optimise the method for greater assay sensitivity. We have recently demonstrated that a one-step specific-primed RT-PCR is more efficient than a two-step random-primed method when applied to faecal samples with high virus template levels (Jothikumar *et al.*, 2005) and this approach may also help to overcome the problems of low virus template levels in shellfish samples, and thus demands further investigation. It will also be necessary to develop a full suite of controls, including an internal standard, to address sample extraction efficiency and potential PCR inhibition. However, the results obtained in this study clearly demonstrate the potential of real-time PCR for a standardised, fast, accurate and reliable test for detection of noroviruses in molluscan shellfish.

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COMPARISON OF METHODS FOR DETERMINATION OF BREVETOXINS AND THEIR METABOLITES IN NSP-TOXIC BIVALVE MOLLUSCS

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Abstract
Neurotoxic Shellfish Poisoning (NSP) is caused by consumption of shellfish which have accumulated toxins from the dinoflagellate, *Karenia brevis*, the causative organism for the Florida red tide. Recent studies have shown that *Karenia* toxins (brevetoxins) are rapidly metabolised by shellfish and that these metabolites are at least partially responsible for NSP. The standard procedure for assessing the public health risk from toxic shellfish is the mouse bioassay, which is non-specific with regard to toxin identity. Efforts are underway to replace it by *in vitro* assay or instrumental method. The present study is part of a larger effort to isolate the major brevetoxin metabolites from clams (*Mercenaria mercenaria-campechiensis*) and oysters (*Crassostrea virginica*) exposed to natural *K. brevis* blooms, to verify metabolite structures by NMR, and to produce analytical standards for methods development. Various assay methods for determining brevetoxin residues and toxicity were compared with results of the mouse bioassay in NSP-contaminated shellfish. LC-MS, ELISA, and receptor binding assay data correlated well with mouse bioassay, while cytotoxicity assay exhibited enhanced responses relative to the other methods. Results exhibited a persistence of brevetoxin metabolites in shellfish long after dissipation of the red tide bloom.

Introduction
Neurotoxic shellfish poisoning (NSP) is the intoxication resulting from consumption of shellfish contaminated with brevetoxins (PbTx) from the toxic dinoflagellate, *Karenia brevis* (formerly *Gymnodinium breve*, Davis) (Daugbjerg *et al.*, 2001). Filter-feeding bivalve mollusks, such as clams, oysters, and mussels accumulate high concentrations of biotoxins as they ingest *Karenia* cells from the water (Steidinger and Baden, 1983; Dickey *et al.*, 1999). Identification of the toxic compounds responsible for NSP is complicated by their extensive metabolism of parent algal toxins to toxin-conjugates, the toxicity of which is largely unknown (Poli *et al.*, 2000; Plakas *et al.*, 2002; Wang *et al.*, 2004). Evidence for shellfish metabolism of PbTx appeared following a 1992-1993 outbreak of NSP in New Zealand (Ishida *et al.*,1996). Metabolic conjugates of PbTx, with mass to charge (m/z) ratios (MH^+) of 1018 and 1034 (representing cysteine and cysteine sulfoxide conjugates, respectively), along with PbTx-3, were isolated from NSP-toxic oysters, *Crassostrea virginica* (Dickey *et al.*, 1999; Poli *et al.*, 2000) and in oysters and clams, *M. merceneria*, (Pierce *et al.*, 2004). More recent LC-MS analyses of NSP-contaminated oysters revealed more than 20 potential metabolites, the most abundant compounds were cysteine and cysteine-sulfoxide conjugates of brevetoxins PbTx-1 and PbTx-2 (Wang, *et al.* 2004). This present study investigates PbTx residues in clams (*Merceneria, merceneria-campenchenesis*) and oysters (*Crassostrea, virginica*)

exposed to a *K. brevis* bloom occurring in Sarasota Bay FL (January 12 to February 27, 2004), by using various *in vitro* assays and LC-MS, and compared to mouse bioassay.

Methods and Materials

Clams (*Merceneria, merceneria-campenchenesis*) and oysters (*Crassostrea, virginica*) were collected from a common site in Sarasota Bay, Florida, during and after a moderate intensity (100,000 < 1,000,000 cells/L) *K. brevis* bloom, that lasted from January 12 to February 27, 2004. Water samples were collected concurrently with shellfish and also in the immediate vicinity (up-current and down-current) of the shellfish collection site, for *K. brevis* cell counts and PbTx analyses. Cell counts were performed by microscopic identification and enumeration from 20 ml water samples, fixed with Utermohls solution. Brevetoxins and associated compounds were recovered from 1-liter water samples within 4 hours of collection, by elution through a C-18 solid phase extraction disc (Spec 47 C-18AR, Ansys Diagnostics Inc., Lake Forest, CA), eluted with 20 ml methanol and brought to a final volume of 3 ml for liquid chromatography-mass spectrometry (LC-MS) (Pierce *et al*, 2004).

Shellfish were placed on ice and shipped overnight to the FDA, Gulf Coast Seafood Laboratory (GCSL), Dauphin Island, AL, for processing. A minimum of 18 clams and 24 oysters were opened under clean conditions, the liquid drained off and the tissue homogenised as one composite sample for each species. The homogenate was separated to 100 g aliquots for mouse bioassay and to 1 g aliquots for extraction in preparation for *in vitro* assays and LC-MS. Brevetoxins and metabolites were extracted from shellfish tissue in acetone with additional separation and clean up by solid-phase extraction (Plakas *et al*. 2002) and sent to participating labs for analysis. Toxicity of the shellfish tissue was determined by standard mouse bioassay, reported as mouse unite/100g (APHA, 1970). PbTx and PbTx-metabolite assays included LC-MS, ELISA, receptor binding assay, and cytotoxicity assay. LC-MS analyses were performed using Agilent Technologies (Palo Alto, CA) Model 1100 LC system and Thermo Finnigan (San Jose, CA) Navigator AQA MS detector with electrospray ion source, on a YMC J'Sphere ODS-L80 S-4, 2.0 x 250 mm column (YMC Inc., Wilmington, NC) under gradient conditions (Plakas *et al*, 2002). MS data were acquired by selected ion monitoring (SIM). Standards of pure PbTx-2 and PbTx-3 were provided by the Center for Marine Science, UNC, Wilmington. Although standards were not available for the metabolites, metabolite structures were previously verified by LC/MS/MS and accurate mass measurements (Plakas *et al.*, 2002; Wang *et al.*, 2004). Quantitation of the metabolites was estimated as µg PbTx-3 equiv./g shellfish tissue. Competitive ELISA assays were performed at FWCC-FWRI, on acetone extracts of the 1g aliquots, according to the protocol of Naar *et al* (2002). Cytotoxicity assay and receptor binding assay were performed at GCSL/FDA, according to previously described procedures (Dickey *et al.*, 1999; Trainer and Poli, 2000). Data are reported in µg PbTx-3 equiv./g shellfish tissue.

Results and Discussion

K. brevis cell counts monitored at the shellfish study site indicated that a low level bloom (<100,0000 cells/L) occurred from September18, 2003 to October 22, 2003, with no further exposure until the January 2004 red tide bloom. *K. brevis* cell counts and concentrations of the most abundant brevetoxins (PbTx-2 and -3) are given in Table 1 for seawater samples collected at the shellfish collection site during and following the January to February 2004 red tide bloom. Brevetoxins remained in the water column for at least one week after *K. brevis* cell counts

diminished. PbTx-3 was the most abundant water-borne algal toxin observed, and the only one after *K. brevis* cells diminished.
LC-MS analysis (Table 1) showed the accumulation of cysteine conjugates of PbTx-1 (*m/z* 990 and 1006) and PbTx-2 (*m/z* 1018 and 1034) in *K. brevis*-exposed clams and oysters. Little or no PbTx-3 and no intact PbTx-1 or pbTx-2 was observed in the tissue extracts, consistent with previous studies (Plakas *et al.*, 2002; Wang *et al*, 2004). Shellfish collected prior to the bloom (November 25, 2003-December 16, 2003) exhibited low concentrations of various metabolites, suggesting a "background" level, probably retained from a previous red tide bloom that ended about one month prior to the November 25 sample. Upon exposure to the red tide, a marked increase was noted in metabolite concentrations. Higher concentrations were found in oysters compared with clams during exposure to the bloom; however oysters eliminated these metabolites more rapidly after the bloom ended on February 27, 2004.

A comparison of results from different assay methods for tissue extracts from oysters and clams is given in Figures 1a and b for oysters and clams, respectively. Mouse bioassay results showed shellfish toxicity (> 2MU/10g tissue) for about 4 weeks following the bloom, indicating a persistent public health risk. Although clams and oysters were toxic by mouse bioassay, little or no parent PbTx was detected, suggesting that PbTx metabolites, including those reported here, comprise the toxins responsible for NSP. Results of the various assays within species exhibited good general agreement in the time-course of toxin uptake and elimination. All assays on oyster tissue exhibited a maximum value in concert with the high point of *K. brevis* cell counts (2/28/04), approximately 5 weeks after bloom initiation, with the exception of cytotoxicity that peaked about 2 weeks later. Assays in clam tissue did not exhibit a pronounced maximum and decline with the cell counts, rather a sustained level of activity for all assays followed the *K. brevis* cell count maximum. Most pronounced was the cytotoxicity that clearly peaked weeks after the cell count maximum. This lag in cytotoxicity maximum indicates continued production of cytotoxic compound(s) after the known metabolite compounds reached their maximum concentration.

Table 1. Concentrations of brevetoxins and major metabolites (as µg PbTx-3 equiv./g tissue) in oysters and clams, *K. brevis* cell counts and major algal toxins (PbTx-2 and -3) in seawater samples from the shellfish collection site. PbTx LOD = 0.05 µg/L; 0.013µg/g tissue; K. brevis LOD = 1,000 cells/L

Collect Date	Shellfish	m/z Pbtx-3 897	Cys PbTx-2 m/z 1018	Oxo-cys PbTx-2 m/z 1034	Cys PbTx-1 m/z 990	Oxo-cys PbTx-1 m/z 1006	Water Samples		K.brevis Cells/L
							PbTx-2	PbTx-3	
11/25/03	Oyster	nd	2.78	0.85	0.49	Nd			nd
	Clam	nd	1.46	0.67	0.42	Tr			
12/16/03	Oyster	nd	2.69	0.63	0.52	Nd			nd
	Clam	nd	1.79	0.67	0.61	0.40			
1/12/03		Red	Tide	Bloom	Start				2,500
1/22/04	Oyster	nd	3.44	0.75	0.54	0.37	0.06	0.14	5,000
	Clam	nd	1.87	0.64	0.60	0.40			
2/4/04	Oyster	tr	12.65	1.31	0.71	Nd	1.49	2.05	73,000
	Clam	nd	2.12	0.68	0.64	0.41			
2/18/04	Oyster	0.38	25.78	2.02	1.44	Nd	1.34	1.89	131,000
	Clam	nd	4.63	1.48	1.08	0.47	nd		
2/27/03		Red	Tide	Bloom	End				3,000
3/4/04	Oyster	nd	12.44	1.36	0.89	Nd	nd	3.28	nd
	Clam	nd	4.26	1.08	1.01	0.53	nd		
3/17/04	Oyster	nd	10.73	1.52	0.97	0.45	nd	0.45	nd
	Clam	nd	3.22	1.51	0.93	0.52	nd	nd	
3/31/04	Oyster	nd	4.12	0.90	0.59	Nd	nd	nd	nd
	Clam	nd	7.42	1.29	1.15	0.43	nd	nd	
4/14/04	Oyster	nd	2.75	0.64	0.46	Tr	nd	nd	nd
	Clam	nd	2.25	1.21	0.68	0.42	nd	nd	

Comparisons between species suggest significant differences in accumulation, metabolism, and elimination of toxin. Most notable is that the oysters exhibited rapid reduction in assay results (except for cytotoxicity), where-as clam assays exhibited longer-term responses with less dramatic reduction following reduction in cell counts. These results suggest that these two species may be producing different concentrations (and possibly different compounds) of NSP-toxic components.

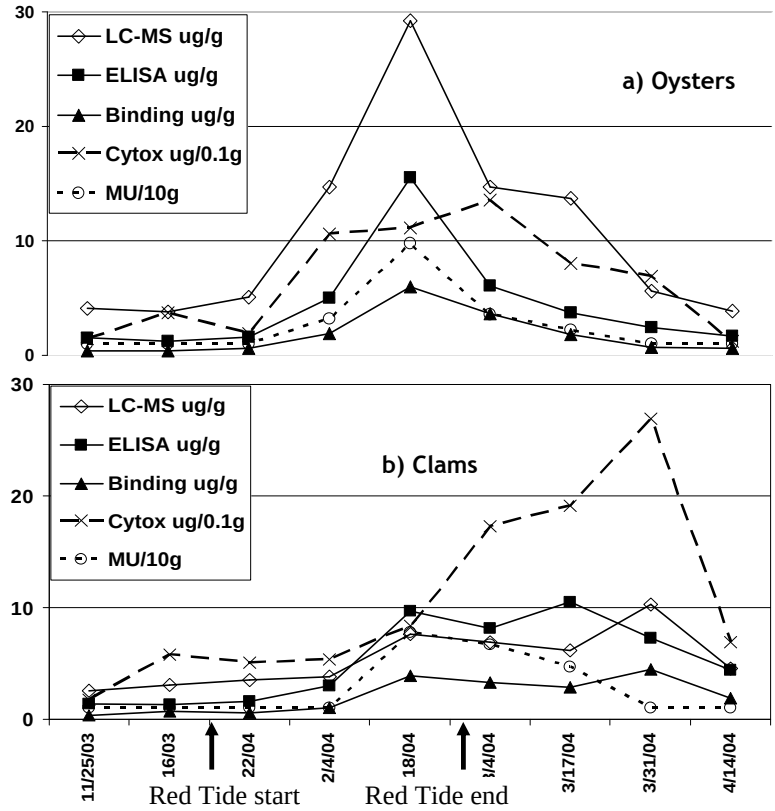

Figure 1. a) Oysters b) Clams: Comparison of assay methods for NSP-contaminated oysters and clams before, during and after exposure to a *K. brevis* bloom in Sarasota Bay, Florida. The bloom lasted from January 12, 2004 to February 27, 2004 analyses continued through April 14, 2004. Mouse Units are give in MU/10g to bring the graph on scale, non-toxic assays are depicted as 1 MU/10g to indicate that the value is less than 2, but greater than 0 MU/10g.

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MOLECULAR METHODS TO DETECT ENTERIC VIRUSES IN MOLLUSCAN SHELLFISH

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Abstract
Enteric virus-contaminated molluscan shellfish continue to cause illness throughout the world. The advent of molecular biological methods has enhanced the ability to detect many of these viruses. Prior to analysis, viruses must be extracted from shellfish tissues and concentrated. We developed a method to extract and concentrate the RNA from hepatitis A and hepatitis E viruses and from noroviruses isolated from oysters and clams. The concentrated RNAs are then assayed by reverse-transcription polymerase chain reaction (RT-PCR) and the presence of PCR product signals virus presence. The RT-PCR method works well on the hepatitis viruses which have conserved genomes, but fails to amplify many of the noroviruses which have highly diverse genomes. We developed real-time RT-PCR (rt RT-PCR) procedures to detect hepatitis A and E viruses. We also developed methods to detect a wide array of genogroup I and II noroviruses using broadly reactive primers and SYBR green detection. Product verification is rapid and simple, and is accomplished by means of first derivative melt curve data. The extraction and assay procedures allow for the detection and semi-quantitation of noroviruses from at least thirteen different genetic clusters. This rt RT-PCR technique has been used on stool preparations in epidemiological investigations to identify the sources of norovirus transmission and is under evaluation for use on shellfish extracts.

Introduction
Shellfish-borne enteric viruses continue to threaten the consumer of raw and lightly-cooked molluscan shellfish. Among the most notable of these viruses are hepatitis A virus (HAV) and a broad group of human caliciviruses known as the noroviruses, formerly referred to as Norwalk-like viruses or small round structured viruses. Shellfish have the unique ability to bioconcentrate these viruses from contaminated water in which they grow. Since shellfish are often eaten raw or are only lightly cooked, these viruses have been responsible for hundreds of thousands of documented cases of illness (Halliday *et al.*, 1991, Richards 1985). Hepatitis E virus (HEV) is another enteric pathogen that is commonly found in Asia. It represents a potential emerging pathogen in many parts of the world.

Traditional methods to assay for many of the enteric viruses involves their propagation in cell culture systems. Unfortunately, the noroviruses, HEV, and most wild-type HAV do not replicate in cell cultures or in animal models. Consequently, molecular biological procedures have become the methods of choice for their detection. The most commonly used assay is reverse-transcription polymerase chain reaction (RT-PCR). An extension of this method involves the use of real-time RT-PCR (rt RT-PCR) for the detection and semi-quantitation of viruses. Both methods involve the amplification of viral RNA into complementary DNA (cDNA) copies.

Whereas standard RT-PCR detects amplified products by gel electrophoresis, real-time methods are more direct, allowing a fluorescent dye or probe to detect cDNA copies as they form. Furthermore, real-time methods can be quantitative or semi-quantitative by means of the development of standard curves. The incorporation of an intercalating dye, like SYBR green, into the RT-PCR reaction mix, allows melt curve data to be generated and this reduces the need for electrophoretic gels and hybridization assays. Here we report the development of rt RT-PCR procedures for the detection of HAV, HEV, and a wide variety of genetically-diverse noroviruses found in human stools and shellfish.

Materials and Methods

Viruses

A panel of genogroup I and genogroup II noroviruses were provided by Stephan Monroe and his colleagues from the Centers for Disease Control and Prevention, Atlanta, GA. The classical strain of genogroup I cluster I Norwalk virus was from a human volunteer study (Graham *et al*. 1994; Richards *et al*. 2004a). HEV was provided by Junkun He, Walter Reed Army Institute for Research, Silver Spring, MD, and was obtained from a patient stool sample collected during a hepatitis E outbreak in Pakistan in 1994. The diagnosis of hepatitis E was confirmed serologically. HAV was the HM-175 cell culture-adapted strain obtained from the American Type Culture Collection, Manassas, VA, as VR-1402. The HAV was propagated in fetal rhesus monkey kidney (FRhK-4) cells provided by Stanley Lemon, University of Texas Medical Branch, Galveston, TX. The FRhK-4 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 0.1 mM non-essential amino acids plus 10 % fetal bovine serum (Gibco-Invitrogen, Grand Island, NY). HAV was propagated in these cells and virus stocks were prepared as previously described (Richards and Watson, 2001).

Virus extraction from stools

Noroviruses and HEV were extracted from human stools using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) as previously described (Richards *et al*., 2004b). In essence, 50-80 µg of stools were weighed, diluted in nuclease-free water, and vortexed for 30 sec. Samples were clarified by centrifugation and the viral RNA was extracted from 140 µl of the supernatant according to the column centrifugation procedure described by Qiagen. For this procedure, 140 µl of each 10 % stool suspension was denatured, adsorbed to a silica column, washed, and then eluted to give a final volume of 60 µl. Using this method, viral RNA was purified within 20 min. The RNA extracts were stored on ice pending assay by molecular methods.

Virus extraction from oysters and clams

Oysters and clams were experimentally challenged with cell culture-propagated HAV in a laboratory uptake system. Viral RNA was extracted using a glycine elution/polyethylene glycol precipitation/Tri-reagent purification/poly dT magnetic bead (GPTT) separation method as previously described (Kingsley and Richards, 2001). This method produces a final volume of
~ 100 µl of viral RNA-containing extract.

Real-time RT-PCR

Real-time RT-PCR for the noroviruses was performed using a SYBR Green Quantitative RT-PCR Kit (Sigma Chemical Co., St. Louis, MO) that contained SYBR Green Taq ReadyMix (with Taq DNA polymerase, SYBR green, dNTP, and $MgCl_2$), DuraScript Reverse Transcriptase, and PCR buffer. Degenerate, "universal" primers for the genogroup I (primers designated MON 432 and 434) and genogroup II (primers

designated MON 431 and 433) noroviruses were added to final concentrations of 640 nM each (Richards *et al.*, 2004b). The formulation for the master mix is as recently described (Richards *et al.*, 2004b). This method permitted all four primers to be combined in a single reaction tube for the detection of both genogroup I or II noroviruses.

Real-time RT-PCR was performed using a Smart Cycler (Cepheid, Sunnyvale, CA). Cycling parameters were optimised including the times and temperatures for the anneal, extension, and denaturation steps, and the temperature of the optical read step as recently described (Richards *et al.*, 2004b). The optical read step was performed at 77°C, a temperature just slightly lower than the melting temperatures of the norovirus amplicons. This allowed the selective detection of amplicon without the detection of lower melting temperature primer dimer or spuriously-produced products.

HAV and HEV were detected by rt RT-PCR using a QuantiTect SYBR green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. For HAV, we used primers designated 2172 and 2415, listed as internal (nested) primers by Hutin *et al*. (1999). For HEV, the primers were designated B3R and B5F as described by Im *et al*. (2001). Amplification was performed using 0.25 µM of each primer. Cycling parameters were as follows: reverse transcription (50°C, 30 min), Taq activation (95°C, 15 min), and 40-50 cycles with denaturation (95°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min) with the optical read performed at 72°C.

Optics graphs were obtained for each run showing the fluorescence intensity of each reaction plotted against PCR cycles. Optics graphs provide cycle threshold (Ct) data, which indicates the number of cycles required for the fluorescence to cross a pre-assigned threshold. We used a threshold of 30 fluorescence units, which is the default value for the Smart Cycler. Samples containing the most viral RNA template have the lowest Ct, while those with the least generally have the highest Ct, provided there are no PCR inhibitors present. Immediately after PCR cycling, first derivative melt graphs were obtained by plotting the fluorescence intensity against temperature as the temperature was increased from 60 to 95°C at 0.2°C/sec.

Results

Viral RNA was successfully extracted from shellfish using the GPTT procedure and from stools using the QIAamp Viral RNA Mini Kit. Both HAV (Figure 1) and HEV (Figure 2) were detected by rt RT-PCR from shellfish and stool samples, respectively, producing optics graphs and first derivative melt curves. HAV was easily detected in oyster and clam extracts (Figure 1A) with Ct values of 27.36 and 26.65, respectively, and with melting temperatures of 77.44 °C and 77.07°C, respectively (Figure 1B). The melting temperature is the temperature at which one-half of the double-stranded amplicon separates into single strands. The single peak for each sample on first derivative melt curves (Figure 1B) indicates the absence of primer dimer or spuriously-produced product. HEV was isolated from stool extract and produced an optic graph and first derivative melt curve as shown in Figures 2A and B, respectively, with a Ct value of 29.52 and a melting temperature of 80.86°C. Like the HAV assays, no primer dimer or spuriously produced products were detected for HEV, as indicated by a stable baseline and the absence of additional peaks on first derivative melt curves. The Ct values may be used in the development of dilution-end point standard curves (Richards *et al.*, 2004a) or for relative comparison of the levels of virus present among multiple samples. We tested a strain from each of thirteen different clusters of norovirus, as described in Table 1. Five genogroup I and eight genogroup II noroviruses were readily detected by rt RT-

PCR of the stools (Figure 3). Problems were experienced in the assay of shellfish extracts by this method, because of the presence of substances inhibitory to the RT-PCR reaction. Substances inhibitory to RT-PCR are a common limitation with molecular biological methods (Richards, 1999). The likely reason that the norovirus assay may be more sensitive to inhibitors than the HAV and HEV assays is because of the highly degenerate nature of the norovirus primers. Nevertheless, viral RNA extraction from stools using the QIAamp Viral RNA Mini Kit provided sufficient purification for amplification and detection by rt RT-PCR of noroviruses and HEV. An examination of the first derivative melt curves in Figures 1-3 shows only one peak for each virus-containing sample. Primer dimmer or spuriously produced products, if present, would have produced multiple peaks. Only the negative controls show peaks associated with primer dimer formation or the presence of spuriously-produced product. The melting temperature is between 81.2°C and 84.9°C (Richards *et al.*, 2004b); therefore, peaks produced within this temperature range are likely from norovirus. To further confirm products as true amplicon, products may be run on an electrophoretic gel to determine if the appropriate size amplicon was generated.

Fluorescence intensities varied greatly in assays of the hepatitis viruses versus the noroviruses. Optics graphs for HAV and HEV (Figure 1A and 2A, respectively) showed low fluorescence intensity, while the corresponding graphs for the noroviruses (Figure 3) showed strong fluorescence intensities. These assays differed in that rt RT-PCR for the hepatitis viruses was performed using the Qiagen RT-PCR amplification kit while the noroviruses were amplified with a Sigma kit. Both kits contain SYBR green; however, the amounts incorporated into the kits are not disclosed by the manufacturers.

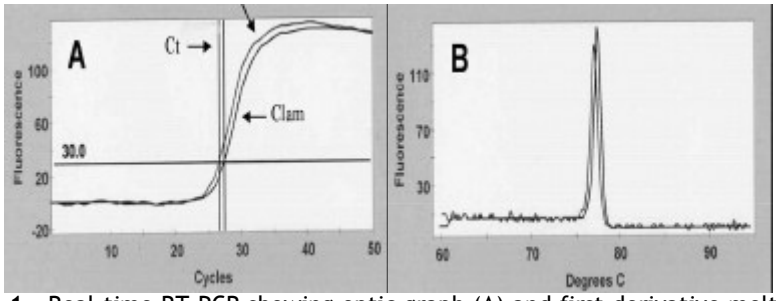


Figure 1. Real-time RT-PCR showing optic graph (A) and first derivative melt graph (B) of hepatitis A virus extracted from oysters and clams.

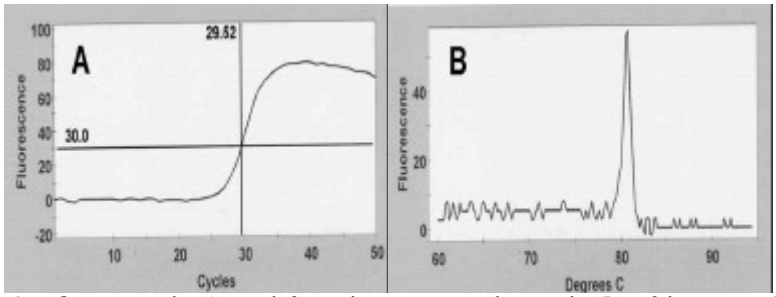
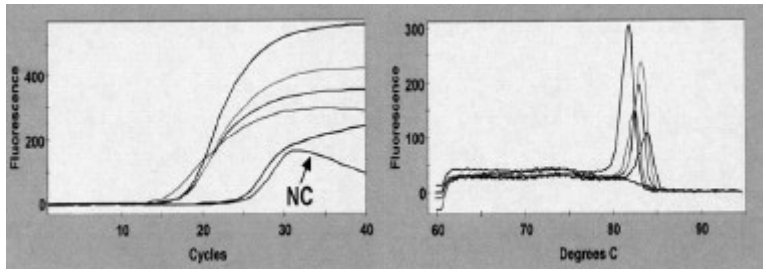


Figure 2. Optic graph (A) and first derivative melt graph (B) of hepatitis E virus extracted from stool.

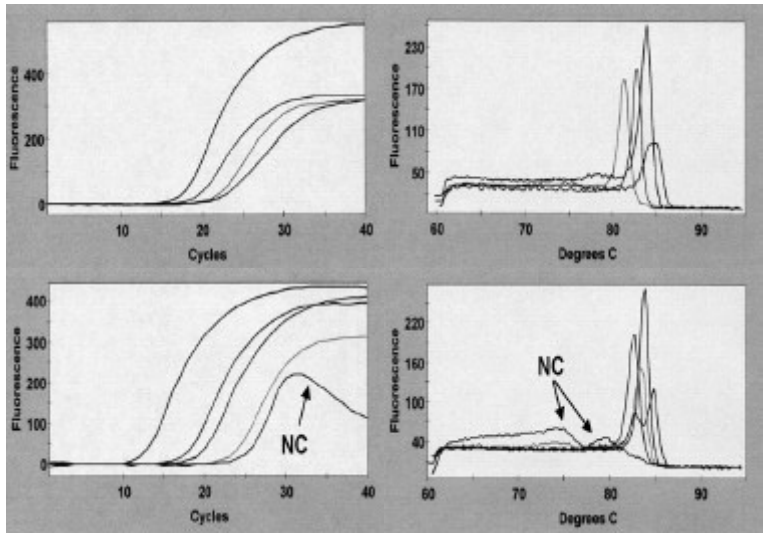
Table 1. Norovirus clusters identified by real-time RT-PCR.

Genogroup I, Cluster	Common name	Genogroup II, Cluster	Common name
1	Norwalk virus	1	Hawaii virus
2	Southampton virus	2	Snow Mountain virus
3	Desert Shield virus	3	Toronto/Mexico virus
4	Chiba virus	4	Bristol/Lordsdale/Grimsby
5	Musgrove virus	5	Hillingdon virus
		6	Seacroft virus
		7	Leeds virus
		8	Amsterdam virus

Five Genogroup I Noroviruses



Eight Genogroup II Noroviruses



Optics Graphs 1st Derivative Melt Graphs

Figure 3. Optic graphs and first derivative melt graphs of five genogroup I and eight genogroup II noroviruses. NC = negative control.

Discussion

We began our research for real-time detection methods using the classical Norwalk virus (genogroup I, cluster I norovirus), which is a commonly used laboratory strain of norovirus. We developed a SYBR green method and the concept of dilution end-point standard curves for the quantitation of Norwalk and other viruses (Richards *et al.*, 2004a). Since genogroup I and II noroviruses can be further broken down into genetic clusters, we evaluated the application of SYBR green rt RT-PCR to detect five clusters of genogroup I and eight clusters of genogroup II noroviruses. Together, these clusters represent over 90 % of the noroviruses circulating in the world today. We set out to develop a rapid and simple method to detect these clusters in a single reaction tube using degenerate PCR primers and SYBR green detection. It was necessary to first evaluate methods to extract the viral RNA from the stool samples. Another prerequisite to developing an rt RT-PCR method was to identify primers capable of detecting all the clusters of noroviruses. This was accomplished through a search of the sequence databases at the Centers for Disease Control and Prevention (CDC) in Atlanta. The CDC personnel identified consensus sequences and developed "universal" primers which we expected to bind to all thirteen clusters of norovirus RNA. Using only 1 µl of the 60 µl of stool extract, we developed and optimised rt RT-PCR procedures. We are currently working to adapt this rt RT-PCR method for use with shellfish extracts.

Real-time RT-PCR was also useful to detect HAV in clam and oyster extracts and HEV in stool extracts. Only a small amount of HEV-containing stool was available to develop the rt RT-PCR methods for HEV and there was insufficient stool to challenge shellfish for subsequent extraction and assay by rt RT-PCR. Our concern about HEV is motivated by the fact that shellfish have been epidemiologically linked to HEV infections. Capopardo *et al*. (1997) identified shellfish ingestion as a significant risk factor for sporadic HEV infection in Eastern Sicily. Likewise, lightly boiled mussels and partially cooked cockles were linked with hepatitis E in India (Tomar 1998). Although our results show that HAV and HEV can be detected using SYBR green-based rt RT-PCR, their conserved genomes make them good candidates for rt RT-PCR detection using fluorescent probes or primers, as well. In contrast, the high diversity of the norovirus genome makes SYBR green the preferred method for the real-time detection of noroviruses (Richards *et al*. 2004b). We have used our norovirus assay to assist in epidemiological investigations of norovirus outbreaks in Delaware where, in one case, an outbreak in a school for the deaf was linked to a food-handler. Together, these methods provide tools for the detection of HAV, HEV, and a broad suite of noroviruses in various biological matrices. Our research is continuing as we seek to develop rapid, cost effective, and practical methods to monitor the safety of shellfish.

Acknowledgements

The author wishes to thank Michael Watson (USDA) for technical assistance, Steve Monroe, Rebecca Fankhauser, Leslie Hadley and Suzanne Beard (CDC) for the selection of noroviruses, Junkun He (Walter Reed Army Institute for Research) for hepatitis E virus, and Stanley Lemon (University of Texas Medical Branch, Galveston, TX) for the FRhK-4 cells.

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DEVELOPMENT OF NEW PCR MARKERS FOR THE ANALYSIS AND THE DETECTION OF TOXIC PHYTOPLANKTON SPECIES.

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Abstract
The characterisation and the detection of toxic phytoplankton species are two major steps in the study and the management of harmful algal blooms. Morphological identification of most of the toxic species is difficult and arduous and even impossible with only light microscopy. Molecular tools, mainly based on rDNA analysis, have found increasing application for the detection of toxic phytoplankton however these techniques are not easy to standardise and in some instances do not provide enough polymorphism to discriminate all the toxic species. In order to evaluate new molecular tools we have developed for the first time two kinds of PCR-based markers using the presence of micro-satellites through phytoplankton genomes. The first one, a multi-locus fingerprinting technique called ISSR (Inter Simple Sequence Repeat), has been used to characterise and to study genetic relationships among toxic and non-toxic cultured species of diatoms, dinoflagellates and flagellates. The second one, mono-locus markers called SCAR markers (Sequence Amplified Characterised Region), has been used for PCR detection of toxic species of *Pseudo-nitzschia* and of *Alexandrium* in water samples from French coasts. Those specific PCR-based markers were revealed to be applicable for quick and low cost detection of toxic strains in the context of coastal phytoplankton monitoring.

Introduction
Early warning of harmful algal blooms is an important requirement in phytoplankton monitoring networks, and nowadays molecular markers are more and more in demand as fast, easy to use and low cost tools for toxic strain detection and identification. To date, the majority of molecular genetic studies in algae have used a fairly limited range of markers such as the plastid rbcL gene and spacer or the nuclear ribosomal DNA and spacers (Scholin *et al.*, 1993; 1994; Chesnick *et al.*, 1996; Tyrrell *et al.*, 2001; Guillou *et al.*, 2002). Markers available lack for intra-species identification of toxic microalgae in which toxicity varies within the species. Extensive work has been made in higher plants based on the occurrence of microsatellites through their genome to study their genetic diversity and to generate specific markers (Zietkiewics *et al.*, 1994; Bornet *et al.*, 2001; 2002; Arnedo-Andres *et al.*, 2002; Bautista *et al.*, 2003), but this property is scarcely investigated in phytoplankton genomes.

Microsatellites, also known as Simple Sequence Repeats (SSR), are tandem repeats of short sequence motifs (no more than six bases long) more or less uniformly distributed across eukaryotic genomes. Their study does not need prior genomic information because the nucleotide repeats are usually the same for many organisms. For phytoplancton species very few studies concerning microsatellites have been published, therefore we began this study to look for their presence in toxic phytoplankton genomes by making PCR-amplifications with an arbitrary microsatellite primer. This unique primer was composed of a few nucleotide repeat representing part of a presumed microsatellite region. Amplifications of inter microsatellite regions (ISSR, Inter Simple Sequence Repeat) were obtained each

time the primer encountered the repeat in inverted position on the DNAs and not too far for PCR amplification.In this work we present the experimental process of ISSR and of the deducted SCAR (Sequence Characterised Amplified Region) methods devoted to a set of 12 phytoplankton strains (diatoms, dinoflagellates and flagellates) and the tools that they can provide for species identification.

Methods

Total nucleic acid extraction

For the study, total nucleic acids were extracted from monoclonal cultures of 12 phytoplankton species (Table 1). The cultured phytoplankton species were grown in ESP medium of Provasoli (12) or f/2 medium of Guillard and Ryther (1962) under controlled conditions (16 °C, 12/12 hour light/dark photoperiod and 50 ± *x*µE m^{-2} s^{-1} light intensity). DNA extractions of cultured microalgae were performed from mid exponential growth phase cells according to a CTAB protocol as described by Bornet *et al*. (2004). DNA concentrations were quantified with a fluorometer (DyNA Quant 200, Hoefler, Amersham Biosciences, UK) using bisbenzimide (Hoechst 33258, Amersham Biosciences, USA) as the fluorescent dye and DNA quality was checked by agarose (1 %, w/v) electrophoresis stained with ethidium bromide.

PCR-amplifications with arbitrary microsatellite primers (ISSR fingerprints and deducted specific SCAR markers)

Five primers without anchored end and one with a 5' anchored end, chosen among primers tested during a previous study by Bornet *et al*. (2002) were used to PCR amplify the phytoplankton DNA (Table 2). The optimal annealing temperature was adapted in order to get the clearest patterns. When the optimal PCR conditions were defined each reaction was repeated at least three times, and with different batches of DNA to check the reproducibility of the reaction. The mix of PCR amplified products, which resulted, was submitted to electrophoretic separation on a 2 % agarose gel in 1X TAE buffer and stained with ethidium bromide. The patterns obtained were Inter Simple Sequence Repeat fingerprints (ISSR).

In each well a clear and separated band was selected as marker and scored as being present or absent in each species profile. Comparison of the profiles was made using pairwise comparisons with Jaccard's coefficient (Jacard, 1901). The similarity values were used to generate a consensus tree by UPGMA analysis and to construct a phenogram.

After comparison of all the ISSR fingerprints, each band occurring especially in the pattern of a species, was purified and sequenced. After BLAST analysis of the sequence, to exclude existing analogies, two primers flanking a short sequence were deducted. They were chosen in order to have an experimental melting temperature equal or higher than 50°C, more than 19 base pairs length and a rich G-C 3' end. These criteria were selected in order to provide high specificity and reproducibility to the reaction. They were assayed with the whole DNA of the study for PCR amplification of the specific genome region they enclosed. Only the pairs of primers giving a positive reaction with the targeted species and without cross reaction with the other strains were retained as species specific SCAR markers. The resulting PCR product was sequenced again and checked to be in accordance with the SCAR marker designed initially. Then they were applied to total DNA extracted from natural sea water samples for which species composition have been microscopically determined. Again the positive amplifications obtained were sequenced and verified to be in conformity with the expected SCAR marker.

Results and Discussion

The ISSR fingerprints obtained showed the presence of microsatellites in phytoplankton genomes. They revealed that polymorphism was abundant among the 12 species tested and that it reflected well their genetic diversity. Each ISSR primer gave rich and clear patterns, distinct enough to discriminate all the tested strains (Figure 1). They were polymorphic enough to provide an identification tool at the specific level. A total of 223 clearly detectable amplified ISSR bands were obtained with the six primers used, ranging from 100 to 2500 base pairs in size. The phenogram drawn from their analysis showed the genetic relationships among the 12 toxic and non-toxic cultured species of diatoms, dinoflagellates and flagellates. It proved to be reliable to cluster the strains in accordance with their taxonomic classification. We found the technique stable across a wide range of PCR parameters and reproducible for a species and for different batches of DNA of that species (Bornet *et al.*, 2004).

The SCAR markers were inferred from the ISSR profiles and were mono-locus markers. Not all the selected polymorphic bands conducted to a highly specific SCAR marker. Six fragments were retained, four for the diatom *Pseudo-nitzschia pseudodelicatissima* and two for the dinoflagellate *Alexandrium catenella*. From these six sequences analysed we have ensured three highly specific SCAR marker for *Pseudo-nitzschia pseudodelicatissima* and one for *Alexandrium catenella*. They have been tested with success to the detection and identification of these toxic species in sea water samples from the French coasts. The results obtained were in conformity with the microscopic observations of the samples (Bornet *et al.*; submitted in J. Phycol.).

Table 1: List of monoclonal cultured phyto-plankton strains

Genus	Species	Strain Designation
Alexandrium	*A. catenella*	ATTL01 (Thau, France)
Alexandrium	*A. catenella*	Tarragone, Spain
Alexandrium	*A. minutum*	AM89BM (Morlaix, France)
Alexandrium	*A. fundyense*	GONY (Canada)
Alexandrium	*A. tamarense*	MOG (Japan)
Alexandrium	*A. tamarense*	Plymouth (UK)
Pseudo-nitzschia	*P. pseudodelicatissima*	France
Pseudo-nitzschia	*P. multiseries*	France
Pseudo-nitzschia	*P. pungens*	Spain
Pseudo-nitzschia	*P. fraudulenta*	Spain
Skeletonema	*S. costatum*	France
Tetraselmis	*S. suesica*	UK

Table 2 : List of the six primers used to amplify ISSR markers from phytoplankton DNA

Primer	T_A (°C)
$(CCA)_5$	57
$(CAA)_5$	52
$(CAG)_5$	60
$(ATG)_5$	45
$(GACA)_4$	52
$GTC(CT)_8$	55

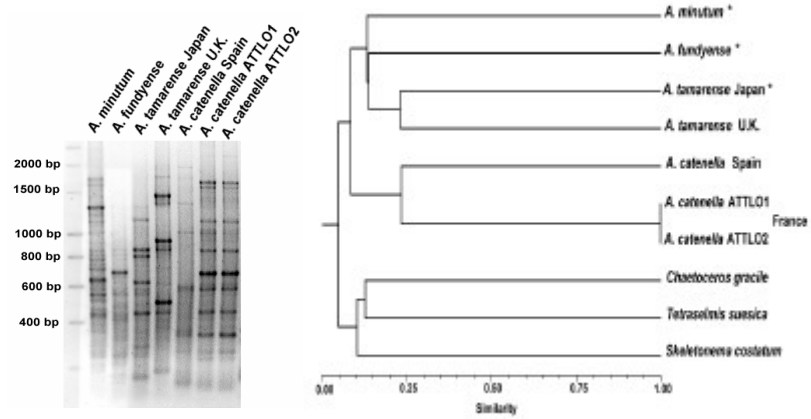

Figure 1 : Example of ISSR fingerprints and genetic relationships obtained by 148 polymorphic markers amplified with 5 primers (* = toxic strains)

Conclusion

Based on the presence of microsatellites in the eukaryotic genomes the ISSR fingerprinting method is an interesting way to identify specific markers for the phytoplankton species. It is simple to apply and does not need prior study of the genomes. Owing to the fact that microsatellite sequences are universal in the eukaryotic genomes, the PCR primers for ISSR fingerprints are arbitrarily defined according to the most frequent repetitions. The primers giving the richest and clearest patterns are retained. This method allows to enlarge the search for genetic markers for the species for which the databases comprise essentially of ribosomal DNA sequences. This method addresses the whole genome and reveals its polymorphism. It mitigates the disadvantages of the conserved genes (as the ribosomal DNAs) which do not always provide sufficient discrimination between species.

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DEVELOPMENT AND PRELIMINARY VALIDATION OF A LATERAL FLOW MEMBRANE ASSAY FOR DOMOIC ACID IN SHELLFISH

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Abstract
Reliable, rapid, on-site, semi-quantitative test methods for specific algal toxins would provide shellfish producers with immediate assurance prior to harvesting that their product will pass the formal regulatory assessment and is safe to put on the market. The lateral flow membrane immunoassay format is rapid, easy to use and has proven ability to give reliable, semi-quantitative data for a variety of analytes. The development and validation of a monoclonal antibody-based lateral flow assay for domoic acid is described. A panel of 62 scallop tissue extracts were tested with the lateral flow devices, and compared to HPLC results. Around the critical cut-off point of 20 mg domoic acid per kg shellfish tissue (equivalent to 5 µg/ml extract), the test had a sensitivity of 100 % and specificity of 75 %. Using the more rigorous cut-off point of 4.6 mg/kg (equivalent to 1.15 µg/ml), the sensitivity and specificity of the test were 87.5 % and 86.8 %, respectively. The absence of false negatives using the 5µg/ml cut-off point and the low level for the lower cut-off point means this assay is suitable for use as a screening assay for domoic acid in scallop extracts.

Introduction
Domoic acid (DA) is a neuro-excitatory algal toxin produced by the P*seudonitzchia sp.,* which is responsible for amnesic shellfish poisoning (ASP) in humans through the ingestion of contaminated shellfish. For food safety reasons, levels of DA in shellfish are under regulatory control. DA concentration in shellfish extracts is normally measured with the laboratory-based HPLC method and UV detection (Quilliam *et al.*, 1995). However, a supplementary screening test method is desirable for DA that would provide producers with immediate assurance prior to harvesting that their product will pass the regulatory assessment procedure. Antibody-based assays provide the only realistic alternative for such a test, offering at the same time the possibility of high levels of specificity and sensitivity and of rapid, reliable and easy-to-use formats for field use.

A number of laboratory-based microtitre plate enzymeimmunoassays have been described for domoic acid, based on polyclonal (Smith and Kitts, 1994; Garthwaite *et al.*, 1998) and monoclonal (Kawatsu *et al.*, 1999; Keaveney *et al.*, 2002) anti-DA antibodies, and have been applied to shellfish analysis. Some more rapid immunoassay formats have also been reported, (Mackintosh and Smith, 2002; Micheli *et al.*, 2004). However, as far as we know none of these assays have yet been adopted by the shellfish industry to assist them in farm management. This paper describes the development and validation of a semi-quantitative, reliable and easy to use lateral flow assay for DA, based on an in-house monoclonal antibody.

Materials and Methods
Materials
DA used to prepare the conjugates, standards and the spiked samples was obtained from Calbiochem (cat. no. 324378). The National Research Council (Canada) DA calibration solution (cat. no. DACS-1c). Nitrocellulose membrane (cat. no. HF2004) and glass fibre were a gift from Millipore Ireland BV. Whatman number 3 paper (cat. no. 1003-917) was used for the sample pad and sink. Cassettes were a gift from

Xenith Biomed (Ireland). Gold colloid was purchased from British Biocell International (cat. no. EM GC 40). Rabbit anti-mouse immunoglobulin (RAM) was obtained from Dako (cat. no. Z109). Polyvinylalcohol and all other chemicals were from Sigma-Aldrich (Ireland). The monoclonal antibody used was produced in-house following immunization of Balb/C mice with a DA-bovine serum albumin (BSA) conjugate prepared according to the method of Kawatsu *et al.*, (1999).

Immobilisation of antibody on gold colloid
The antibody was purified from tissue culture supernatant by Protein G affinity chromatography. Protein concentration was determined using the BCA assay (Pierce Biotechnology). Gold colloid (average diameter: 40 nm) was coated with antibody in 5 ml batches by adding 50 µL antibody solution (27.5 µg/ ml) dropwise to the gold suspension, stirring for 1 min, and then blocking with 10 % human serum albumin. The antibody concentration used was determined as the lowest amount which would sufficiently stabilise the gold colloid. Following centrifugation, the suspension was resuspended to give an optical density of 2.0 at 544nm. Glass fibre pads were then impregnated with the coated gold by immersion in the suspension and dried at 20°C.

Membrane coating
Lines of DA-BSA conjugate (0.2 mg/ ml) and rabbit anti-mouse antibody (RAM, 1.8 mg/ ml) were applied approximately 11 and 17 mm from the lower end of a 25 mm wide strip of nitrocellulose membrane using a BioDot™ XYZ3000 dispensing platform fitted with a Biojet™ 3000 dispenser. The membrane was then blocked with 1 % polyvinylalcohol, stabilised with 3 % sucrose and dried.

Assembly of the test device
The device was assembled using adhesive cardboard backing as follows: the nitrocellulose strip was placed down first on the backing. The gold-impregnated pad (5 mm wide) was arranged so that it overlapped the lower end of the nitrocellulose strip. The sample application pad (5 mm wide) was then placed over the gold pad, ensuring it did not make direct contact with the nitrocellulose strip. A strip of Whatman number 3 paper was placed overlapping the top end of the nitrocellulose strip to act as a sink. Following assembly, the long strip was cut into 5 mm wide strips and installed in the cassette holder such that the two lines applied to the nitrocellulose would be visible in the viewing zone. The cassette was then closed and stored vacuum packed with desiccant at room temperature.

Assay procedure
The principle of the test is that when the standard/sample (100 µL) is applied to the sample application zone, it wicks along the membrane strip carrying the antibody-coated gold with it. In negative samples, the antibody-coated gold binds to the DA-BSA in the test line and also to the RAM in the control line giving two red lines. In positive samples (when DA is present above the cut off-level of 1 µg/ ml) DA binds to the antibody-coated gold, making it unavailable for capture by the DA-BSA in the test line. The gold particles can still be captured by the RAM in the control line. The presence of DA above the cut-off level is indicated by the occurrence of only one red line. The test is read when the sample solvent front has reached the end of the viewing window, which takes approximately 3 minutes.

Sample analysis
Sixty-two random extracts of scallop tissues were supplied by the Marine Institute, Galway, Ireland from their routine DA monitoring programme. Crude extracts were prepared as recommended for routine analysis by HPLC (Quilliam *et al.*, 1995).

Briefly, shellfish tissues (whole or specific tissues) were homogenised in 50 % methanol using Ultra-Turrax homogeniser (4g of tissue/16 ml of solvent). HPLC analysis of these crude extracts was carried out as described by Hess *et al.*, 2004. Extracts were stored at 4°C in dark bottles and diluted 1 in 2 with 0.01 M phosphate-buffered saline (PBS) containing 0.1 % Tween prior to analysis on the lateral flow assay. Results were scored as either positive or negative by a single trained operator depending on the number of red lines visible at the end of the test.

Assay performance analysis

Assay sensitivity was calculated as the percentage of true positives as defined by the HPLC analysis that tested positive in the assay, and specificity as the percentage of true negatives testing negative. Positive predictive value was the percentage of all samples testing positive that were actually positive and the negative predictive value, the percentage of all samples testing negative that were true negatives.

Results

Assay characteristics

Assay parameters were optimised to give a cut-off point of approximately 1 µg DA per ml extract. The cut-off point of the final test device was defined by repeated analysis (n = 10) of a negative scallop extract spiked with four different concentrations of domoic acid, ranging from 0.1 to 4.0 µg/ ml (Table 1). The 0.1 µg/ ml sample always tested negative and the 4.0 µg/ ml sample always gave a positive result. However, the concentrations, 0.5 µg/ ml and 1.5 µg/ ml, gave a positive result 80 % and 90 % of the time, respectively, indicating that these lay within the 'grey zone' or arbitrary zone of the test.

Table 1. Determination of the 'grey zone' of the lateral flow assay

DA concentration (µg/ ml)	No. of positive results	No. of negative results
0.1	0	10
0.5	8	2
1.5	9	1
4.0	10	0

Analysis of scallop extracts

A panel of 62 crude scallop extracts were tested using the lateral flow test devices and results compared to DA concentrations of the same extracts determined by HPLC. They included extracts of 4 different tissue types: gonad, hepatopancreas, adductor muscle and remaining tissues. Twenty-six samples tested positive with the lateral flow test, their DA concentrations by HPLC ranging from 0 to 790 µg/ ml. The DA concentration of the 36 negative samples ranged from 0 to 2.7 µg/ ml. Figure 1 shows a scattergraph of all samples with DA concentration less than 5 µg/ ml. This shows clearly that all samples with DA concentration greater than 3 µg/ ml tested positive, while 8 samples with DA concentration below 3µg/ ml and 3 with concentration below 1 µg/ ml gave positive results.

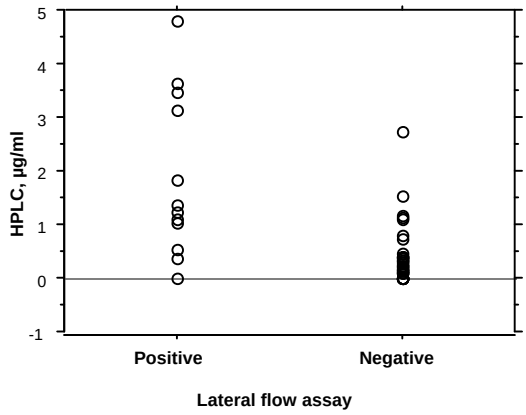

Figure 1. Scattergraph of the lateral flow assay results for samples with DA concentration less than 5µg/ ml. Negative samples (n = 36). Positive samples (n = 13). Thirteen of the positive results are not shown (DA concentration > 5µg/ ml).

Assay performance

The regulations specify two critical levels of DA in shellfish extracts. In most countries, the regulatory upper limit for DA in shellfish is set at 20 mg/kg. In 2002, the EU introduced an additional limit of 4.6 mg/kg for the edible part of scallops, where the concentration of DA in the whole body of the scallops lies between 20 mg/kg and 250 mg/kg (Decision 2002/226/EC). The performance of the lateral flow assay was therefore assessed at two critical points, 20 mg/kg and 4.6 mg/kg, relative to the HPLC analysis (Table 2). The assay performed very well around the 20 mg/kg cut-off point, which corresponds to 5 µg/ ml in the extract. The 4.6 mg/kg level, corresponding to a concentration of 1.15 µg/ ml extract, is within the grey zone of the test explaining the less reliable performance characteristics at this critical point.

Table 2. Summary of the performance of the lateral flow test relative to the HPLC analysis at two critical points.

Critical point	**20 mg/kg**	4.6 mg/kg
Sensitivity	100 %	87.5 %
Specificity	75 %	86.8 %
PPV*	53.8 %	80.8 %
NPV*	100 %	91.7 %

*PPV = positive predictive value: NPV = negative predictive value.

Discussion

The occurrence of toxins in shellfish is a continuous threat to the shellfish industry with serious consumer health and economic consequences. Regulatory analysis ensures that the product reaching the market place is safe to eat. However, shellfish farmers need additional support mechanisms to help reduce their losses when contamination occurs. The availability of convenient screening test systems for these toxins, which could be used in the field, would facilitate better decision making with regard to timing of harvests and overall better management of shellfish farms.

The development and preliminary validation of a convenient lateral flow assay device for DA is described. A monoclonal anti-DA antibody developed in-house forms the basis of the test. This antibody has already been shown to be specific for DA and to have low cross-reactivity for compounds structurally similar to DA, such as kainic acid and glutamic acid (Keaveney *et al.*, 2002). The assay is easy to perform and read and minimal training would be necessary. It also works with a simple sample preparation procedure involving solvent extraction, which is routinely used as the first step in preparation of samples for HPLC analysis (Quilliam *et al.*, 1995) and should be feasible in the field. The goal was to design an assay that had a cut-off point of approximately 1µg/ ml, equivalent to 4 mg/kg shellfish flesh. This level is lower than either of the critical DA concentrations (4.6 mg/kg and 20 mg/kg) laid down in the regulations (EU decision 2002/226/EC) and was selected to allow for the 'grey zone', i.e. the concentration range where there would be some uncertainty about the results. Although not always specified, all qualitative/semi-quantitative test systems have a 'grey zone' around the cut-off point (Feldsine *et al.*, 2002). The data presented show that at 4µg/ ml concentration, the test always gave a positive result. This ensured that there would be no false negatives at the critical point of 5 µg/ ml (20 mg/kg). At concentrations of 1.5 µg/ ml and 0.5 µg/ ml, there was a small degree of uncertainty about the results and hence about the test's ability to discriminate around the second critical point of 1.15 µg/ ml (4.6 mg/kg).

The performance parameters of the test when compared to the HPLC method confirmed the ability of the test to discriminate around the most important critical point of 20 mg/kg. At this point, the test had 100 % sensitivity and a 75 % specificity. The absence of false negatives (indicated by the 100 % sensitivity) is an absolute requirement for a screening assay, while some false positives can be tolerated. Thus, the assay performed very well around this most important critical point. There was a slight decrease in sensitivity and corresponding increase in specificity to 87.5 % and 86.8 %, respectively, at the lower cut-off point of 4.6 mg/kg. For application at this critical point, further optimisation of the test would be needed.

In conclusion, the preliminary validation results presented here for the lateral flow assay for DA confirm that the device is suitable for use as a rapid screening assay for the detection of shellfish contaminated with DA and warrants further large-scale evaluation in the field.

Acknowledgements
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AN IMPROVED METHOD FOR THE RECOVERY AND DETECTION OF ENTERIC VIRUSES FROM PACIFIC OYSTERS

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Abstract
Enteric viruses are a significant cause of shellfish associated foodborne disease. Current methods, whilst able to detect these viruses are not sufficiently sensitive for routine quality assurance purposes. This paper outlines a novel concentration and RT-PCR detection method for a range of enteric viruses (Noroviruses, Hepatitis A, Enterovirus) in pacific oysters. Tank experiments for the study of naturally accumulated virus by live oysters using bacteriophage F X174 as a surrogate enteric virus were conducted. Dissection of the oysters digestive gland and viral precipitation followed by a nested RT PCR method were used. Results comparing the surrogate with a phage plaque assay indicated that this method was capable of detection of approximately 1 PFU of virus per oyster. This limit of detection represents a significant improvement over current published methods. Shellfish associated foodborne disease is often caused by bacterial or viral etiological agents. Routinely *Escherichia coli* are used as indicators of the potential for contamination by other pathogens associated with shellfish borne disease, including enteric viruses, as they are communicated from fecal material and via similar routes of transmission.

Inadequacies of the current indicator for oyster safety
There are characteristics of *E. coli* that limit its suitability as an indicator organism. Firstly, the survival of *E. coli* in the marine environment is not comparable to the survival of other pathogens, particularly enteric viruses (Jiang et. al., 2001). Secondly, viruses are retained within shellfish following ingestion for longer periods of time than bacteria. This is believed to result from the association of viral particles with shellfish digestive tissues. Conversely, bacteria seem to pass through the shellfish digestive system more readily (Dore and Lees, 1995).

Methods for the detection of viral pathogens
In response to these problems, methods have been developed to detect the presence of viral pathogens in shellfish. As many of the viral pathogens related to shellfish associated illness are non-culturable, these methods have mainly centered on the use of polymerase chain reaction (PCR) techniques for the detection of viral nucleic acids in shellfish. The sensitivity of these methods is critical to their value as the infectious doses of enteric viruses are very low while the capacity for shellfish to accumulate these pathogens, and the volumes of meat consumed by individuals is very high.

We propose a novel methodology involving:

1. the concentration of viruses from oyster meat, followed by;
2. the extraction of viral nucleic acids from the resulting viral concentrate, and
3. the detection of viral nucleic acids by nested PCR.

This method achieves a sensitivity of 0.24 to 2.44 plaque forming units (PFU) per oyster, as assessed in oysters allowed to naturally ingest infected marine water.

Method Development – 1. Viral concentration from oysters

Sensitivity of known methods

To determine how the sensitivity of the known viral concentration method could be improved, the relative recovery of the enteric virus surrogate, coliphage F X174, from oysters was determined at each stage of the method.

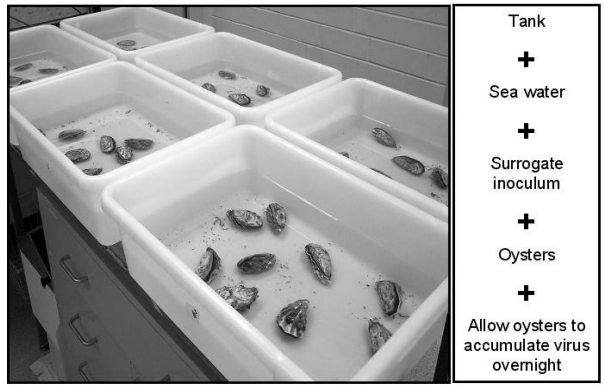

Figure 1. Method for the inoculation of filter feeding oysters.

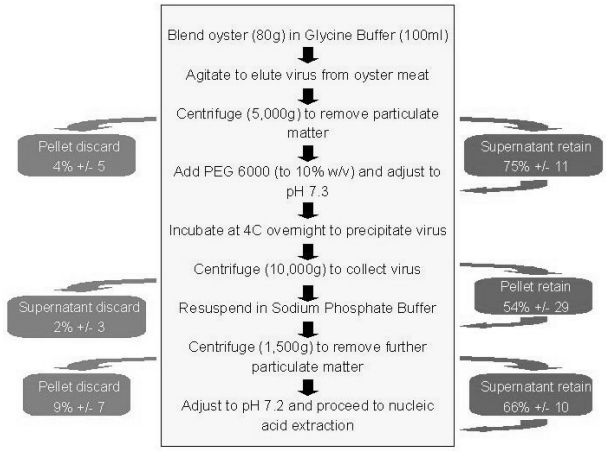

Figure 2. Relative recovery of coliphage F X174 at each step of the Traore et. al. (1998) method for the concentration of virus from oysters.

This method gave a final recovery of 66 % of the initial concentration of virus present in the oyster meat. To determine the sensitivity of the method, viral nucleic

acids present in the concentrate were detected by reverse transcriptase (RT) PCR, which gave a sensitivity range of 55.6-555.6 PFU/g of oyster meat.

Sensitivity improvement strategies - incorporation of a second PEG 6000 precipitation

In order to improve the sensitivity of this method it was necessary to concentrate virus from oyster tissue further so that a greater sample size could be assayed and to further eliminate PCR inhibiting compounds that may be compromising PCR efficiency. A second PEG 6000 precipitation was introduced to the virus concentration method to achieve this.

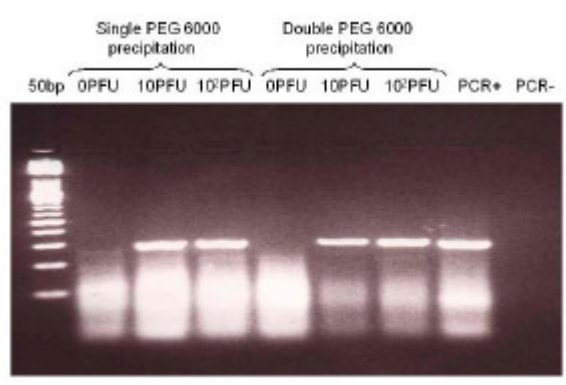

Figure 3. RT-PCR detection of virus at three initial concentrations following viral concentration by single PEG 6000 precipitation (lanes 2-4) and double PEG 6000 precipitation (lanes 5-7).

The introduction of a second PEG 6000 precipitation reduces the carry over of material from the viral concentration step to the PCR step. Furthermore, the final recovery of the viral concentration method only decreased 2 % to 64 % but gained a 2.5x concentration of virus, which improves sensitivity by increasing the volume of oyster meat assayed.

Sensitivity improvement strategies - exclusion from assay non infected oyster tissues

The sensitivity of the assay is further improved by excluding oyster tissues from assay that do not accumulate virus so that a greater volume of virus accumulating tissue may be submitted for assay. Oysters that had accumulated phage were assayed to confirm that the oyster digestive tissues accumulate virus.

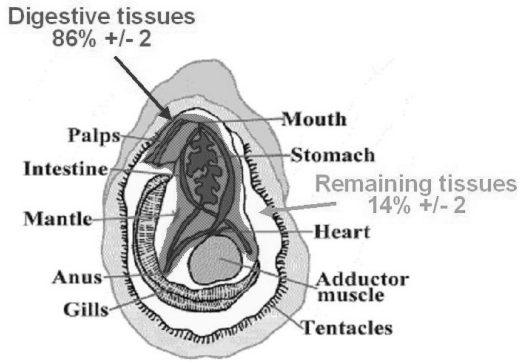

Figure 4. Location of virus in oyster tissues following filter feeding (modified from The Assateague Naturalist, 1997).

The digestive tissues are shown to accumulate greater than 85 % of the viral content of the oyster in only 27 % of the total tissues.

Sensitivity improvement strategies - length of viral precipitation time
The length of incubation time allowed for the precipitation of viral particles was compared.

Table 1: Comparison of a 2 hour and 18 hour incubation period for the precipitation of virus by PEG 6000.

Length of precipitation	Percentage recovery of virus at each PEG 6000 precipitation step	
	First precipitation	Second precipitation
2 hours	99.83 +/- 0.16	98.36 +/- 1.69
18 hours	86.1 +/- 4.54	99.41 +/- 0.48

A two way analysis of variance shows an extremely significant improvement in recovery at the first PEG 6000 precipitation with the adoption of a 2 hour precipitation length (P value = 0.0003) with extremely significant interaction between length of precipitation time and PEG 6000 precipitation step (P value < 0.0001). Therefore a 2 hour incubation time was adopted for the first PEG 6000 precipitation and an 18 hour precipitation time was adopted for the second, which improves overall recovery and reduces operation time by 16 hours.

Comparison of model organism with enteric virus
To ensure that the model organism is representative of the enteric viruses to be detected in the natural environment, the relative recovery of coliphage F X174 was compared with the recovery of poliovirus seeded to oyster tissue.

Table 2: Comparison of the relative recoveries of F X174 and poliovirus during the virus concentration protocol.

Virus concentration method step	Percentage recovery of virus	
	ϕX174[1]	Poliovirus[2]
Initial	100 +/- 0	100 +/- 0
First centrifugation	95.54 +/- 2.42	73.57 +/- 27.39
First precipitation	95.07 +/- 1.68	64.04 +/- 31.77
Second centrifugation	76.5 +/- 9.2	24.13 +/- 19.02
Second precipitation	74.76 +/- 8.04	23.21 +/- 19.43

[1] determined by plaque assay

[2] determined by fluorescence microscopy

The similarity in behaviour of the two viruses throughout the virus concentration protocol confirms that F X174 is an adequate model for the enteric virus poliovirus.

Final viral concentration method

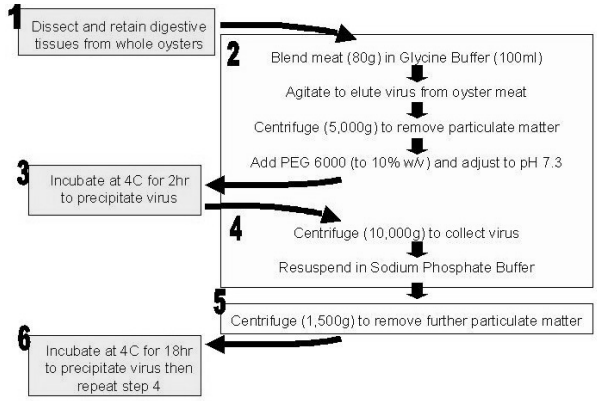

Figure 5. Virus concentration method with changes adopted.

Method Development – 2. Extraction of nucleic acids from oyster concentrate
RNA was initially extracted from the viral concentrate using the Trizol (Gibco BRL) product and accompanying method. An extraction method capable of eliminating plant material from the concentrate (RNeasy Plant Minikit, Qiagen) was found to be more effective for the extraction of nucleic acids due to the presence of algae in oysters.

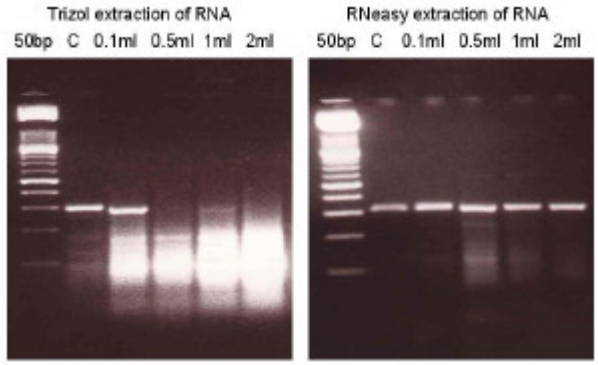

Figure 6. RT-PCR detection of virus at four concentrations following Trizol (Gibco BRL) extraction of RNA from the viral concentrate (left) and RNeasy Plant Minikit (Qiagen) extraction of RNA from the viral concentrate (right).

The RNeasy Plant Minikit was able to eliminate PCR inhibiting compounds that had prevented nucleic acid detection using Trizol. Furthermore it permitted the extraction of RNA from a greater volume of viral concentrate, resulting in a 14-fold improvement in sensitivity.

Method Development – 3. Detection of viral nucleic acids
To improve specificity and possibly further improve sensitivity, a nested RT-PCR was compared with the standard RT-PCR for viral nucleic acid detection.

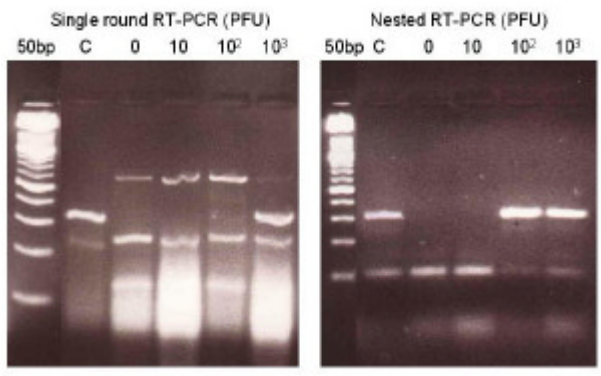

Figure 7. RT-PCR detection of virus at four concentrations (following viral concentration and subsequent RNA extraction) by standard RT-PCR detection (left) and nested RT-PCR detection (right).

Not only was an improvement in specificity observed but a 10 fold improvement in sensitivity was also achieved.

Final detection limit
The improvements made to the method initially adopted have resulted in a greater than 4500-fold improvement in sensitivity over the original method.

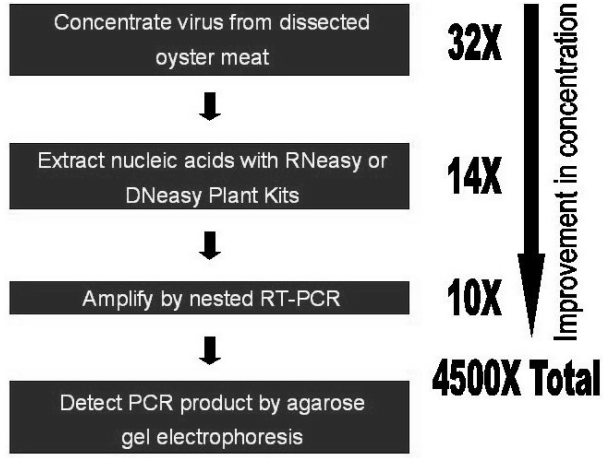

Figure 8. Steps involved in the final improved viral detection method and relative improvement at each step.

The detection range for this method as assessed in oysters allowed to naturally ingested infected marine water is 0.012 PFU/g to 0.122 PFU/g of oyster meat or 0.244 PFU/oyster 2.44 PFU/oyster.

Conclusions

- Improved limits of detection for viral particles were achieved by modifications to viral concentration, nucleic acid extraction and PCR methods.
- Detection ranges of 0.24- 2.4 PFU per oyster were achievable by this method.

This methodology represents a significant improvement on previously published detection limits that may in due course permit quantitative risk assessment.

Acknowledgements

The authors would like to gratefully acknowledge the continuing support of the Australian Fisheries Research and Development Corporation in funding this research and the South Australian Oyster Growers Association.

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SAMPLE PREPARATION AND ANALYSIS OF ALGAL TOXINS BY ALTERNATIVE METHODS

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Abstract
Novel, sensitive and accurate analytical strategies for a reliable control of phycotoxins naturally occurring in the aquatic environment involving features such as sensitivity, selectivity as well as shorter time of analysis are clearly demanded. These features are often compromised as a consequence of an inefficient sample pre-treatment, since the protocols required for the preparation of the complex sample matrices where these toxins are usually present, are tedious and complicated and usually the main source of errors in the analytical procedure, therefore the search for simple and efficient protocols is an important task for scientists working in this field of research. This work reports on the application of some sample preparation strategies alternative to the ones conventionally used for such particular applications. Examples of Accelerated Solvent Extraction (ASE) and Microwaves for the extraction of PSP toxins will be shown, while Immunoaffinity extraction and Solid Phase Microextraction will be used for the extraction of Microcystins. Regarding to the analysis of these toxins High Performance Capillary Electrophoresis and High performance Capillary Electrochromatography will be also applied and results of the analysis of Yessotoxins and Amnesic Shellfish Poisoning (ASP) toxins are also discussed. The potential of this technique for such particular applications is clearly shown after the improvement on the sensitivity by modern on-line strategies. It can be concluded from the results that these alternative methods can simplify and improve the analytical protocols usually applied for the mentioned applications.

Introduction
The aquatic environment has been seriously affected by the presence of natural toxins produced by several phytoplanktonic species. Seafood contamination is an important socio-economic problem worldwide. The high toxicity of these compounds at very low levels has made it necessary to search for sensitive analytical approaches for adequate analysis of these toxic compounds at the required levels. The complexity of the biological matrix makes it necessary to search for efficient strategies of sample preparation with the aim of increasing the selectivity as well as the enhancement of the sensitivity at a pre-concentration step. This study focused on the development and application of modern analytical strategies for sample preparation and analysis of seafood toxins such as ASP, Paralytic Shellfish Poisoning (PSP) and Yessotoxins (YTX's) as well as Cyanotoxins such as microcystins (MC's). Accelerated solvent extraction (ASE) and Microwaves assisted process (MAP) have been applied for a fast and efficient extraction of algal toxins. Accelerated solvent extraction uses conventional liquid solvents at elevated temperatures to increase the efficiency of the extraction process while and MAP is based on a basic physical principle, namely the fact that different chemical substances absorb microwave energy to different levels resulting in a powerful tool for a selective and rapid extraction. An example of the application of these strategies for the PSP extraction is described in this paper. Sample extraction has been carried out using novel strategies such as Solid Phase Microextraction (SPME), a simple and effective

adsorption and desorption technique. This approach has been applied to the extraction of analytes such as MC's prior to HPLC (Villar González *et al*) Immunoaffinity (IAC) extraction has also been applied in this work for a fast extraction of MC's as well as for a specific removal of interferences, based on the specificity of the antigen–antibody interaction (Aguete *et al.*, 2003) used in IAC. Examples of the application of modern analytical Electroseparation techniques such as Capillary Electrophoresis (CE) and Capillary Electrochromatography (CEC) for the analysis of certain algal toxins such as YTX's, ASP's present in different matrices are described.

Experimental

Sample Preparation

Microcystins

A 20 ml volume of methanol:water (75:25) was added to a 3 g portion of fish (skinless and boneless) sample in a centrifuge tube and the contents mixed for 5 min using an Ultraturrax homogeniser. The mixture was then centrifuged at 4500rpm for 15 min at room temperature. The supernatant was transferred to a clean glass tube and the residue was remixed with 20 ml of the methanol-water (75:25) and re-centrifuged at 4500rpm for 15min at room temperature. The supernatant was combined with the previous one and the volume was reduced to 4 ml under a nitrogen stream.

Immunoaffinity clean-up

Immunoaffinity columns (Microcystin ImmunoSepTM) were purchased from Abkem Iberia S.L. (Spain). The SPE columns contained anti-microcystin LR monoclonal antibodies on sepharosa-based column. Columns were conditioned with 3 ml of water and 3 ml of PBS (pH 7.4). Prior to sample percolation, 380 µL of sample was evaporated to dryness and re-disolved in 1 ml PBS (pH 7.4) and finally it was loaded into the column. The cartridge was rinsed with 3 ml of PBS, followed by 3 ml of water and 3 ml of methanol:water (25:75). The microcystins were eluted with 6 ml of methanol:water (80:20) containing 4 % (v/v) acetic acid. The effluent was evaporated to dryness in rotavapor at 40ºC and then re-dissolved in 0.2 ml of methanol for injection into the HPLC system.

Solid-phase Microextraction

Before extraction by SPME, 380µL of sample was evaporated to dryness and re-dissolved in 3 ml of Milli Q water (pH 2 and 20 % NaCl (w/v)). The extraction was performed by immersion of the PDMS/DVB 60µm fiber in this sample solution, with permanent magnetic stirring (1000 rpm) and temperature control at 56ºC for 60min. Then the fiber was then placed in the desorption chamber (Supelco), which replaced the injection loop of the HPLC system, and the desorption was carried out in dynamic mode for 15 min.

Paralytic Shellfish Poisoning (PSP Toxins)

Microwaves Assisted Process (MAP) Extraction of PSP toxins. Different parameters affecting the Microwaves assisted process, such as irradiation time, irradiation power, type of solvent and solvent volume have been evaluated. The optimal conditions include the use of 3 ml of 0.25 M acetic acid, an irradiation time of 15 seconds with an irradiation power of 270 W, and were applied for the extraction of PSP toxins present in contaminated samples.

Accelerated Solvent Extraction (ASE)
An ASE 200 automated extractor (Dionex) was used for the extraction of GTX 2/3 from contaminated mussel tissues. For the extraction, 4-5 g of mussel tissue was combined with 6-7 g of Hydromatrix (Varian) sorbent material, mixed very well in a beaker, transferred to a 11 ml volume capacity cell extractor. The material was packed strongly into the cell, which was sealed and placed in the apparatus (ASE 200 automated extractor, DIONEX). The following parameters were used in order to optimise the extraction program: pressure 1500psi, 1-3 cycles for 5 min each, 50-100 % flush volume, 120s purge. The extraction temperature ranged from 60 to 120°C. The extraction solutions used were: water, 1 % and 5 % acetic acid, 30 % ethanol in water and 30 % ethanol in 5 % acetic acid. About 18-20 ml extract was collected and after adjustment of the volume to 25 ml with water, 1 ml of extract was passed into a SPE C18 cartridge for cleanup and then oxidised for HPLC analysis.

Yessotoxins (YTX's) (Yasumoto, pers. comm.).
Yessotoxins extraction was carried out from 1g of shellfish digestive gland or 1 ml algal extract by means of homogenization for 3 min with 9 ml of methanol:water (80:20), followed by centrifugation at 3000 rpm for 10 min. SPE cleanup was carried out using LC-18 cartridges (3 mL, 500 mg, Supelco, Bellefonte, PA, USA). At first, the stationary phase was conditioned with 20 ml of MeOH. 5 ml of crude extract mixed with 15 ml phosphate buffer 20 mM at pH 5.8 was immediately loaded onto the cartridge and 6 ml methanol:water (20:80) was used for the washing. Finally the toxin was eluted with 6 ml methanol:water (70:30). After evaporation to dryness and re-suspension in 500 µL methanol, the purified extract was ultrafiltered through 0.45 µm filter (Ultrafree®-MC, Millipore, Bedford, MA, USA) at 14.000 rpm.

Amnesic Shellfish Poisoning (ASP Toxins)
Sample extraction was carried out under the conditions proposed by Quilliam *et al*. (1989) with slight modifications. 16 ml MeOH:H_2O (1:1 v/v) was added to 4 g tissue homogenate of shellfish sample. This mixture was homogenised for 3 min and then centrifuged at 4500 rpm for 10 min. The supernatant was filtered through a 0.45 µm filter (Millex HV) and kept in the fridge.

Solid Phase Extraction (SPE)
C18
The conditions used for the cleanup of the sample prior to CE analysis was a slightly modified version of Quilliam *et al*. (1989) and slightly modified. Through a C18 cartridge, previously conditioned with 6 ml acetonitrile and 6 ml water, 2 ml of sample extract was loaded. The ASP toxins were eluted with 3 ml acetonitrile/water (1:9) and collected in a 5 ml volumetric flask.

SAX
The conditions for the cleanup of the sample prior to CEC analysis initially described by Quilliam *et al* (1995) was slightly modified using Anion Exchange (SAX) cartridges, previously conditioned with 6 ml methanol, 3 ml water and 3 mL methanol:water (1:1). After conditioning the cartridges, 5 ml of sample extract was loaded and washed with 3 ml methanol:water (1:1). The ASP toxins were then eluted with 5 ml 0.1 M formic acid.

Analysis

HPLC-MS Analysis of Microcystins

LC-MS analyses was performed using an Agilent 1100 Series Liquid Chromatograph equipped with an Agilent 1100 series MSD detector with an electrospray ionization source. Separations were performed in a Vydac C18 column (25cm × 2,1mm ID, 5 μm) with a mobile phase consisting of water containing 0.05 % (v/v) formic acid in channel A and acetonitrile with 0.05 % (v/v) formic acid in channel B. Linear gradient elution started from 10 to 50 % B over 12 min, held at 50 % B for 3 min and a post-time of 10min to come back to initial conditions. The flow-rate of the mobile phase was set at 0.4 $ml \cdot min^{-1}$.

MS detection was carried out in the selected ion monitoring (SIM) for the positive ions $[M+H]^+$ (*m/z*: LR= 996; YR=1039; RR=1046), which provides a LOD of 3.8, 8.0 and 64.1 ng/ml for MC-LR, MC-YR and MC-RR respectively.

HPLC-FLD Analysis of PSP toxins

The LC system consisted of 2 Beckman pumps (Model 110B and 114M), a gradient controller (model 421A), and an Altex injection port (model 210A) with a 50 μL loop. The column used was a Supelcosil LC-18 (15cm x 4.6mm id, 5μm) while a LC 240 Perkin Elmer fluorescence detector (ex: 340nm, em: 395nm) was used to monitor the LC effluent. PSP oxidation products were eluted using a step gradient of 0-20 %B in 7.5min; 20-80 % B from 7.5 min to 11 min and 80 %-0 % B from 11min to 12 min, at 2 ml/min. The mobile phase A was composed of 0.1M ammonium formate, pH ajdusted to 6 with 0.1M acetic acid (6.3g of ammonium formate, 6ml of 0.1M acetic acid in 1L of water) and B 5 %(v/v) acetonitrile in 0.1M ammonium formate, pH adjusted to 6 with acetic acid (6.3g of ammonium formate, 6 ml of 0.1M acetic acid, 50 ml of acetonitrile in 1L of water).

CE-UV/DAD Analysis of Yessotoxins

CE analyses of Yessotoxins was carried out using 48,5 cm coated fused-silica capillaries (40cm effective length) with 50 μm inner diameter and 375 outer diameter (Composite Metal Services, The Chase, Hallow, UK. Ref: TSP050375). The capillary was thermostated at 25ºC and direct detection by DAD was set at 230 nm with a reference wavelength of 450 nm. Separations were achieved using a background electrolyte (BGE) prepared with 50 mM Na_2HPO_4 and 40 % MeOH, adjusted at pH 8.5. The background buffer was filtered through 0.45 μm filter (Millex-HV, Millipore, Bedford, MA, USA) and degassed 15 min before use. In order to overcome the lack of sensitivity of CE, a stacking procedure based on Large-volume Sample Stacking, LVSS (Osbourn *et al.*, 2000) was developed for this application. The sample buffer consisted of a MeOH:BGE (70:30) solution, pH 3.0. The sample was hydrodynamically loaded onto the capillary by applying a pressure of 50 mbar during 20 s and then stacked by applying a voltage of -25 kV for 40 s. Separation was then performed at 30 kV. This procedure allows for the concentration of the analytes into a narrow band, thus enhancing the sensitivity of CE for this particular application by a factor of 20. The LOD achieved for YTX after application of the present preconcentration procedure was 0.12 μg/ ml, which fulfils the EU sensitivity requirements (EC Regulation, 2004) for this toxic compound and can be compared with the one obtained in HPLC-FLD analysis.

CE-UV/DAD Analysis of ASP Toxins

The CE analysis of ASP toxins was performed using fused-silica capillaries, 62.5 cm (54 cm effective length) x 50 μm i.d. obtained from Agilent Technologies. The capillary was thermostated at 25ºC and UV detection was set at a wavelength of

242nm. Prior to the run, the capillary was conditioned with a three-step protocol (0.1N NaOH for 10 min followed by 5 min of ultrapure water and 20 min of background electrolyte) and the current was stabilised by application of 30 kV for 120s in order to improve the repeatability and performance. The background electrolyte used was 25 mM phosphate buffer at pH 7.0 filtered through 0.45 μm filter (Millex-HV) and degassed 15 min before use. A stacking procedure based on LVSS was also developed in order to overcome the lack of sensitivity. The sample solution consisted in a MeCN:H_2O (1:9). The sample was hydrodynamically loaded onto the capillary by applying a pressure of 50 mbar during 75s and then stacked by application of -5 kV for 130s. After that, separation was performed at 30 kV. This procedure allowed the concentration of the analytes into a narrow band, thus enhancing the sensitivity of CE for this particular application by a factor of 5. The sensitivity provided by the application of this approach in terms of LOD was 0.23 μg/ ml, which is also comparable with the one provided by HPLC-UV method.

CEC-UV/DAD Analysis of ASP Toxins

Capillary Electrochromatography of ASP toxins was performed using a 100 μm i.d./375 μm o.d. capillary column with a packed bed length of 25 cm (CEC-Hypersil C18, 3 μm particle size) obtained from Agilent Technologies. For all separations, the total column length was the packed bed length plus 8.5 cm of poly-imide-coated fused-silica tubing. The column was conditioned with mobile phase by first pressurizing the inlet at 10 bar and ramping the voltage to 12 kV over a 40 min period. Electrokinetic injection of the sample was done using 10 kV for 10 seconds and the applied voltage during the run was 12kV at 25ºC. Both inlet and outlet were pressurised at 10 bar during the run for avoid bubble formation. The UV detection was performed at 242 nm. The mobile phase (5 mM phosphate buffer pH 2.5: MeCN (40:60)) was prepared daily and filtered through a 0.2 μm LC PVDF acrodisc filter and degassed for 25 min by ultrasonication before use. In this case, the LOD achieved for DA was 0.07 μg/ ml.

Results and Discussion

Different clean-up procedures were used for the analysis of a fish sample spiked with MC-LR standard and the results can be observed in Figure 1. IAC is an efficient tool for simple sample extraction allowing the specific extraction of MC's present in different matrices, including fish samples, contributing to an efficient trace enrichment and therefore contributing to an increase of the sensitivity, while SPME efficiency is clearly dependent on the matrix.

Both ASE and MAP offer a good potential for an efficient extraction of PSP toxins with a considerable time reduction and minimal sample degradation. An example of the application of both ASE and MAP for the pre-column HPLC/FLD analysis of PSP toxins, in samples spiked with GTX2,3 standard solution, is shown in Figure 2. The main advantages of these extraction procedures are associated with the efficient extraction they provide in short times and the lack of conversion between toxins during the extraction, especially for the MAP extraction procedure. Indeed, the extraction process using the ASE technique, resulted in the production of traces of STX as a result of the conversion of GTX2,3 (Figure 2).

Figures 3 and 4 show the high performance of CE for the analysis of Yessotoxin and ASP toxin respectively, in different complex matrices. The sensitivity of this technique has been clearly enhanced by using an on-line pre-concentration mode based on Large Volume Sample Stacking. From the result obtained it can be concluded that CE resulted in a simple, efficient, fast and low consumption

alternative for the analysis of Yessotoxin and ASP toxins, which allows the determination of this compound in such complex matrices at the low required levels.

Figure 5 shows the presence of Domoic Acid and some of its isomers as determined by CEC. From these results we can conclude that CEC is a promising alternative for an efficient determination of ASP toxins, nevertheless further improvements are still required, especially regarding to the lack of robustness associated with this approach, as well as for the limited stationary phases commercially available for CEC. The main advantage of using CEC is the higher selectivity and sensitivity compared to CE, as well as the higher efficiency compared to HPLC.

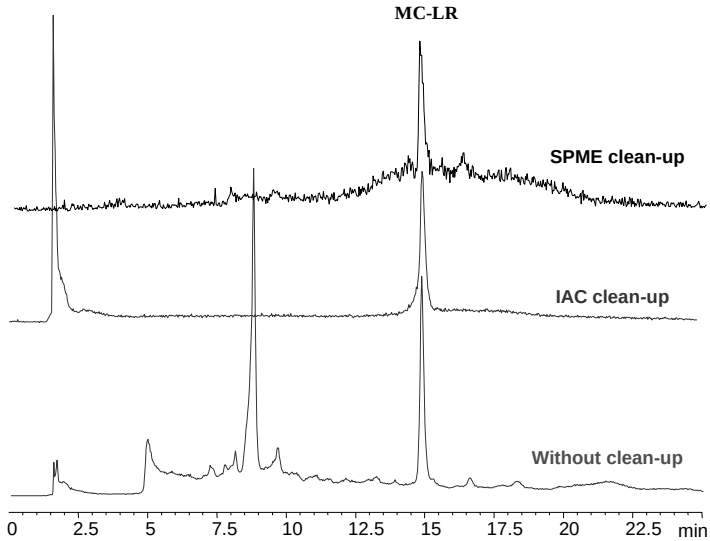

Figure 1. Comparison of the different clean-up procedures for the HPLC-MS analysis of a fish sample spiked with MC-LR standard. *IAC Conditions:* Conditioning: 3 ml water and 3 ml PBS (pH 7.4). Load: 380 µL crude extract in 1 ml PBS (pH 7.4). Wash: consecutive 3 ml of PBS (pH 7.4), 3 ml water and 3 mL methanol:water (25:75). Elute: 6 ml of methanol:water (80:20), 4 % (v/v) acetic acid. *SPME Conditions: 3*80µL crude extract in 3 ml of Milli-Q water (pH 2.0, 20 % NaCl (w/v)). Extraction conditions: Immersion mode, PDMS/DVB 60µm fiber, 1000 rpm magnetic stirring, 56ºC temperature, 60min extraction time. Desorption conditions: dynamic mode for 15 min.

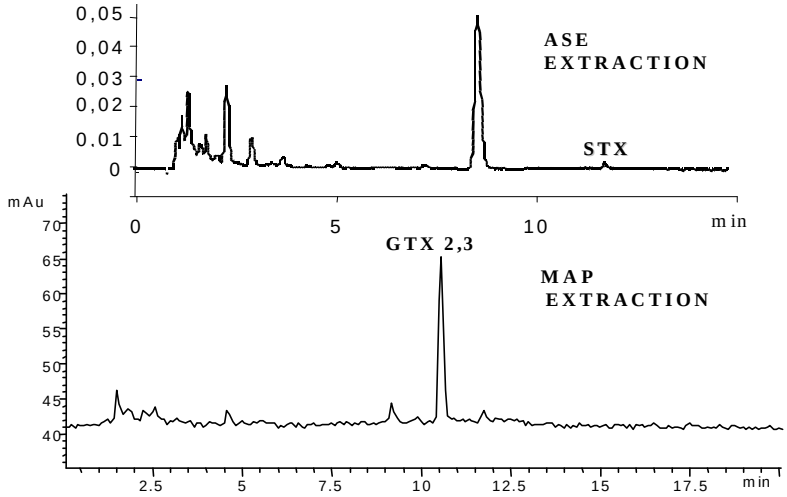

Figure 2. Comparison of MAP versus ASE extraction procedures for the PSP toxins. *MAP Conditions:* 3 ml 0.25 M acetic acid. Irradiation: 270 W for 15 s. *ASE Conditions:* 5g of mussel tissue combined with 6 g of Hydromatrix (Varian) sorbent. Program conditions: Pressure 1500 psi, 3 cycles for 5 min each, 100 % flush volume, 120s purge, 100ºC. Extraction solution: 5 % acetic acid (v/v).

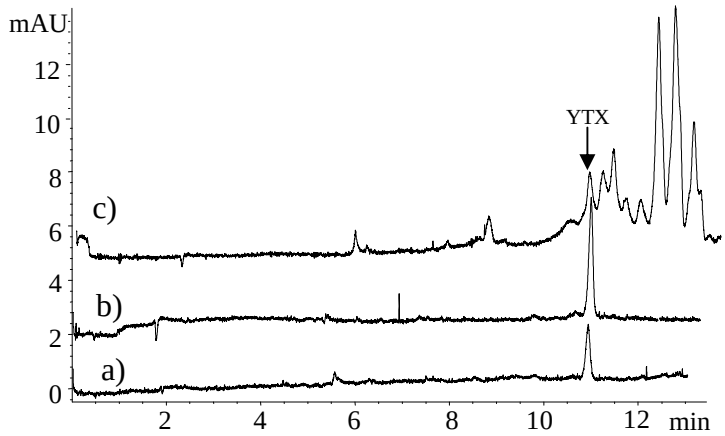

Figure 3. Electrophoregrams obtained for YTX analysis: a) standard of YTX 2 µg/ml, b) naturally contaminated mussel hepatopancreas from *P. canaliculus*, c) phytoplankton sample from culture *P. reticulatum*. Conditions: BGE: Sodium monohydrogen phosphate/methanol (60:40) 50 mM, pH 8.5. Sample solution: methanol/BGE (70:30), pH 3.0. Injection: 50 mbar, 20s. Sample Stacking: -25 kV during 40 s. Run: 30 kV, 25ºC. UV detection: 230 nm.

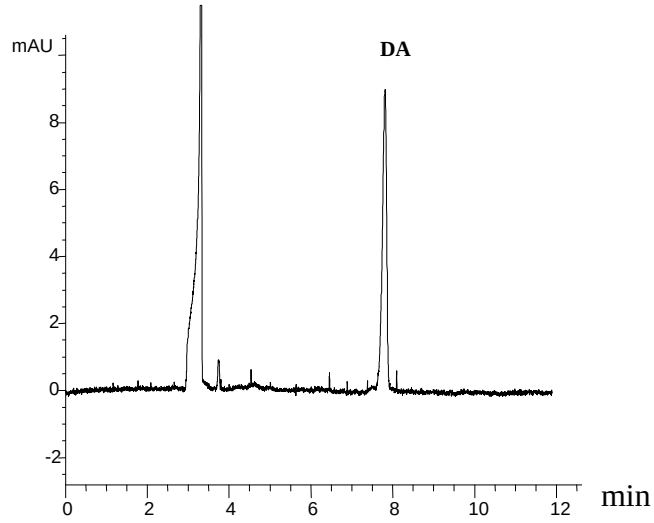

Figure 4. CE-UV/DAD analysis of a shellfish sample naturally contaminated with DA. Conditions: BGE: 25 mM phosphate buffer, pH 7.0. Sample solution: acetonitrile/water (10:90). Injection: 50 mbar, 75s. Sample Stacking: -5 kV during 130 s. Run: 30 kV, 25ºC. UV detection: 242 nm.

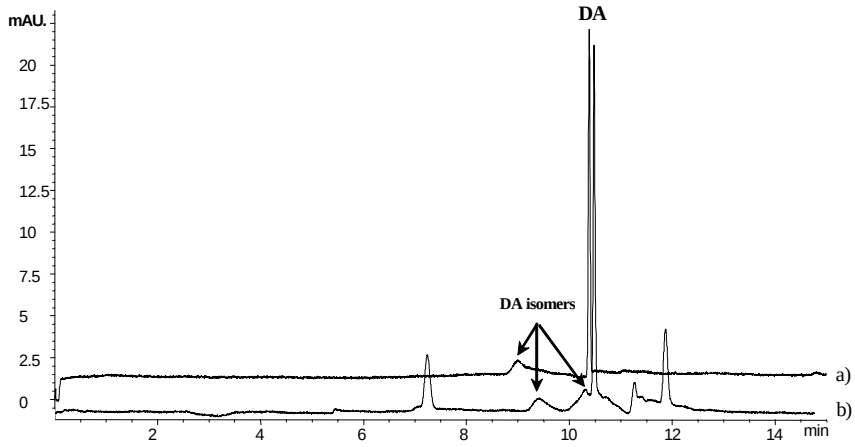

Figure 5. CEC-UV/DAD analysis of (a) Domoic acid standard solution and (b) Mussel Tissue Reference Material. Conditions: Mobile phase: 5 mM phosphate buffer/acetonitrile (40:60), pH 2.5. Injection: 10 kV, 10 s.
Run: 12 kV, 25ºC (Both inlet and outlet 10 bar pressurised). UV detection: 242 nm.

Acknowledgements
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IMPACT OF HPLC-UV METHODS (SOLID-PHASE EXTRACTION/UV-DETECTION AND PHOTODIODE-ARRAY DETECTION) FOR THE DETERMINATION OF DOMOIC ACID ON QUALITY OF RESULTS AND SAMPLE TURN-AROUND TIME

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Abstract:
The maximum concentration permissible for DA in marketed shellfish or shellfish products is set to 20 mg/kg of the whole flesh or any edible part separately. In Ireland the routine monitoring entails testing of ca. 700-800 tissues per annum, mostly arising from the control of scallops (*Pecten maximus*) landed as fisheries products, although other shellfish species are also tested at reduced frequency. The highest concentrations of DA in shellfish from Irish waters are typically found in scallops, in particular the hepatopancreas, leading to situations where scallops must be processed to achieve edible parts such as adductor muscles and gonads below regulatory limits. Due to the food safety aspects and the requirement for freshness, quality of results and a fast sample turnaround are important factors in the official control of DA. This paper describes an attempt to improve both factors through the validation of a method based on direct analysis of crude extracts without a solid-phase extraction (SPE) clean-up step prior to determination by HPLC-UV, as described in methods developed earlier. The performance characteristics and resulting sample turnaround are described for the SPE-HPLC-UV method and for the HPLC-photodiode-array (PDA) method. The PDA-method showed better recovery (95 %) and between-batch reproducibility (CV of 4.4 %) than the SPE-method, which had a recovery of 92 % and a between-batch reproducibility with a CV of 7.1 %. The sample turnaround time achieved with the SPE-method was 3.0 days on average over a 6-month period while the turnaround time for the PDA-method was 1.7 days on average over a 6-month period.

Introduction
The content of domoic acid (DA) in shellfish intended for human consumption is regulated in the EU by Council Directive 91/492/EEC, as amended by Council Directive 97/61/EC and Commission Decision 2002/226/EC. While EU legislation specifies the use of HPLC for the detection of domoic acid, the actual protocol to be used is not given. The only officially validated method for Domoic Acid (DA) by HPLC uses an HCl extract (in which DA is not stable) and has a determination limit which equates to the regulatory limit, Lawrence *et al.*, 1991. A recent method validation was conducted by German official laboratories on a variation of the method by Quilliam *et al.*, 1995. This method is currently being standardised by the European Committee for Standardisation (CEN). This paper compares a method similar to the one described by Quilliam *et al.* (method 1) with a slightly modified version of the method to be standardised (method 2) in terms of sample turnaround, recovery and precision in a routine monitoring setting.

Methods and Materials
Method 1 follows closely the procedure published by Quilliam *et al.*, 1995, with some modifications. Briefly, a 4g-aliquot of the homogenised tissue is extracted with 16 ml of 50 % aqueous methanol (1:1, v:v), using a high-speed blender for 3 min at 11,000 rpm. The sample is then centrifuged and a 2 ml aliquot of the supernatant subjected to a pre-conditioned 6 ml SAX cartridge (Schleicher and Schuell). The cartridge is washed with 10 % acetonitrile and the sample eluted with 5 ml of citric acid buffer. The eluent is analysed by HPLC using an isocratic mobile phase of 10 % acetonitrile in water with 0.1 % trifluoro acetic acid (TFA). Method 2

uses the same extraction procedure and centrifugation step, however, the SAX clean-up step is omitted. Instead the sample is filtered through a 0.2 µm syringe filter and analysed by HPLC directly. The HPLC mobile phase is reduced to 9 % acetonitrile to improve separation of DA from tryptophan to > 1.5 min, and a wash with 91 % aqueous acetonitrile is attached to the isocratic part in order to flush the HPLC column between chromatographic runs, both mobile phases contain 0.1 % TFA. Phase A is 100 % from 0-14 min, 0 % from 14.01-17 min, linearly increased from 0-100% during 17.01-19 min and held until 29 min at 100 %. Phase B is 0 % from 0-14 min, 100 % from 14.01-17 min, linearly decreased from 100-0 % during 17.01-19 min and held until 29 min at 0 %.

Both methods were accredited by the Irish National Accreditation Board (INAB), according to ISO 17025. Currently however, all routine analysis is carried out using the PDA-method. Samples were obtained at the Marine Institute as part of the routine national monitoring system. The sample turnaround is expressed in days, starting from the time when the sample arrives at Marine Institute premises and ending with the time when a report is published, using the automated database. Recovery of each of the 2 methods was estimated from the analysis of a certified reference material (CRM) obtained from the National Research Council Canada (MUS-1b). Precision of method 1 was estimated from the repeated analysis of the MUS-1b CRM, while precision of method 2 was estimated from the repeated analysis of a laboratory reference material (LRM), due to temporary shortage of the CRM. The homogeneity of the LRM was estimated as a relative standard deviation (RSD) of the analysis of 20 randomly chosen aliquots in a single batch, RSD = 4.3 %.

Results

The sample turnaround for method 1 is shown in Figure 1. During the early part of 2003, there were 3 periods in January, Feb-March and June when medium term instrument-downtime led to unacceptable delays in sample turnaround. These downtimes were not related to the method used but due to the age of the HPLC used in this analysis (> 6 years). 269 samples were analysed with system 1 from January to June. The average sample turnaround was 3.0 days with this method.

Figure 2 shows the sample turnaround for method 2, on which 427 samples were analysed from Jul-Dec. The average sample turnaround with this method was 1.7 days.

The PDA-method showed better recovery (95 %) and between-batch reproducibility (CV of 4.4 %) than the SPE-method, which had a recovery of 92 % and a between-batch reproducibility with a CV of 7.1 %.

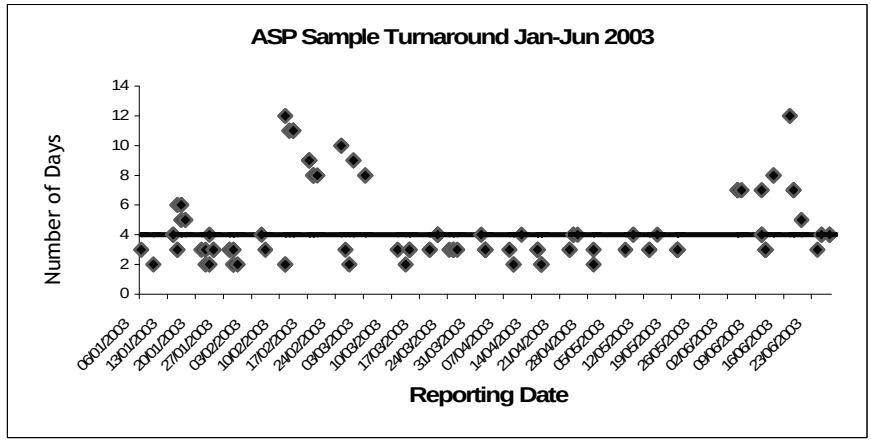

Figure 1. Batch-wise sample turnaround of method 1 (extraction with 1:1 MeOH: H_2O, SAX-SPE, 10 % ACN in mobile phase, UV detection at 242 nm), note that outlier periods in January (5-6 d), March (8-12 d) and June (5-12 d) were due to instrument failure, not method performance.

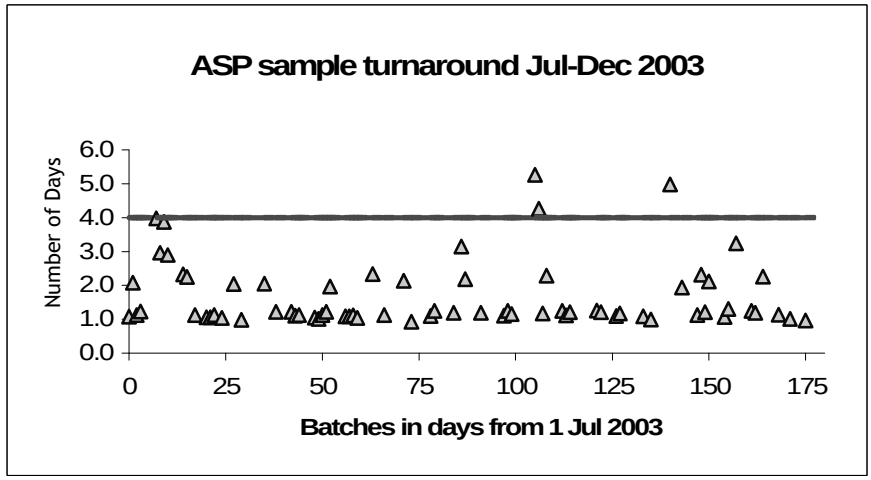

Figure 2. Batchwise sample turnaround of method 2 (extraction with 1:1 MeOH: H_2O, 9 % ACN in mobile phase, plus 91 % ACN wash, PDA detection)

Discussion

Not only were more samples turned around with method 2 in a period of the same duration (427 from Jul-Dec, compared to 269 from Jan-Jun) but these samples were also reported much faster. The typical sample turnaround time of 2-3 days with method 1 dropped to 1-2 days with method 2. The sample turnaround over the latter six months was less than 4 days for 96 % of all samples received in this period, which exceeds the target required by the MI service contract with the Food Safety Authority of Ireland. Indeed, 93 % of samples were turned around in less than 3 days while 69 % of samples were reported in less than 2 days. This is clearly explained by the fact that method 2 has a faster sample preparation. The improvement in recovery and precision can also be explained by the removal of the clean-up step

from the procedure. Both methods have been accredited and performed comparably when reference samples were analysed blindly. We consider both methods to have a similar potential of confirmation since one eliminates interferences through the SPE clean-up step while the other decreases the potential for interference through improved HPLC separation and additionally confirms the identity of the compound through the spectral comparison. In our view, the improvement in the sample turnaround time when using the PDA-method is significant and should be considered in further developments and comparisons with alternative assays. Additionally, the better performance characteristics in terms of bias and precision improve the potential of the PDA-method for research applications.

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A MONOCLONAL BIOSENSOR-BASED IMMUNOASSAY FOR DOMOIC ACID IN SHELLFISH AND PHYTOPLANKTON

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Abstract
Domoic acid (DA) is produced by the diatom *Pseudonitszchia sp*. and can cause amnesic shellfish poisoning (ASP) in humans if ingested via DA-contaminated shellfish. The level of DA in shellfish placed on the market is therefore highly regulated and a number of analytical methods for determination of DA have been described. To facilitate more convenient high throughput screening of both shellfish and phytoplankton samples, we have developed a rapid automated immunoassay, using the BIACORE™ surface plasmon resonance biosensor, to measure DA. The assay was designed as an inhibition assay with DA covalently immobilised to the carboxymethyl dextran matrix of a CM5 sensor chip. A fixed amount of the monoclonal anti-DA antibody is mixed with the sample/standard and the amount of free antibody is then determined using biomolecular interaction analysis (BIA) by injection of the mixture over the immobilised DA sensor surface. Preliminary validation of the assay for determination of DA in scallop extracts is presented.

Introduction
Domoic acid is a potent neuroexcitatory toxin produced by the diatom *Pseudonitszchia* sp. It was identified as the causative agent of amnesic shellfish poisoning (ASP) during the first outbreak in 1987 in Prince Edward Island, Canada (Addison and Stewart, 1989, Perl *et al*, 1990). Outbreaks were then reported worldwide. ASP is caused by the ingestion of contaminated shellfish in humans, and is characterised by gastro-intestinal symptoms such as vomiting, cramps, diarrhoea and neurological symptoms (headaches, seizure, and memory loss). Thus, the level of DA in the shellfish product placed on the market must be strictly controlled. For proper management of shellfish farms, an early warning of the presence of DA in phytoplankton samples would also be highly desirable. A high throughput and automatic screening procedure for DA in shellfish and phytoplankton samples would facilitate these procedures. There are currently a number of analytical techniques available for detection of DA, for instance HPLC and LC-MS. However, although relatively quick and reliable (Quilliam *et al*, 1989, Furey *et al*, 2001, Hess *et al*, 2004), the extensive cleanup steps necessary are time consuming.

Biomolecular interaction analysis (BIA) from Biacore Ab uses the optical phenomenon of surface plasmon resonance (SPR) to monitor biomolecular interactions in real time without labelling (Liedberg *et al*.,1983). Any change in mass at the surface of a gold sensor chip creates a change in the refractive index, which is detected in real-time by SPR (Jonsson *et al*, 1991) and gives rise to a sensorgram. BIACORE instrumentation has been used widely, with a variety of applications, such as analysis of chemical and biochemical molecular interactions, environmental monitoring and food analysis. It has proven to be a very useful analytical tool for determination of a range of analytes in biological fluids (e.g. Dillon *et al*, 2002, Gillis *et al*, 2002 and Fitzpatrick *et al*, 2004). We describe the development of a rapid automated immunoassay to measure DA, using the BIACORE 2000™ SPR biosensor, which will be applicable to either shellfish or phytoplankton extracts.

Materials and methods

Instrument

The BIACORE 2000™ biosensor (Biacore Ab, Uppsala, Sweden) was controlled by BIACORE control software version 3.1.1 running under Windows 95 and BIAevaluation software was used for data analysis.

Materials

CM5 research grade sensor chips, reaction vials, HBS buffer, amine-coupling kit and surfactant P-20 were obtained from Biacore Ab, Uppsala, Sweden. DA used to prepare the standards and the spiked samples was obtained from Calbiochem (cat. no. 324378). The National Research Council (NS, Canada) supplied DA calibration solution (cat. no. DACS-1c).

Antibody

The monoclonal antibody (F11) used was produced in-house following immunization of Balb/C mice with a DA-bovine serum albumin conjugate prepared according to the method of Kawatsu *et al.*, (1999). The myeloma fusion partner was the SP2/0 cell line. The antibody was purified from tissue culture supernatant by Protein A affinity chromatography. Protein concentration was determined using the BCA assay (Pierce Biotechnology). The antibody was diluted at 1/1000 in HBS-EP buffer before use.

Coating of the sensor chip

The immobilisation of DA to the carboxymethyl dextran matrix at the surface of a CM5 research grade sensor chip was performed on the bench following the method described by Gillis *et al.*, 2002.

Assay

The BIA assay was designed as an inhibition assay. Antibody was mixed with the sample/standard prior to injection over the sensor chip surface. The DA present in the sample/standard binds to the antibody and therefore inhibits it from binding to the DA immobilised at the surface of the chip. The greater is the amount of DA in sample/standard, the lower the binding of the antibody on the chip and therefore the lower the response of the biosensor. Antibody and sample/standard were mixed at a set ratio to give a total volume of 100 µl in reaction vials. The instrument was programmed to inject 25 µl of the mixture over the chip surface over a minute. After each injection, the surface of the chip was regenerated with 25 µl of 100 mM NaOH regeneration buffer at a flow rate of 25 µl/min (Traynor *et al.*, 1999). The standard curve (response at each concentration divided by the response at zero concentration (B/Bo) versus log standard concentrations) were fitted using a four-parameter equation with BIAevaluation software (Biacore AB).

Samples

Scallop tissues - gonads, hepatopancreas, adductor muscle and remainding tissues - were obtained from the Marine Institute, Galway, Ireland from their routine DA screening programme. Extracts of shellfish tissues were prepared following the protocol used for HPLC analysis (Quilliam *et al*, 1995). Briefly, 4g of tissue was homogenised for 3 min in 16 ml of extraction buffer (50 % methanol, 50 % water) with the Ultra-Turrax. The extracts were collected by centrifugation and filtered at 0.2 µm prior to being analysed with the BIACORE 2000™. Separate extracts were analysed by HPLC (Quilliam *et al.*, 1995)

Results

Standard curve

Standard curves for DA were established under two different sets of conditions. Using an antibody:sample/standard ratio of 9:1, the range of the assay was 0.1 to 50 μg/mL of DA, with an ED-50 of approximately 1.3 μg/mL and a working lower detection limit of 0.1 μg/mL (Figure 1). The precision of the response for each standard was <5 % coefficient of variation (CV). These conditions were selected for analysis of DA in scallop tissue extracts, as the critical concentration of 4.6 mg/kg (equivalent to 1.15 μg/mL extract) was close to the centre of the standard curve.

As a more sensitive assay would be required for analysis of DA in phytoplankton samples, the antibody:sample/standard ratio was adjusted to 3:1. This gave an assay with linear range of 0.025 to 10 μg/mL and an ED-50 of 0.5 μg/mL (Figure 1). Again the precision of the responses for each standard was very good (<5 %).

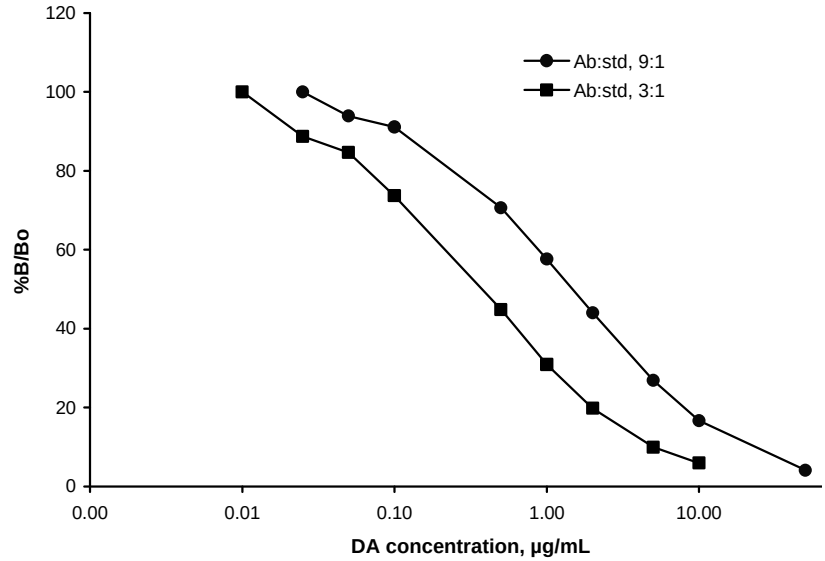

Figure 1. Composite standard curve for DA assay produced on the Biacore 2000™. The standard curves represent the mean of ten sets of standards prepared in HBS-EP buffer, each analysed in duplicate, using either a 9:1 antibody:standard ratio or a 3:1 antibody:standard ratio.

Shellfish analysis

The BIA assay was then applied to the analysis of scallop extracts using the 9:1 antibody:sample/standard ratio protocol. Firstly, the assay was shown to be compatible with 50 % methanol, the normal extraction buffer used for HPLC analysis of DA in shellfish. Standard curves given by standards prepared in 50 % methanol and HBS buffer were almost co-incident (data not shown). Six negative scallop extracts were run in the assay and the binding observed ranged from 99 to 102 % of that given by the zero standard. Three known positive samples were analysed neat and following dilution at 1:2, 1:4, 1:6, 1:8 and 1:10 with HBS-EP buffer. Figure 2 shows

that the results obtained were independent of the volume assayed. The recovery of standard added to negative extracts was then examined. Sample extracts were spiked with a low, medium and high concentration of DA and analysed in the assay. The mean recoveries were in the range of 61 % – 71 % (Table 1), which was lower than desirable. However, overall these results suggested that there were no significant matrix effects from the scallop extracts.

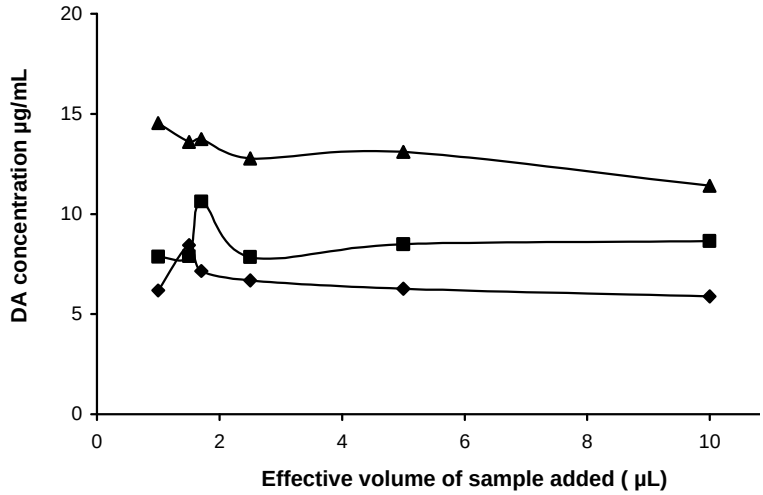

Figure 2. Relationship between the effective volume of three scallop extracts assayed and the concentration of DA measured in the BIA assay.

Table 1. Estimation of the recovery of added standard to negative extracts.

Conc. of DA added (µg/ml)	Mean % recovery (n = 3)
0.1	61.19
1	64.29
10	71.31

A panel of sixteen random scallop extracts were then assayed in the BIA assay. The concentration of DA in these samples, as determined by HPLC, ranged from 0 to 140 mg/kg. The results given by the BIA assay ranged from 0 to 50 mg/kg. There was, therefore, a considerable underestimation by the BIA assay (Figure 3), significantly more than was expected from the recovery experiments (Table 1). However, despite the low results given by the BIA assay, there was a very good correlation between the two sets of results (R^2 = 0.98; Fig. 3). The reason for this underestimation of DA in the assay is currently under investigation.

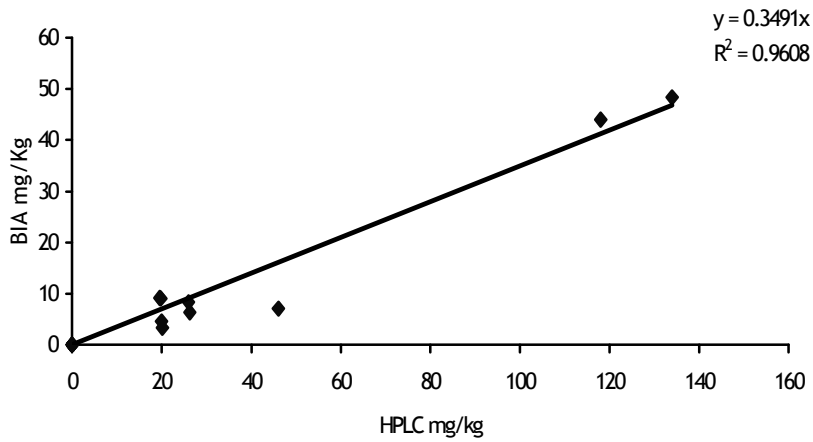

Figure 3. Correlation between results given by HPLC analysis and the BIA assay for scallop tissue extracts (n = 16).

Discussion

The commercially available BIACORE ™ surface plasmon resonance biosensor has considerable potential as a high throughput platform for immunoassays. It is a fully automated system with an autosampler for precision liquid handling and integrated microfluidics and detection, which permits precise, rapid and simple-to-operate analysis of samples. Moreover, our current model, the BIACORE 2000™, has the potential for simultaneous analysis of up to 4 different analytes during the one run, and newer models have up to 12 flow-cells. It is conceivable therefore that an initial screen for a panel of algal toxins could be carried out on this instrument. As a first step in the application of this technique for algal toxin testing, this study has described the development of a BIA assay for DA using a monoclonal antibody produced previously in our laboratory. By simple adjustment of the test parameters and using the same sensor chip, it was possible to set up standard curves over two different ranges. The two critical DA concentrations for shellfish extracts, 4.6 mg/kg (equivalent to 1.15 µg/mL) and 20 mg/kg (equivalent to 5 µg/mL), were both within the linear range of one of the standard curves (Crooks *et al.*, 1998; Gillis *et al.*, 2002). BIA assays are noted for being highly reproducible and this was confirmed here under both assay conditions, with coefficients of variation being less than 5 % in all cases. This performance level is achievable because of the precision liquid handling capability of the instrument and also the stability of the coated sensor chip to repeated regeneration cycles. The same DA-coated chip was used throughout this study and similarly coated chips have been reported to withstand over 3,000 regeneration cycles without showing any deterioration (Gillis *et al.*, 2002). Preliminary evaluation of the suitability of one of the assays for determination of DA in scallop extracts gave promising results. The extraction solvent did not affect the standard curve. Negative extracts did not interfere with binding of the antibody to the immobilised DA and the response obtained with positive samples was independent of the volume assayed. However, the recovery of DA added to negative extracts was not as good as expected and absolute DA concentrations were underestimated in a small panel of scallop extracts. Nevertheless, because of the good correlation with HPLC results, we are optimistic that further optimisation of the assay will yield a very useful tool for high-throughput toxin screening of shellfish and phytoplankton samples.

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PREPARATION OF STABLE, HOMOGENOUS SHELLFISH TISSUE MATERIALS, NATURALLY CONTAMINATED WITH DINOPHYSISTOXINS AND AZASPIRACIDS

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Abstract

Shellfish tissue reference materials (RMs) were prepared contaminated with diarrhetic shellfish poisoning (DSP) and azaspiracid poisoning (AZP) toxins. The RMs were prepared using mussel tissues (*Mytilus edulis*) which had been obtained by the Marine Institute during several toxic episodes (2000-2003), on the west and southwest coasts of Ireland. The materials were naturally contaminated with Azaspiracids (AZA)–1, -2 and -3, as well as Okadaic acid (OA) and DTX-2. Steps in the method included sterilization of the tissues, preparation of modules, and combination of specific quantities of modules to achieve adequate toxin concentrations in the finished materials. Water content of the final homogenates were adjusted to reflect natural matrices. Homogenisation was carried out using blenders and fluidisers. Problems observed initially in obtaining sufficiently homogeneous materials were overcome using heat treatment, addition of small percentages of ethanol and a peristaltic pump for dispensing the materials. Evaluation of toxin concentrations in aliquoted materials was carried out using LC-MS. The coefficients of variation (CVs) obtained for AZA-1, -2, -3 as well as OA and DTX-2 were within 5 % for several materials that have been prepared on a pilot scale (2-4g aliquots, 1-3kg total weights). The stability of these materials is currently being investigated and we intend to make them available for use in national and international ring trials.

Introduction

Azaspiracid poisoning is a recently discovered human toxic syndrome that is caused by the consumption of shellfish contaminated with AZAs (Hess *et al*. 2001, McMahon and Silke 1996 and Satake *et al*., 1998). Azaspiracids were first detected in mussels in 1995 (McMahon and Silke 1996) and have since been detected in numerous other bivalve species including oysters (*Crassostrea gigas, Ostrea edulis*), scallops (*Pecten maximus*) and razor fish (*Ensis siliqua*) (Ito *et al*., 2002). In 2003, the Marine Institute and University College Dublin began a collaborative project called Azaspiracids Isolation and Toxicology (ASTOX). ASTOX is funded by the National Development Plan (NDP) under the marine RTDI programme. One of the aims of this project is the preparation of stable, homogeneous shellfish tissue materials naturally contaminated with azaspiracids (AZA 1-3) as well as with dinophysistoxins (OA and DTX-2). Monitoring of these toxin groups is necessary to prevent or minimise the risk of intoxications. In monitoring RMs have an important role as they enable development, validation and quality control of the entire analytical method used. Reference maretials are also necessary for the assessment of the entire analytical process including sample extraction, clean up and concentration as well as the final separation and detection. They are equally helpful in the assessment of the proficiency of official laboratories, one of the main tasks of the Marine Institute as a National Reference Laboratory for marine biotoxins in Ireland. The materials are prepared using shellfish tissue retrieved as part of the ASTOX project during previous toxic events.

Reference Material Design Criteria
Various modules of mussel tissues need to be combined to produce reference materials with a suitable toxin profile. The materials have to demonstrate good homogeneity and stability with regard to the toxins.
They should contain the following toxins:
1. AZA-1, AZA-2, AZA-3.
2. DTX-2, Okadaic Acid.
These were selected as they commonly co-occur in mussels harvested from toxic areas.

Preparation of materials
To date a total of nine materials have been prepared. These consist of seven pilot scale materials (800g-1kg) and two medium scale materials (1kg-10kg). Throughout the preparation of these various materials the method has been progressively changed and new steps were included to improve the homogeneity of the toxins within the tissue matrix. The method at this point consists of the following phases:

- Mussel tissues acquired during previous toxic episodes in Ireland and from Norway were selected for use in a preparation.
- The tissues were stabilised by heat treatment/sterilisation using an autoclave.
- Once the tissues had been stabilised they were homogenised using a series of blending and fluidising steps. Various apparatus including blenders, liquidisers and sample dividers were used in this phase. Ethanol was added during the homogenisation step in order to disperse the lipophilic toxins within a predominantly hydrophilic tissue matrix.
- The last step involved division of the homogenised materials into aliquots suitable for characterisation of the material and for use in stability studies on the material. The aliquoting step was performed using a peristaltic pump and the aliquots are typically dispensed in 2g or 4g portions.

The following three materials have been selected to illustrate method development to date:

a) This pilot material was the second tissue prepared. Steps omitted in this preparation were; use of the autoclave and addition of ethanol. Aliquoting was performed manually.
b) This pilot material was the eighth tissue prepared. This material was prepared using the optimised method outlined above.
c) This medium scale material is the latest material prepared. The method above was used, however this preparation is on a medium scale.

Extraction and analysis of materials
To select a representative number of samples for homogeneity testing the formula $3(n)^{1/3}$ was used, where n is the total number of portions aliquoted from a preparation (Bermudo *et al.*, 2001). The materials were characterised using the following extraction and instrumental analysis procedures.
4g aliquots of the tissue materials extracted using 15ml of 80:20/MeOH:H_2O *or:*
2g aliquots extracted using 18ml of 90:10/MeOH:H_2O. Different extraction procedures were used as a result of method development which was on-going at the time of work. Analysis of all the materials was carried out using LCMS instruments from both the Marine Institute laboratories in Galway and Dublin.

In the Galway laboratory an isocratic LC method was used (Figure 3), whereas, in Dublin a gradient elution system was employed (Figure 4). Chromatograms of the AZP and DSP toxins using these two systems are shown below.

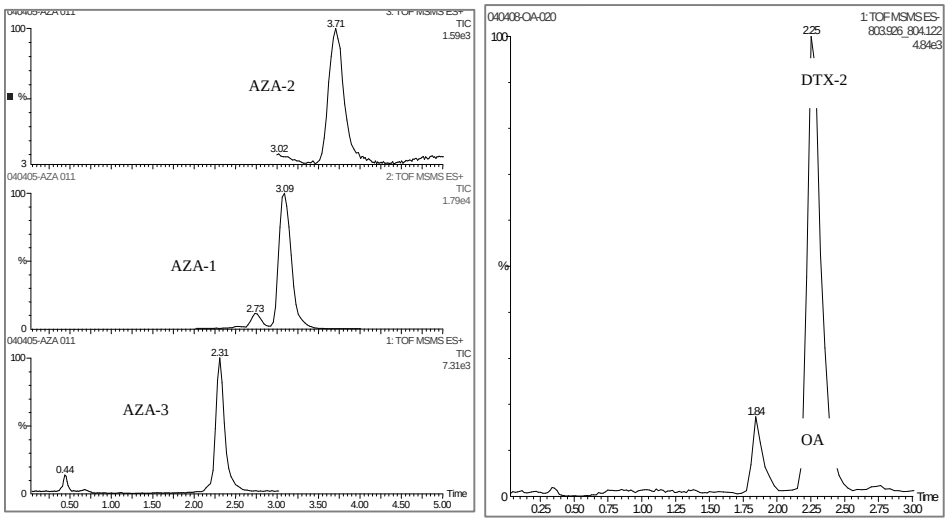

Figure 3. LC-MS-MS (ESI-QTOF): Isocratic system
Chromatographic conditions: AZA: 40 %A (H20 containing 5 % ammonium formate) – 60 %B (Acetonitrile containing 5 % ammonium formate). Flow rate is 0.25ml/min. DSP: 45 %A– 55 %B. Flow rate is 0.2ml/min.

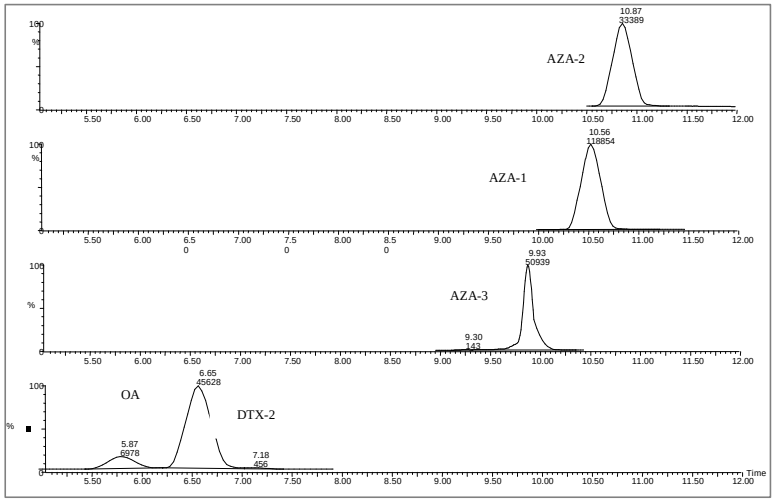

Figure 4. LC-MS-MS (Triple-Quadrupole): Gradient system.
Chromatographic conditions: 70 % A (H20 containing 5 % ammonium formate) – 30 % B (acetonitrile containing 5 % ammonium formate) → 10 % A (8mins), hold (2.5mins) → 70 % A (0.5mins), hold (3mins). Flow rate is 0.2ml/min.

The extraction and instrumental procedures outlined above also apply to work looking at the stability of the materials. The triple quadrupole instrument with the gradient system is used in the routine monitoring exercises of the Marine Institute.

Results

The results of the three different materials (A, B and C) are presented in tables 1-3.

Table 1. Concentration. (µg/g), toxic equivalent and % RSD of (A), n=170

	Conc (µg/g)	Toxic Equivalent (µg/g)	RSD (n=17)
OA	0.19	0.19	17.5
DTX-2	1.15	1.15	10.2
Sum		1.34	9.7
AZA-1	0.17	0.17	13.4
AZA-2	0.03	0.05	18.5
AZA-3	0.04	0.06	10.9
Sum		0.28	13.3

Table 2. Concentration (µg/g), toxic equivalent and% RSD of (B), n=198

	Conc (µg/g)	Toxic Equivalent (µg/g)	RSD (n=18)
OA	0.16	0.16	6.6
DTX-2	0.76	0.76	5.1
Sum		0.92	5.1
AZA-1	0.19	0.19	5.2
AZA-2	0.05	0.09	6.9
AZA-3	0.02	0.03	6.1
Sum		0.31	5.0

Table 3. Conccentration (µg/g), toxic equivalent and % RSD of (C), n=1105

	Conc (µg/g)	Toxic Equivalent (µg/g)	RSD (n=31)
OA	0.22	0.22	10.0
DTX-2	1.35	1.35	4.6
Sum		0.92	5.0
AZA-1	0.32	0.32	4.5
AZA-2	0.10	0.19	5.2
AZA-3	0.12	0.17	4.4
Sum		0.68	4.2

For analytes to be satisfactorily homogeneous within a material, a guidance value of 5 % CV is used. This is similar to the analytical variability of the method at the level of interest. Table 1 shows how the early method produced a material which displayed poor homogeneity with regard to AZP/DSP toxins. This poor homogeneity is probably due to AZP and DSP toxins being lipophilic toxins within a matrix with a very high water content (ca. 81 %). In addition, manual dispensing of the material was quite slow using a pipette. Table 2 shows the results of the homogeneity study on a material prepared using the optimised method. Solubility of the toxins in ethanol, and use of a peristaltic pump to hasten the dispensing step, most likely attribute to the good homogeneity observed. Table 3 shows that scaling up from a pilot scale material to a medium scale material does not have a detrimental effect on homogeneity. Materials B and C were also heat-treated. While this had no influence on the moisture contents, it did make the tissues more fluid and may have

contributed slightly to the improved homogeneities. The material summarised in table 3 has since been used as a second LRM in the routine monitoring of AZP/DSP toxins in commercial samples of shellfish from Irish coastal areas. Figure 5 below is a control chart of the results obtained for the material ran for 26 batches. This data, which spans 7 weeks, indicates that the material is stable when stored in the freezer. Work examining the stability of the material at several temperatures is underway.

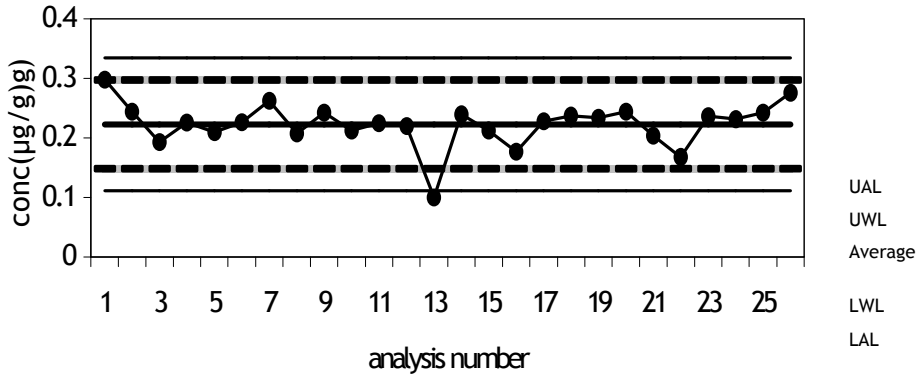

Figure 5. AZA-1 Control Chart from (C) UAL: upper action level (avg + 3 stdev). UWL: upper warning level (avg + 2 stdev). LAL: lower action level (avg - 3 stdev). LWL: lower warning level (avg – 2 stdev).

Conclusion

Work to date on the preparation of reference materials has resulted in an optimised procedure for the production of materials which have satisfactory homogeneity with regard to AZP and DSP toxins. Work on the stability of these materials is on going. The method of preparation still requires fine-tuning and an aspect, which requires particular attention, is the selection of suitable containers for sub-division of the materials. Scaling the preparations up to bulk scale (>10kg) is also a major aim of the work, as only on this scale could a material be considered for certification as a reference material (CRM).

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VALIDATION OF AN AUTOMATED PROCEDURE OF SOLID PHASE EXTRACTION OF MUSSELS DIGESTIVES GLANDS FOR IDENTIFICATION AND QUANTIFICATION OF DINOPHYSISTOXINS IN LC/ESI/MS^2 BY QUADRUPOLE ION TRAP

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Abstract
We present a new extraction method for the dinophysistoxins (DTXs), confirmed by quantification using high-performance liquid chromatography coupled to mass spectrometry with an ion trap and electrospray interface (HPLC/ESI/MS^2-LCQ™ ThermoFinnigan Corporation). The method consists on the adaptation of DTXs basic extraction procedure (liquid/ liquid) to a solid phase extraction (SPE) via a robotic station (GILSON). This method was validated according to two normative referentials. Comparison of the automated method with the classical liquid/liquid extraction shows clear advantages for the former. In an analytical method, extraction is generally considered to be the most labour-intensive and error-prone step. This new procedure allowed us to increase throughput, to improve the reproducibility and to reduce the error risks due to the individual manual treatments.

Introduction
Powerful tools, which are fast and very sensitive, are available for phycotoxins detection. Nevertheless, their extraction from biological matrix (digestive glands) remains a challenge: the procedure is labour-intensive and time consuming. Owing to the increasing number of analyses required for consumer protection and the performance requirements of quality assurance, our laboratory was provided with an ASPEC XL automated system controlled by the 735 Sampler Software (Gilson SA, Roissy Ch De Gaulle, France). This equipment was used to develop a new method of Solid Phase Extraction (SPE) for diarrhetic shellfish toxins, more suitable to automation than traditional liquid/liquid extraction method (Suzuki and Yasumoto, 2000, Vale and Sampayo, 2002).

Material and methods
SPE automation.
An experimental design allowed determination of the method ruggedness. Selected factors were evaluated at different levels (solvents, type of solid phase for SPE column) along different steps of experiment (sample dissolution, conditioning, washing and elution of the analyte on SPE cartridges) according to the following performance characteristics: recovery, reproducibility and the signal improvement. Investigation of different fractions collected at each stage enabled estimation of the toxin amount lost and evaluation of recovery. Interpretation of results allowed optimisation of significant parameters and development of the following operating procedure: Weigh approximately 30 g of digestive glands and grind and homogenise with Ultra Turrax at 8000 rpm. Weigh accurately 4.0 g digestive glands in a (Greiner)

tube. Homogenise and extract successively with 10, 8 and 6 ml Acetone 100 %. Centrifuge 10 min. at 3000 rpm and remove the supernatant phase. Combine the acetone extracts and adjust to 25 ml with acetone solvent. Evaporate the extract and dissolve the residue in Hexane/Chloroform (50:50). Start SPE (SI 500mg 3mL) with the robotic station using task parameters:

1) SPE Dry (N2) and SI particles compression, (Drying duration = 4 min)
2) SPE Condition with 20 ml Chloroform 100 % and then with 10 ml Hexane/Chloroform (50/50) both at 8 ml/min
3) Load 1.50 ml of extract at 2.50 ml/min
4) Wash with 3 ml Hexane/Chloroform (50/50) at 3 ml/min
5) Elute toxins with 3 ml Chloroform/Methanol (65/35) at 3 ml/min. Then evaporate collected extracts and dissolve the residue in 1 ml of Methanol and inject to LC/MS.

HPLC/ESI/MS^2 DTXs detection

Development and validation of the automated SPE method, detection and quantification of toxins were carried out on LC/MS^n Ion Trap. The HPLC Instrumental conditions were: Column and Pre-column: Kromasil C18 (250x2.0mm I.D.) 5 µm; Temperature: 40°C; Isocratic Mobile Phase: Acetonitrile/water + 0.1 %TFA (75: 25); Flow-rate: 0.2 mL/min. Sensitivity of HPLC/ESI/MS^2 depends on parameters optimisation: Electro spray source, ion optics and ion trap. Most suitable conditions leading to a good specific signal were obtained using a "T system" flushing the sample transfer line, sample tube and ESI probe. Optimal values related to Chromatography and to molecular structure of different standards okadaic acid (OA) and DTX1, were obtained. This LC/MS method uses experimental and instrumental conditions developed in our Laboratory (Mondeguer, 2002), (Table 1).

Table 1. Experimental and instrumental conditions of LC-MS

ESI Source	*Ion trap spectrometer*
Ion Polarity Mode: positive Sheath gas flow rate (N_2) : 75 units	Alternation: Full-MS/Full-MS^2 Full-MS Scan range (m/z): 750 - 825
Auxiliary gas (N_2) : 26 units Spray Voltage: 4.50 kV	Full-MS^2 AO Scan ranges (m/z): 220 - 806 Full-MS^2 DTX1 Scan range (m/z): 225 - 802
Spray Current: 6.10 µA Capillary Temperature: 183°C -	Total Microscans: 2 Maximum Injection time: 200 x ms
Capillary Voltage: 32	Collision energy: AO = 22 % - q_z: 0,250 Collision energy DTX1 = 20 % - q_z : 0.250
Ions optic transmission	Activation Time: 30 msec x ms
Lens voltage: -16 V	Isolation Width: 10 = maximum (m/z)
Octapole 1 offset: -4 V	Full MS Target: 5.10^7
Octapole 2 offset: -7,50V	Full MS Target: 2.10^7
Octapole RF Amp: 710 (Vpeak to peak)	Electron Multiplier Voltage (set point): -1100

Data acquisition was performed in positive mode, alternating Full/MS and Full/MS^2. Alternatively combined scan mode and scan type combinations enable detection of molecular ions according to mass charge ratio m/z (Full/MS) and allowed precursor

toxin ion of interest to be trapped and then fragmented into products ions all of which were scanned out and quantified (Full/MS^2). This provides two mass spectra that allow specific identity of the toxin according to molecular ion MH^+ and products ions. Products ions fragmented from okadaic acid were caused by loss of water molecules: $[MH\text{-}H_2O]^+$, $[MH\text{-}2H_2O]^+$ and $[MH\text{-}3H_2O]^+$ and the mass to charge ratio m/z are respectively: 805 – 787 – 769 – 751. In only one injection, this combination allows universal detection by Full/MS and high specific and selective detection by Full/MS^2. Quantification was carried out on products ions. This technique provided an increase of sensitivity. Thus for example for 10.7 µg OA g^{-1} digestive gland (dg), the ratio signal to noise changes from 46 to 177.

Results

The accuracy and reliability of this automated procedure were statistically demonstrated. This automated technique of extraction for toxins and their detection by mass spectrometry was validated according to standards of SFSTP, guidelines of analytical validation 92 and AFNOR, 1998. Linearity assessment was first performed using a wide range of external standard concentrations of OA/DTX1. For each set of experiments, eight calibration solutions evenly spaced with six replicate measurements (n = 6, p = 8) were selected within the concentration range 0.05 ng to 50 ng to be validated. Linearity in the range was demonstrated using simple linear regression. No weighting coefficient was applied to regression. Nevertheless, the experiment results have shown non-homogeneity of variances among different data groups. Therefore, we decided to reduce the concentration range of the calibration curve. The new range was set from 0.05 ng to 11.5 ng (with n = 3; p = 9) and enabled achieving better results (Figure 1) and lower detection and quantitation limits (Table 2).

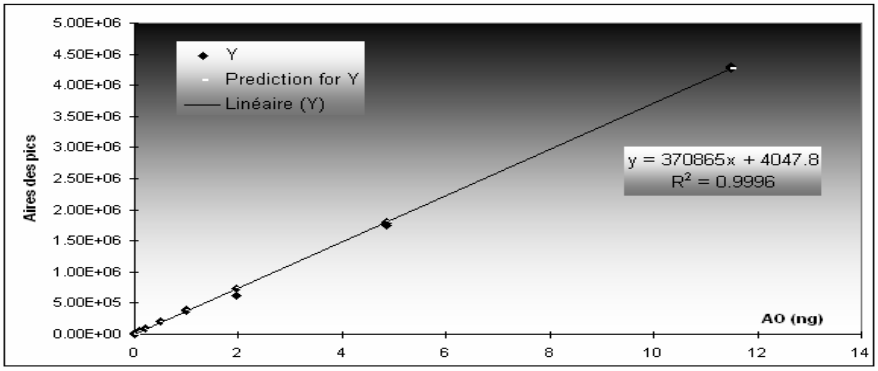


Figure 1. Calibration curve

Linearity and matrix effect assessment was second performed using three independent MUS2 and 3 ampoules containing 0.5 mL OA (25.3µg/mL).

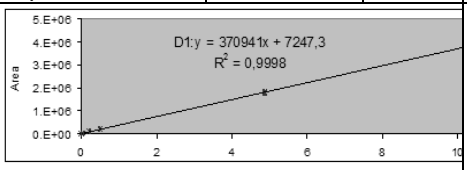
We were able to record significant variances intra-group (table 3) between three extracts of certified reference material (MUS2) however identically respectively certified to 11 µg and 1 µg of AO and DTX1. This heterogeneity was however, not limiting in the quantification of matrix toxins compared to standards quantification since we showed that the method was exact with a confidence interval of average going from 97 to 105 %, for a degree of confidence of 99 %. The validation of our analytical method enabled us to define its fidelity.

Table 2. Statistical data validation**.**

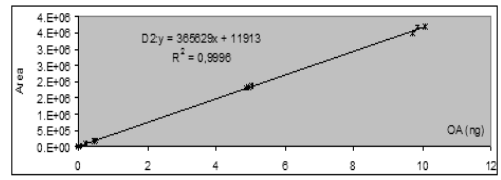
Statistical parameters	Unit	Range	Observed value	Critical value a = 5 %	Conclusion
Linear regression OA standard					
Testing homogeneity of variances	Cochran Test	OA	0.4633	0.4775	Not significant
Comparison of intercept with zero		OA	0.62	2.06	Not significant
Slope of regression line	ng^{-1}	OA	370864.80		
Intercept of regression line		OA	4047.80		
Correlation coefficient		OA	0.9996		
F for regression testing			78543.27	4.414	Acceptable
F error for estimated regression line			2.22	2.577	Acceptable
Calibration OA standard					
Number of levels			9		
Total number of measured samples			27		
Sensitivity	Response	370864.80	0.989	ng	x=y-b/a
Blank	Response	4047.80	-2.59E-15	ng	
Standard deviation for sensitivity	Response	1532.19	-0.007	ng	
Standard deviation for Blank	Response	6482.53	0.007	ng	
Detection and quantification limit: Standard OA					
Experimental standard deviation	Response	28561.57269	0.066	ng	
Detection limit	ng		0.063		
Quantification limit	ng		0.186		

Table 3: Statistical data linearity-matrix effect for 3 independent MUS2

Statistical parameters	Range	Test	Risk	Observed Value	Critical value	Conclusion
Slope of regression line	D_1 - Standard			370940.50		
	D_2 - MUS-2			365629.279		
Intercept of regression lines	D_1 - Standard			7247.253		
	D_2 - MUS-2			11912.852		
Correlation coefficient	D_1 - Standard			0.9998		
	D_2 - MUS-2			0.9996		
Comparison of intercept with zero	D_1 - Standard	Student	1 %	1.23	2,91	NS
	D_2 - MUS-2			1.40		
Comparison of intercept with D_1, D_2		Student	1 %	0.45	2,75	NS
Comparison slopes		Student	1 %	2.40	2,75	NS



Linearity of the method on a range of calibration of AO standard (D1) calculated starting from dissociation of ion MH^+ (m/z 787) in SM/SM for n=3 ranges and six levels (p)



Linearity of the method on a range of calibration of MUS2 (D2) calculated starting from dissociation of ion MH^+ (m/z 787) in SM/SM for n=3 ranges and six levels (p)

Conclusion.

The robotic station ASPEC-Xli can be used for many applications and presents many advantages. High reproducibility of solid phase extracts is obtained due to good control of the flow through different cartridges (SI, C18…). The ASPEC-Xli uses standard SPE columns of 1, 3 and 6 ml or plates with 96 wells proposed by many manufacturers. Besides this benefit, ASPEC-Xli is nowadays the only one able to control multiple fractions collection. Moreover, this technique uses small volumes of solvents that reduce significantly the cost of extraction. With the robotic station, the extract can be injected either directly on line on to the "Analyser" or by the vials in the auto sampler of HPLC system. Automation of extraction and clean-up procedures of organic compounds with the robotic station ASPEC-Xli include column conditioning, loading sample and elution with a risk of cross contamination level between samples less than 0.005 %. Programming ASPEC-Xli by microcomputer allows development, implementation and easier modifications of different methods. For adaptation of liquid/liquid extraction to Solid Phase Extraction and optimisation of different parameters of automation, we investigated the accuracy using a MUS2 reference material (Quilliam, 1995) certified with OA (okadaic acid) at level 11.03 µg/g digestive glands, by means of quantitative LC/ESI/MS^2. Recovery obtained from silica cartridge was 110 %. Statistical data analysis and validation of the automated

SPE of OA/DTX1 in shellfish were carried out according to SFTP and AFNOR (AFNOR, 1998, Algranti, 1992) standards, using some performance criteria such as linearity, accuracy and precision. For a risk error of 1 %, validation showed that method limits of detection and quantification were respectively 0.063 and 0.186 ng/g for AO and DTX1. This automated SPE of OA/DTX1 by LC-ESI/MS^2 technique enabled tackling the problem of a great number of samples with good reliability. Manual procedures are labour intensive and error prone, a serious drawback unknown to automated protocols. Thus, within our laboratory, with our equipment (ASPEC-Xli automat modified and arranged within this study), the method that we developed gave respectively coefficients of repeatability and reproducibility of 1,3 % and 2,1 %.

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DETERMINATION OF ASP AND DSP TOXINS IN MUSSELS BY LC-ESI-MS

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Abstract
A multitoxin method was developed using ion trap mass spectrometry LC-ESI-MS-MS. This method allowed rapid and specific determination of different ASP and DSP toxins including domoic acid (DA), okadaic acid (OA), dinophysistoxins (DTX-1, DTX-2), azaspiracids (AZA-1, AZA-2, AZA-3) and pectenotoxins (PTX-2). Determination was by reversed phase chromatography using acetonitrile solvent and water containing 0.05 % trifluoroacetic acid additive and an Electrospray source (ESI) in positive mode. For specificity improvement, the relative collision energies were optimised for each toxin and MS^2 analysis was implemented to achieve extensive fragmentation of the selected ions. A specific example of validation on domoic acid according to ISO 17025 is shown. The multitoxin method was applied to a naturally contaminated mussel sample. The experimental results obtained are presented and discussed.

Introduction
Filter-feeding bivalve molluscus such as mussels consume the plankton thereby accumulating toxins in their tissues. These phycotoxins are not toxic to shellfish, unfortunately they are harmful to human health upon consumption of contaminated seafood. In most European countries, the Mouse Bioassay (MBA) has been used in monitoring seafood for DSP toxins. Although the MBA is recommended by the European Commission as the official method, it raises numerous problems. One of them is that the inherent variability can exceed ± 20 %. Another one is the slaughter of too many mice and the length of observation time required (24 hours). Many countries have banned animal bioassays due to protest by ethical and animal rights groups. There is a considerable pressure to develop alternative methods to MBA. A rapid, sensitive and specific LC-MS quantitative method is proposed as an alternative method to the MBA.

Materials and Methods
Chemicals
Standard solutions of domoic acid, okadaic acid and pectenotoxin-2 were purchased from the Institute of Marine Bioscience, National Research Council of Canada (NRC, Halifax). A semi-purified extract of AZA-1, AZA-2 and AZA-3 was provided by Dr. Philipp Hess (Marine Institute, Galway, Ireland). DTX-2 was provided by the EU-CRL (Vigo, Spain). Acetonitrile, methanol and n-hexane (HPLC grade), acetone, chloroform and formic acid 98-100 % (analytical grade) were supplied from Merck (Darmstadt, Germany). Diethylether (grp) and acetic acid glacial (pa) were purchased from BDH (Poole, England). Trifluoroacetic acid (TFA) 99 % (spectrophotometric grade) was obtained from Aldrich (Milwaukee, WI, USA). Water was purified with Milli-Q filtration system (Millipore, Molsheim, France). Strong Anion Exchange cartridges (Supelclean LC-SAX SPE tubes 3ml) and silica cartridges (Supelclean LC-Si SPE tubes 3ml) were purchased from Supelco (Bellefonte, PA, USA).

Reference materials and sample collection
Three different mussel samples were used. The first sample was toxin free and was obtained from a supermarket of Brussels. The second sample, collected from the Belgium market was contaminated by DSP toxins. The last sample was a mussel tissue reference material NRC for DSP toxins (MUS-2). The certified toxin level was 11.0 µg/g for okadaic acid and 0.96 µg/g for DTX-1.

LC-MS-MS analysis of toxins
LC-MS-MS was performed using a surveyor liquid chromatograph coupled with a LCQ Deca XP plus ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The mass spectrometer (MS) was equipped with electrospray ionisation (ESI). The MS was tuned with okadaic acid standard solution (5 µg/ml) infusing 3 µl/min in the mobile phase and monitoring the $[M+H]^+$ ion at 805 m/z. Optimal conditions were set as follows: positive mode; wide band activation applied, ESI needle voltage: 5 kV; spray current: 80 µA; capillary voltage: 38 V; capillary temperature: 275°C; sheath gas flow rate: 66 (arb.); auxillary gas: 30 (arb.); tube lens offset: 44 V; multipole 1 offset: -5.5 V; multipole 2 offset: -11 V; intermultipole lens voltage: (-26 V); entrance lens: -46 V); trap dc offset voltage: -10 V. Separation of toxins was achieved on Xterra MS C_{18} 5 µm 2.1x250 mm (Waters, Milford, MA) and maintained at 40°C. The mobile phase was acetonitrile-water (58:42, v/v) containing 0.05 % trifluoroacetic acid (TFA). The flow-rate was 250 µL/min.

Sample extraction for the simultaneous determination of ASP and DSP toxins (screening method)
An aliquot (4.0 g) of mussel tissue was weighed accurately into a graduated centrifuge tube (50 ml). Methanol-water (16.0 ml, 80:20, v/v) was added and homogenised using ultra-turrax (IKA Labortecnik homogeniser, Staufen, Germany) for 3 min. The homogenate was centrifuged at 3500 rpm for 10 min. The supernatant was decanted and filtered (0,2 µm) and a 5.0 ml portion was extracted twice with 5 ml aliquots of diethyl ether. Diethyl ether extract was evaporated under nitrogen stream (40° C). The residue was dissolved in 1 ml methanol and 10 µl solution was injected into LC-MS.

Sample preparation for data validation (confirmatory method)
DA: a blank mussel sample was fortified at three levels of concentration: 50, 100 and 150 % of the maximum residue level (MRL, 20 mg/kg). Extraction was carried out using procedure described in an internal document (Chrysostome, SOP 22/F/3018). A portion of mussel accurately weighed (4.0 g) was homogenised with 16 ml methanol-water (1:1, v/v) using ultra-turrax for 3 min. The resulting slurry was centrifuged at 3000 rpm for 10 min. The SAX cartridge was successively conditioned with 6 ml methanol, 3 ml water and 3 ml methanol-water (1:1, v/v). A 5.0 ml portion of supernatant was loaded onto the conditioned SAX cartridge. The column was washed first with 5 ml acetonitrile-water (1:9, v/v) and then 0.5 ml formic acid 0.1 M. Domoic acid was eluted with formic acid 0.1 M in the volumetric tube just until 2.0 ml is reached. 5 µl of solution was injected into the LC-MS system.

Okadaic acid extraction used for the data validation (confirmatory method)
OA was extracted from a mussel tissue reference material (MUS-2) according to a method previously reported by Mondeguer (2003). A 4.0g aliquot of mussel tissue reference material was weighed accurately and homogenised with 10 ml of acetone for 5 min using ultra-turrax. The resulting mixture was centrifuged at 3000 rpm for 10 min. The extraction procedure was repeated twice with aliquots of 8 ml and 6 ml

of acetone. The acetone layers were combined and made up to 25.0 ml with acetone. This solution was used to prepare three levels of concentration: 50, 100 and 150 % MRL (160µg/kg) by diluting respectively 150µl, 300µl, and 450 µl of the acetone solution to 20.0 ml with acetone solvent. A 5.0 ml portion of each solution was evaporated to dryness. The residue was dissolved with 5 ml chloroform-hexane (1:1, v/v). A silica cartridge was conditioned with 10 ml chloroform and 5 ml chloroform-hexane (1:1, v/v). 3 ml chloroform-hexane extract was loaded onto the cartridge. The cartridge was washed with 3 ml of chloroform-hexane (1:1, v/v) and DSP toxins were eluted by 3 ml chloroform-methanol (65:35, v/v). The eluate was evaporated and the residue was re-dissolved in 1.0 ml methanol. 10 µl solution was injected into the LC-MS system.

Results and Discussion

Data validation on domoic acid followed guidelines for ISO 17025, ISO 11843-1, ISO 5725-2 and the European Commission Decision 2002/657/EC. Optimised conditions (Table 1) for fragmentation of the parent ions into daughter ions were obtained using infusion of standard solutions of interest into the mobile phase. The selected mobile phase resulted in a good chromatographic separation (Figure 1) of all the component ions under investigation. Specific full MS/MS scan and positive ESI mass spectra are presented (Figure 2). The mobile phase optimisation was most valuable for OA and DTX-2 to obtain well-resolved peaks since they have the same molecular weight. A mass chromatogram of blank mussel sample fortified with OA and DTX-2 showed good resolution (Figure 3).

Table 1. Fragmentation of DA and DSP toxins: product ions and conditions

Toxin	MW	Product ions						t_R	Fragmentation	
		$[M+NH_4]^+$	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	$[M+H-3H_2O]^+$	$[M+H-4H_2O]^+$	(min)	Isolation width (m/z)	Collision Energy (%)
DA*	311		312					2.2	4	45
OA	804		805	787	769	751	733	7.8	7	45
DTX2	804		805	787	769	751	733	9	7	45
PTX2	858	876.5	859	841	823	805	787	10.5	7	45
AZA3	827.5		828.5	810.5	792.5	774.5	756.5	11.5	3	40
DTX1	818		819	801	783	765	747	15.5	8	45
AZA1	841.5		842.5	824.5	806.5	788.3	770.5	17.0	3	40
AZA2	855.5		856.5	838.5	820.5	802.5	784.5	21	3	40

*DA 312 $\rightarrow$ 266 $\rightarrow$ 248 $[M+H]^+ \rightarrow [M+H - HCOOH]^+ \rightarrow [M+H - HCOOH-H_2O]^+$

Statistical performance characteristics (Table 2) were evaluated using LC/MS/MS for domoic acid with good reliability. Good linearity was observed with a correlation coefficient r = 0.9992, with n = 7 and m = 3 replicates. Recoveries obtained at the three levels of concentration 50 % MRL, 100 % MRL, 150 % MRL (with m = 3 fortified samples per level measured at 3 different days, n = 27) were 107.96 %, 106.46 %, and 113.44 % respectively. Estimated decision limit (CCa) and detection capability (CCß) **(1)** from the designed experiment were CCa = 23.29 and CCß = 26.63 respectively. Repeatability (*r*) and intermediate precision (or within-laboratory reproducibility, *R*) were investigated (ISO 5725-2). Resultant RSD (%) values at 3 levels 50-100-150 % of MRL were *r = 6.2, 3.78, 4.22 and r = 10.98, 12.58, 4.22* respectively. Validation for okadaïc acid was carried out using NRC reference material homogenate (MUS-2). Good chromatographic separation for OA and DTX-1 daughter ions was demonstrated (Figure 4). Linearity checked by least square regression was observed with a correlation coefficient r = 0.9991 (with n = 6 and m = 3 replicates). Recovery examined at three levels 50, 100, 150 % MRL were 96.2 %, 102.6 % and 84 %respectively.

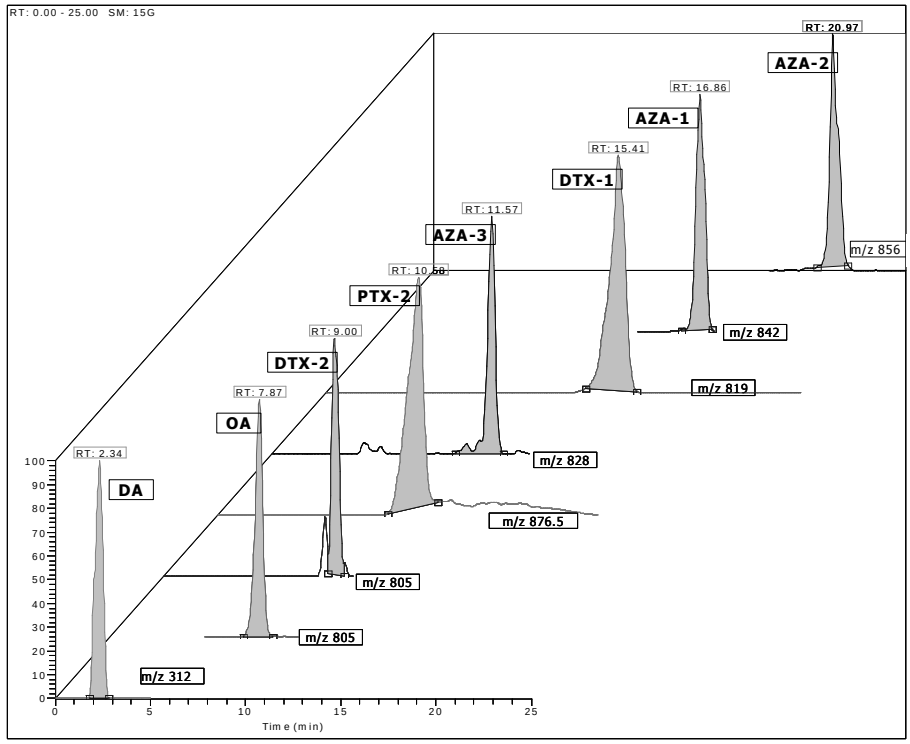

Figure1. Mass chromatogram of a mixture of DA and DSP toxins

Table 2. Validation data on Domoic acid.

Levels of DA	Recovery (%)	Repeatability RSD_r (%)	Intermediate precision RSD_R (%)
50 % MRL	107.96	6.2	10.98
100 % MRL	106.46	3.78	12.58
150 % MRL	113.44	4.22	4.22
Decision limit, CCa = 23.29 mg/kg Detection capability, CCß = 26.63 mg/kg With a = 0.05 ß = 0.05 n = 3 different days (ISO 11843-1), MRL = 20 mg/kg: Maximum residue level.			

The described LC-MS/MS method was applied successfully to a naturally contaminated mussel sample collected from the Belgian market that revealed the presence of large amounts of OA and PTX-2, higher than the regulatory limits and small amounts of AZA-1 and AZA-2 (Figure 5).

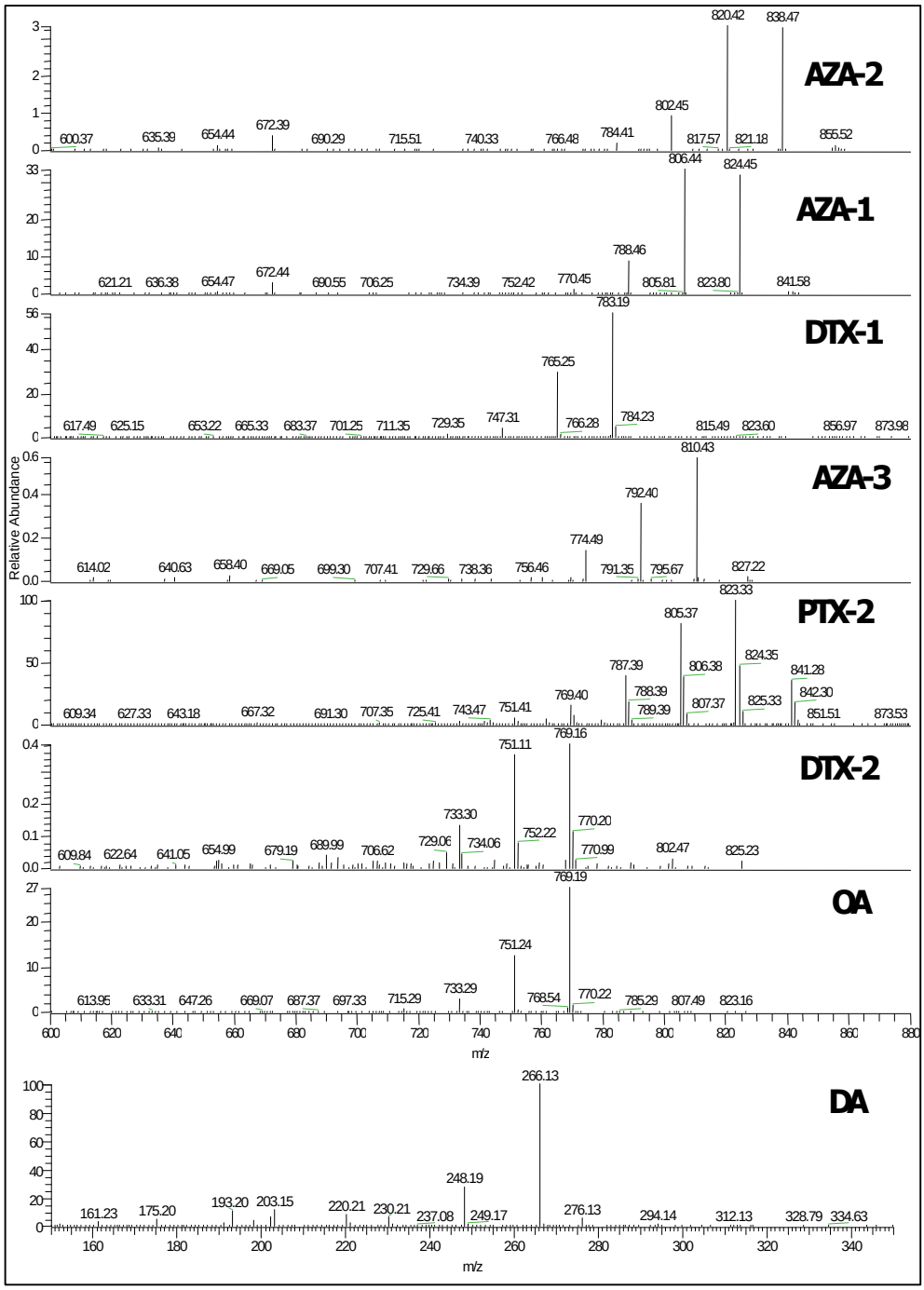

Figure 2. LC-MS^2 mass spectra of DA and DSP toxins

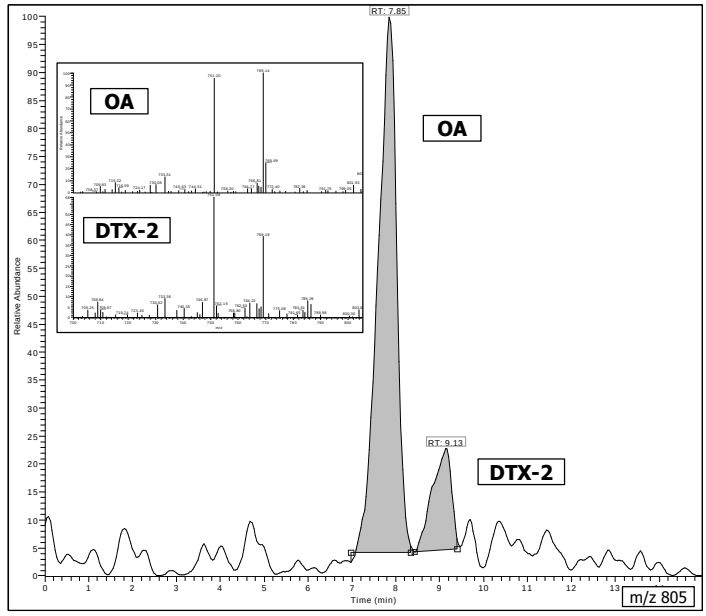

Figure 3. Mass chromatogram of a blank mussel fortified with OA and DTX-2

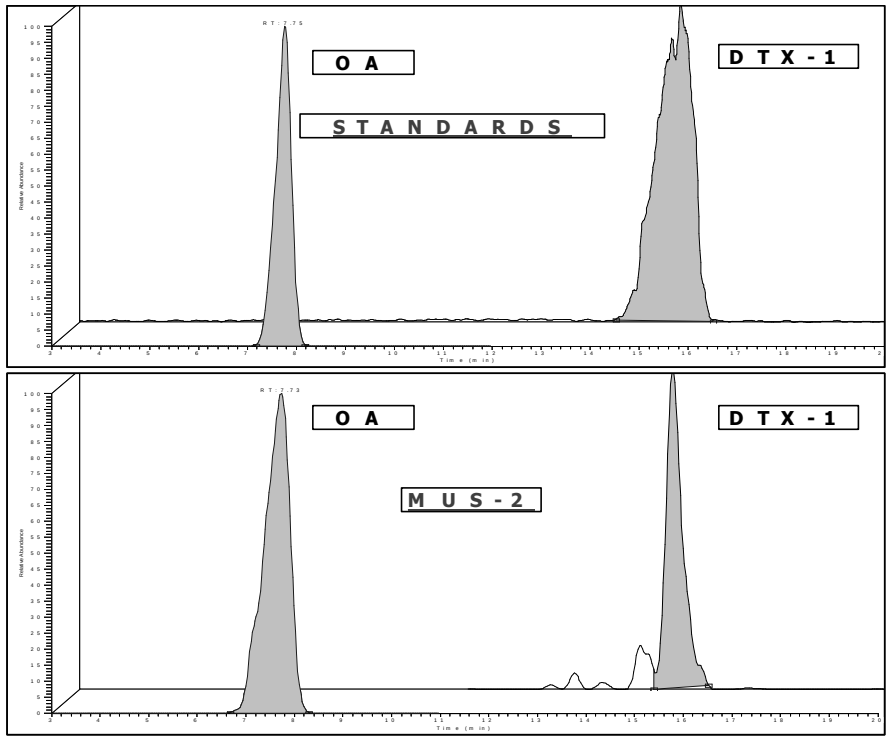

Figure 4. Mass chromatograms of OA and DTX-1 in standard solutions and in MUS-2 extract

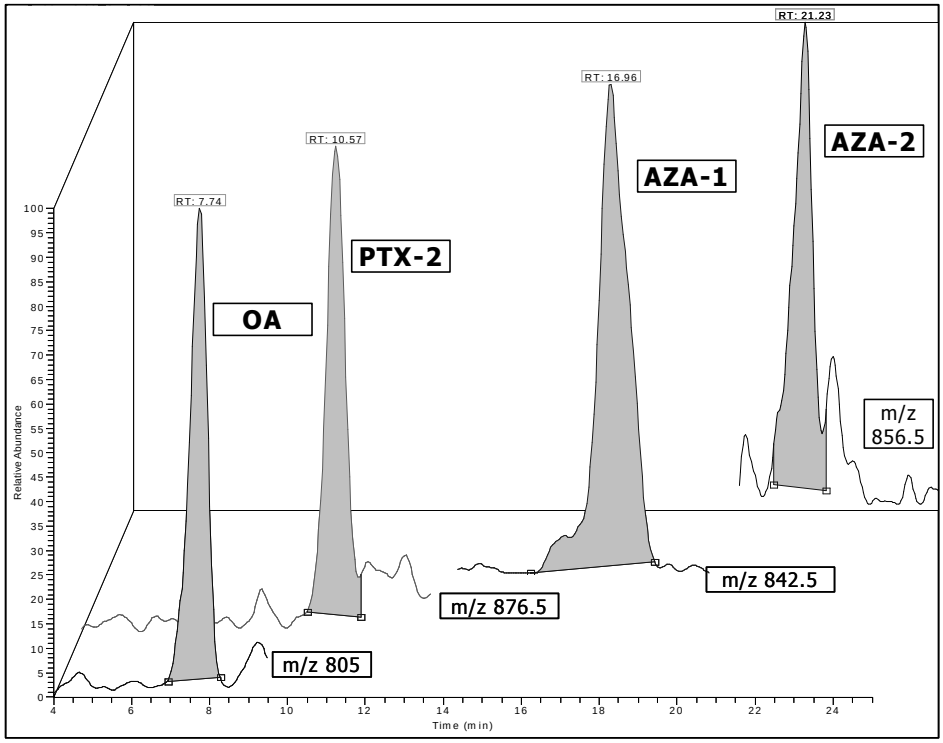

Figure 5. Mass chromatogram of a mussel contaminated with DSP toxins

Conclusion

A multi-toxin LC-MS/MS method has been developed using ion trap mass spectrometry. This method is a significant improvement over the MBA approach in that, it provides a rapid, specific, sensitive, quantitative and unequivocal determination of DSP and ASP toxins in mussels. The described method was successfully tested using a naturally contaminated mussel sample. This method has proven to be a powerful tool to tackle the significant challenges presented by determination of lipophilic toxins (DSP) and domoic acid (ASP) in seafood, due to their complex diverse structures.

This method should be used for routine monitoring programmes of shellfish as an alternative to the conventional mouse bioassay. Future validation work will be focused on the other DSP toxins according to the availability of appropriate calibration standards and reference materials. Further studies will include expanding the method to provide additional testing for DSP toxins in other matrices such as scallops, oysters and clams.

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APPLICATION OF A TISSUE CULTURE ASSAY FOR DETECTION OF TETRODOTOXIN PRODUCING V*ibrio alginolyticus* STRAINS ISOLATED FROM SEA FOOD

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Abstract
50 strains of *Vibrio alginolyticus* iolated from seafood were cultured and examined for tetrodotoxin productivity by a tissue culture assay and mouse bioassay. Using these approaches most of the strains were shown to produce tetrodotoxin.

Introduction
Bacterial production of tetrodotoxin (TTX) was first reported by Yasumoto *et al*. (1986). TTX is produced by a series of marine bacteria, including members of the *Vibrionaceae* which are frequently found in seawater and associated with marine animals (Lee *et al*. 2000; McEvoy *et al*. 1998). V*ibrio alginolyticus,* in particular has been reported as a producer of tetrodotoxin (Noguchi *et al*.1987; Simidu *et al*.1987; Simidu *et al*.1990). Several studies suggest that tetrodotoxin found in animal organs is the product of marine bacteria by a symbiotic relationship. The TTX-containing animals may have absorbed and accumulated TTX and its derivatives produced by several marine bacteria. The paralytic action of these potent sodium channel-blocking neurotoxins would enhance the capture and subsequent feeding capability of those animals that have a suitable delivery system. The biological and chemical standard methods for TTX detection are relatively indiginous and require partial purification of the sample (AOAC, 1984; Simidu *et al*. 1987). Moreover, the chemical methods lacked specificity for TTX (Matsumura, 1995). Whith regard to this, Matsumura (1995) highlighted no ability in microorganisms to produce tetrodotoxin: the contaminants from a bacterial culture medium may confound the identification of TTX which puffer fish produce (Matsumura, 1995). Gallacher and Birkbeck (1992) has developed a tissue culture assay based on the ability of TTX to protect cells treated with veratridine and ouabain. The respective actions of these compounds are to open sodium channels and to block the action of Na/K-ATPase. Thus, TTX prevents the influx of sodium ions and protects the cell from swelling and eventual death. To evaluate the real involvement of marine bacteria in tetrodotoxin production, screening experiments using the Gallacher's method with *V. alginolyticus* strains isolated from seafood were carried out. To confirm the results obtained with the tissue culture assay, the samples were also processed for tetrodotoxin detection by the mouse bioassay.

Materials and Methods
Chemicals
All chemicals were obtained from Sigma unless otherwise stated.

Bacterial strains
To perform the tissue culture test and the mouse bioassay, were used the following ATCC (American Type Culture Collection, Rockville Maryland, USA) strains producing TTX (Simidu *et al*., 1987): *Vibrio parahaemolyticus* ATCC 17802, *Plesiomonas shigelloides* ATCC 14029, *V. alginolyticus* ATCC 17749. The standardised protocols were then applied to 50 strains of *V. alginolyticus* from refrigerated seafood (Table 1). For the tissue culture assay the bacteria were cultured in 10 ml of Luria Broth

(Difco) with 1 % NaCl at room temperature for 7 days. Supernatants were collected after centrifugation at 10 000 x g for 10 minutes and stored at –20 °C until required. In the experiments the 1:2 and 1:4 culture supernatant dilutions, untreated and heated at 100 °C for 30 minutes, were used. For the mouse bioassay the microorganisms were cultured in 1 liter of Luria Broth for 7 days at room temperature. The cells were harvested by centrifugation at 7 000 x g for 15 minutes and extracted with 100 ml of ethanol containing 1 % of acetic acid. After removal of ethanol by evaporation the extract was dissolved in 6 ml of distilled water (Matsumura 1995; Kodama *et al*.1988).

Assay of sodium channel blocking toxins
The mouse neuroblastoma cell line C-1300 (ATCC CCL131) was used. Mouse neuroblastoma cells C-1300 were cultured in RPMI1640 medium containing 25 mM Hepes, penicillin (200 IU/ml), streptomicin (200 mg/ml) and 5 % foetal calf serum. Cell suspensions were prepared from confluent monolayers by tripsinization and were resuspended diluted 1:2 in the above medium containing 0.75 % (w/v) methylcellulose. Volumes (200 µl) were dispensed into the wells of a microtitre plate. After incubation for 24 hours at 37 °C the medium was replaced with the following solution: 50 µl 1 mM ouabain, 50 µl 0.2 mM veratridine and 100 µl test sample. All samples were tested in quadruplicate and controls consisted of (a) 2 sets of 4 wells containing cells with 200 µl RPMI1640 medium, (b) 2 sets of 4 wells containing ouabain-veratridine solution plus 100 µl of RPMI medium, (c) 2 sets of 4 wells containing oubain-veratridine solution plus 100 µl TTX (3.13 µM) 160 ng/ml. After 24 h at 37°C of incubation the medium was removed and replaced with 200 µl of 0.05 % (w/v) neutral red solution in Hank's balanced salt solution (BSS). After incubation at 37° for 30 minutes the dye solution was removed and the cells were carefully rinsed once with BSS to remove free dye. Citrate-buffered ethanol (200 µl 50 mM citrate-acetic acid buffer, pH 4,6, 50 % ethanol) was added to each well, incubated at room temperature for 1 hour and absorbance values were determined at 540 nm in a microtitre plate reader. The percentage protection of cells by tetrodotoxin or test samples was calculated from the relationship D= (A-B)/(C-B) x 100

D = percentage protection
A = absorbance at 540 nm of cells + oubain+ veratridine or test sample
B = absorbance at 540 nm of cells + oubain+ veratridine
C = absorbance at 540 nm of cells alone

Determination of optimum veratridine and ouabain concentrations for tetrodotoxin assay.
In the above assay the concentrations of ouabain and veratridine added to the wells were varied from 0 to 1 mM (ouabain) and 0 to 0.15 mM (veratridine) in a checkerboard assay in presence of 160 ng/ml of TTX (Gallacher and Birkbeck, 1992).

The mouse bioassay
The mouse bioassay, using mice weighing 18 g, was performed by the official method (AOAC, 1984).

Results
The results from the optimised tissue culture assay demonstrated that 40 % of the *V. alginolyticus* isolates produced TTX (Table 1). These results were confirmed by the mouse bioassay. In the mouse bioassay for TTX mouse death was observed five minutes post innoculation with TTX positive isolates of *V. alginolyticus*.

Table 1. The isloated strains used in this study

Isolated strains	Source	Tissue culture assay TTX	*Mouse bioassayTTX*
1. V. alginolyticus	water	+	+
2. V. alginolyticus	anchovy	-	-
3. V. alginolyticus	crawfish	-	-
4. V. alginolyticus	mussel	-	-
5. V. alginolyticus	cod	-	-
6. V. alginolyticus	crawfish	-	-
7. V. alginolyticus	cuttle-fish	+	+
8. V. alginolyticus	mackerel	+	+
9. V. alginolyticus	clam	-	-
10. V. alginolyticus	mussel	-	-
11. V. alginolyticus	mussel	-	-
12. V. alginolyticus	crawfish	-	-
13. V. alginolyticus	cod	+	+
14. V. alginolyticus	clam	-	-
15. V. alginolyticus	cod	+	+
16. V. alginolyticus	clam	+	+
17. V. alginolyticus	mussel	-	-
18. V. alginolyticus	clam	-	-
19. V. alginolyticus	water	-	-
20. V. alginolyticus	anchovy	+	+
21. V. alginolyticus	mussel	-	-
22. V. alginolyticus	cod	+	+
23. V. alginolyticus	mackerel	+	+
24. V. alginolyticus	clam	+	+
25. V. alginolyticus	cod	-	-
26. V. alginolyticus	cuttle-fish	-	-
27. V. alginolyticus	mackerel	-	-
28. V. alginolyticus	clam	+	+
29. V. alginolyticus	crawfish	-	-
30. V. alginolyticus	sole	+	+
31. V. alginolyticus	mussel	+	+
32. V. alginolyticus	water	-	-
33. V. alginolyticus	anchovy	-	-
34. V. alginolyticus	mussel	+	+
35. V. alginolyticus	crawfish	-	-
36. V. alginolyticus	cuttle-fish	+	+
37. V. alginolyticus	water	+	+
38. V. alginolyticus	crawfish	+	+
39. V. alginolyticus	mussel	-	-
40. V. alginolyticus	cuttle-fish	-	-
41. V. alginolyticus	mussel	-	-
42. V. alginolyticus	mussel	+	+
43. V. alginolyticus	anchovy	-	-
44. V. alginolyticus	mussel	+	+
45. V. alginolyticus	cod	+	+
46. V. alginolyticus	mackerel	-	-
47. V. alginolyticus	clam	-	-
48. V. alginolyticus	cod	-	-
49. V. alginolyticus	cuttle-fish	-	-
50. *V. alginolyticus*	water	-	-

Discussion

The protocol we proposed allows an efficient and rapid identification of TTX producing bacteria. The sensitivity of the assay is such that low concentrations of tetrodotoxin, such as those in bacterial culture supernates, can be quantified directly without the extraction and concentration necessary for analysis by the biological and chemical methods. Matsumura (1995) insists that bacteria do not have the ability to produce TTX, that contaminants from the bacterial culture medium may confound the identification of the TTX, and that puffer fish do produce the toxin. The results obtained in this study, using biological methods, confirmed that bacterial production of TTX is still a valid proposition. Although, high-performance liquid chromatography or gas chromatography-mass spectrometry might detect the same peaks as those obtained from the bacterial medium, positive toxicity in a mouse bioassay or in a tissue culture assay would not be detected. However, our extracts purified from the bacterial culture cells showed a strong toxicity by biological tests. Moreover only a few *V. alginolyticus* strains isolated from seafood produce TTX. Although the role of TTX in the bacteria themselves is still unclear, it was postulated that TTXs regulate the transfer of sodium ions through biological membranes (Simidu *et al*. 1987), and this fact may have some relevance to the function of the toxin in marine bacterial cells. Further research should be aimed at elucidating the exact mechanism of production and the biological functions of TTX produced by microorganisms.

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PREPARATION OF REFERENCE MATERIALS FOR AZASPIRACIDS

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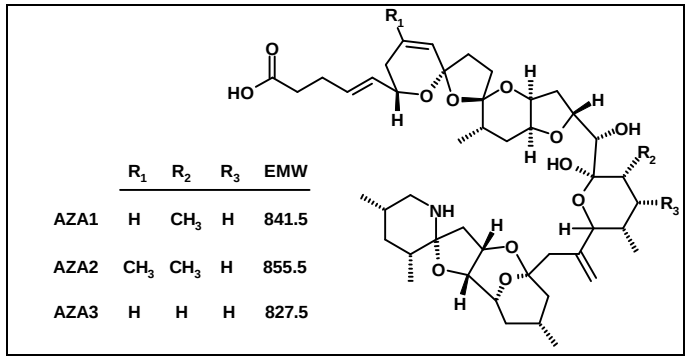
Abstract
Azaspiracids (AZAs) are a family of polyether toxins, first discovered in Ireland (1995) and now observed throughout Europe. These compounds have a high oral toxicity to humans and have been responsible for incidents of shellfish poisoning. This leads to a need for accurate calibration solutions as well as tissue reference materials, as they play important roles in method development, validation and quality control. The Certified Reference Materials Program (CRMP) at the National Research Council of Canada (NRC) has prepared solutions containing AZA1 and AZA1-3 as well as a tissue reference material. This paper presents our work towards the preparation of calibration solutions and tissue reference materials for azaspiracid toxins.

Introduction
Azaspiracids (AZAs) are a family of polyether toxins, first discovered in Ireland (1995) and now observed throughout Europe (James *et al.*, 2002, Ofuji *et al.*, 1999 and Satake *et al.*, 1998) (Figure 1). These compounds have a high oral toxicity to humans and have been responsible for incidents of shellfish poisoning. Enforcement of regulatory directives on food contaminants such as AZAs and other shellfish toxins requires fully validated analytical methods which in turn require certified reference materials (CRMs). Accurate calibration solution CRMs are required for both method development and calibration of instruments and assays. Shellfish tissue CRMs play an important role in method development, validation and quality control, as they allow the testing of an entire method from extraction through to analysis. This paper will present our work towards the development of calibration solutions and a mussel tissue CRM for AZAs.

Preliminary calibration solutions for azaspiracid-1 (AZA1) and a mixture of azaspiracids-1, -2 and -3 (AZA1-3) have been prepared. This involved developing methods for the purification of AZAs, conducting a large-scale preparative isolation of AZAs from contaminated shellfish tissues, and analyzing the products by LC-MS and nuclear magnetic resonance (NMR) techniques. NMR was used to determine purity and to quantify the toxin in a concentrated stock solution, prior to accurate dilution to the micromolar level and dispensing into flame-sealed glass ampoules. Stability studies have been conducted with these solutions.

A preliminary, pilot scale mussel tissue reference material (RM) has also been prepared. This involved homogenization of AZA-contaminated mussel tissues, packaging into plastic bottles, and thermal sterilization in a steam retort. Stability studies are now being conducted on this RM as part of a feasibility study on the preparation of a large-scale tissue CRM.



	R_1	R_2	R_3	EMW
AZA1	H	CH_3	H	841.5
AZA2	CH_3	CH_3	H	855.5
AZA3	H	H	H	827.5

Figure1. Structures of the azaspiracids.

Azaspiracid isolation and purification
Ground mussel tissue was divided into lots of approximately 500 g each and stored frozen until processed with a multi-step clean-up method for the various AZAs present. Emphasis was placed upon the isolation of the three most common compounds, AZA1, AZA2 and AZA3. At each purification step an aliquot was removed and analysed by LC-MS for AZA purity and content employing an Agilent HPLC1100 connected to an API300 mass spectrometer. A 95 % ethanol extract of thawed mussel tissue was partitioned against 1N NaCl and ethyl acetate. The resulting organic layer was then partitioned between methanol and hexane to remove the apolar compounds. Vacuum liquid chromatography of the methanol fraction on a silica gel column eluted with hexane, ethyl acetate (0.1 % acetic acid), and methanol/ethyl acetate (0.1 % acetic acid) yielded azaspiracid-containing fractions, which were pooled and applied to a Sephadex LH-20 column. Elution with methanol resulted in the isolation of five fractions containing various AZA analogues of interest. Flash chromatographic purification of these fractions on RP-8 sorbent eluted with methanol, and various acetonitrile/water (0.2 % acetic acid) compositions yielded fractions containing predominately AZA3, AZA1, and AZA2. Further purification of these fractions using semi-preparative HPLC (Agilent 1100 series HPLC-DAD-MSD) equipped with a 4.6 mm X 150 mm Hypersil BDS C-8 column and eluted isocratically with 75 % acetonitrile/water (1.0 mM ammonium formate and 0.05 % formic acid) yielded purified AZA1, AZA2 and AZA3. The purified AZAs were submitted for NMR to ascertain purity and for quantitation.

Quantitation by NMR
A stock solution of purified AZA1 in CD_3OH was prepared and quantified by ^1H NMR spectroscopy at 500.13 MHz using a Bruker DRX-500 spectrometer. The AZA1 sample and weighed caffeine standards of known concentration were contained in separate Wilmad 535 pp precision glass tubes. Spectra of AZA1 and standards were obtained at constant receiver gain over a spectral width of 7507 Hz using 90^o pulses and low-level presaturation of the solvent resonance during the delay (15s) between acquisition periods (2.182 s). Data accumulation was preceded by 8 dummy scans. All samples were individually tuned and matched at the observing frequency. Pulses lengths were calibrated precisely for each sample by finding the null corresponding to a 360^o pulse. Spectra were baseline corrected and integration performed with Bruker XWINNMR software version 2.6 using constant integral scale throughout. Integrated intensity values I were multiplied by the 360^o pulse length p_{360} for each individual sample to compensate for probe Q-damping effects. The integral ranges

chosen for the AZA1 sample (at 5.7, 3.9 and 2.8 ppm) were across isolated multiplets free from overlap with minor impurity resonances. Integrals of the three methyl resonances were used for the caffeine standards. 2D ^{1}H COSY, ^{1}H TOCSY (160 ms and 10 ms mixing time), ^{1}H/^{13}C HSQC and ^{1}H/ ^{13}C HMBC spectra of AZA1 were recorded to confirm ^{1}H assignments. Integrals were corrected for the numbers of protons contributing to each integral range and for the numbers of scans accumulated. The AZA1 solution concentration was then determined by comparing the corrected I x p_{360} response (per proton per scan, average of 3 ranges from each of 3 spectra) of AZA1 to that of the known concentration of caffeine (41.3 mM). The ranges chosen for NMR integration would correspond to the sum of AZA1 as well as the AZA3 and AZA6 present in the prepared solution.

NRC RM-AZA1
After the concentration was determined, the solution was diluted with high purity methanol into a calibrated volumetric flask. The weights of aliquot and final solution were determined using three balances (as a check on sources of error such as static charge). The prepared RM solution was dispensed in 500 µL aliquots into amber glass ampoules pre-filled with argon and then flame sealed. The prepared RM has a final AZA1 concentration of 1.42 ± 0.08 µM and low concentrations of AZA3 and AZA6 impurities at 0.025 µM and 0.022 µM respectively. A total of 429 ampoules were prepared.

NRC RM-AZA123
During purification of AZA1, various fractions were collected that contained other AZAs. These fractions were selected and blended in methanol to produce a final solution with AZA1, AZA2 and AZA3 in similar concentrations. This solution was diluted with high purity methanol and the prepared RM solution was dispensed in 500 µL aliquots into amber glass ampoules, pre-filled with argon and flame sealed. The prepared RM has final AZA1, AZA2 and AZA3 concentrations of 243 nM, 160 nM and 114 nM, respectively. These concentrations were determined using RM-AZA1 as the calibrant and assuming equimolar response factors. A total of 528 ampoules were prepared.

NRC RM-AZA-Mus
AZA-contaminated mussels from Killary Harbour were provided by the Marine Institute (Ireland). They were shucked and homogenised by the FRS Marine Laboratory and shipped to NRC-IMB in a frozen state. Approximately 1 kg of this tissue was thawed and mixed with 500 mL water in a Robot Coupe RSI6. The presence of byssus thread fibres and small pieces of shell required that the material be passed through a screen to avoid clogging of dispensing equipment. This material was placed on a hot plate and brought to 90°C for 10 min. The water content was measured and adjusted to 85 % to produce a final mass of 1.8 kg of homogenate.

The prepared material was taken to the Canadian Institute of Fisheries and Technology at Dalhousie University in Halifax to be packaged and processed. The fluid delivery system was calibrated to pump 3.8 g of the material. Polypropylene bottles were filled, checked for weight and any weighing <3.7 g were rejected. Acceptable bottles were heat sealed (seal was strips of metal laminate) in groups of four and processed in a steam retort. Bottles were removed from the retort, cooled, and had their seals inspected. Seals were trimmed, labelled and then the bottles were sealed into laminate pouches. A total of 379 bottles were successfully filled and processed. The RMs were transported to NRC and stored at -80°C.

The AZA concentrations in the prepared mussel RM were determined by LC-MS to be 440 ± 40 µg/kg, 127 ± 12 µg/kg and 5 ± 2 µg/kg for AZA1, AZA2 and AZA3 respectively. These concentrations were determined using RM-AZA1 as the calibrant and assuming equimolar response factors. The LC-MS analysis of the processed RM-AZA-Mus revealed one problem: AZA3 seems unstable to the high temperature used in the RM preparation. Most of it rearranged to an isomer, which showed greater retention on the LC column. Some slight rearrangements of AZA1 and AZA2 are also observed.

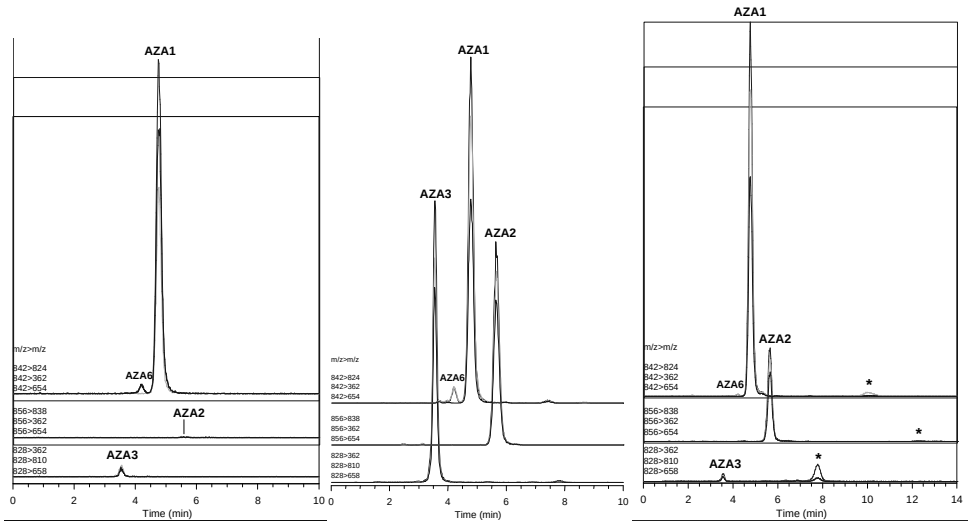


Figure 2. LC-MS analyses of RM-AZA1 (left), RM-AZA123 (middle), and RM-AZA-Mus (right).

Stability studies

AZASPIRACID-1

The stability of AZA1 was tested in 3 types of solvents at a concentration of 200 ng/mL: (a) methanol; (b) acidified methanol (0.05 % TFA); (c) acetonitrile. These solutions were sub-divided and placed into various temperature-controlled environments (37°C, 20°C, 4°C, -12°C, -80°C). Some AZA1 was also stored as a dry residue in glass vials. At increasing times, samples were removed and analysed. The results showed good stability in methanol with no detectable decomposition over 60 days at temperatures up to +4°C. There was an increased decomposition rate observed in the acetonitrile samples and rapid decomposition in acidic solution. The dried residues were also found to be unstable.

RM-AZA-MUS

We are investigating the stability of azaspiracid contaminated mussel tissue using bottles of RM-AZA-Mus as well as lyophilised material. Lyophilised samples were prepared by freeze-drying samples of RM-AZA-Mus directly in their individual bottles. The bottles were then recapped and the mass of water loss recorded. Prior to extraction, the residues were first rehydrated with the same mass of water lost during freeze-drying. Samples of both wet and dry tissue have been placed into various temperature-controlled environments (37°C, 20°C, 4°C, -12°C, -80°C) and are being monitored at increasing time intervals. Stability testing of the RM-AZA-Mus

is not yet completed, but results are encouraging. There was no detectable decomposition of AZA1 at temperatures up to 20°C over 60 days. About 5 % decomposition was observed at 37°C after 60 days. AZA3 does appear to be unstable at higher temperatures, as indicated above. Lyophilised tissues seem relatively stable but extraction yields from replicate bottles seem much more erratic than from the wet homogenates.

Conclusions

1) Purification of azaspiracids from shellfish tissues was difficult and the yield of material was low.
2) AZAs showed good stability in refrigerated methanol solutions but are unstable in acidic solution.
3) A wet, thermally-sterilised tissue homogenate proved best for the shellfish tissue CRM.
4) AZA3 presents problems as it was found to be thermally unstable, rearranging to an unidentified isomer. Extraction yields proved erratic with lyophilised material.
5) More contaminated material is required to facilitate production of full-scale calibration solution and tissue CRMs.

Acknowledgements

The authors thank CRMP staff, particularly K. Thomas, S. Crain, and D. Marciniak, for technical assistance. The packaging and processing of the tissue RM was contracted to the Canadian Institute of Fisheries and Technology, Dalhousie University, and the contribution of their staff is gratefully acknowledged. Azaspiracid-contaminated mussels were provided by the Marine Institute, Ireland. Partial funding for this project was provided by UK Food Standards Agency.

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A MUSSEL TISSUE CERTIFIED REFERENCE MATERIAL FOR PARALYTIC SHELLFISH POISONING TOXINS

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Abstract
Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins, produced by several marine dinoflagellates. Over 20 structural analogs have been identified, each having a different specific toxicity. Enforcement of regulatory directives on PSP toxins in shellfish requires a validated method, accurate calibration standards, and a shellfish tissue certified reference material (CRM). CRMs are important in method validation and quality control, as they allow the testing of an entire method from extraction through to analysis. This paper presents our preliminary work towards the development of a mussel tissue CRM for PSP toxins.

Introduction
Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins, produced by several marine dinoflagellates. These toxins are tetrahydropurine derivatives based on the parent compound, saxitoxin (STX) and can be divided into three classes: carbamate, N-sulfocarbamoyl and decarbamoyl toxins (Figure 1), with the carbamate toxins being the most toxic and the N-sulfocarbamoyl toxins being the least toxic (Oshima *et al*. 1984). Enforcement of regulatory directives on food contaminants such as PSP toxins requires validated analytical methods. The AOAC mouse bioassay is the method used in most regulatory laboratories today, but it has been banned in some countries. The two most commonly used chemical analysis methods for PSP toxins are liquid chromatography (LC) coupled with post-column oxidation and fluorescence detection (LC-pcr-FLD) (Oshima, 1995) and LC-FLD with pre-column oxidation (Lawrence *et al.*, 1991). Another method gaining popularity is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Quilliam *et al.*, 2001). All of these LC methods require the use of accurate calibration standards for each individual toxin. Shellfish tissue certified reference materials (CRMs) are also required as they can play an important role in method validation and quality control by testing an entire method from extraction through to analysis. Although the European Commission has recently announced a lyophilised mussel tissue CRM for decarbamoylsaxitoxin (VanEgmond *et al.*, 2001), it is not being distributed currently. Furthermore, a reference material (RM) based on a liquid slurry of homogenised whole mussel tissue with a wider range of toxins would provide a better match to natural samples. At the National Research Council's Institute for Marine Biosciences we have set a goal to develop a mussel tissue CRM containing 8 of the principal PSP toxins. Results from a pilot scale (named RM-PSP-Mus-p) will be presented.

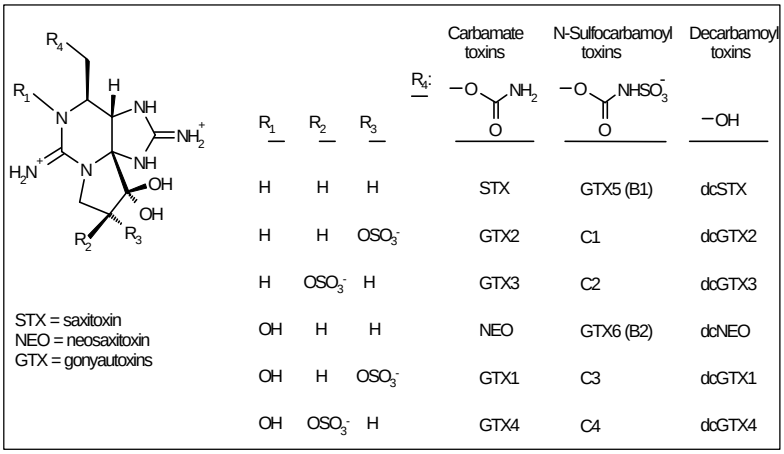

Figure 1. Structures of the principal PSP toxins.

Results and Discussion

RM design criteria: The purpose of this work was to design a wet, thermally sterilised mussel tissue homogenate, similar to our other CRMs for amnesic shellfish poisoning and diarrhetic shellfish poisoning (NRC CRM-ASP-Mus and CRM-DSP-Mus). A blend of mussel tissue and cultured plankton was selected as the best means of producing a suitable toxin concentration and profile as any natural sample is in fact a blend of shellfish tissue and plankton that the animal consumed. The target toxin level was to be approximately 5 times the North American regulatory level of 800 µg STX equivalents per kg whole tissue. Although this is a relatively high concentration, it is required for accurate determination of CRM values and also allows the blending of the RM with negative control tissues to achieve a range of concentrations. The toxin profile was to include STX, NEO, GTX2, GTX3, dcGTX2, dcGTX3, GTX1, and GTX4. The N-sulfocarbamoyl toxins would not be present as these can readily decompose. It was decided dcSTX would not be part of the toxin profile as it is not produced by either plankton.

Preparation of plankton: Our algal research group at IMB has produced large of quantities of two PSP-producing cultures, *Alexandrium tamarense* (AL18b) and *Alexandrium minutum* (AL1V). *A. tamarense* produces primarily C2, GTX3, NEO and STX, whereas *A. minutum* produces GTX4, a small amount of GTX3 and no C toxins (Figure 2). There are problems with blending such biomass directly with mussel tissue to produce a CRM, mainly the gradual conversion of the C toxins to dcGTXs and GTXs (Figure 3), as well as the epimerisation of the GTXs themselves. It is best to stabilise the epimer ratios and to eliminate C toxins prior to blending the plankton with the mussel tissue. The final product must be adjusted to an acidic pH (3-4) for overall stability of the GTX, STX and NEO toxins. The role of *A. minutum* was to provide the GTX1 and GTX4 epimers. Our calibration solution CRM for GTX1 and 4 (CRM-GTX1 and 4) has a GTX1/GTX4 concentration ratio of 3, which was set as our goal for the ratio in mussel tissue RM. The first step therefore was to stabilise the epimer ratio, which had already reached 2 during storage, prior to blending with the mussel tissue. The effect of pH on the toxin epimerization in plankton was investigated. Epimerization is favoured at a high pH (7.6), but under such conditions the GTXs were rapidly destroyed (Figure 4). At a natural pH (5) an equilibration was achieved in a short period of time (30 min) with relatively little decomposition.

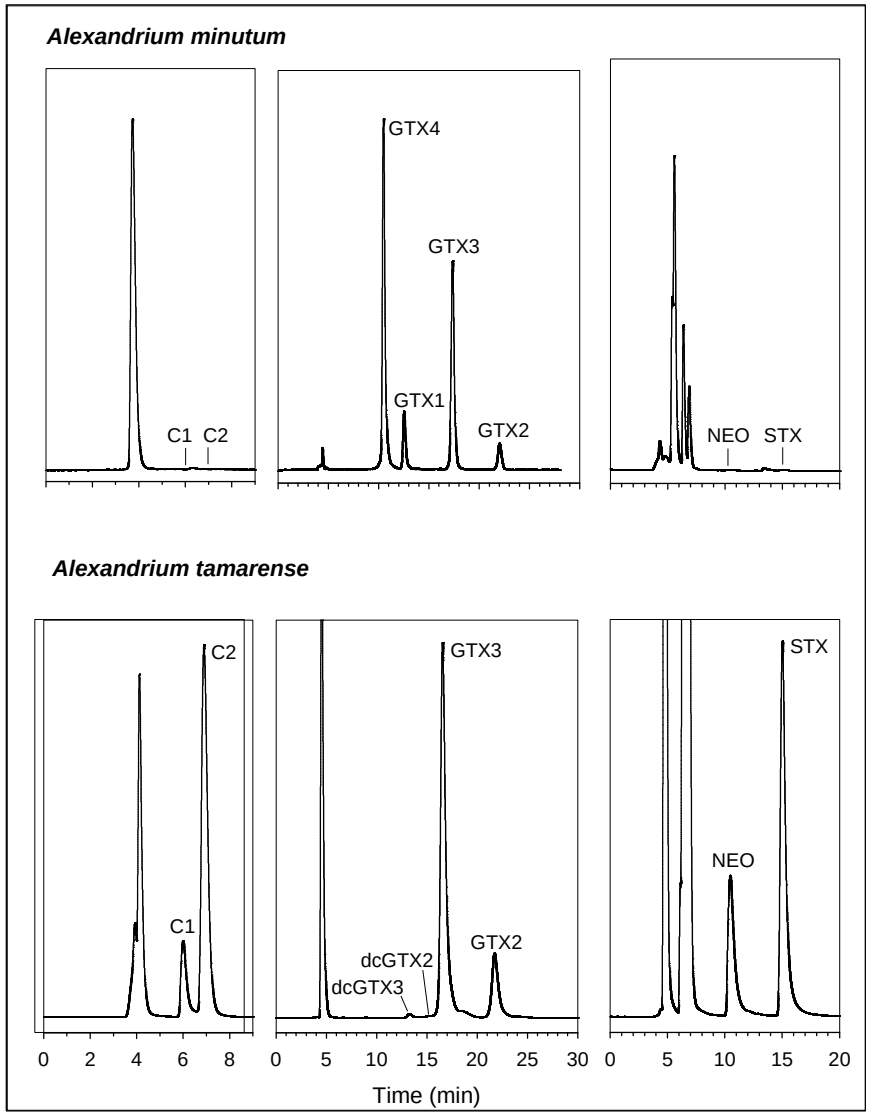

Figure 2. Plankton analyses using LC-pcr-FLD (modified Oshima method) (Oshima, 1995).

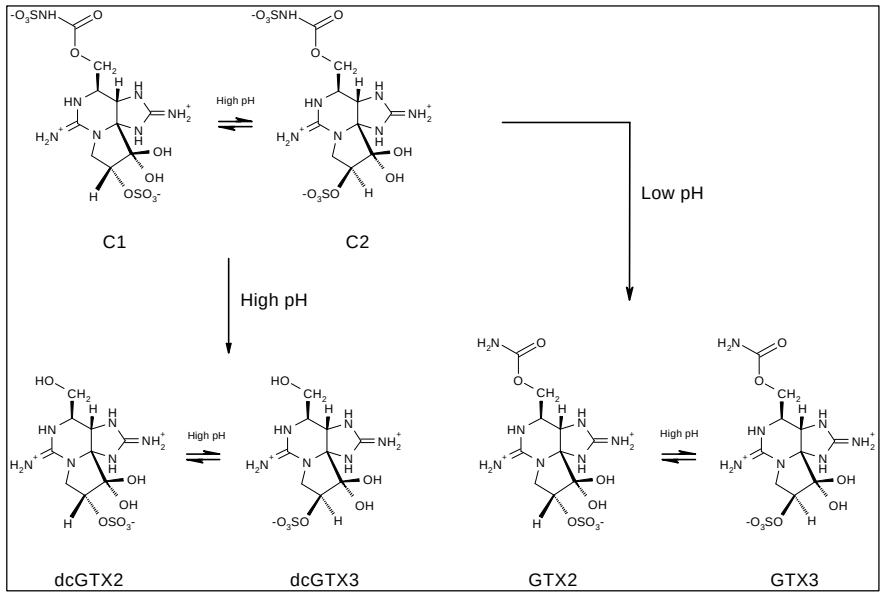

Figure. 3. Schematic of toxin interconversions within *A. tamarense*.

The role of *A. tamarense* was to provide GTX2 and 3, dcGTX2 and 3, NEO and STX. Our current calibration solution CRMs for the GTXs have stable concentration ratios of 3:1 (CRM-GTX2 and 3-b) and 3.5:1 (CRM-dcGTX2 and 3). These values were set as our goals for the ratios in the mussel tissue RM. In order to achieve these ratios the C toxins were first epimerised to a stable ratio by heating the plankton biomass. The plankton was then acidified and heated (100°C) until the C toxins were all converted to GTXs. Initial experiments with heating the AL18b plankton at a high pH of 7.6 resulted in the epimers reaching equilibrium quickly but this also resulted in a rapid conversion of the C toxins to dcGTXs. Heating at a natural pH of 5, equilibrium was reached with only a partial conversion of C toxins to dcGTXs (Figure 5). STX and NEO also decomposed partially during the treatments but especially under basic pH, which we concluded should be avoided during the epimerization.

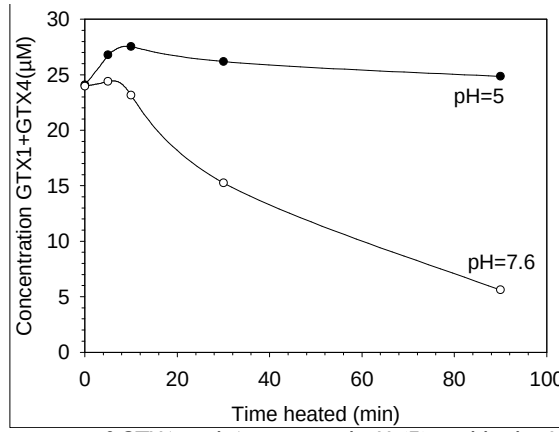

Figure. 4. Destruction of GTX1 and 4 at natural pH (5) and high pH (7.6) in AL1V plankton heated at 100°C.

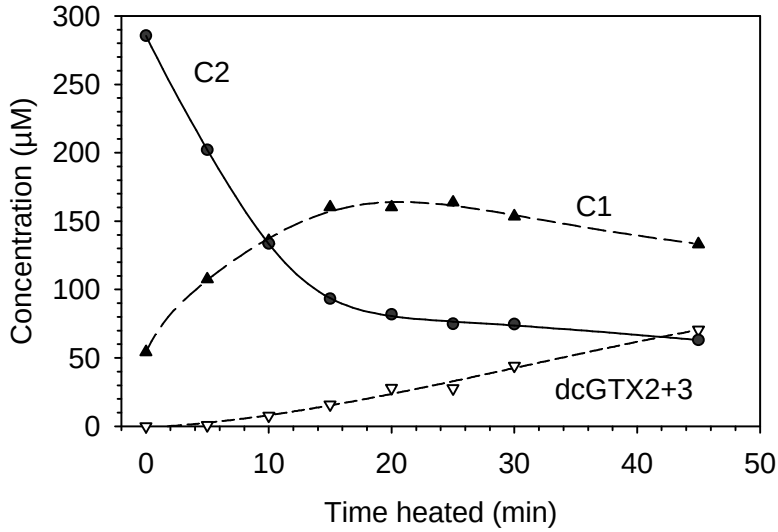

Figure. 5. Epimerization of C toxins and production of dcGTX2 and 3 in AL18b plankton at natural pH (5), heated at 100°C.

After epimerization of the C toxins and partial conversion to dcGTXs, the plankton must be hydrolysed to convert the unstable C toxins to the more stable GTXs. This may be accomplished by heating the plankton at low pH. We determined that an equal volume of 0.5 M HCl added to the plankton was sufficient to convert the C toxins in a reasonable amount of time without excessive dilution (Figure 6).

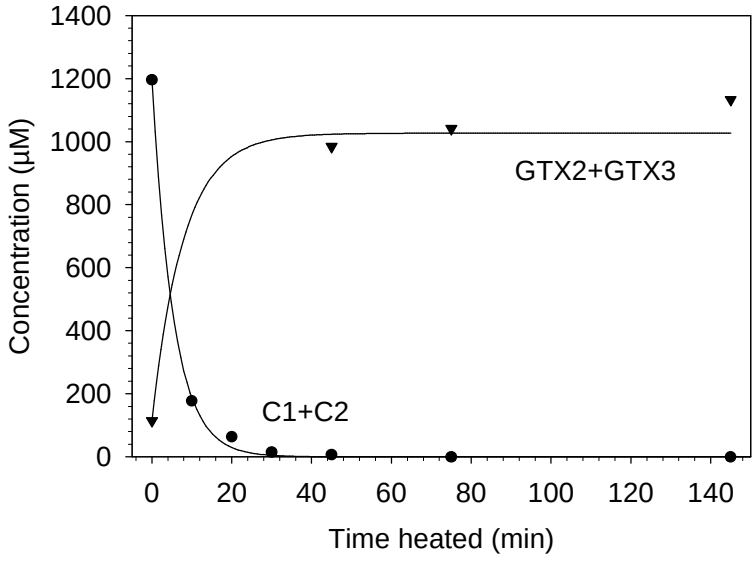

Figure. 6. Hydrolysis of C toxins and production of GTX2 and 3 in AL18b plankton at natural pH (5), heated at 100°C.

Preparation of RM-PSP-Mus-p: A. minutum (60 g) was diluted 1:1 (v/v) with deionised H_20 and heated. The temperature and toxin profile was monitored by LC-pcr/FLD until the GTX1/GTX4 ratio reached a stable equilibrium. A sample of *A. tamarense* (25 g) was diluted 1:1 (v/v) with deionised water and heated. The temperature and C toxin area ratios were monitored by LC-pcr/FLD until the C1/C2 ratio reached equilibrium. After a stable C1/C2 ratio had been reached, 0.5 M HCl was added to the plankton (1:1 v/v). It was heated and monitored by LC-pcr/FLD until C toxins were destroyed. The prepared plankton was stored overnight until the mussel homogenate was ready for plankton addition.

The mussel tissue used for this pilot study was a 9:1 mixture of cooked mussels from the Netherlands and Prince Edward Island, respectively. The former contained okadaic acid and the latter contained domoic acid. The presence of these additional toxins will be useful for our research projects on "universal" extraction. It was necessary to remove byssus thread fibres from the Dutch mussels to prevent tubing obstruction during filling of the bottles. The tissues were homogenised in a Robot Coupe RSI6 and the moisture content was determined by drying an aliquot of the tissue at 100°C. The prepared AL1V and AL18b plankton was added to the mussel homogenate along with deionised water to bring the final moisture content to 85 %. The final homogenate contains approximately 3 % plankton by weight. The pH of the prepared homogenate was adjusted with HCl from 5.5 to a final pH of 3.9 and was dispensed into polypropylene bottles, which were then sealed and thermally sterilised in a steam retort.

Extraction and analysis: Different extraction procedures are being developed and tested on this pilot scale RM for the eventual certification of the final CRM. A modification of the AOAC extraction procedure (boiling with 0.1 M HCl) was employed for this study but with an increased v/w ratio and volumetric control for accuracy. Initial extraction work on RM-PSP-Mus-p consisted of mixing 0.1 M HCl with the contents of 1 bottle of the RM-PSP-Mus pilot (approximately 8 g). The samples were exhaustively extracted by boiling the HCl/mussel tissue homogenate. The decanted supernatant was brought to a final volume of 25 mL. Samples were filtered and analysed by LC-pcr/FLD to determine the final toxin concentration. Our prepared pilot RM had a toxin level of approximately 4600 µg STX equivalents per kg whole tissue (see Table 1 and Figure 7).

Table 1. Toxin concentrations measured in RM-PSP-Mus-p.
[1]Oshima, 1995; some errors in the original data values have been corrected (Y. Oshima, personal communication)

Toxin	Concentration (µmoles/kg tissue)	Relative Toxicity (1)	µg STX equivalent per kg tissue
GTX-4	0.69	0.7261	186
GTX-1	2.73	0.9940	1009
dcGTX-3	2.19	0.3766	307
dcGTX-2	8.26	0.1538	473
GTX-3	1.64	0.6379	389
GTX-2	5.61	0.3592	750
NEO	1.42	0.9243	488
STX	2.61	1.0000	971
		Total µg STX equivalents/kg tissue:	**4600**

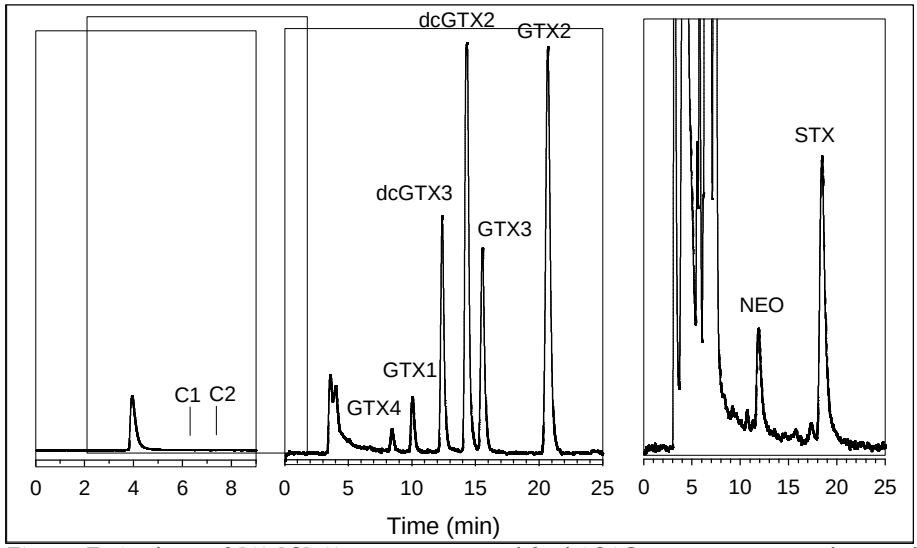

Figure 7. Analysis of RM-PSP-Mus-p using a modified AOAC extraction procedure and LC-pcr/FLD (modified Oshima method) (Oshima, 1995).

Conclusions

Our preliminary research has determined that a mussel tissue RM for PSP toxins can be produced by blending plankton and mussel tissue, after conversion of toxins in the plankton to a stable mixture. After further method development work and a stability study, a large-scale CRM will be prepared, hopefully in 2005.

Acknowledgements

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ISOLATION AND PURIFICATION OF AZASPIRACIDS

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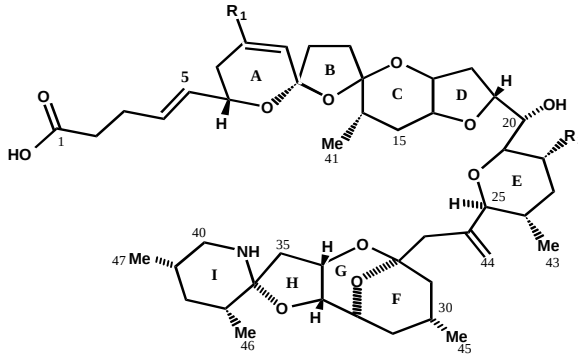
Abstract
Azaspiracids are recently discovered marine biotoxins known to accumulate in molluscs, such as blue mussels (*Mytilus edulis*) and oysters (*Crassostreas gigas*). Recent *in vivo* and *in vitro* studies indicate that a consumption of these toxins pose a serious threat to human health. A continuous monitoring of molluscan shellfish for human consumption is necessary to minimise the risk of shellfish poisonings. The aim of the present study to isolate azaspiracids in mg yields from naturally contaminated shellfish or algae to provide material for instrument calibration and further toxicology studies. The materials used for the isolation are the digestive glands of contaminated blue mussels from Irish waters. The isolation scheme follows a method developed by Satake, and includes extraction and partitioning steps and a number of different chromatography steps. So far, five isolation batches have been performed and a total of 1807mg AZA-1 has been obtained. Purity increased throughout batches with 70 % purity achieved in the fourth isolation batch. Several difficulties were encountered for those isolation steps involving normal phase open column chromatography, low pressure reversed phase chromatography (Develosil material), and the cation-exchanger step as well as the final separation on the ODP material. The overall recovery was very poor with the highest yield achieved being 18 % in the 5th batch. During the 5 performed batches several changes have been made to overcome these problems. Notable differences have been observed for mussels harvested from different sites and times of year.

Introduction
In 2003 the Marine Institute started a collaborative project funded by the NDP under the Marine RTDI programme called Azaspiracid Standards isolation and Toxicology (ASTOX). One of the aims of this project is the isolation of azaspiracids in their pure form for standard material and further toxicological research. The isolation and structural elucidation of azaspiracid was first described by Satake in 1998 (Figure 1). The reported scheme involved extraction with acetone in triplicate, a partitioning step with hexane and 85 % aq. methanol, a silica chromatography using a step gradient with three different mobile phases (acetone, acetone-methanol, methanol), gel permeation chromatography and ion exchange chromatography. A final clean up was achieved on gel permeation material. A very similar procedure was reported by Ofuji (1999). A further reverse phase chromatographic step was introduced using a Develosil ODS material. The final clean up was performed on a C18 polymer material which managed to separate further analogues of the toxin, AZA-2, and -3. The first aim of the project was to reproduce this scheme and then further optimise the steps.



	R_1	R_2
Azaspiracid-1	*H*	*Me*
Azaspiracid-2	*Me*	*Me*
Azaspiracid-3	*H*	*H*

Figure 1. Structure of azaspiracids as reported by Satake

Materials and Method:

1kg of digestive gland from blue mussels (*Mytilus edulis*) was obtained from 6kg of pre-cooked and shelled mussels. The hepatopancreas was extracted three times for the first two batches (1x acetone 3L, 2x methanol 3L) and twice (2x 3L methanol) for batches 3-5. The extract was evaporated to dryness. The extract was then partitioned between ethyl acetate (1L) and water (1L) and the aqueous phase was re-extracted with ethyl acetate (1L). The two ethyl acetate phases were combined and evaporated. The extract was then partitioned between hexane (1L) and 80:20 v/v methanol/water (1L). The hexane phase was re-extracted with 80:20 v/v methanol/water (1L). The aqueous phases were combined and evaporated. The sample was suspended in acetone and loaded onto a silica gel column (500 x 40mm) (Silica Gel 60 Merck). The column was eluted with a step gradient 100 % acetone/100 % methanol. The toxin was found in the methanol fraction. The sample was again evaporated and dissolved in 700:300:1 v/v/v propanol/water/acetic acid. It was then chromatographed on Toyopearl HW-40 (Tosoh) with 700:300:1 v/v/v propanol/water/acetic acid as mobile phase at 1mL/min. 5mL fractions were collected and the toxins were found to elute around fraction 6-9 (30-45min).

The sample was then further chromatographed on Develosil ODS (C_{18}) (Phenomenex) using 850:150:1 v/v/v methanol/water/acetic acid as mobile phase at 1mL/min. The toxic fractions were combined, evaporated and passed through a DEAE anion-exchanger (Tosoh). A step gradient was run from 80:20 v/v methanol/water to 850:150:1 v/v/v methanol/water/acetic acid at flow-rates of 0.5ml/min (60min) and 1mL/min (30min) respectively. The toxins were eluted in the latter fraction. The fraction was evaporated again and passed through a CM650 cation-exchanger (Tosoh) in the same manner. The final clean up was carried out on an ODP-50 (Asahipak) column. The mobile phase was 50:50 v/v methanol/water containing 0.1 % acetic acid at 0.5mL/min for five minutes and then a linear gradient to 100 % methanol containing 0.1 % acetic acid over 45 minutes (Figure 2)

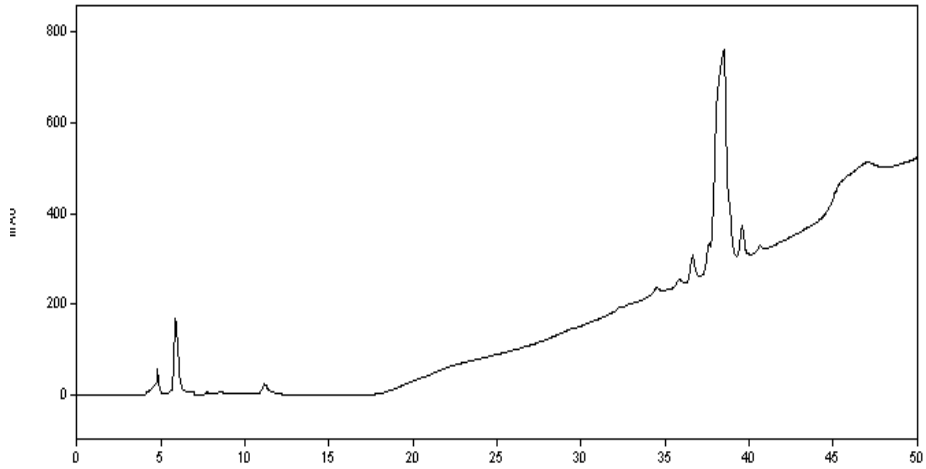
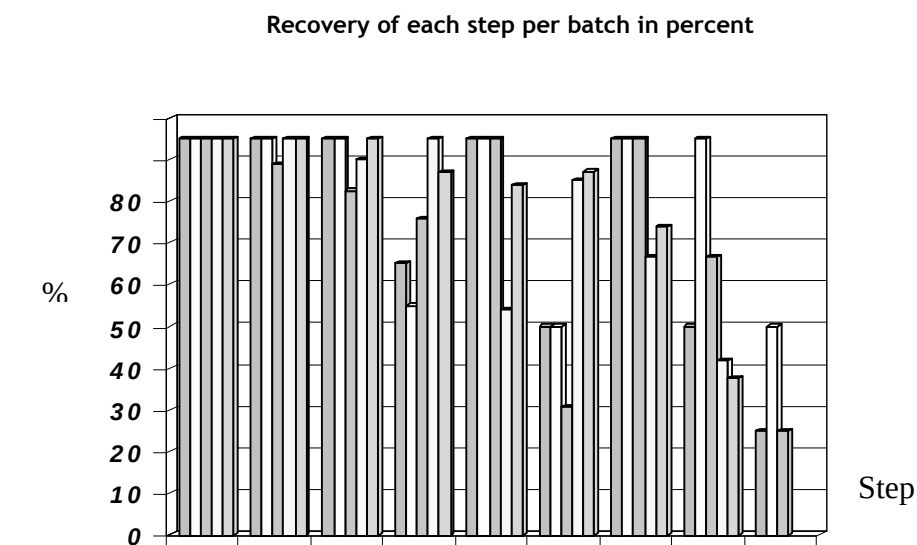


Figure 2. Chromatogram of the final clean up on ODP-50

Chromatography was carried out on Shimadzu Avp with Class VP 6.0. The toxic fractions were identified by LC-MS-MS (Waters 2795/ Q-tof Ultima Micromass) using an ACE C8 column 30 x 2.1mm i.d. (Advanced Chromatography Technologies). All solvents were obtained from LabScan.

Results.

The recovery for the extraction and the partitioning steps was 95 % and all of these procedures were carried out without problems. Suspension of the sample for the silica column involved great difficulties and a loss of toxin occurred in this step. The HW-40 column again had a recovery of 95 % and a very good clean up effect on the sample. When using the Develosil column with a rather dirty sample a good separation is not achieved. The toxins elute over a long period of time. Changing the flow-rate did not have a positive effect on the separation. While the anion exchanger retained the toxins as expected and eluted them in the desired fraction the cation exchanger did not seem to work as well. A major amount of azaspiracid eluted within the neutral fraction and was therefore not separated from any cationic compounds. The final separation on the ODP-50 material resulted in a significant loss of toxin and gave impurities in the final compound (See Figure 3 for recovery chart). The yield of AZA-1 was 150, 257, 130, 470, 800µg for batches 1, 2, 3, 4, 5 respectively. The purities of the obtained azaspiracid were determined to be between 30-70 %. Another observation made was the different behaviour of mussel material from different locations throughout the isolation. Batches 1, 2, 3, 5 were all done with mussel material from the same sampling location. Batch 4 was carried out with material from a different location. This material showed a different behaviour in the isolation process. During extraction the material foamed up. The ethyl acetate partitioning was hard to achieve as an emulsion formed that hardly separated. The chromatography on Develosil however, gave better results than any other batch. The anion exchanger did not perform as good as in other isolation batches.



	1	2	3	4	5	6	7	8	9
Batch 1	95	95	95	65	95	50	95	50	25
Batch 2	95	95	95	55	95	50	95	95	50
Batch 3	95	88.9	82.5	75.8	95	30.7	95	66.7	25
Batch 4	95	95	90	95	54	85	66.7	41.7	
Batch 5	95	95	95	87	83.8	87.1	74	37.5	

Figure 3[*]. Recovery chart for batches 1-5

Discussion:
The problems mentioned above were partly overcome by either changing parameters of the relevant step or replacing the step altogether. In order to increase the flow capabilities of the silica column the width of the column was changed from 20 to 40mm. This increased the flow-rate as well as eliminating problems of blockage due to a to thick layer of sample at the top of the stationary phase. Attemps at changing the flow-rate for the Develosil column in order to achieve better separation failed. Further to improve the cation-exchangers performance, experiments examining the influence of temperature were carried out. Increasing the temperature from 15°C to 35°C seemed to improve the performance significantly in batch 2, 95 % of the toxin eluted in the acidic fraction. Unfortunately this result could not be reproduced. Observation of the behaviour of different mussel tissues during various steps, indicate that the mussel tissue itself can have an effect on the success of an isolation batch

[*] Batch 4 and 5 have not been chromatographed on the ODP-50 column

Acknowledgments:
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COMPARISON OF METHODS FOR DETECTION OF NOROVIRUS IN SPIKED OYSTERS.

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Abstract
In the absence of culture methods for noroviruses, detection in foods relies on molecular detection by Reverse Transcription –Polymerase Chain Reaction (RT-PCR) on extracted viral RNA. We have tested four RNA extraction protocols for their sensitivities and robustness to detect norovirus in spiked oysters. One method seemed to be more sensitive than the others using RT seminested-PCR. Not much difference was observed between the four methods by a RT single-PCR approach.

Introduction
It is well documented that outbreaks of viral disease related to consumption of bivalve shellfish harvested in faecal-polluted areas occur (Le Guyader *et al.,* 1996, Lees 2000). Since oysters can filter 10-24 litres of water per hour - pathogens can be concentrated in the meat and give rise to human infections. In Europe, the pathogens of main concern are the gastrointestinal viruses like norovirus, which are known to cause outbreaks involving a large number of people, and Hepatitis A virus, which is known for its severe symptoms (Lees 2000).

Identification of enteric viruses from oysters implicated in gastroenteritis outbreaks can be difficult due to insufficient viral recovery and presence of PCR inhibitors. Several extraction procedures of viral nucleic acid from oysters have been described but few comparisons of their performance have been done. A typical protocol contains the following steps; homogenisation of tissue and viral elution, extraction and concentration of virus, RNA extraction and finally amplification of virus by the use of RT-PCR. In each of these steps there is a broad variation of procedures used by different laboratories, which calls for international consensus. During the implementation of methods for detection of virus in oysters, we evaluated different protocols for extraction of viral RNA to be used in the following RT-PCR detection. Thus the objective of the present study was to identify the most appropriate method for detection of norovirus (NoV) in oysters with respect to 1) sensitivity, 2) robustness and 3) ease of handling. This was done by comparing four different protocols for their ability to detect norovirus in spiked oysters.

Materials and Methods
Oysters from the Danish market, *Crasosstrea gigas* and *Ostrea edulis* harvested in France and Denmark respectively, were collected during the winter 2002-2003. Digestive glands were cut out, mixed and aliquoted in 1.5 g portions and stored at -80°C for further use. The 1.5 g portions were later spiked with 100 µl of 10-fold serial dilutions (range 10^{-1} to 10^{-4}) of stool samples earlier found positive for NoV and determined by sequencing to contain a genogroup II strain. The positive stool samples were kindly provided by Dr. Blenda Böttiger, Statens Serum Institute, Denmark. Before spiking with virus, all batches were examined and found negative for natural content of NoV using the method described by Le Guyader *et al.*, (1996) for RNA extraction and the genotype specific RT- seminested PCR primers

G1SKR/COG1F + G1SKR/G1SKF and G2SKR/COG2F + G2SKR/G2SKF for NoV amplification and detection (Kojima *et al.*, 2002 and Nishida *et al.*, 2003). The four selected protocols for detection of virus in oysters are shown in Table 1. The four protocols A, B, C and D are essentially according to Le Guyader *et al.*, 1996; De Roda Husman *et al.*, 2004; Mullendore *et al.*, 2002; and Beuret *et al.*, 2002, respectively. NoV amplification and detection was carried out by RT-PCR and using the generic primers JV13I/JV12Y targeting the RNA polymerase gene (Vinjé *et al.*, 1996; and Vennema *et al.*, 2002) and the genogroup II specific RT-seminested PCR primers G2SKR/COG2F + G2SKR/G2SKF in the capsid region (Kojima *et al.*, 2002; and Nishida *et al.*, 2003). RT-PCR reactions were run on a PTC-225 Peltier Thermal Cycler, (MJ Research). The level of recovery was based on the highest virus dilution giving a clear positive signal after RT-PCR, which was visualised on a 2 % ethidium bromide stained agarose gel and subsequent confirmed by sequencing (MWG-Biotech, Germany). All four protocols were randomly applied on triplicates of oyster tissue seeded with the different dilutions (10^{-2} – 10^{-4}) of NoV positive stool samples.

Results and Discussion

All the methods could be implemented in the laboratory and detect NoV from 1.5 g of seeded oyster tissues see table 2. Method B was found to be the most sensitive method. Thus using method B we were able to detect NoV in all 3 samples spiked with NoV positive stool samples diluted 10^{-4} when the RT seminested - PCR primers was applied for detection of viral RNA. RT seminested – PCR is known to have an advantage compared to ordinary RT- PCR when samples contain high amounts of inhibitors. None of the other methods were able to detect NoV in this dilution. In addition method B was faster and easier to handle than any of the other methods in this study. On the other hand, little difference was observed between the four methods when a single RT-PCR approach was used for detection of NoV. This may indicate that the better sensitivity obtained with method B is more likely due to a good viral recovery rather than to removal of inhibitors. A possible improvement of inhibitor removal could be to include a chloroform extraction step prior to the use of the RNeasy Plant and Fungi kit. Method A and B are chosen for further use in surveillance of naturally contaminated molluscan shellfish from Danish harvest areas.

Table 1. Principles of the four RNA extraction methods for virus-oyster processing used for recovery of norovirus in the present study.

	Methods			
Processing steps	A (Le Guyader *et al.*, 1996)	B (De Roda Husman *et al.*, 2004)	C (Mullendore *et al.*, 2002)	D (Beuret *et al.*, 2002)
Homogenisation / virus elution	Glycine pH = 9.5	Tissue disruption in a Mixer Mill (Bead Beater) with Lysisbuffer, RTL and zirconia beads, followed by centrifugation	Acid absorption of viral particles to tissue solids. Glycine elution pH=7.5 Re-elution of pellet in threonine. PEG precipitation.	Glycine elution pH=7.5 Re-elution of pellet in threonine. PEG precipitation.
Virus extraction	Chloroform/ Butanol + CatFloc-T.	-	PBS+Chloroform Chloroform-phase re-extracted with threonine.	PBS+ Chloroform.
Concentration	PEG precipitation	-	PEG precipitation followed by GITC wash	PEG precipitation followed by GITC wash
RNA extraction	Proteinase K + phenol/ chloroform Ethanol precipitation Cethyltriamin borat wash Ethanol precipitation	Rneasy® MiniKit, Plant and Fungi, QIAgen.	QIAamp Viral RNA Mini Kit, QIAgen.	QIAamp Viral RNA Mini Kit, QIAgen.

Table 2. Detection of norovirus from seeded oyster tissue processed by four different RNA extractions methods, A, B, C and D. For detection of virus two conventional detection formats RT-PCR and RT-seminested PCR were used.

Detection format	Artificially contaminated oyster tissue	RNA processing methods [1)]			
	(ml stool/1.5 g oyster)	A	B	C	D
RT- PCR	1×10^{-2}	2/3	3/3	2/3	3/3
	1×10^{-3}	2/3	3/3	2/3	1/3
	1×10^{-4}	0/3	1/3	0/3	0/3
RT-seminested PCR	1×10^{-2}	3/3	3/3	2/3	2/3
	1×10^{-3}	3/3	3/3	1/3	1/3
	1×10^{-4}	0/3	3/3	0/3	0/3

1: See table 1 for keys.

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ANALYSIS OF PSP TOXINS BY LIQUID CHROMATOGRAPHY WITH POST COLUMN OXIDATION AND FLUORESCENCE DETECTION

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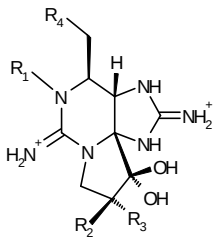
Abstract
One of the most commonly used chemical methods for the analysis of PSP toxins is liquid chromatography with post-column oxidation and fluorescence detection (LC-pcr-FLD). Unfortunately, to achieve complete separation of all the PSP toxins with the existing method, it is necessary to use three separate analyses on each sample, each using a different mobile phase and/or column. Described here is work towards the development of an improved gradient method, which will allow the analysis of GTXs, STX and NEO in a single run, thus reducing the number of runs for each sample from three to two. In addition, the development of a microbore column system based on a miniaturised post-column reaction system enabling the analyses of smaller samples will be shown. An alternate oxidant was also investigated.

Introduction
Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins, produced by several marine dinoflagellates. These toxins are tetrahydropurine derivatives based on the parent compound, saxitoxin (STX) and can be divided into three classes: carbamate, N-sulfocarbamoyl and decarbamoyl toxins (Figure 1). There are 18 principal structural analogs but many more have been identified.



STX = saxitoxin
NEO = neosaxitoxin
GTX = gonyautoxins

R_1	R_2	R_3	R_4: Carbamate toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins
			$-O-C(=O)-NH_2$	$-O-C(=O)-NHSO_3^-$	$-OH$
H	H	H	STX	GTX5 (B1)	dcSTX
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	H	H	NEO	GTX6 (B2)	dcNEO
OH	H	OSO_3^-	GTX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Figure 1. Structures of the principal PSP toxins.

The enforcement of regulatory directives on food contaminants such as PSP toxins requires validated analytical methods. The AOAC mouse bioassay is the reference method used in most regulatory laboratories today. The technique is a reliable means of protecting human health but does suffer from a poor detection limit (just half the regulatory level of 800 µg/kg edible tissue), poor precision (20-30 % CV), matrix effects that can result in negative bias at low levels and false positives due

to other contaminants (e.g., zinc in oysters). It also provides no information on the toxin profile. Due to the cruel nature of the test, it has been banned in some countries. Therefore it is urgent that a suitable replacement method be developed.

Earlier work by Bates and Rappoport (1978) showed that an oxidizing agent under basic conditions could oxidise saxitoxins to form fluorescent purines. This very selective chemistry led to the development of a method based on the combination of liquid chromatography with post-column oxidation and fluorescence detection (LC-pcr-FLD) (Oshima, 1995). There is an alternative method (Lawrence, 1991) that involves oxidizing the PSPs prior to LC-FLD. This pre-column oxidation method is simpler and faster, but some of the toxins give rise to the same oxidation products (Quilliam *et al.,* 1993). This can increase sample preparation time, as some of the toxins must be separated prior to oxidation. Data processing can also be arduous.

Unfortunately, to achieve complete separation of all the PSP toxins, it is necessary to use three separate analyses for each sample, each one using a different mobile phase and/or column. One is used for STX and NEO, another for GTXs and yet another for C toxins. Our investigation had the following objectives:

1) Develop and optimise a gradient separation of PSP toxins with ion-pair chromatography that allows the analysis of GTXs, STX and NEO in a single run.
2) Optimise the post-column oxidation reaction including investigate an alternate oxidation reagents.
3) Develop a microbore column system based on a miniaturised post-column reaction system that could save on reagent use and enable analysis of much smaller samples such as plankton.
4) Characterise the system in terms of detection limits and linearity and apply the optimised system to various samples.

Experimental
Experiments were performed on an Agilent HP1090 HPLC equipped with ternary solvent delivery system and variable injector (0 to 25 µL). The post column reaction system consisted of a Waters Post Column Reaction Module. The reaction coils used were knitted Teflon tubes (Supelco KRC 5-25, 5 m x 0.25 mm i.d.) with a total volume of 250 µL or a 1000 µl coil (Waters PN 030805)). Reagents for the post column oxidation chemistry were delivered using Eldex Recipro PEEK metering pumps (Model # A-10-S-PK). Gradient methods were performed using a Zorbax Bonus column (2.1mm X 150mm or 4.6mm X 250mm, Agilent, CA, USA). Analysis of C-toxins were performed separately using a Beta Basic-C8 column (2.1mm X 250mm or a 4.6mm X 250mm, Thermo Electron Corporation, MA, USA). Mobile phase compositions and post column reaction temperatures are described in the figure captions. The fluorescence detector was a Shimadzu RF551 detector fitted with either a large or micro flow cell (12 µL or 2 µL, respectively). PSP toxins were detected by excitation and emission wavelengths 330 and 390 nm, respectively.

Results and Discussion
Optimization of post column reaction: The optimal conditions for periodate oxidation were found to be the following: (a) maintenance of the reaction coil at 80°C; (b) oxidation reagent: 100mM ammonium phosphate with 5mM periodate at pH 7.8; and (c) acidification of effluent with 0.75M nitric acid prior to fluorescence detection. We also investigated *tert*-butyl hydrogen peroxide (tBHPO) in sodium hydroxide as an alternative oxidation reagent as it has the advantage that it does

not contain high levels of insoluble salts that could block the reaction coil. Different conditions were investigated including concentration of tBHPO, reaction temperature, concentration of sodium hydroxide and addition of ethanol to the oxidant. The optimum conditions for this oxidant were found to be 0.3 % tBHPO in 0.5 M NaOH with no losses in sensitivity compared to the periodate oxidant. A maximum temperature of 80°C was used for reaction because much beyond this causes bubbles to form. The tBHPO oxidant seemed very reliable with wide bore column system and was easy to use, but when we switched to the micro system, there was formation of air bubbles in our reaction coil that were impossible to eliminate. Therefore, we returned to using the periodate oxidant for the micro gradient work.

Column selection: A variety of columns were tested in our investigation. The Lichrosphere 100 RP18 gave good selectivity for separation of dcSTX and STX, as well as adequate separation of GTXs. The Betabasic could not resolve dcSTX from STX. The Zorbax Eclipse exhibited the best separation in the area of dcGTX2and3 and GTX5. However, we experienced problems with phase collapse after as little as 100 injections with some of these columns. Other columns tested include Phenomenex Aqua C18, Waters X-Terra, Supelco LC-308, Supelco LC-318, Zorbax Bonus, Keystone Beta Basic C8, and Vydac C18. After discussions with Agilent Technologies, they recommended the Zorbax Bonus-RP column.

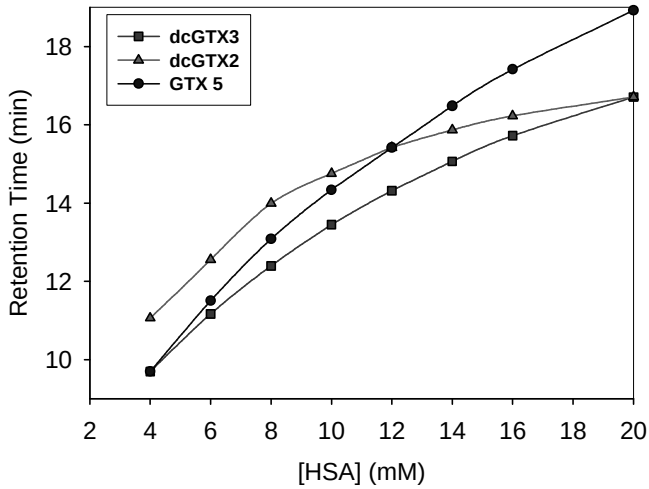


Figure. 2. The effect of heptane sulfonate (HSA) concentration on the separation of dcGTX2, dcGTX3 and GTX5.

Optimisation of mobile phase: The effects on separation selectivity of different concentrations of heptane sulphonate and phosphate in the mobile phase were investigated. The graph in Figure 2 illustrates one such detailed study of the effect of heptane sulphonate concentration on the separation of dcGTX2, dcGTX3 and GTX5. Various gradient curves were tested and we finally selected a step gradient because it was the easiest system in which to optimise separations at different points in the chromatograms. It also lends itself to use with a less expensive HPLC system in which just an isocratic pump and a switching valve could be used. Figure 3

shows the final separation on a 25 cm x 4.6 mm i.d. column. A faster analysis time is possible by executing the gradient step earlier but the separation of NEO and dcNEO begins to collapse. If that separation is not important, then an analysis time of less than 50 min is possible.

Optimisation of detection limits: The analysis of small samples can benefit greatly from the use of microbore LC systems. Much smaller sample sises are required when using narrow bore columns as long as the detector sensitivity does not have to be modified and if there is no loss of resolution due to the volume of the post column reaction system. Usually to accommodate micro columns, detector cell volumes must be reduced in size to avoid band broadening thus reducing fluorescence sensitivity. We therefore tested the use of small and large cell sizes as well as different injection volumes with a 2 mm i.d. column. In addition, we scaled down the post column reaction system to minimise band broadening (Figure 4). It appears possible to use the more sensitive large cell sise with only a slight loss of resolution and to be able to push the injection volume up as high as 10 µL (this gives a result equivalent to 50 µL injected on a 4.6 mm column). Good detection limits were measured (see Table 1) and responses for the different toxins were very linear with concentration in injected sample.

Application to real samples: The micro column LC-pcr-FLD method has been applied successfully to a variety of samples. Figure 5 shows the analysis of extracts of two different plankton samples and a mussel tissue reference material.

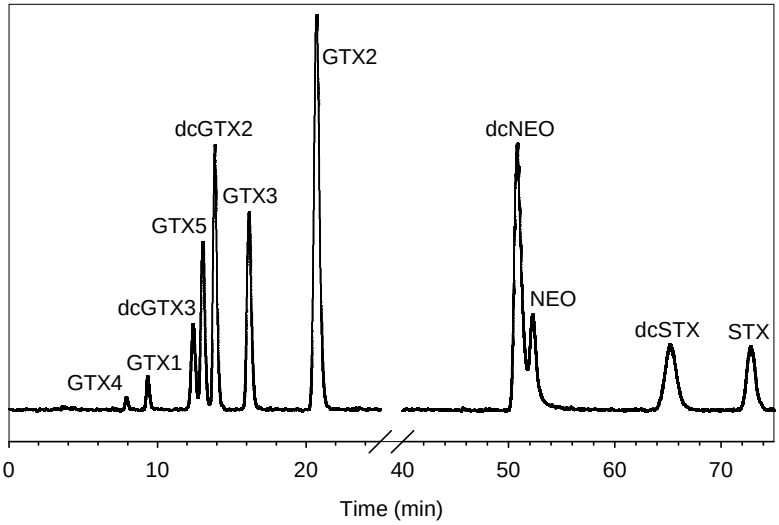


Figure 3. PSP analysis using a Zorbax Bonus-RP column (25 cm x 4.6 mm i.d., 5 µm particle size). Mobile phase A: 20 mM heptane sulphonate, 10 mM ammonium phosphate, pH 7.1. Mobile phase B: 30 mM ammonium phosphate, 11 % acetonitrile. Mobile phase C: 10 mM ammonium phosphate, pH 7.1. Gradient: 40 % A + 60 % C, hold to 17 min, step to 40 % A + 60 % B at 17.1 min, hold to 75 min. Ox = tBHPO, 0.4 ml/min; H^+ = 0.75M nitric acid, 0.4 ml/min.

Table 1. Detection limits of micro (2 mm i.d.) system using LC-pcr-FLD with large flow cell compared to the relative toxicities of different PSP toxins.

	Relative Molar Response	Limit of Detection (µM)	Relative Toxicity
dcGTX3	100	0.009	38
GTX3	91	0.008	64
GTX2	43	0.02	36
dcGTX2	21	0.03	15
dcSTX	11	0.2	51
GTX4	10	0.05	73
STX	5.9	0.2	100
GTX1	3.7	0.1	99
NEO	3.6	0.2	92
GTX5	3.6	0.4	6.4
dcNEO	3.0	0.5	*not available*

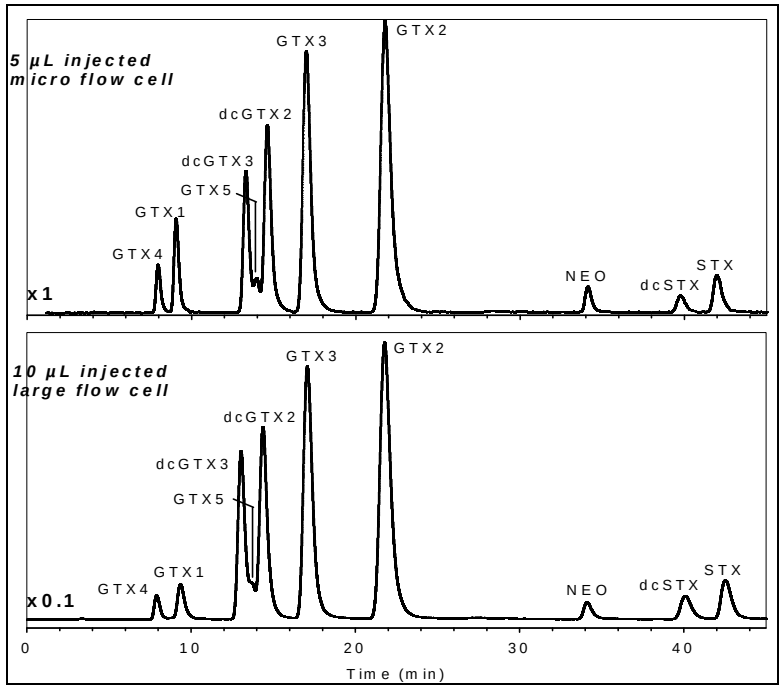


Figure. 4. PSP analysis using a microbore Zorbax Bonus-RP column (10 cm x 2 mm i.d., 3.5 µm particle size) using both small and large detector flow cells. Mobile phase A: 20 mM heptane sulphonate, 10 mM ammonium phosphate, pH 7.1. Mobile phase B: 30 mM ammonium phosphate, 23 % acetonitrile. Mobile phase C: water. Gradient: 55 % A + 45 % C until 22 min, step to 55 % A + 35 % B + 10 % C at 22.1 min and hold. Ox = periodate, 0.1 mL/min, H^+ = 0.75 M HNO_3, 0.1 mL/min.

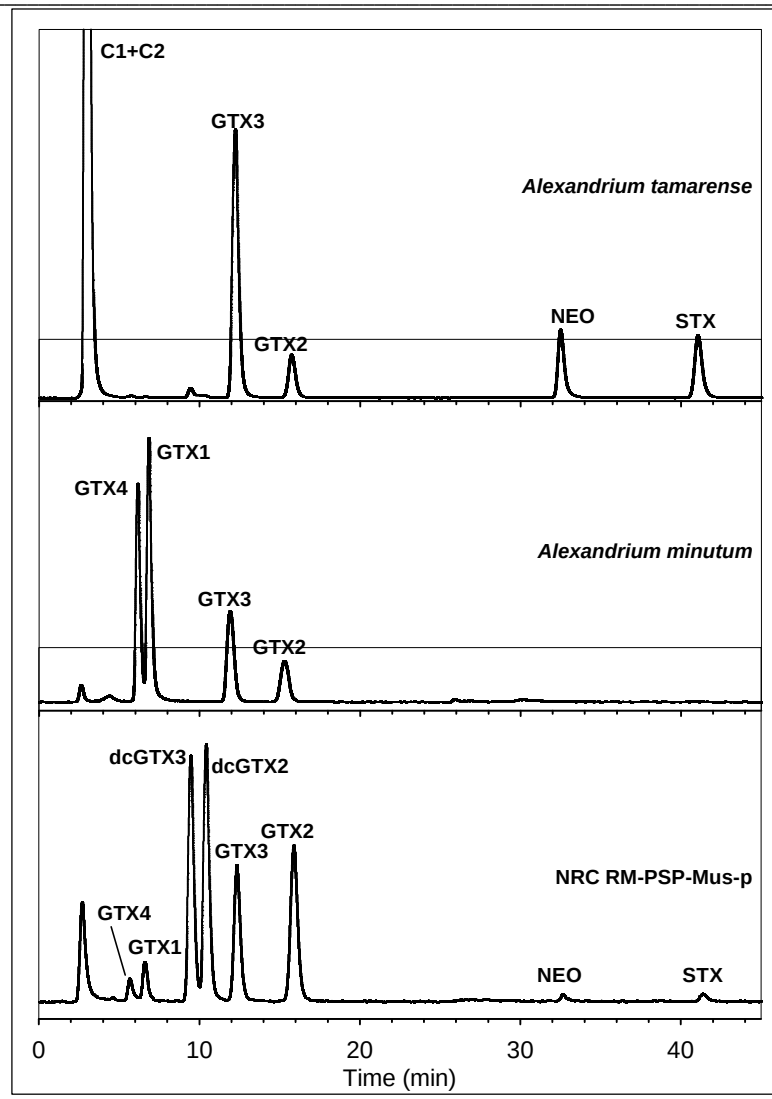


Figure 5. Analysis of PSP toxins in two plankton samples and a mussel tissue reference material using the microbore Zorbax Bonus-RP column.

Conclusion

The gradient system described herein allows the determination of GTX1-6, dcNEO, NEO, dcSTX and STX in the same analysis. The common problem of column collapse was remedied by switching to the Bonus RP column, which also provided the necessary separation selectivity. The micro post column reaction system would be advantageous when attempting to analyse small samples of limited size such as plankton or cysts. Future work will include a microbore system for the analyses of C toxins, as well as a column switching technique for determination of all PSP toxins in one analysis.

Acknowledgements

We thank Dominik Weschler for technical assistance. We are grateful for funding from the Trade and Investment Liberalisation and Facilitation fund of the Asia-Pacific Economic Cooperation (APEC) and the National Research Council of Canada.

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CERTIFIED REFERENCE MATERIALS FOR MARINE TOXINS

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Abstract
The main focus of National Research Council's Certified Reference Materials Program (CRMP) in Halifax is the production of certified reference materials (CRMs) for toxins of marine algal origin. These include both calibration solutions and shellfish tissue homogenates. Proper calibration standards are crucial to ensure accurate analyses are performed in a regulatory setting. The preparation of calibration standards requires careful attention to purification, stability determination and quantitation. Periodic checks on certified concentrations, controlled storage conditions, an efficient distribution system and a process to replenish CRMs are also required. A description of CRMs and their use will be presented, along with procedures involved in the production and certification of CRMs.

Introduction
The National Research Council's Certified Reference Materials Program (CRMP) began producing toxin standards in response to the domoic acid crisis in Canada in 1987. Since then, the program has expanded to include calibration solution CRMs and shellfish tissue CRMs for a variety of toxins of marine algal origin, including those responsible for amnesic, diarrhetic and paralytic shellfish poisoning (Table 1). The lack of accurate calibration standards for algal toxins has been and still is a significant problem in the development and implementation of analytical methods for routine monitoring of seafood. In addition, regulatory labs now face the need to operate under GLP and ISO guidelines, which require validated methods, accurate calibration standards, and CRMs. The goals of CRMP include the production and marketing of CRMs as well as the development and validation of methods for the reliable analysis of phycotoxins in plankton and shellfish, all of which will help analysts obtain more accurate results. CRMP works to disseminate information on methods, standards, and reference materials to analysts worldwide with product certificates and publications, and through collaborations with a number of other organizations. This work is being performed in part with funding from the Trade and Investment Liberalisation and Facilitation fund of the Asia-Pacific Economic Cooperation (APEC). Our collaborating organizations (FRS [UK], Marine Institute [Ireland], Cawthron Institute [NZ] and AgResearch [NZ]) provide in-kind contributions such as plankton or shellfish tissues, purified toxin or technical expertise. Collaborators may also receive part of the final product in return for their efforts. By definition, a reference material (RM) is a material or substance for which one or more property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values. A *CRM* is an RM that is accompanied by a certificate and for which one or more property values are certified by a procedure that establishes its traceability to the units in which the property values are expressed (an uncertainty value must also be provided). A CRM must be representative of the sample matrix of interest, homogeneous, and stable with respect to analyte and matrix. It must be accurately certified using at least two independent, definitive methods. Alternatively, an interlaboratory consensus or a mixture of interlaboratory and independent methods can be used to achieve certification. RMs are useful for the verification of the correct application of

standardised methods, as well as the development and validation of new methods of measurement. CRMs can be used in the laboratory for the calibration of instruments and assays, as well as the calibration of secondary standards and in-house RMs. CRMs are crucial to quality assurance and quality control of routine analyses and the validation of entire methods in routine operation. Tissue RMs are important for method development, determining extraction and cleanup recoveries, and testing the full implementation of a method. The preparation of calibration solution CRMs requires careful attention to toxin purity and stability. Accurate quantitation involves a cross-comparison of results from different procedures, including gravimetry, nuclear magnetic resonance (NMR) spectroscopy, and separation methods, such as liquid chromatography (LC) and capillary electrophoresis (CE) coupled with diverse detection systems such as ultra-violet (UVD), fluorescence (FLD), mass spectrometry (MS) and chemiluminescence (CLND). Periodic checks and eventual replenishment of CRMs are part of the CRMP program.

Table 1. Toxin calibration solution CRMs and mussel tissue CRMs from NRC-CRMP

Amnesic Shellfish Poisoning (ASP) Toxins	
NRC CRM-DA-d	domoic acid calibration solution
NRC CRM-ASP-Mus-c	mussel tissue CRM for ASP toxins
Diarrhetic Shellfish Poisoning (DSP) Toxins	
NRC CRM-OA-b	okadaic acid calibration solution
NRC CRM-DSP-Mus-b	mussel tissue CRM for DSP toxins
Paralytic Shellfish Poisoning (PSP) Toxins	
NRC CRM-STXdiAc	saxitoxin calibration solution
NRC CRM-STX-d	saxitoxin dihydrochloride calibration solution
NRC CRM-dcSTX	decarbamoylsaxitoxin calibration solution
NRC CRM-NEO-b	neosaxitoxin calibration solution
NRC CRM-dcNEO	decarbamoylneosaxitoxin calibration solution
NRC CRM-GTX1and4-b	gonyautoxin-1 and -4 calibration solution
NRC CRM-GTX2and3-b	gonyautoxin-2 and -3 calibration solution
NRC CRM-GTX5	gonyautoxin-5 (B1) calibration solution
NRC CRM-dcGTX2and3	decarbamoylgonyautoxin-2 and -3 calibration solution
NRC CRM-C1and2	N-sulfocarbamoylgonyautoxin-2 and -3 calibration solution
Other Toxins	
NRC CRM-PTX2	pectenotoxin-2 calibration solution
NRC CRM-SPX1	13- desmethyl-spirolide C calibration solution
NRC CRM-GYM	gymnodimine calibration solution
Under development:	
Paralytic Shellfish Poisoning (PSP) Toxins	
NRC CRM-YTX	yessotoxin calibration solution
NRC CRM-C3and4	N-sulfocarbamoylgonyautoxin-1 and -4 calibration solution
NRC CRM-GTX6	gonyautoxin-6 (B2) calibration solution
NRC CRM-dcGTX1and4	decarbamoylgonyautoxin-1 and -4 calibration solution
NRC CRM-PSP-Mus	mussel tissue CRM for PSP toxins
NRC CRM-Zero-Mus	mussel tissue CRM with no toxins present
Other Toxins	
NRC CRM-PTX2sa	pectenotoxin-2 seco acid calibration solution
NRC CRM-AZA	azaspiracid calibration solution

Results and Discussion

Production and purification of toxins: Toxic strains of algae are grown in large quantities to produce the biomass needed to isolate the toxins for the calibration solutions. In some instances, the required toxin may be isolated from toxic shellfish. Occasionally, toxins are derived from others via semi-synthetic operations. For example, the primary PSP toxin produced by our culture of *Alexandrium tamarense* is C2. The following conversions have been performed: C2 → C1 → GTX2/3 → STX → dcSTX; C2 → dcGTX2/3; and C2 → GTX5 (Laycock *et al.*, 1995). Toxins are taken through several stages of preparative chromatography in order to achieve a high degree of purity (Laycock *et al.*, 1994). *Purity analyses:* Isolated toxins are analysed with a variety of methods to ensure there are no significant impurities that will interfere with the intended use of the CRM. For example, the PSP toxin CRMs are designed mainly for LC-FLD and LC-MS analyses, so it is important that there be no unresolved impurities. The purity of the GTX5 used in the NRC CRM-GTX5 calibration solution was determined using a combination of analyses (Figure 1.) including LC-FLD, CE-UVD, LC-CLND, LC-MS and NMR.

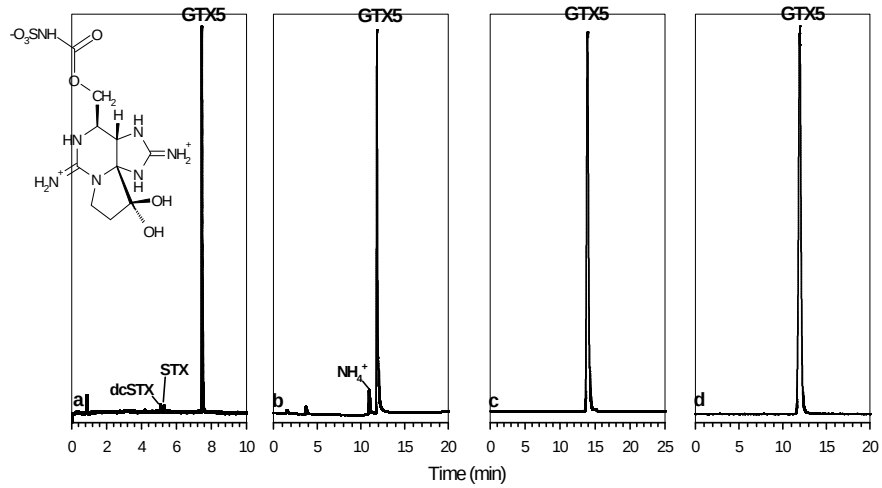

Figure 1. Purity analyses of GTX5 using: (a) capillary electrophoresis with diode-array detection; (b) LC with chemiluminescence nitrogen detection; (c) LC-fluorescence with post column oxidation; and (d) LC-MS.

Stability studies

Stability studies are important in preparing a CRM. Valuable quantities of toxins are used in the production of a product and we need to be confident of the stability of the final product during storage (which may be 5 years) and shipment. Parameters investigated include type of solvent and sensitivity to oxygen, light, pH, and temperature (Figure 2). Increased temperatures are used to accelerate decomposition which otherwise may be difficult to detect over relatively short periods of time. For the PSP toxins, special consideration must be given to toxins that exist as epimeric pairs that can interconvert (e.g., GTX2 and GTX3). It is important to stabilise the pair to an equilibrium ratio and to measure their individual molar concentrations. It should be noted that relative molar responses vary between individual PSP toxins, so it is important to have standards for all individual toxins.

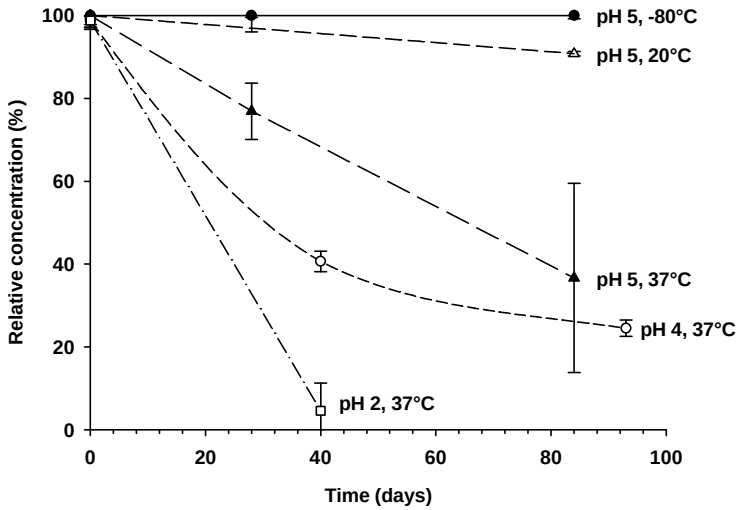

Figure 2. Stability of GTX5 under various storage conditions.

Quantitation

For many toxins, which may be isolated in only 10-100 mg quantities, it is difficult to determine the amounts of associated salts, counter-ions or water of hydration and therefore simple weighing of materials may be difficult to do reliably. PSP toxins are particularly difficult because they cannot be crystallised easily, are hygroscopic and are in the form of salts. Quantitation of a CRM is done using at least two independent methods. Molar responsive systems are used to determine the concentration of the toxin in a stock solution, which is then diluted accurately. In Table 2, three PSP toxin calibration solution CRMs were quantitated using NMR (Walter *et al.*, 2005) and LC-CLND (Quilliam *et al.*, manuscript in preparation). A routine check 6 months later revealed good agreement with previous measurements.

Table 2. Assignment of concentrations (μM) of some PSP toxin CRMs.

	CRM-STX-d	CRM-NEO-b	CRM-dcSTX
NMR	66 ± 3	63.8 ± 0.9	62.1 ± 0.8
LC-CLND	65 ± 1	65.7 ± 0.5	62.5 ± 0.5
Certified value	65 ± 3	65 ± 2	62 ± 2
6-month check (LC-CLND)	65 ± 1	65 ± 3	62 ± 3

Production of standards

When a solution has been prepared using the purified toxin, it is then placed into ampoules using an automated ampouling machine. Each ampoule is pre-purged with argon, filled with a small amount of solution, then immediately flame sealed to prevent any evaporation. After the ampouling process is completed, each individual ampoule is inspected for proper volume and seal, then labelled with a number that indicates the order of filling. A representative number of ampoules (usually 1.5 % of the total prepared) are selected to test for homogeneity of the analyte concentration throughout the whole set. Each product is then stored under specific conditions that are continuously monitored. Careful planning is needed to ensure

each product is replaced when stocks are depleted. As well, concentrations must be checked on a regular basis to ensure the certified values hold true.

Production of tissue CRMs
For any analytical method, it is important to test the entire method for accuracy with a tissue CRM. To produce such a CRM, large amounts of naturally incurred tissue homogenates are treated with antioxidant, de-aerated under vacuum, and dispensed into small bottles. These are sealed and taken through a steam retort process for sterilization. After inspection and receiving a unique number according to the order of filling, each bottle is individually heat-sealed in trilaminate pouches. Toxin concentrations are measured using at least two independent and complimentary analytical methods. Each method of quantitation uses an independent calibrant.

Distribution
CRMP products are sold around the world, therefore shipments must be planned and monitored carefully to ensure the products are held under the appropriate conditions should the package be held up in customs or encounter other unusual circumstances. Once a product is sold and shipped to the customer, the certified values are typically guaranteed for one year, providing the product has not been opened and has been stored under the specified conditions.

Conclusions
The National Research Council's CRMP is addressing the lack of reference materials for algal toxins by expanding its product line of calibration solution CRMs and shellfish tissue CRMs. Particular care is taken to ensure accuracy of concentrations and to investigate the stability of the CRMs. CRMP is now the world's primary distributor of toxin CRMs.

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MONITORING DSP TOXINS WITH IMMUNOASSAY, LC-MS AND MOUSE BIOASSAY

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Abstract
In Portugal, diarrhetic shellfish poisoning (DSP) is a very recurrent gastrointestinal illness resulting from consumption of contaminated shellfish. We compared a commercial immunoassay kit – 'DSP-Check' - against LC-MS, both techniques employing the same whole flesh final extract, subjected to prior alkaline hydrolysis in order to detect simultaneously the esterified and non-esterified toxin forms. A good correlation was found when analysing raw LC-MS data, and was further improved when taking into account the specific cross-reactivity of dinophysistoxin-2, which is lower than okadaic acid. Mouse bioassays using whole flesh and acetone extractions followed by ethyl acetate partitioning were performed for three prominent vectors: blue mussel, common cockle and donax clams. For blue mussels a good correspondence between survival times and LC-MS was obtained. For donax clams and cockles the mouse bioassay often failed to detect levels close or higher than the current EU limit of 16 µg DSP/100g. One explanation for this discrepancy might be the high percentage of acyl esters present in these species, which are poorly solubilised by the detergent used for ressuspending the final dried residue.

Introduction
Diarrhetic shellfish poisoning (DSP) is a very recurrent gastrointestinal illness resulting from consumption of contaminated shellfish, causing annually prolonged harvest closures in lagunar areas of the northwest and open sea areas of the south coast of Portugal. Okadaic acid (OA) has been found to contaminate shellfish from spring until autumn, while dinophysistoxin-2 (DTX2) is found particularly in late summer and autumn. *Dinophysis acuminata* has been found to be responsible only for the OA contamination, while *Dinophysis acuta* is responsible for simultaneous OA and DTX2 contamination (Vale and Sampayo, 2000; Vale *et al*, 2004).
In the Portuguese monitoring program the employment of fast, selective and quantitative assays is very important for a proper risk management of this recurrent toxicity. We compared a commercial enzyme-linked immunosorbent assay (ELISA) based on the assay developed by Usagawa *et al*. (1989), against liquid chromatography-mass spectrometry (LC-MS). Both techniques employed the same whole flesh final extract, subjected to prior alkaline hydrolysis in order to detect simultaneously the esterified and non-esterified toxin forms (Vale and Sampayo, 1999b). Mouse bioassay (MBA) data was also evaluated against LC-MS data.

Materials and Methods
Shellfish analyses reported here by LC-MS and ELISA were from samples harvested throughout the Portuguese coast and belonging to the 2003 monitoring series. Three prominent vectors from endemic areas (blue mussel (*Mytilus galloprovincialis*), common cockle (*Cerastoderma edule*) from Aveiro lagoon and donax clams (*Donax* spp.) from Algarve offshore coast) were additionally analysed by MBA. For LC-MS and ELISA, shellfish whole flesh was extracted with 80 % aqueous MeOH and hydrolysed with NaOH to release the toxin's esters, neutralised with HCl, cleaned with hexane and partitioned into dichloromethane. The final dried residue was resuspended with 80 % aqueous MeOH (aq) and analysed simultaneously by the commercial ELISA "DSP-Check" from Panapharm Laboratories Company, Japan (Vale and Sampayo, 1999b)

and by LC-MS (Vale and Sampayo, 2002). Okadaic acid (OA) from NRC-CRM-OA-b was used to prepare calibration solutions for both techniques. For ELISA, final extracts were diluted with variable proportions of water/methanol to achieve a final concentration of 40 % aqueous MeOH. ELISA analyses were performed in duplicate, together with a 2-point standard curve plus a blank. Phytoplankton counts and trends in bivalve toxin accumulation were used as a guideline for the selection of the appropriate dilution factor. Dilutions up to 40-fold were used. For LC-MS, after isocratic separation, a single quadrupole mass spectrometer with an atmospheric pressure ESI interface operated in the negative ion mode was used to monitor the $[M-H]^-$ ion. A 3-point calibration curve was run every 6-10 samples. For mouse bioassays, whole flesh was extracted with acetone followed by ethyl acetate partitioning (modified Yasumoto method, 1984). MBA results were considered positive when 2/3 mice died in 24 hours.

Results and Discussion

According to the sample preparation scheme, the ELISA detection limit was set up at 2.5 µg/100 g and LC-MS at 0.4 µg/100 g. Only samples that gave results above the ELISA detection limit were chosen to compare quantitatively with LC-MS (Figure 1a). Many samples from late summer had a high DTX2 percentage. DTX2 data obtained by LC-MS was corrected using the antibody cross-reactivity of 40 % towards DTX2 (Carmody *et al.*, 1995). In consequence, underestimation and data dispersion observed decreased and an agreement closer to 1:1 was obtained (Figure 1b). Qualitatively, all samples tested were classified as above or below the current EU limit of 16 µg/100g (Anonymous, 2002) (Table 1). When LC-MS data was corrected for antibody sensitivity towards DTX2, the percentage of results positive by LC-MS and negative by ELISA (false negatives by ELISA technique) decreased from 3.7 % to 0.5 %. The overall agreement increased from 93.9 % to 96.3 % (Table 1). A good temporal agreement between LC-MS and ELISA was obtained for blue mussel (Figure 2a) and common cockle (Figure 2b). For blue mussel, ELISA results were systematically lower than LC-MS in late summer and autumn. The selective retention of DTX2 by blue mussel (Vale, 2004) can lead to an underestimation because the cross-reactivity towards DTX2 is only 40 %. In spring, levels obtained in blue mussels by ELISA were often above the levels obtained by LC-MS, probably due to matrix effects. The data in Table 2 show that MBA results do not always corresponded to the real DSP content as determined chemically. In particular, for cockle and donax clams, in several samples the biological assay did not have the sensitivity to detect levels above the current EU limit of 16 µg DSP/100g. Values up to 6 times this limit could be obtained by chemical methodology without detection by MBA. At the detection level of the MBA (24 h survival time), the DSP levels found ranged considerably, mussel: 19.7 – 41.8 µg/100g, cockle: 28.5 – 128.6 µg/100g, Donax: 13.5 – 56.4 µg/100g. While for mussel only 2.3 % of samples were positive by LC-MS and negative by MBA (false negatives by MBA), in cockle and Donax clams this discrepancy ranged from 8.7 to 11.4 %, respectively (Table 2).

Table 1. Agreement between LC-MS and ELISA results for all samples tested by both techniques (n=375) (Pos = positive; Neg = negative).

Regulatory criteria	Raw LC-MS data (OA + 100% DTX2)	Corrected LC-MS data (OA + 40% DTX2)
Pos LC-MS and Pos ELISA	28.0 %	27.2 %
Neg LC-MS and Pos ELISA	2.4 %	3.2 %
Neg LC-MS and Neg ELISA	65.9 %	69.1 %
Pos LC-MS and Neg ELISA	3.7 %	0.5 %

Table 2. Overall agreement of DSP toxicity analysed by LC-MS and MBA, in blue mussel and common cockle from Aveiro lagoon, and donax clam from Olhão – VRSA offshore coast (Pos = positive; Neg = negative).

Regulatory criteria	Blue Mussel	Common Cockle	Donax Clam
Pos LC-MS and Pos Bio	47.7 %	17.4 %	20.5 %
Neg LC-MS and Pos Bio	0.0 %	2.2 %	2.3 %
Pos LC-MS and Neg Bio	2.3 %	8.7 %	11.4 %
Neg LC-MS and Neg Bio	50.0 %	71.7 %	65.9 %

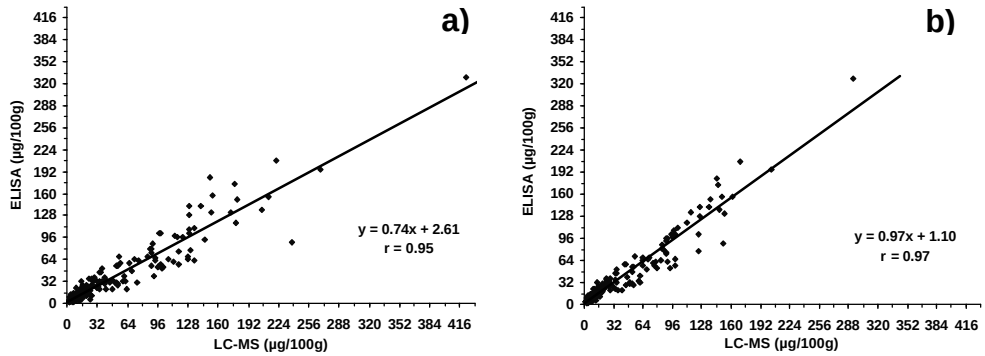

Figure 1. Comparison between LC-MS and ELISA results for samples above 2.5 µg/100g (n=216): a) raw LC-MS data (OA + 100 % DTX2); b) corrected LC-MS data (OA + 40 % DTX2).

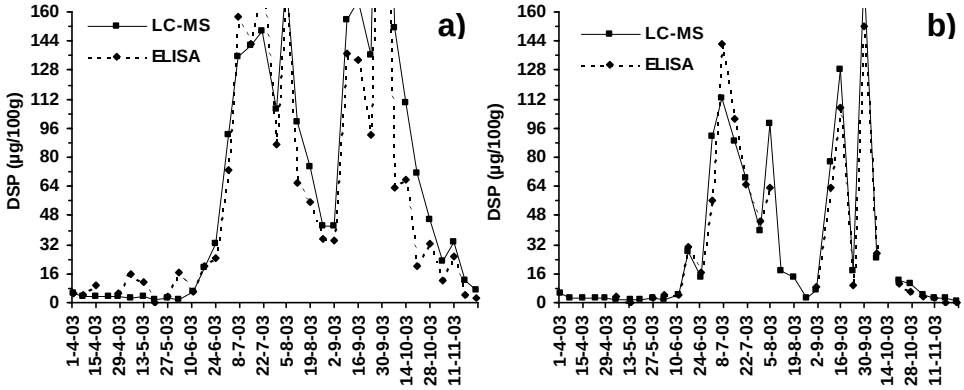

Figure 2. Temporal variability of DSP toxicity analysed by LC-MS and ELISA from April to November 2003: a) blue mussel from Aveiro lagoon; b) common cockle from Aveiro lagoon.

Conclusions

A good correlation was found when comparing the commercial "DSP-Check" kit with raw LC-MS data, and it was further improved when taking into account the specific cross-reactivity of dinophysistoxin-2, which is lower than Okadaic acid. A very high agreement between ELISA and LC-MS results was obtained when classifying the samples positive or negative for management purposes. The results obtained using ELISA tended to underestimate the DSP toxin content, in particular for species presenting a specific retention of DTX2, such as mussels or donax clams. This is especially problematic in late summer/autumn, when DTX2 percentage (over the sum OA+DTX2) increases from 40 % up to 80 %. In other bivalve species, DTX2 proportion remains similar to the producing microalgae, *D. acuta*, i.e., 40 % (Vale, 2004; Vale *et al*, 2004). The results of this study show that Mouse bioassays are not sufficiently sensitive to adequately fulfil current EU regulatory requirements for DSP toxins. At the detection level of the MBA, the range of DSP toxins found in cockles was greater than for mussel or donax clams. This is probably due to the DSP toxin being completely conjugated with fatty acids, while in mussels or donax clams a greater proportion of free toxin is present. It is probably more difficult to solubilise the ester form with the detergent used for resuspending the final dried residue in the mouse bioassay and this can lead to a significant underestimation of toxicity.

The results of this work show that fast, selective, sensitive and quantitative assays are more efficient for a proper risk management of DSP toxicity than the traditional slow, unspecific, insensitive and qualitative mouse bioassay.

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FIRST REPORT ON DETECTION OF OKADAIC ACID 7-O-ACYL-ESTER DERIVATIVES (DTX-3) IN FRENCH SHELLFISH

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Abstract
In the context of the health monitoring program on shellfish likely to be contaminated by diarrhetic shellfish poisoning (DSP) via okadaic acid (OA)-producing *Dinophysis cf. acuminata*, the French Phytoplankton and Phycotoxin Monitoring Network (REPHY) is using the mouse bioassay for DSP toxin detection. For this study, the DSP mouse bioassay extraction protocol was initially optimised in order to adapt it to chemical analysis of mussel samples from southern Brittany during the 2001 *D. cf. acuminata* outbreak. The results has revealed the presence, in addition to okadaic acid, of OA acyl-ester derivatives (DTX-3) in all contaminated mussels, in proportions ranging from 50 % to 100 % of total OA-equivalent concentration. These findings suggest an overall consistency between the development of *D. cf. acuminata* in seawater and the toxicity of mussel extracts.

Introduction
Since 1983, shellfish produced in French farm areas have regularly been contaminated by microalgae of the okadaic acid (OA)-producing *Dinophysis cf. acuminata* (Lassus *et al.,* 1988; Masselin *et al.,* 1992). Once accumulated in the shellfish, a portion of the okadaic acid may be acylated to generate acyl-ester OA derivatives called dinophysistoxins-3 (DTX-3) (Figure 1) (Yasumoto *et al.*, 1985; Yanagi *et al.,* 1989 ; Marr *et al.*, 1992; Suzuki *et al.*, 1999). From the onset of a *D. cf. acuminata* outbreak, the IFREMER Phytoplankton and Phycotoxin Monitoring Network (REPHY), in charge of the french shellfish monitoring program, has proceed weekly during a risk period to use the mouse bioassay for DSP toxin detection (according to the modified Yasumoto method (Yasumoto *et al.*, 1984)). A method for chemical analysis of OA and its methylated derivative DTX-1 by liquid chromatography/fluorimetric detection (LC/FLD) was initially devised by Lee *et al.* (1987) and subsequently adapted to a MUS-2 certified standard of DSP-contaminated mussel (Quilliam, 1995). The preparation of hepatopancreas (HP) extracts for chemical analysis uses successive liquid/liquid partitioning steps hexane/ CH_2Cl_2/MeOH 70 % in order to analyse OA/DTX-1 in CH_2Cl_2 fraction by LC/FLD. The OA derivatives (DTX-3) were eliminated in hexane washing steps. For this reason, the hexane phase is then subjected to alkaline hydrolysis in order to release the OA for subsequent LC/FLD analysis (Fernandez *et al.,* 1996; Yasumoto and Suzuki, 2000).

DTX-3 detection via chemical analysis has become a lengthy and fastidious task since several other DTX-3 derivatives have been detected, more polar and soluble in the CH_2Cl_2 and MeOH 70 % phases (Vale *et al.,* 1998 ; Vale and Sampayo, 1999). A comprehensive assessment of all DTX-3 derivatives would consequently require an alkaline hydrolysis on all three fractions, i.e. hexane, CH_2Cl_2 and MeOH 70 %. Furthermore, the multiplicity of fractions to be analysed would constitute a potential source of error in assessing the DTX-3 derivatives, since losses are likely to occur in the course of successive liquid/liquid partitioning steps.

Hence it would seem more sensible to chemically analyse all dinophysistoxins (OA, DTXs) from a single extract prepared according to the DSP mouse bioassay extraction protocol. For this reason, the DSP mouse bioassay extraction protocol was optimised initially in order to adapt it to chemical analysis. In the case of OA and its DTX1 derivative, we used MUS-2 certified mussel standards. As for DTX3, due to the lack of any reference material, we optimised the indirect analysis (i.e. via alkaline hydrolysis to release the OA for subsequent measurement) using contaminated shellfish samples. The results were expressed in total OA-equivalent (OA + DTX-3). The optimised extraction protocol was used to extract OA and DTX derivatives from shellfish samples previously tested using the DSP mouse bioassay. Shellfish samples were obtained at a time coinciding with high concentration of *D. cf. acuminata* cells in the water column. OA and DTX derivatives in the extracts were identified and quantified using LC/FLD. This study was performed with mussel samples from southern Brittany collected by the REPHY in 2001.

Materials and Methods

Reference materials and samples

- Certified reference materials (Institute of Marine Biosciences in Halifax, IMB, NRC, Canada) : 1) OACS-1, standard okadaic acid solution 25.3 µg/ml, 2) MUS-2, mussel standards containing 11 µg OA and 1 µg DTX-1 per gram of hepatopancreas.
- Samples of mussels, from three representative sites in southern Brittany, previously mouse-assayed during the 2001 *D. cf. acuminata* outbreak.
- Samples of mussels mouse-assayed during the 2002 *D. cf. acuminata* outbreak.
- Control samples of uncontaminated shellfish (oysters and mussels).

Mouse bioassay for DSP toxin detection

Out of 30 g of hepatopancreas (HP) homogenate, 20 g were used for the mouse bioassay according the modified method of Yasumoto *et al.* (1984) and 10 g stored frozen for subsequent LC/FLD chemical analysis. The HP homogenate was subjected to triple acetone extraction (3 x 50 ml). Following acetone evaporation, the residual aqueous phase underwent liquid/liquid separation with CH_2Cl_2 (3 x 50 ml). The combined CH_2Cl_2 phase containing the DSP toxins was then separated with water (2 x 15 ml) to prevent any interferences with polar toxins whenever present, i.e. paralytic shellfish toxins (PSP). After evaporation of the CH_2Cl_2 phase, the extract was mixed with 4 ml of Tween 60 at 1 % solution, and inoculated into three male mice, each weighing approximately 20 $\pm$ 1 g. One ml of test extract solution is injected intraperitoneally into each mouse. The mouse bioassay is regarded as positive if at least two out of three mice die within 24 hours. The remaining 10 g of HP homogenate was later used for LC/FLD analysis of okadaic acid (free OA and in DTX-3 form) in positive mouse bioassay extracts.

LC/FLD chemical analysis of OA/DTX-1

Preparation of extracts

Figure 2 shows the DSP mousse bioassay extraction protocol optimised and adapted for OA/DTXs chemical analysis. DSP toxins were extracted from 2 g HP homogenate. The resulting CH_2Cl_2 phase was then dried and diluted in MeOH (1 ml). One 35 µl aliquot was used for OA measurement, and a second 500 µl aliquot for DTX-3 alkaline hydrolysis for OA release and subsequent analysis of DTX3.

Alkaline hydrolysis of acyl-ester OA derivatives (DTX-3)

The 500 µl aliquot (see above) was mixed with 500 µl NaOH 0.5 M dissolved in methanol/water (9:1). The reaction medium was heated at 50°C and agitated for 30

minutes, and subsequently neutralised with ca. 750 µl HCl 0.25 M. Two procedures were compared before selecting a recovery protocol for the OA released by hydrolysis (Fernandez *et al.*,1996; Yasumoto and Suzuki, 2000).

LC/FLD analysis of OA/DTX-1

One hundred µl of 9-anthryldiazomethane (ADAM) 0.2 % (W/V) in MeOH are added to: i) 35 µl of each concentration in the calibration range of OA/methanol solutions (1 - 2.5 - 5 - 10 - 12,5 µg/ml); ii) 35 µl of methanolic extract without hydrolysis step, for native (free) OA measurement; iii) 35 µl of methanolic extract after hydrolysis, for total OA measurement (native OA and OA released by DTX-3 hydrolysis). The reaction medium was heated at 37°C for 2 hours, then purified through a silica cartridge (Supelclean, LC-Si-SPE, 3ml/500mg), previously conditioned with $CHCl_3$ and $CHCl_3$/hexane (1/1) successively. After sample deposit, any surplus of ADAM reagent was eliminated via two successive elutions with 5 ml $CHCl_3$/hexane (1/1) and 5 ml $CHCl_3$ respectively. Toxin derivatives (OA-ADAM) were eluated with 5 ml $CHCl_3$/MeOH (9/1), dried and diluted in 500 µl MeOH for subsequent LC/FLD analysis, using a C_{18} reverse-phase (Vydac 201TP54) 250 x 4.6 mm in size placed in a thermostated oven at 40°C. The mobile phase consists of an acetonitrile/water (80/20) at 1 ml/min flow rate. Detection was performed at excitation and emission wavelengths of 254 nm and 412 nm respectively.

Results and Discussion

Optimisation and adaptation of DSP mouse bioassay extraction protocol for LC/FLD chemical analysis

We used certified reference mussel standards (3 triplcates) containing both OA (11 µg/g HP) and DTX-1 (1 µg/g HP) in an attempt to optimise the DSP mouse assay extraction protocol and make it suitable for LC/FLD chemical analysis. Our purpose was to reduce the volumes and/or number of steps in order to save time necessary for solvent evaporation. Various trials were performed to determine the required volume of acetone extractions (4, 3, 2 and 2 ml), the water volume for extract dilution (1 or 2 ml) and the number of water/CH_2Cl_2 washing steps. Okadaic acid was then measured in the different fractions. Figure 2 is a schematic diagram of the procedure ultimately selected : triple acetone extraction (4, 3, and 2 ml), extract dilution in 2ml H_2O followed by triple CH_2Cl_2 partitioning (4, 3, and 2 ml). After CH_2Cl_2 evaporation, the extract is diluted in MeOH, for subsequent derivatives extraction with ADAM reagent, and final LC/FLD analysis. OA extraction yield reaches 95.0 % ± 8.9 %.

The optimised extraction procedure was applied concurrently to the MUS-2 standard and to non-contaminated (control) mussels and oysters samples to determine the absence of any peaks likely to interfere with OA or DTX-1. Results demonstrated (figure 3): 1) no peaks in the vicinity of OA and DTX-1 peaks in the reference standard (MUS-2); 2) the absence of any peaks related to shellfish matrix compounds naturally present in uncontaminated shellfish.

Assessment of DTX-3 derivatives as OA-equivalent

Following alkaline hydrolysis of DTX-3 derivatives, we compared two OA recovery protocols for selection of the most suitable in order to ensure maximum OA recovery. These protocols differed mainly in the elimination of acyls released by DTX-3 derivatives: 1) the Fernandez *et al.* (1996) protocol starts with a hexane wash of the hydrolysed phase in order to eliminate acyls, leading to a risk of concurrent OA losses in the hexane phase. The aqueous phase then undergoes $CHCl_3$ separation for OA recovery; 2) the Yasumoto and Suzuki (2000) protocol begins with $CHCl_3$

separation, enabling a quasi-total recovery of OA along with acyls, which are then eliminated in the subsequent hexane/MeOH 80 % separation. The MeOH 80 % phase is then acidified for OA extraction with $CHCl_3$.

Both protocols were subjected to parallel- and double-trials on two 2001 southern Brittany mussels samples, each previously mouse-assayed by the REPHY as highly toxic and non-toxic respectively. The LC/FLD chromatograms revealed 1) that both samples contained a much higher proportion of OA in DTX-3 form than in free form, hence corroborating the investigations of Vale and Sampayo (1999) who detected 80 to 90 % of OA in DTX-3 form in Portuguese mussels; 2) the total OA-equivalent content in the mouse-negative sample was below the mouse assay detection limit (0.8 µg OA-equivalent/g HP). The OA recovery is better with the Yasumoto and Suzuki protocol (2000), while the Fernandez protocol leads to a 31 % overlap reduction on the first sample and 40 % on the second. Okadaic Acid losses are probably due to the hexane/hydrolysed phase separation (organic phase/aqueous phase) as previously suggested. After validation on a non-contaminated (control) mussel sample in order to check for the absence of any OA-interfering peak(s), we opted for the Yasumoto hydrolysis procedure: the hydrolysed phase is separated from $CHCl_3$ (2 x 1 ml) to recover the quasi-total OA content along with acyls. Following dry evaporation, the $CHCl_3$ extract is diluted in methanol 80 % and subsequently hexane-washed (2 x 1 ml) to eliminate the acyls released by DTX-3. The MeOH 80 % phase is acidified with 0.2 % acetic acid, followed by separation from the $CHCl_3$ phase from which the quasi-total OA content is recovered. The extract is then diluted in methanol for subsequent LC/FLD chemical analysis of OA/DTX-1. Figure 4 presents chromatograms of the non-hydrolysed and hydrolysed fractions of a contaminated mussel sample (positive mouse bioassay). An overlay of both chromatograms reveals the presence of DTX-3 derivatives, showing an increase in the OA peak in the hydrolysed fraction, corresponding to the total amount of OA released (free OA and DTX-3 forms). The OA-equivalent amount released from DTX3 may be calculated by deducting the free OA content measured in the non-hydrolysed fraction from the total OA content measured in the hydrolysed fraction.

OA and derivatives DTX-3 chemical evaluation on positive mousse bioassay extracts during D. cf. acuminata outbreak

Within the frame of this study, we selected the southern Brittany samples most often affected by *D. cf. acuminata*. Three representative sites were covered: Groix Island, Men er Roue (bay of Quiberon), and Dumet Island (bay of Vilaine). The overall findings from this study are illustrated in the results with two types of graphs:

Graph (a) represents the OA concentration (free OA + DTX-3 form) measured by LC/FLD in mussel hepatopancreas (HP) samples. Graph (b) shows : i) the mouse activity of these extracts, as measured according to the DSP mouse bioassay. Usually, the mouse-toxicity of a substance is a function of survival time (st). For convenience purposes, we decided to represent mouse-activity on the graph, i.e. inversely proportional to median survival time (A = 1/st x 1000). ii) the *D. cf. acuminata* cell concentration in seawater in 2001. The primary finding was that all positive mouse-assayed shellfish samples tested during the 2001 *D. cf. acuminata* outbreak contain OA and/or DTX-3. Furthermore, a qualitative analysis of the methylated OA derivative (DTX-1) in the MUS-2 certified standard confirmed once again the lack of any DTX-1 in all tested samples.

As illustrated by figures 5a, 6a and 7a corresponding to chemical analysis results on samples from various sites, systematic detection of acyl-ester OA derivatives (DTX-3) revealed presence of DTX-3 in all samples in portions more or less equivalent to 50 % of the total OA-equivalent concentration. This proportion may reach 90 % in some samples, and up to 100 % in low-contaminated samples (figures 5a, 6a), hence making DTX-3 detection essential to properly assess actual toxicity. These findings alone confirm the use of a 24 hour observation limit of inoculated mice in DSP screening assays, in order to take into account the toxicity of the DTX-3 derivatives: unlike free OA, the toxic effect of DTX-3 inoculated intraperitoneally is time-influenced due to the peritoneal barrier against apolar compounds such as DTX-3 (Ito and Terao, 1994). According to figure 5b, a concentration of 400 *D. cf. acuminata* cells/L is sufficient to cause toxin accumulation, thereby making mussels unfit for consumption on Groix Island. Nevertheless, this finding should not be extrapolated to other sites due to the geographical variability of *D. cf. acuminata* toxicity and to the relative abundance of *D. cf. acuminata* cells in relation with the total phytoplankton population (Kat, 1989; Marr *et al.*, 1992b; Sato *et al.*, 1996; Marcaillou *et al.*, 2001).

In addition, the graphs illustrating *D. cf. acuminata* cell concentrations in seawater (figures 5b, 6b, 7b) indicate that the main season for *D. cf. acuminata* growth in Brittany in 2001 extended from April to June. On the contrary, as usually observed by REPHY since 1983, a *D. cf. acuminata* outbreak occurred in the autumn of 2002, due to favourable weather conditions, leading to a DSP outbreak for shellfish late in the year. Furthermore, the decreasing *D. cf. acuminata* cell concentration was not matched by any shellfish depuration: mussels remained contaminated for three weeks, although over the same period *D. cf. acuminata* was only found at concentrations close to the detection limit (100 cells/L). Chemical analysis (data not shown) found that all mussel samples contained OA both in free form (minority) and in acyl-ester forms (DTX-3) (majority). Similar to the 2001 samples, the proportion of DTX-3 derivatives in these 2002 samples varied from 80 % to 89 % of the total OA-equivalent concentration (AO + DTX-3) during the final depuration phase in the mussels (analysed samples were collected at the end of the *D. cf. acuminata* outbreak). These observations would seem to suggest that the OA depuration process in mussels occurs in two successive steps: 1) bioconversion of the okadaic acid into its acyl-ester derivatives (DTX-3), equally toxic (thereby explaining the late contamination, since it would be equivalent to a "re-contamination"; 2) elimination of DTX-3 derivatives. Consequently this leads to a much longer decontamination period for bivalves. Some observations and experiments on the depuration of DSP toxins have been performed (Sevensson, 2003; Sevensson and Forlin, 2004). These studies have suggested that, on a seasonal basis, the lipid content in the digestive glands may play a role in determining the levels of bioaccumulation of the lipophilic DSP. Increased usage of lipid stores, which occur during starvation, may accelerate the release of OA. The rate of depuration of OA in mussels is not positively correlated with digestive activity and fecal production. Their results showed the difficulties to accelerate the mechanism of depuration by short-term manipulation of external factors. They suggest that endogenous processes, intensive to immediate changes in the surrounding environment. Further investigations including *in situ* measurements during a future *D. cf. acuminata* outbreak are required in order to monitor the kinetics of OA bioconversion into DTX-3, a necessary step to improve our understanding of the contamination/decontamination processes in shellfish. In addition, we need to prepare reference material with mussels affected by *D. cf. acuminata* outbreaks, without DTX-1 but full of acyl-derivatives of OA.

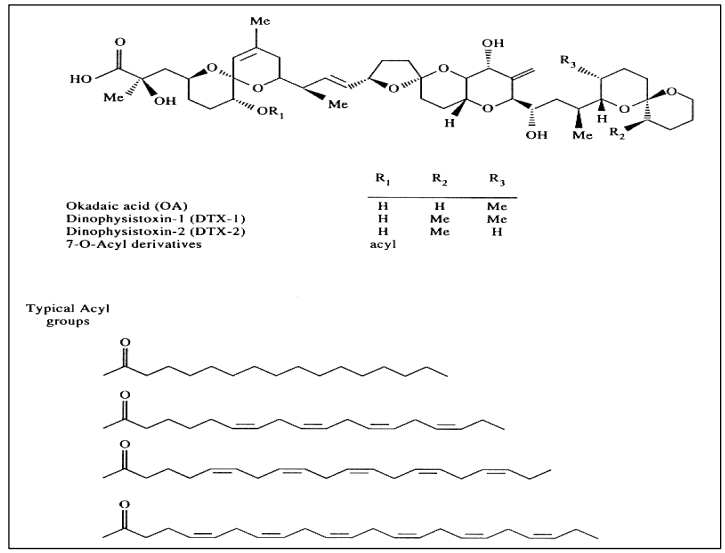

Figure 1. Structures of the main toxins, responsible for diarrhetic shellfish poisoning

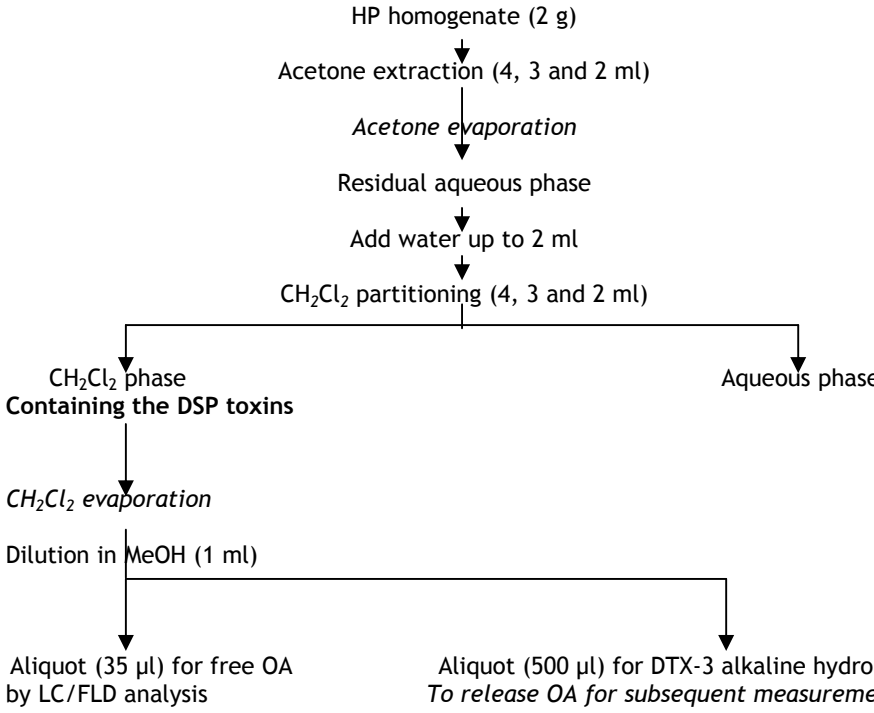

Figure 2. Modified official mouse bioassay for DSP toxins extraction procedure suitable for OA/DTXs chemical analysis by LC/FLD

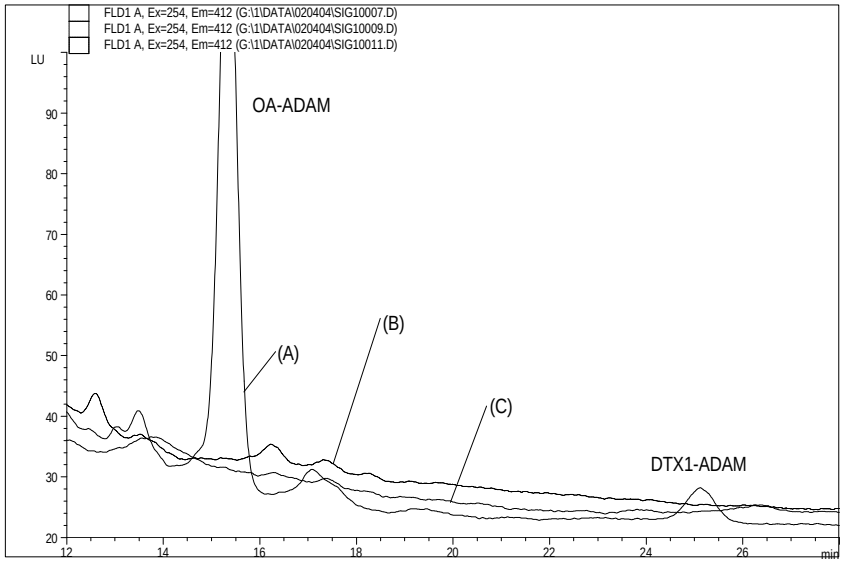

Figure 3. Overlaid LC/FLD chromatograms of
(A) MUS-2 reference material containing OA and DTX-1,
(B) uncontaminated mussel
(C) uncontaminated oysters showing no interfering peaks for OA and DTX1

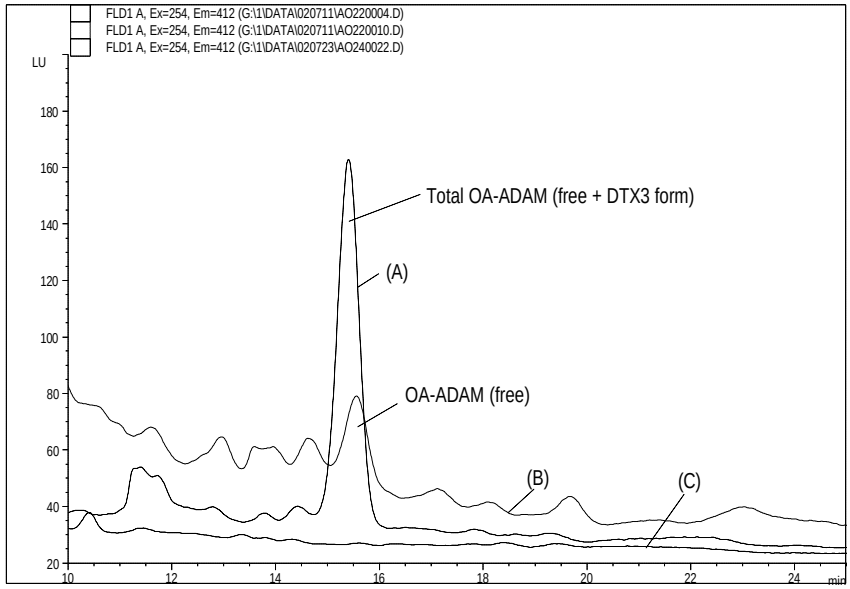

Figure 4. Overlaid LC/FLD chromatograms of hydrolysed (A) and non hydrolysed (B) fractions of a *Dinophysis cf. acuminata* contaminated mussels sample ; (C) hydrolysed fraction of uncontaminated sample.

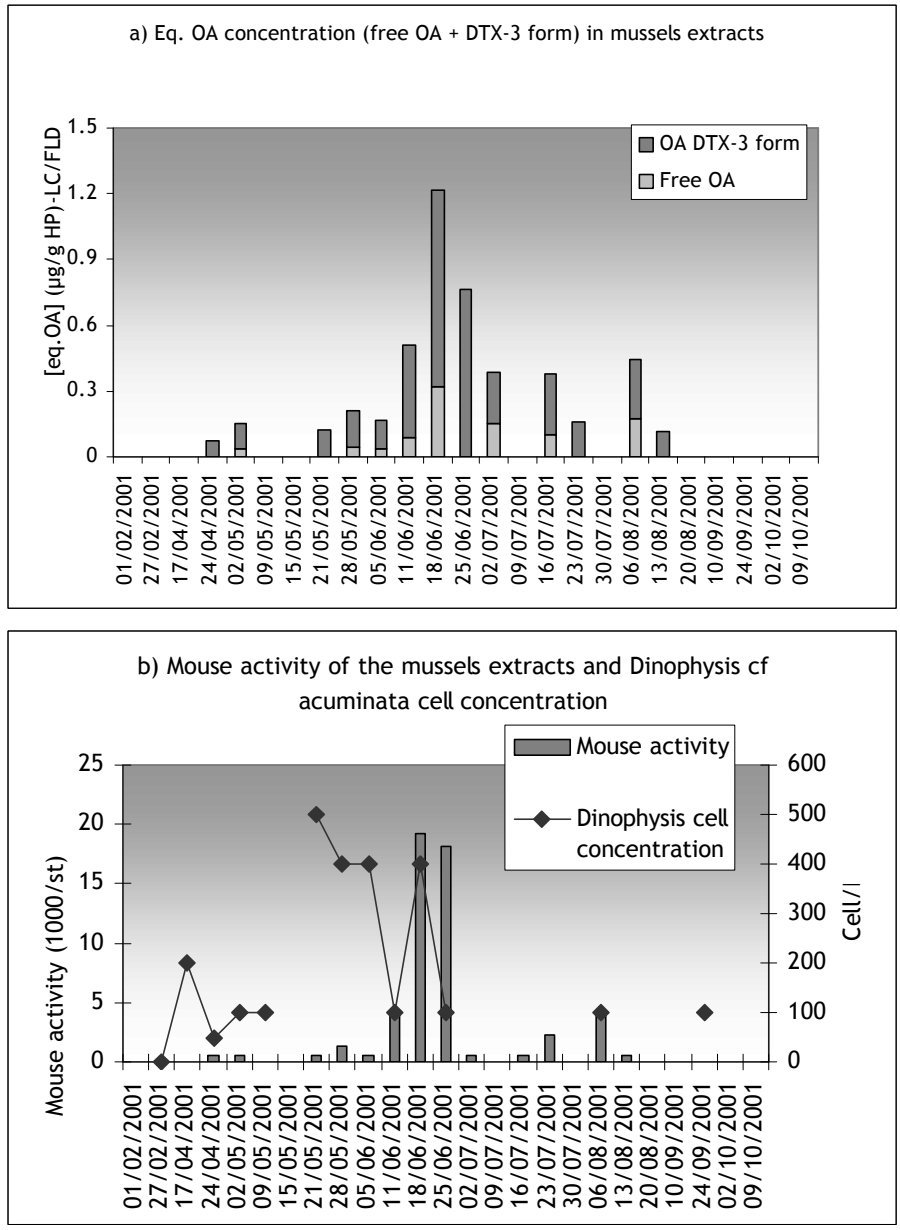

Figure 5. A one-year survey (2001) of Groix Island (South Brittany) for *Dinophysis cf. acuminata* and mussels samples

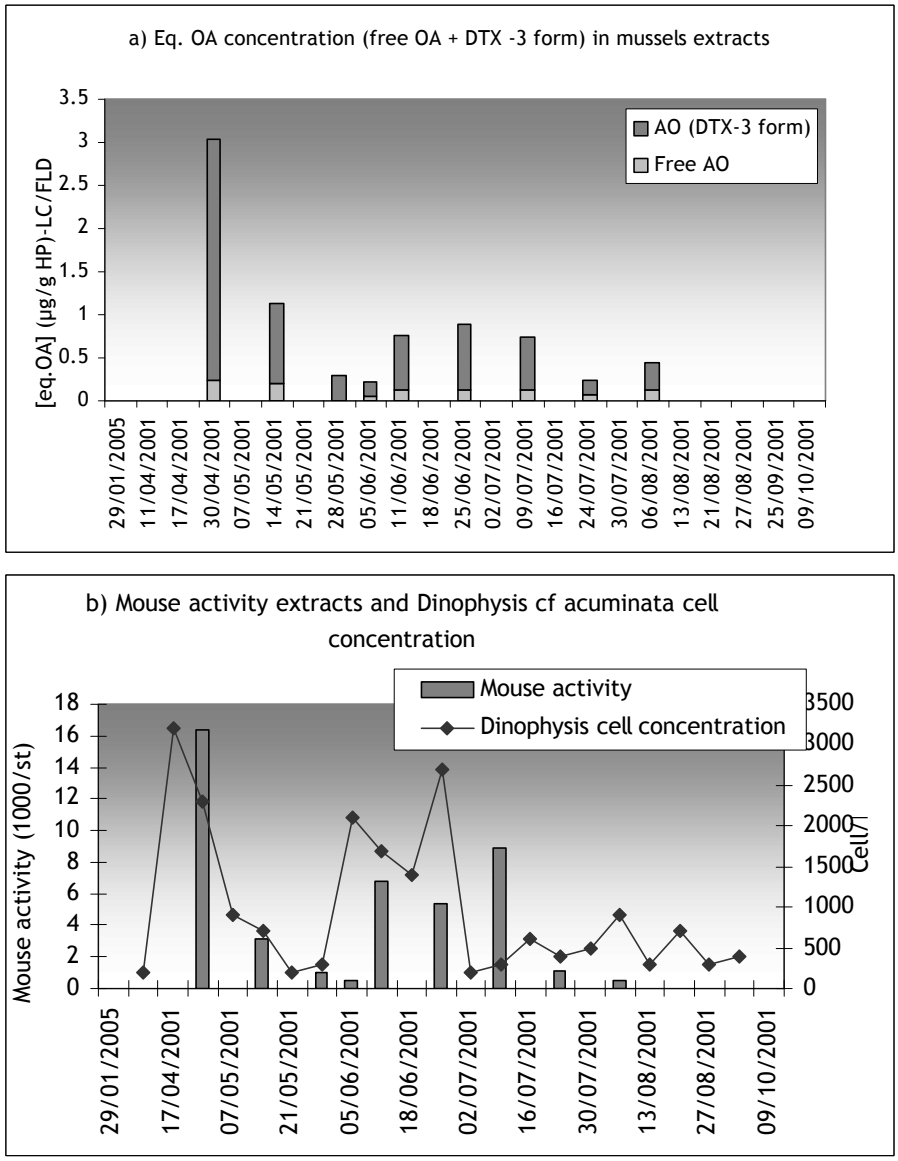

Figure 6. A one-year survey (2001) of Dumet Island (South Brittany) for *Dinophysis cf. acuminata* and mussels samples

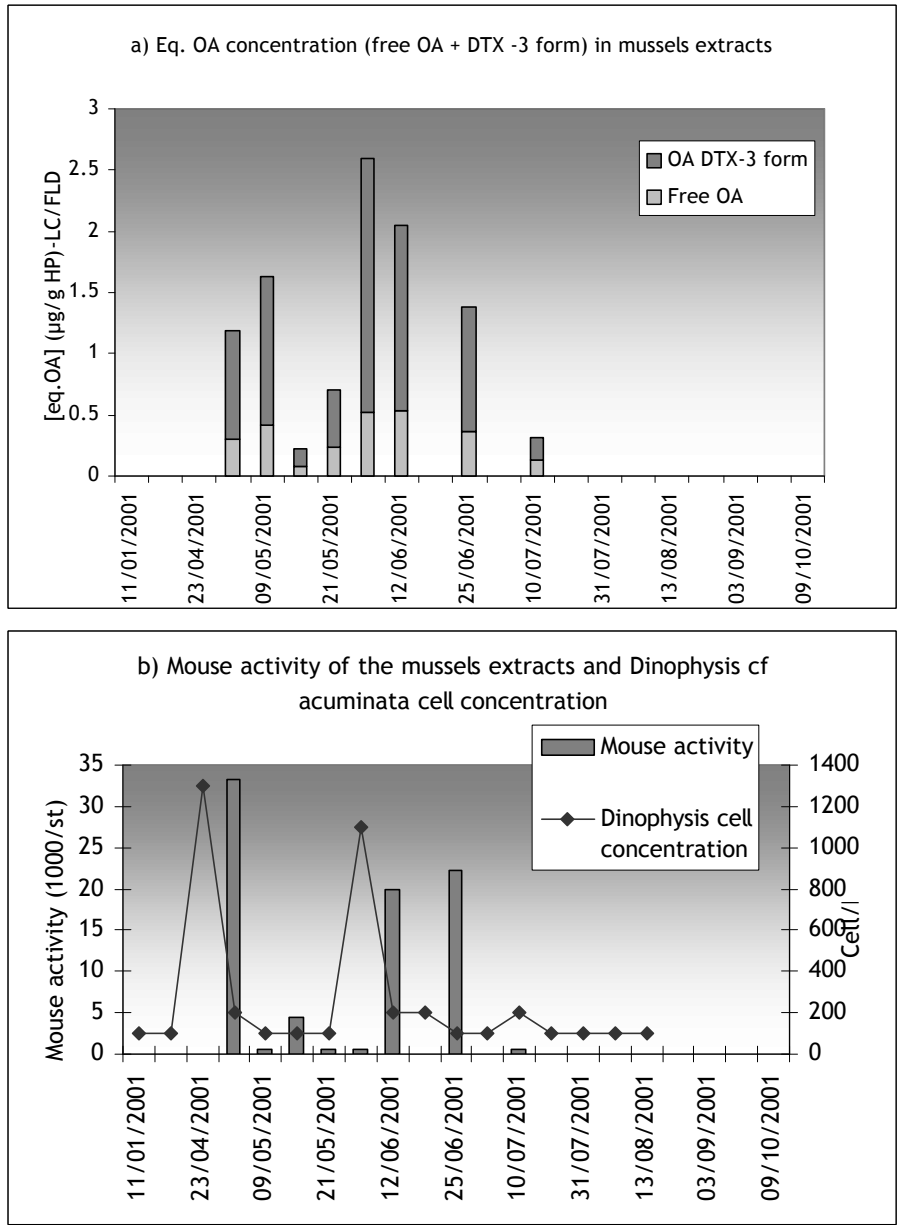

Figure 7. A one-year survey (2001) of Men er Roue (South Brittany) for *Dinophysis cf. acuminata* and mussels samples

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CONFIRMATION OF AZASPIRACIDS IN MUSSELS IN NORWEGIAN COASTAL AREAS, AND FULL PROFILE AT ONE LOCATION.

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Abstract
In the fall of 2002 and the winter 2002/2003 the first closures of mussel farming areas in southern Norway occurred due to the presence of azaspiracids (AZAs) above the regulatory limit in blue mussels (*Mytilus edulis*). The azaspiracids reoccurred in mussels in the fall of 2003 and the winter of 2003/2004. Identification of azaspiracids was initiated by positive response in the mouse bioassay for lipophilic toxins in the absence of other lipophilic toxins by LC-MS. The symptoms in the mice were consistent with those described for the AZAs, and their identity was confirmed with LC-MS. Additional LC-MS/MS analysis of a sample from one location (Flødevigen) was performed for a full profile of AZAs present, confirming AZA-1 to AZA-6 present with indications of AZA-7 to 10.

Introduction
Azaspiracids were first discovered in mussels from Ireland in 1995 (Satake *et al.*, 1997; Satake *et al.*, 1998). AZAs in mussels were recorded by LC-MS (in-house data) at the South-Western costal areas in Norway in 2001, but only at trace level amounts. Analysis of stored material from the Sognefjord revealed presence of AZAs in the Sognefjord already in 1998 (James *et al.*, 2002). In more recent years, an increase in the geographical spreading of the AZA toxin complex has been observed. Today, AZAs are found in mussels from the Swedish border, along the southern coast, and at the west coast up to the Sognefjord. In addition, AZAs are also found in mussels at two locations in northern Norway (Harstad and Alta). Following the geographic spreading of AZAs, there was an increase in the amount of toxin found in mussels, from trace levels to amounts exceeding the regulatory limit of 160 µg/kg (EC, 2002). The presence of AZAs has resulted in increased number of closures of mussel beds for harvesting, based on both positive mouse bioassay for lipophilic toxins (Stabell *et al.*, 1992; Yasumoto *et al.*, 1984) and on LC-MS data from routine sampling (Quilliam *et al.*, 2001). The length of the closures has been for up to three months, while the toxins may remain in the mussel for a longer period but below the regulatory limit. According to the literature, the AZAs found in mussels in Ireland are produced by the algae *Protoperidinium crassipes* (James *et al.*, 2003a).

Materials and methods:
The extraction processes for routine analyses are as follows:
Analysis by LC-MS:
1g sample homogenates (hepatopancreas, HP) from blue mussels were extracted once with 9 ml methanol by mixing for 10 minutes, and centrifuged for 5 minutes at 3000 rpm. 500 µl aliquots of the supernatants were diluted to 80 % methanol with water and after filtration through 0.22 µm centrifuge filters, the samples were ready for injection on LC-MS. The LC system consisted of an Agilent 1100 LC system (Palo Alto, CA, USA) with binary pump, auto sampler and column oven. The Mass spectrometer consisted of a Sciex API 2000 (Concorde, ON, Canada), equipped with an electrospray interface (Turboionspray®). The eluent from the LC was led directly into the MS, the flow rate was 300 µl/min and the injection volume 20 µl. Liquid

chromatography was performed according to a modified version of Quilliam *et al*. (2001), using isocratic elution with acetonitrile : water (65 : 35) containing 50mM formic acid and 2mM ammonium formate on a Varian Omnispher 5 C18 column (5 μm, 150mm x 2.0mm id.) for routine analysis. Selected Ion Monitoring (SIM) was used to record the signals from the $[M+H]^+$ ions for AZAs (AZA-1 to AZA-6). Analyses were also conducted according to Aasen *et al*. (2003), on a gradient method with an X-terra C18MS (50mm x 2.1mm id.) column with guard (10mm).

Analysis by LC-MS/MS:
10 g of hepatopancreas samples were extracted with 40 mL 80 % methanol for 3 min on a Brinkmann Polytron (Westbury, NY, USA) homogeniser. 5 mL extracts were diluted to 20 % methanol and cleaned on an OASIS-HLB SPE-column (500mg) preconditioned with 5 mL methanol, followed by 3mL H_2O. The samples were washed with 50 % methanol and eluted with 100 % methanol, dried and re-dissolved in 100 µl 100 % methanol (10g tissue/ml). A 3 µl aliquot was separated on an Hypersil C8 (50 * 2.0mm, 5µm). A gradient from 35 % B to 100 % B was run for 10 min, held at 100 % B for 10 min, followed by 10 min equilibration at 35 % B. (A: 50mM formic acid and 2mM ammoniumformate in water, B: 50mM formic acid and 2mM ammoniumformate in 95 % acetonitrile) according to Quilliam *et al*. (2001) The eluent was analysed on an API-4000 triple quadrupole instrument from Sciex (Concorde, ON, Canada), with Analyst® software from Applied Biosystems. The MS/MS analyses were conducted with Selected Reaction Monitoring (SRM) of masses of different known AZAs to the fragment ion m/z 362.5, product ion scan of the $[M+H]^+$ ions and on precursor ion scan of the common fragment ion at m/z 362.5 for AZAs (Brombacher *et al*., 2002; Lehane *et al*., 2004).

Bioassay:
The mouse bioassay for lipophilic toxins was carried out according to a Norwegian accredited method, based on (Yasumoto *et al*., 1984; Stabell *et al*., 1992) and according to Norwegian law on animal welfare.

Algal monitoring:
High resolution monitoring data for algae were available from Flødevigen (Figure 1) at the southern coast of Norway (Institute of Marine Research, Flødevigen Research Station). The concentration of selected algae collected at 0–3 m in Flødevigen Bay, has been quantified routinely three times a week (Monday, Wednesday and Friday) since 1989 (Dahl and Johannessen, 1998). *Protoperidinium spp.* has been included among the enumerated species since 2001. Blue mussels were also collected from Flødevigen Bay every second week for toxin analysis.

Figure1. Map of Norway showing the location of Flødevigen, and the locations from Table 1, where the samples of blue mussels were collected.

Results and Discussion:
In Ireland, *Protoperidinium crassipes* has been suggested as producer of AZAs, resulting in toxin accumulation in mussels (James *et al.*, 2003a). This *Protoperidinium* species is not very common in Norwegian waters, and does not occur in high density along the coast. Preliminary analyses of phytoplankton data collected at Flødevigen over the last years show that there is no correlation between AZAs in mussels and the abundance of *Protoperidinium* spp, see Figure 2. The same has been found in the Hardanger fjord at the west coast (P. Hovgaard, personal communication). Consequently, it is possible that other algae are the true producers of AZAs in this region. More detailed analysis of phytoplankton data and special studies are planned in the future to try to reveal the source organism for AZAs at Flødevigen.

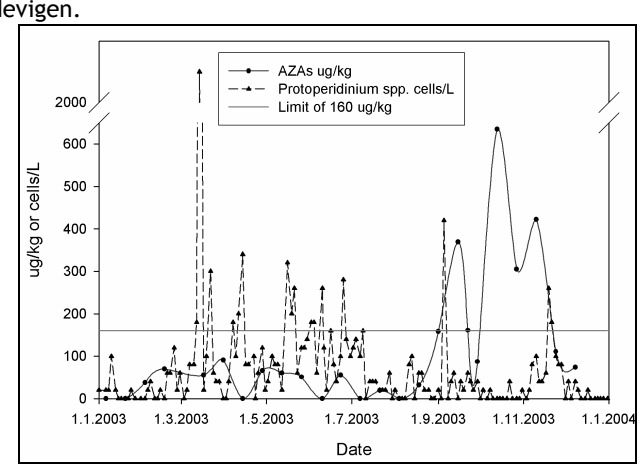

Figure 2. AZA-1 toxin equivalents in µg/kg plotted against the *Protoperidinium* spp.

In 2002/2003, presence of AZA-1, AZA-2 and AZA-3 was confirmed (AZA-1 by standard, AZA-2 and -3 by corresponding retention times to a positive sample obtained from Ireland). Their identity was also confirmed by samples from Norway, analysed at Marine Institute, Ireland. Results exceeding 160 µg/kg AZA-1 equivalents and positive bioassays led to closures for up to three months. AZAs stayed in mussels until late spring, but below the tolerance level. Slow detoxification in winter might be due to reduced metabolic activity in cold water. Mussels from the areas that were positive with the mouse bioassay in the fall/early winter of 2003 were analysed by LC-MS, and the toxin levels are given in Table 1. The results indicate the highest reported values in AZA-1 equivalents at these locations and the times of the peaks in toxicity. All mussel samples reported to be positive in the mouse bioassay were found to have toxin levels close to or exceeding 160 µg/kg AZA-1 equivalents. This corresponds with the approximate detection level in mice (Expert working group-EU, 2001). Death times of mice at 50-60 min were associated with high concentrations of AZAs, while low concentrations (around 160 µg/kg mussel) resulted in death of the mice within 24 hours. AZA levels in mussels from those areas were all below the regulatory limit by the end of December, but a few locations in the Hardanger Fjord area had a reoccurrence of AZAs in early February 2004.

The results indicate a geographical spreading of AZAs covering the entire southern coast of Norway up to the area around the Sognefjord. Further north, AZAs have only been found at a few locations, Alta and Harstad (see Map in Figure 1 and Table1), at the most northern part of the country.

Table 1: Stations monitored for AZAs in 2003. Dates and levels of peaks of AZAs in mussels.

Location:	Map	Peaked at Date:	Amount AZA-1 eq (ug/kg)
Harstad	**1**	06.10.2003	373
Hvaler	**2**	12.10.2003	149
Flødevigen	**3**	13.10.2003	635
Dalskilen	**4**	13.10.2003	460
Alta	**5**	27.10.2003	115
Flekkefjord	**6**	10.11.2003	158
Risør	**7**	11.11.2003	175
Lindesnes	**8**	08.12.2003	170
Hardangerfjorden	**9**	16.12.2003	213

A routine LC-MS method has been established at our institute for analysis of AZAs, based on Quilliam *et al*. (2001). With this routine method, only the main AZAs, AZA-1, AZA-2 and AZA-3 are quantified. In addition, samples from one location have been analysed to elucidate the full azaspiracid profile by using gradient elution and a sensitive LC-MS/MS system. The sample from Flødevigen was analysed by precursor ion scan of the ion of m/z 362.5, that is a common fragment ion of AZAs originating from the dissociation of ring E (see Figure 3) (Brombacher and Volmer, 2003; Diaz *et al.*, 2003; Brombacher *et al.*, 2002; Lehane *et al.*, 2004). In addition to AZA 1-3, the precursor ion scan revealed the presence of AZAs 4, 5 and 6. There were also possible AZAs 7-10 (Lehane *et al.*, 2004; Blay *et al.*, 2003). The scan is presented in Figure 4 as a contour plot with time as x-value, m/z as y-value and intensity for number of cps. The possible masses of AZAs were analysed by SRM

(Figure 5) and for each mass a product ion scan was made of the possible molecular ion found by the precursor ion scan (Figure 6) (MSMS of AZA1). This confirms the finding of these structures by identical mass spectra with literature (James *et al.*, 2003b; Brombacher *et al.*, 2002). Similar MSMS spectra confirming the structure for AZA-2, 3, 4, 5 and 6 were also found (not shown). AZA-7 to AZA-10 (James *et al.*, 2003b) were not conclusive, but are possible matches.

The profile of AZAs found seems to be very similar to the profiles found in Ireland, Spain and UK (James *et al.*, 2002; Magdalena *et al.*, 2003; Volmer *et al.*, 2002).

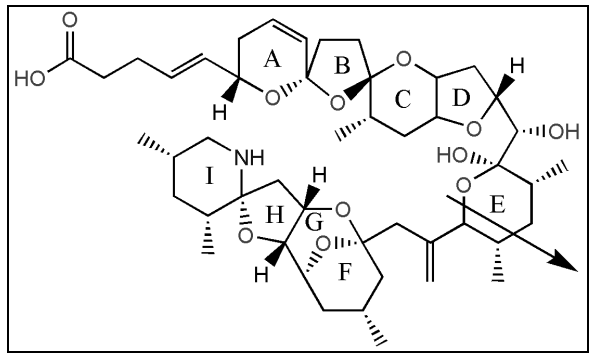

Figure 3. Structure of AZA-1 and fragmentation point for the E-ring.

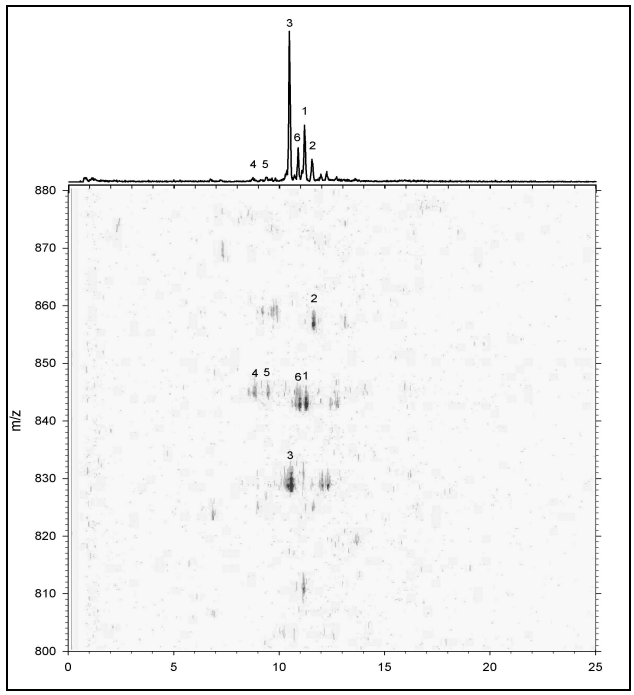

Figure 4. Contour plot of possible AZAs after precursor ion scan of m/z 362.5. AZA-1 to AZA-6 marked 1-6 in the plot.

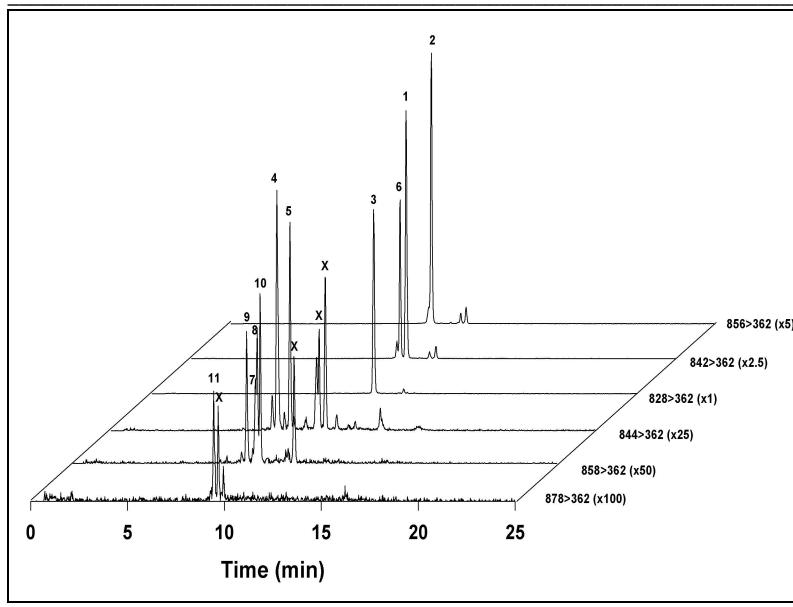

Figure 5. Selected reaction monitoring (SRM) chromatogram of possible AZAs. AZA-1 to AZA-10 numbered in the chromatogram. (x: isomer masses of other peaks).

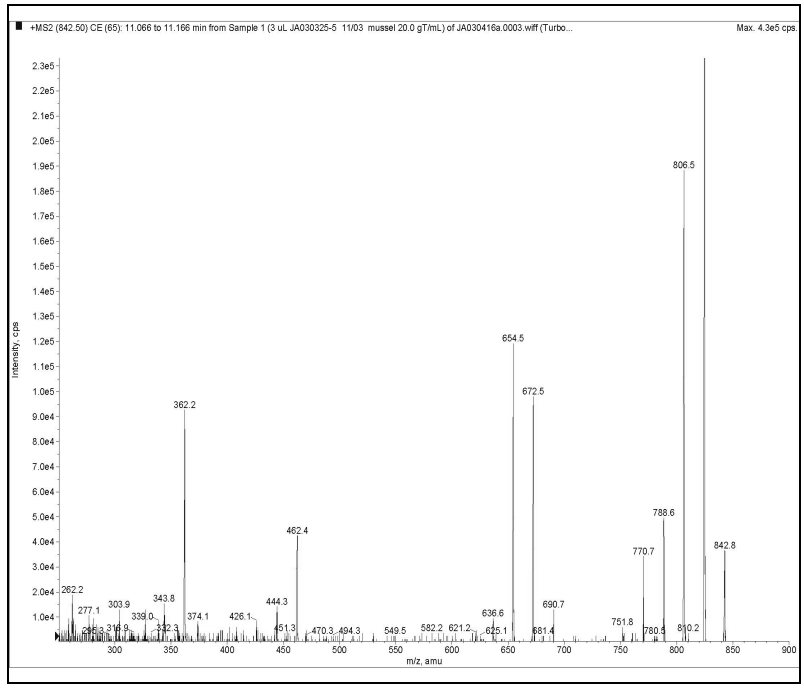

Figure 6. Product ion scan of m/z 842.5, AZA-1 (peak 1, Fig. 4 and 5).

Conclusion:
AZAs were found in mussels from Hvaler in eastern Skagerrak to the Sognefjord at the west coast, as well as at two locations in the northern Norway. The presence of AZAs in the Norwegian costal areas has clearly an impact on mussel farming and the problem seems to be increasing. The source organism of AZAs is still unknown in Norwegian waters, but our results strongly indicate that *Protoperidinium* is not the source of this toxicity group in Norwegian waters.

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RAPID RESPONSES OF JUVENILE OYSTERS EXPOSED TO POTENTIALLY HARMFUL PHYTOPLANKTON

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Abstract
During the summer of 2001, a mass mortality of early juveniles of Pacific oysters, *Crassostrea gigas* Thunberg, was detected at a farm site in Jervis Inlet, British Columbia. During this episode, several toxin producing and potentially harmful algae were detected within the phytoplankton, with *Protoceratium reticulatum* (Claparède *et* Lachmann) Buetschli exhibiting the closest match. Searching for a cause we experimentally examined the rapid response behaviour of similar oysters, which ranged from a strong rejection that diminished and interrupted feeding activity when exposed to cultures of the toxic *P. reticulatum*, *Alexandrium tamarense* (Lebour) Balech, *Amphidinium carterae* Hulburt, *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara, *Karenia mikimotoi* (Miyake *et* Kominami ex Oda) Hansen and Moestrup, *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle, and *Gonyaulax spinifera* Diesing, to normal feeding on controls: *Dunaliella tertiolecta* Butcher, *Isochrysis galbana* Parke, *Phaeodactylum tricornutum* Bohlin and *Chaetoceros calcitrans* Paulsen. Our study suggests that several of these species could have contributed to the oyster mortality in 2001 by causing starvation, and contributing to the environmental stress. In order to avoid losses, the phytoplankton composition should be considered in farm sites. This suite of reactions may also explain why adult oysters are slow in toxin uptake among bivalves.

Introduction
There have been numerous reports of summer mortalities of adult and juvenile farmed Pacific oyster (*Crassostrea gigas*) at sea-farms on the coasts of the Pacific Ocean (Chew, 1996, Landsberg, 2002, Meyers and Short, 1990, Shumway *et al.*, 1990). Several hypotheses exist that try to explain this recurring phenomenon, including environmental stress due to high temperatures, high amount of energy expended during their gametogenic cycle and opportunistic diseases in larval and adult oysters (Cheney *et al.*, 1999, Purdue *et al.*, 1981). Only limited information exists on juveniles and their trophic behaviour when faced with harmful algae and their blooms, most of the studies being based on experiments with adults.

Juvenile oysters (3-10mm) were affected by massive mortalities during the summer of 2001, in the vicinity of Sykes Island in Jervis Inlet, British Columbia. Environmental parameters, composition and abundance of the phytoplankton were obtained and analysed for this period, in order to investigate the possible involvement of harmful phytoplankton in this episode. The dinoflagellate *Protoceratium reticulatum* (=*Gonyaulax grindleyii* Reineke) was the dominant species in the phytoplankton community during the mortality of oysters. Although it has not been previously related to damage of cultured shellfish, it is known to produce yessotoxin (Seamer *et al.* 2000). Also present were lower concentrations of the dinoflagellates *Ceratium fusus* (Ehrenberg) Dujardin and the saxitoxin-producing *Alexandrium* spp.

Twelve species including toxic algae, potentially harmful algae (pHA), well accepted controls, and the most abundant algal species present during the oyster mortalities were isolated and cultured. These algae were utilised to observe the reaction and general behaviour of juvenile oysters using qualitative and quantitative methods.

Materials and methods

A sampling station was maintained near Sykes Island, on the mouth of Sechelt inlet, British Columbia. From April 2001 until April 2002; water samples from three depths (1, 5 and 15m) were obtained biweekly by means of oceanographic sampling bottles. In addition, net phytoplankton, water temperatures, salinities and Secchi disk transparencies were taken. The site contained a suspended tray grow out operation and a Floating Upwelling System intensive nursery raft (Ralonde, 1998). The mortalities were observed and recorded during the normal sizing operations of the farm every week. The samples were analysed quantitatively for microphytoplankton and small zooplankton, by the Utermöhl method (Sournia, 1978). Net phytoplankton (20µm mesh) was analysed qualitatively, for further species identification.

The rapid response was observed by placing one 5mm shell-length oyster in a 25ml settling chamber, with 10ml of .45µm filtered seawater, then adding 2ml of concentrated cultures of *Dunaliella tertiolecta*. When feeding started, 2ml of the pHA algae are placed on the surface or through subsurface injections using micropipettes. The results were recorded with a video camera attached to an inverted microscope.

The clearance rate experiments were performed in triplicate: 300ml beakers with .45µm filtered seawater were inoculated with high abundance algal cultures and an oyster. A control was kept and used to correct for the reproduction of the algae. To avoid damage to dinoflagellate cells, no aeration was provided. Only a gentle homogenisation was produced with a spiral glass rod before sampling, every two hours. The abundance of the algae was measured using a Coulter Counter Z2 version 1.02. Clearance rates were calculated using Coughlan's 1969 formula, modified by Laabdir and Gentien (1999). Toxin analysis the *P. reticulatum* cultures was performed by Bill Hardstaff and Mike Quillam of the Institute of Marine Biosciences of the National Research Council, Canada.

Results and Discussion

Between June 25 and September 10, 2001 *P. reticulatum* was the predominant species in water samples at the oyster farm. During its August bloom it reached a maximum abundance of $2.3x10^5$ cells/l (Fig. 1), it was accompanied by lesser quantities of three other pHAs: *Ceratium fusus*, *A. catenella* (Wheldon et Kofoid) Balech and A. *tamarense*, the first of which has been experimentally demonstrated to harm oyster larvae (Landsberg, 2002), while the latter are PSP producers in local waters (Taylor and Harrison, 2002).

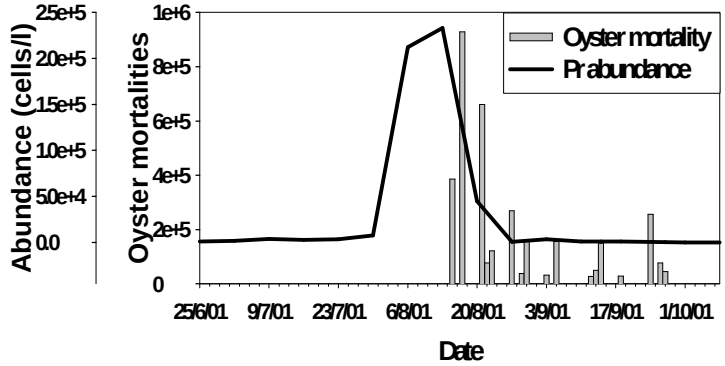

Figure 1: Oyster mortality (total individuals) and *Protoceratium reticulatum* abundance (cells/l) over time, during the summer of 2001.

High mortality of oyster juveniles was detected during the latter part of the P. *reticulatum* bloom and in the following weeks, with most being observed a short time after the peak algal abundance (Figure 1). The smaller oysters (Sizes 2-3, 3-5mm) were the most sensitive, with cumulative mortalities over 80 %. Larger oysters (Sizes 4-6, 6-10mm) died in smaller numbers (approximately 30 %), while no visible harmful effect was noted on adult oysters.

Experimental observations on the rapid response behaviour of juvenile oysters, suggest that high abundance of toxic algae contribute to starvation of oysters, even when high abundance of more acceptable species exist at the time. The algae were classified into five groups based on the responses they elicit on the oysters; 1) highly toxic and causative of mortality when in high densities, 2) moderately toxic, but elicit violent rejection, 3) moderately toxic, generate a mild reaction and are expelled in pseudo feces, 4) moderate toxin production, produce slow feeding or feeding arrest when in high concentrations, but no violent rejection is observed, 5) "food algae" well accepted by the oysters, and taken in high speed continuous feeding currents, even at extremely high concentrations ($6x10^5$ cells/ml). The most violent reactions were observed with most toxin producing species and large spiny phytoplankton. (Table 1)

The feeding response of the oysters was severely hindered when exposed to toxic algae of groups 1 and 2, normally with clearance values near zero. *A. tamarense* killed 30 % of the subjects when presented in extremely high concentrations ($1.5x10^6$ cells/l), rarely achieved in natural blooms. The mechanism involved is not clear, since the oysters remained closed, although haemolytic compounds could be an option (Gainey and Shumway, 1987). *P. reticulatum* elicited the most violent reactions, as the presence of only one or two algal cells within the pallial cavity of the oysters was enough to cause severe clapping and closure. The local strain of this species is toxic, producing high quantities of yessotoxin. The oysters exposed to these algae normally emerged from the experiments in a debilitated state, brittle shells and overgrown with epiphytic algae, but were able to feed shortly after. The algal species of groups 3 and 4 reduced the feeding rate, mostly by clogging the gills and being expelled as pseudo feces. The response to *H. akashiwo* cultures in exponential growth were well accepted while senescent cultures produced a slow filtration or even stopped feeding completely.

Table 1. Qualitative rapid responses of 5mm shell length oysters to selected cultures of phytoplankton (Y= yes, N= no, n.c.= non conclusive)

Species	Clapping	Total closure	Pseudo feces	Feces	Feeding arrest	Slow feeding	Normal feeding	Digested	Group
Alexandrium tamarense	Y	Y	N	N	Y	N	N	N	1
Protoceratium reticulatum	Y	Y	N	N	Y	N	N	N	2
Gonyaulax spinifera	Y	Y	Y	N	Y	Y	N	N	2 (2.5)
Amphidinium carterae	Y	Y	Y	N	Y	Y	N	N	3
Large phytoplankton	Y	N	Y	N	N	Y	Y	N	3
Silt and dust	Y	N	Y	N	N	Y	Y	N	3
Pseudo-nitzschia pseudodelicatissima	Y	N	Y	N	Y	Y	N	N	4
Cyanophyte	Y	N	N	N	Y	Y	N	N	4
Heterosigma akashiwo	Y	N	Y	N	n.c.	n.c.	n.c.	n.c.	4 (5)
Karenia mikimotoi	N	N	Y	Y	N	n.c.	Y	Y	4 (5)
Chaetoceros calcitrans	N	N	Y	Y	N	N	Y	Y	5
Dunaliella tertiolecta	N	N	Y	Y	N	N	Y	Y	5
Isochrysis galbana	N	N	Y	Y	N	N	Y	Y	5
Phaeodactylum tricornutum	N	N	Y	Y	N	N	Y	Y	5

Conclusions

Although summer mortalities are of common occurrence in Pacific oyster (*Crassostrea gigas*) farms throughout the Pacific Ocean, the causative factors involved are still poorly understood. In addition to the suspected high temperatures that can lead to environmental stress, low nutritional value, low abundance of the phytoplankton during extended periods, overloading of the Floating Upwelling System, poor seed quality, hinge ligament and other opportunistic diseases, we have identified the yessotoxin producing dinoflagellate *Protoceratium reticulatum* as a new causative agent, as it prevents feeding in oyster juveniles. This species produced an intense bloom preceding and during the die offs registered at Sechelt Inlet, it was accompanied by much smaller abundances of several other toxin producing algae.

The oysters presented a variety of reactions to medium and high concentrations of selected algae. These species were grouped into five levels, as the response varied from severe rejection, impeding feeding, normally exhibited with toxin producing species, to full acceptance of known innocuous and commercially important species. *P. reticulatum* cells elicited a very strong rejection reaction, rendering the oysters

unable to feed, appearing debilitated after four days, even when this dinoflagellate was present in the water representing only 10 % of the total algae.

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LIPOPHILIC TOXIN PROFILES OF SCALLOPS, PATINOPECTEN YESSOENSIS, AND MUSSELS, MYTILUS GALLOPROVINCIALIS AND MYTILUS CORUSCUS, COLLECTED IN VARIOUS PRODUCTION AREAS IN JAPAN

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Abstract
In 2003 in Japan, harvesting of scallops Patinopecten yessoensis, and mussels Mytilus galloprovincialis and Mytilus coruscus, ceased due to contamination with lipophilic toxins. During the event, a total of 196 samples of scallops and mussels were collected from almost all the production areas and were analysed by liquid chromatography mass spectrometry (LC-MS) for the presence of lipophilic toxins. Okadaic acid (OA), dinophysistoxin-1 (DTX1), 7-O-palmitoyl dinophysistoxin-1 (DTX3), pectenotoxin-1 (PTX1), pectenotoxin-2 (PTX2), pectenotoxin-6 (PTX6), pectenotoxin-2 seco acid (PTX2sa), yessotoxin (YTX), and 45-hydroxy yessotoxin (45OH YTX) in bivalves were quantified by LC-MS. There were no remarkable differences observed in the Dinophysis-toxin (DTXs and PTXs) profiles in the same shellfish species collected from different areas, however toxin profiles were quite different between scallops and mussels. PTX6 and DTX1 were the dominant toxins in the scallops and the mussels, respectively. Yessotoxins (both YTX and 45OH YTX) originating from Protoceratium reticulatum, were detected in scallops and mussels. The proportion of yessotoxins in comparison to the Dinophysis-toxins (DTXs and PTXs) differed substantially among samples even within the same shellfish species collected in the same production area. This could be explained by differences in the relative proportions of Dinophysis spp and P. reticulatum cell densities between the sampling areas or periods.

Introduction
Diarrhetic shellfish poisoning (DSP) is seafood poisoning resulting from the ingestion of shellfish contaminated with DSP toxins (Yasumoto *et al.*, 1978, Yasumoto *et al.*, 1985 and Yasumoto and Murata, 1993. Okadaic acid (OA) and dinophysistoxin (DTX) analogues are the only toxins to be associated with the DSP syndrome, whereas it is now commonly agreed that pectenotoxins and yessotoxins are separate toxins that were originally associated with DSP toxins (Figure 1). Okadaic acid (OA), dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2) are produced by toxic dinoflagellates, Dinophysis spp. and Prorocentrum spp (Yasumoto *et al.*, 1980 and Lee *et al.*, 1989). These toxins have been shown to be potent phosphatase inhibitors (Takai and Mieskes, 1991), a property which can cause inflammation of the intestinal tract and diarrhea (Terao *et al.*, 1986). They have also been shown to have tumour-promoting activity (Fujiki *et al.*, 1988). Dinophysistoxin-3 (DTX3) is 7-O-acyl-DTX1 that is converted from DTX1 by esterification with free fatty acids in bivalves (Yasumoto *et al.*, 1985 and Suzuki *et al.*, 1999). The 7-O-palmitoyl (16:0)-derivative is a predominant compound in bivalves (Marr *et al.*, 1992). Pectenotoxins are suggested to be highly hepatotoxic (Terao *et al.*, 1986). Pectenotoxin-2 (PTX2) is a potent natural actin depolymerizing compound with unique mode of action (Hori

et al., 1999). The latest study showed that pectenotoxins do not cause diarrhoea to mice by the oral route at doses up to 5 mg/kg (Miles *et al.,* 2004), thereby suggesting that pectenotoxins do not belong in the DSP toxins. PTX2 is produced by Dinophysis spp. and the other pectenotoxin analogues are metabolites converted from PTX2 in bivalve tissues (Lee *et al.*, 1989, Suzuki *et al.*,1998, Suzuki *et al.*, 2001a and Suzuki *et al.,* 2001b). Yessotoxin (YTX) is produced by Protoceratium reticulatum and Lingulodinium polyedrum (Satake et al 1997, Tubaro *et al.*, 1998). 45-Hydroxyl yessotoxin (45OH YTX) is thought to be a metabolite converted from YTX in bivalves because 45OH YTX is not detected in P. reticulatum (Yasumoto *et al.*1997). Because YTX was found to be far less toxic to mice via the oral route compared to intraperitoneal injections (Ogino *et al.,* 1997 and Aune *et al.,* 2002), the European Authorities recently set a higher control limit for yessotoxins than for OA and pectenotoxin groups (Garthwaite *et al.,* 2001). Recently several enzyme-linked immunosorbent assays (ELISA) for OA or YTX groups have been developed (Tubaro *et al.,* 1996). Protein phosphatase inhibition assay is also applicable to quantification of OA groups (Mountfort et al., 2001 and Suzuki and Yasumoto, 2000). These assays are potentially useful as screening methods of DSP toxins in the routine monitoring of bivalves as an alternative to mouse bioassay. To introduce these assays in the routine monitoring, toxin profiles of bivalves are important. However toxin profiles of bivalves in Japan had not been investigated yet, except for certain production areas (Quilliam *et al.,* 2001). In 2003 in Japan, harvesting of scallops P. yessoensis, and mussels M. galloprovincialis and M. coruscus, ceased due to contamination with lipophilic toxins. It was the most serious year, in the last decade, in terms of number of areas where harvesting was prohibited within. During the event, a total of 196 samples of scallops and mussels were collected from almost all of the production areas and lipophilic toxins in the bivalves were analysed by liquid chromatography mass spectrometry (LC-MS). To promote efficiency of the LC-MS analysis for many samples, the LC-MS conditions reported previously (Goto *et al.,* 1998) were modified in the present study.

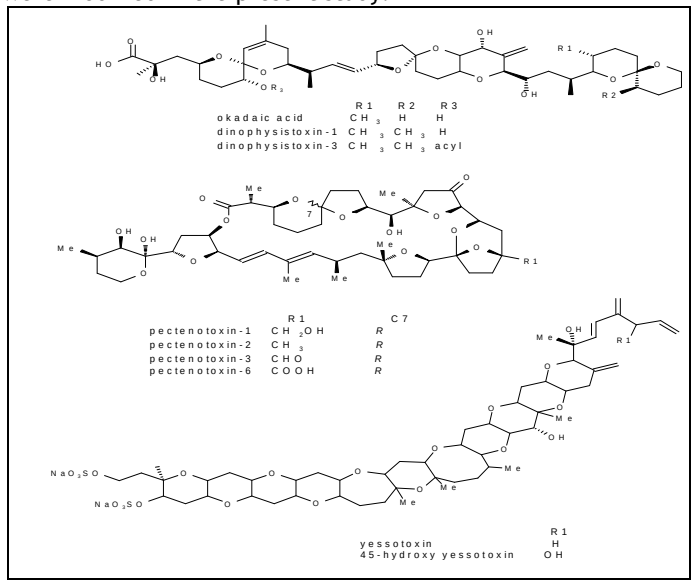

Figure 1. Structure of typical DSP toxins found in Japanese bivalves. Okadaic acid (OA) is a rare toxin in Japanese bivalves.

Methods

Standard toxins

The standards of okadaic acid (OA), dinophysistoxin-1 (DTX1), 7-O-palmitoyl dinophysistoxin-1 (DTX3), pectenotoxin-1 (PTX1), pectenotoxin-2 (PTX2), pectenotoxin-6 (PTX6), and yessotoxin (YTX) were provided by Japan Food Research Labs (Tama, Tokyo, Japan) (Suzuki *et al.*, 2003). Each toxin was dissolved in methanol at a concentration of 100 ng/mL.

Samples

A total of 196 samples were collected from six production areas during a DSP outbreak from May to September 2003 (Figure 2). Scallops P. yessoensis were collected from several production sites in Hokkaido (5 sites), Aomori (10 sites), Iwate (8 sites) and Miyagi (12 sites). Mussels M. galloprovincialis were collected in Aomori (2 sites), Iwate (1 site), and Miyagi (3 sites). Mussels M. coruscus were collected in Akita (1 site), Yamagata (2 sites), and Niigata (1 site). For each sample, hepatopancreas of more than five individuals were combined and kept frozen at -30 °C until being used for the extraction of toxins.

Extraction

Combined hepatopancreas (1 g) were extracted with 9 ml 90 % methanol. The methanol extracts were centrifuged at 3000 rpm for 5 minutes. An aliquot of the supernatant was passed through a 0.5 μm filter for HPLC sample preparation (Millipore, MA, USA), then directly injected into LC-MS.

LC-MS

LC-MS was performed on a Hewlett-Packard 1050 Series liquid chromatograph coupled to a Finnigan MAT SSQ-7000 instrument (San Jose, CA, USA) equipped with an atmospheric pressure electrospray ionization (ESI) interface. Separations were performed on Quicksilver cartridge columns (50 mm x 2.1 mm i.d) packed with 3 μm Hypersil-BDS-C8 (Keystone Scientific, Bellefonte, PA, USA) maintained at 35 °C. Eluent A was water and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 50 mM formic acid (Goto *et al,*. 1998). Gradient elution from 20 % to 100 % B was performed over 10 min and then held at 100 % B for 20 min for LC separation. The flow rate was 0.2 mL/min and the injection volume was 5 μL. By using a divert valve, LC eluent between 8 and 13 minutes, and between 19 and 21 minutes were introduced into an ESI interface. The other eluent fractions were discarded to avoid pollution of the MS detector by bivalve matrices. Toxins were scanned with selected ion monitoring (SIM) of negatively charged ions for [M-H]- of OA (m/z 803.5), DTX1 (m/z 817.5), DTX3 (m/z 1055.7), PTX2 seco acid (m/z 875.5), and for [M+HCOOH-H]- of PTX1 (m/z 919.5), PTX2 (m/z 903.5), PTX6 (m/z 933.5), and for [M-H]-of of YTX (m/z 1141.5), 45OH YTX (m/z 1157.5). The SIM scan width for each toxin was 0.3 U (unit mass). The voltage on the ESI interface was maintained at approximately 4.5 kV. The temperature of heated capillary was set to 200 °C. High-purity nitrogen gas was used as a sheath gas (nebuliser gas) at an operating pressure of 70 p.s.i. and an auxiliary gas at 5 p.s.i., respectively (1 p.s.i. = 6894.76 Pa).

In the present study, the same LC-MS method was also performed on a HP 1100 liquid chromatograph (Agilent, Palo Alto, CA, USA) coupled to an Q TrapTM mass spectrometer (PE-SCIEX, Thornhill, ON, Canada) to confirm the applicability of the method for different equipments.

Figure 2. Production areas of bivalves where harvesting was prohibited as a result of contamination of bivalves with lipophic toxins in 2003.

Results and Discussion

LC-MS

Figure 3 shows the SIM LC-MS chromatogram obtained from a mixture of standard toxins. Clear chromatogram was obtained for all analysed toxins. All the toxins except for DTX3 (7-O-palmitoyl DTX1) eluted between 10 to 13 minutes. The retention time of DTX3 was 21 minutes. Although the peak of YTX, PTX1, and PTX6 overlapped, it was not problematic for the quantification of these toxins as different ion traces are monitored in SIM as shown in Figure 3. The sensitivity of YTX and pectenotoxins were lower than that obtained for OA analogues. The lowest sensitivity was obtained for PTX6. PTX6 has a carboxyl group and [M-H]- ion is detectable. Sensitivity of the [M-H]- ion of PTX6 was higher than that of [M+HCOOH-H]- ion in the present method, however we found that detection of the [M+HCOOH-H]- ion is more useful in terms of the reproducibility. The [M-H]- ion was detected in PTX2 seco acid which is an acidic compound, however the [M+HCOOH-H]- ion was not detected in contrast to the other pectenotoxins. This suggests that the macrolide structure of pectenotoxins is essential to form the [M+HCOOH-H]- adduction. It is reported that positive mode is applicable to detection of OA and pectenotoxin analogues (Goto *et al,*.1998 and Yasumoto et al., 1995). Sensitivity of [M+NH4]+ ion of OA analogues and pectenotoxins in the positive mode was obviously higher than that obtained in the negative mode, however sensitivity of YTX in the positive mode was too low to quantify it. In addition to this, serious matrix effects for quantification of toxins, especially for DTX3, were observed in the positive mode, therefore toxins were detected by the negative mode in our present study. Detection limit of PTX6 which gave the lowest sensitivity was approximately 25 pg (S/N=3). Because 1/2000 aliquot of one gram hepatopancreas extracts is injected to LC-MS, the detection limit of PTX6 in the hepatopancreas is 50 ng/g. Since the sensitivity of OA analogues is approximately five times higher than that of PTX6 (Figure 3), the detection limit of OA analogues is 10 ng/g hepatopancreas. As 4.0 µg of OA corresponds to one mouse unit (MU) defined in the mouse bioassay method

(Ito and Tsukada, 2002), the present LC-MS method is applicable to the hepatopancreas at a toxicity level of 0.0025 MU/g. This level is 1/200 of the maximum allowance level (0.5MU/g) currently employed for shellfish monitoring. It is reported that biological matrices interfere with quantification of analytes (Goto, 2001) and suitable clean up of samples is recommended (Suzuki, 2001). To investigate the matrix effects in the present LC-MS method, non-toxic 90 % methanol extracts of scallops hepatopancreas were fortified with OA, DTX1, DTX3, PTX1, PTX2, PTX6 and YTX. The 90 % methanol extracts fortified with toxins were analysed by the present LC-MS method. More than 90 % recovery was obtained for each toxin added at a toxin concentration of 0.05 μg/mL in the shellfish extract. Furthermore, detection of toxins was quite stable even when analyses of samples continued for more than 48 hours. These results indicate that the present LC-MS method is applicable to analyzing crude 90 % methanol extracts without clean-up of samples. Similar results were obtained by LC-MS analysis using API Q TrapTM mass spectrometer. This suggests that the present LC-MS method is widely applicable to several types of equipments. Detailed results will be reported elsewhere.

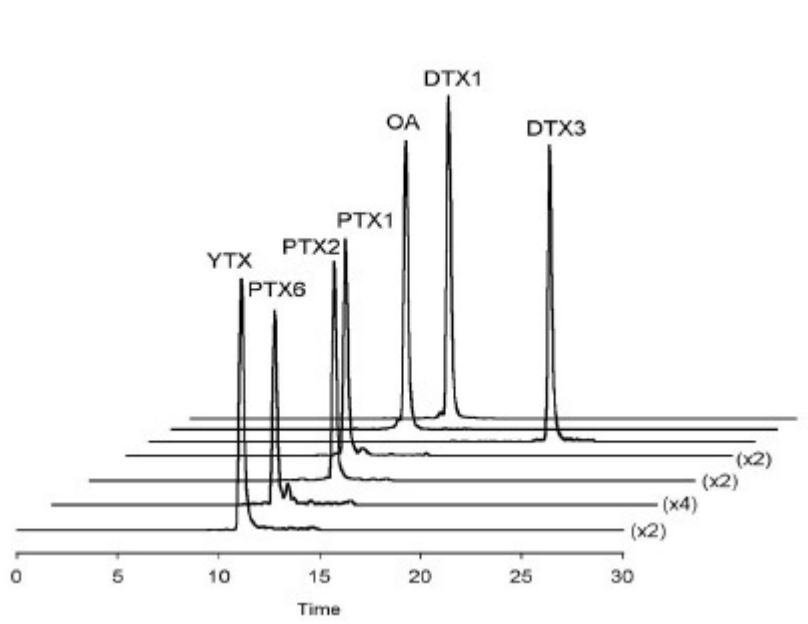

Figure 3. SIM LC-MS chromatogram of DSP toxins. Toxins were scanned with SIM of negatively charged ions for [M-H]- of OA (m/z 803.5), DTX1 (m/z 817.5), DTX3 (m/z 1055.7), and for [M+HCOOH-H]- of PTX1 (m/z 919.5), PTX2 (m/z 903.5), PTX6 (m/z 933.5), and for [M-H]- of YTX (m/z 1141.5). Chromatographic conditions, see text.

Toxin profiles of bivalves

Table 1 lists the toxin profiles obtained from scallops and mussels collected from various production areas in Japan. Because PTX2sa is not toxic to mice when ip injected at the concentration of 5 mg/kg (Miles, 2004) it was excluded from the toxin profiles. OA was not detected in samples, probably because OA was not produced by the causative Dinophysis spp. There were no remarkable differences observed in the Dinophysis-toxin (DTXs and PTXs) profiles in the same shellfish species collected from different areas, however toxin profiles were quite different between scallops and mussels. PTX6 and DTX1 were the dominant toxins in the

scallops and the mussels, respectively. PTX2sa was not detected in the scallops although it was the dominant pectenotoxin analogue in the M. galloprovincialis (data not shown). No pectenotoxin analogues were detected in the mussels M. coruscus, suggesting that this mussel species converts or decomposes PTX2 to unknown metabolites. In a previous study (Quilliam *et al.*, 2001), contents of esterified DTX1 in scallops and mussels collected in Aomori were determined after alkaline hydrolysis of the extracts. The relative contents of esterified DTX1 to DTX1 reported in the previous study is comparable to that obtained by direct quantification of DTX3 (7-O-palmitoyl DTX1) in the present study. Therefore it was suggested that dominant esterfied DTX1 in bivalves in Japan is 7-O-palmitoyl DTX1 as reported previously (Yasumoto *et al.*, 1985). Further study is necessary to clarify the detailed profiles of the esterified DTX1 in bivalves in Japan. We are investigating a direct LC-MS/MS method for quantification of 7-O-acyl DTX1. Yessotoxins (both YTX and 45OH YTX) originating from Protoceratium reticulatum, were detected in scallops and mussels. The proportion of yessotoxins to the Dinophysis-toxins (DTXs and PTXs) differed substantially among samples even within the same shellfish species collected in the same production area. This could be explained by differences in the relative proportions of Dinophysis spp and P. reticulatum cell densities between the sampling areas and/or the sampling periods.

Table 1. DSP toxin profiles of scallops and mussels collected in various production areas in Japan in 2003.

	Scallops (*P. yessoensis*) average (SD[*1])				Mussels (*M. galloprovincialis*) average (SD)			Mussels (*M. coruscus*) average (SD)		
Toxin	Hokkaido n=14	Aomori n=38	Iwate n=45	Miyagi n=50	Aomori n=3	Iwate n=5	Miyagi n=27	Akita n=5	Yamagata n=4	Niigata n=5
OA	-	-	-	-	-	-	-	-	-	-
DTX1	1(4)	7(6)	5(2)	3(2)	83(30)	38(21)	28(22)	80(6)	91(6)	96(5)
DTX3	Tr[*2]	2(3)	10(5)	7(5)	2(3)	11(6)	9(8)	11(5)	8(4)	4(5)
PTX1	7(10)	3(4)	7(4)	7(5)	-	-	Tr	-	-	-
PTX2	-	Tr	3(3)	3(4)	-	-	-	-	-	Tr
PTX6	48(33)	47(17)	55(14)	71(11)	-	-	-	-	-	-
YTX	43(40)	36(20)	14(12)	7(6)	10(18)	36(19)	43(18)	8(6)	1(2)	Tr
45O HYTX	1(3)	5(5)	6(7)	2(2)	5(10)	15(8)	20(9)	1(1)	Tr	-
Total	100	100	100	100	100	100	100	100	100	100

[*1] SD; standard deviation. [*2] Tr; trace less than 0.2 %.
OA, okadaic acid; DTX1, dinophysistoxin-1; DTX3, dinophysistoxin-3 (7-*O*-palmitoyl DTX1); PTX1, pectenotoxin-1; PTX2, pectenotoxin-2; PTX6, pectenotoxin-6; YTX, yessotoxin; 45OHYTX, 45-hydroxy yessotoxin.
Toxins in bivalves were quantified by comparing the peak areas with authentic standard toxins. 45OH YTX was quantified by using authentic YTX.
PTX2 seco acid (PTX2sa) was detected in Mussels, *M. galloprovincialis*.

Conclusions

The results obtained in the present study indicate that PTX6 and DTX1 are the most important Dinophysis-toxins in scallops and mussels respectively in Japan, whereas YTX is an important toxin in both scallops and mussels. It has been reported that the yessotoxins and pectenotoxins are far less toxic than OA and DTX1 analogues when they are orally administered to mice (Miles *et al.,* 2004, Ogino *et al.,* 1997 and Aune *et al.,* 2002). The results obtained in the present study suggest that in Japan, the risk associated to the DSP toxins may be lower in scallops than in mussels, due the different toxin profiles found in these two bivalve species. Complicated toxin profile in bivalves is one of the obstacles to introduce alternative chemical or biochemical methods to mouse bioassay. In the present study, the major toxins in Japanese bivalves were identified. The determination of the dominant toxins in bivalves by chemical or biochemical methods is necessary prior to establishing a rapid and efficient monitoring system. However, the accumulation of more data on toxin profiles in bivalves is essential. We will try to accumulate the data for at least another three years.

Acknowledgements

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FIRST REPORTED CASE OF YESSOTOXINS IN MUSSELS IN THE GALICIAN RIAS DURING A BLOOM OF *Lingulodinium polyedrum* STEIN (DODGE)

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Abstract
During the summer of 2003, a red tide caused by *Lingulodinium polyedrum* was detected in the Ría de Ares-Betanzos (NW, Spain) in the oceanographic stations that are monitored weekly. The area, which is dedicated to the intensive culture of mussels (*Mytilus galloprovincialis* Lmk) in rafts, had already been closed by lipophilic toxins as a result of a previous outbreak of *Dinophysis acuminata*. LC-MS analyses of mussel samples revealed the presence of yessotoxins (YTX) and okadaic acid (OA). YTX and OA were quantified on mussels by HPLC-FD ranging between 0.9-1.3 and 1.1-2.9 $\mu g \cdot g^{-1}$ of digestive gland, respectively. YTX and Homo-YTX were also detected in net haul samples by LC-MS but they were below the HPLC-FD quantifiable limits. This is the first reported detection of YTX's in shellfish in the Iberian Peninsula.

Introduction
The accumulation of marine phycotoxins by bivalve molluscs poses a health risk and creates serious disruptions for the aquaculture industry. Yessotoxins (YTX's) are a group of marine toxins known about since the first diarrheic shellfish toxin (DST) studies were performed (Murata *et al.*, 1987), but whose actual toxic effect in humans is still unclear (Tubaro *et al.*, 2004). These disulphated polyether toxins have been traditionally included in the DST group, although the YTX chemical structure is different from that of okadaic acid (OA) and its group of derivatives and produces neither a diarrhoetic effect nor the same toxicological profile (Terao *et al.*, 1990; Ogino *et al.*, 1997). With this consideration, European Authorities have recently established YTX closure limits that are different to those for DST (EC, 2002). The first species confirmed to be the biogenetic origin of YTX was *Protoceratium reticulatum* (Satake *et al.*, 1997) and recently the suspicion that *Lingulodinium polyedrum* can also produce YTX has been confirmed (Paz *et al.*, 2004). Nowadays, reported detection of YTX in bivalve molluscs arise in an extremely wide geographical range, Japan (Murata *et al.*, 1987), Norway (Lee *et al.*, 1988), The Adriatic Sea (Ciminiello *et al.*, 1997), New Zealand and Chile (Yasumoto and Takizawa, 1997), but taking into account the fact that known YTX-producer species are fairly common, it is likely that YTX may actually be a far more widespread phenomenon than has been reported to date.

The Galician Rías (NW, Spain) are an area of intensive mussel aquaculture recurrently affected by DST episodes. In the summer of 2003 a *L. polyedrum* red tide was observed in the Ría de Ares-Betanzos. At that time harvest of the mussel rafts in this Ría had already been closed by DST of a previous outbreak of *Dinophysis acuminata*. Because of the suspicion of YTX production by *L. polyedrum*, several mussel samples were collected and analysed for YTX using HPLC-FD and LC-MS. The aim of this study was to determine both the profile and content of toxins and its association with this species.

Materials and methods

Water and mussel sampling and phytoplankton count

The water column was sampled weekly at three stations using a 15 m hose (Figure 1). An aliquot of this sample was immediately preserved with lugol's solution, and the phytoplankton cells were counted under an inverted microscope, using the Uthermöhl procedure. At two sampling points (Stations L2 and L3) a concentrated sample was collected by means of a plankton net haul (10 µm mesh size) from a depth of 15 m to the surface. This concentrated sampled was split into two aliquots. The first was preserved with lugol's solution and used to quantify the phytoplankton population. The second aliquot was filtered through Whatman GF/C glass fibre filters and frozen until the extraction of the toxins was performed. Samples of mussels were collected in culture areas Sada A and Sada B (Figure 1).

Toxicity and Toxins analysis in mussels and seawater

Mouse bioassays for DST were carried out according to the Yasumoto 84 method (1984), starting with 20 g of digestive gland (dg). For PSP the AOAC Method 1999 was used.

For HPLC toxins analyses in mussel samples, an extraction process based on the method of Goto *et al*. (2001) was applied, 1g of dg was extracted in 9 mL of MeOH, homogenised and centrifuged at 2500 rpm for 10 minutes. For both YTX and OA analyses, 3 mL of supernatant were dried under a N_2 stream, re-suspended in 3 mL of Methanol:ammonium acetate 0.02 M pH 5.8 (3:7), and loaded onto a Sep-Pak C18 cartridge, the extract was washed with 10 mL of MeOH:H_2O (3:7) and toxins were eluted with 10 mL of PrOH:H_2O (20:80). A 5 mL aliquot from this eluate was dried under an N_2 stream and derivatised for YTX analysis by HPLC-FD, the other 5 mL aliquot was dried and re-dissolved in 0.5 mL of MeOH for YTX and OA analysis using LC-MS.

Extraction of toxins from net haul samples and analysis of YTX by HPLC-FD was performed as per a previous report (Paz *et al.*, 2004). Analysis of OA by HPLC-FD in mussels samples was based on the procedure of Lee *et al*. 1987.

For LC-MS analysis of both YTX and OA, the separation column was an XTerra C8 MS 5µm (150 x 2.1mm). A mobile phase of 2 mM ammonium acetate (pH 5.8) and MeOH (3:7) was used. The flow rate was 0.20 mL·min^{-1}. Mass spectrometric measurements were performed using an ion trap mass spectrometer, Thermo Finnigan LCQ-Advantage, equipped with an electrospray ionisation (ESI) negative interface. ESI was performed by a 4.5 kV spray voltage and 200 °C capillary temperature. A flow rate of 60 mL·min^{-1} was used for the sheath gas and 20 mL·min^{-1} for the auxiliary gas. Data was acquired in full scan mode from *m/z* 500 to 1500, YTX was detected in negative mode for the ion $[M-2Na+H]^-$ at *m/z* 1141 (Draisci *et al*. 1999, Ciminiello *et al*. 2002; 2003) and OA was detected in positive mode for the ion $[M+NH_4]^+$at *m/z* 822 and for ion $[M+Na]^+$ at *m/z* 828 (Draisci *et al*. 1998, Suzuki and Yasumoto 2000). HPLC-FD and LC-MS systems were calibrated with certified solutions of OA (OACS-1) provided by NRC (Canada) and YTX solutions provided by Prof. M. Satake (Japan).

Results

Seasonal evolution of harmful phytoplankton

Dinophysis acuminata was detected in the area, almost throughout the year, showing two main periods of higher development: One during the month of April and the other from mid July to early September (Figure 2). *Lingulodinium. polyedrum* was detected only between the 31st of July and early September, with a maximum concentration of 2.1·10^6 cells·L^{-1} on the 25th August (Figure 2). From 7th August to 1st

September the *L. polyedrum* bloom was almost monospecific: this species representing up to 99 % of the total number of dinoflagellate cells and 77 % of the total phytoplankton community cells. *Protoceratium reticulatum* was not detected in the area during this year.

Toxins in mussels and net haul samples

The mouse bioassay for DST showed neurological symptoms on mice that do not correlate with the presence of OA and/or its derivatives nor PSP toxins. No PSP toxins were detected.

LC-MS analyses of mussel samples from Sada-A and Sada-B revealed the presence of YTX and OA. The associated mass spectrum displayed a signal at *m/z* 1141 resulting from the ion $[M-2Na+H]^-$ from YTX (Figure 3). OA was also detected in the mass spectrum for both associated ions: $[M+NH_4]^+$ at *m/z* 822 and $[M+Na]^+$ at *m/z* 828 (Figure 3). YTX and OA were quantified by HPLC-FD and ranged between 0.89-1.30 and 1.1-2.9 $\mu g \cdot g^{-1}$ of dg, respectively (Table 1).

LC-MS analyses of net haul samples, rich in *L. polyedrum*, revealed the presence of YTX. The mass spectrum displayed a signal at *m/z* 1141 owing to the $[M-2Na+H]^-$ ion in YTX. A high signal was also noted at *m/z* 1155, which could be assigned to the homoYTX, which is 14 mass units larger than YTX. In the SIM at *m/z* 1141 and *m/z* 1155, a peak appeared at 23.1 minutes, as a result of the co-elution of YTX and homoYTX (Figure 4). *L. polyedrum* concentrations in the filtrates were between $5 \cdot 10^6$ and $2.5 \cdot 10^8$ cells$\cdot L^{-1}$, but for all those samples YTXs concentrations were below the quantifiable limits for the HPLC-FD.

Table 1. Toxicity data (mouse bioassay) and OA and YTX concentrations (HPLC-FD) of culture mussels from the Ría de Ares-Betanzos.

Sampling station	Date	OA ($\mu g \cdot g^{-1}$ dg)	YTX ($\mu g \cdot g^{-1}$ dg)	DTX Bioassay	PSP AOAC Bioassay
Sada-A (L1)	July 30th 2003	1.1	1.12	Positive	Negative
Sada-B (L2)	July 30th 2003	2.1	0.89	Positive	Negative
Sada-A (L1)	August 5th 2003	2.9	1.28	Positive	Negative
Sada-B (L2)	August 5th 2003	Not	1.30	Positive	Negative

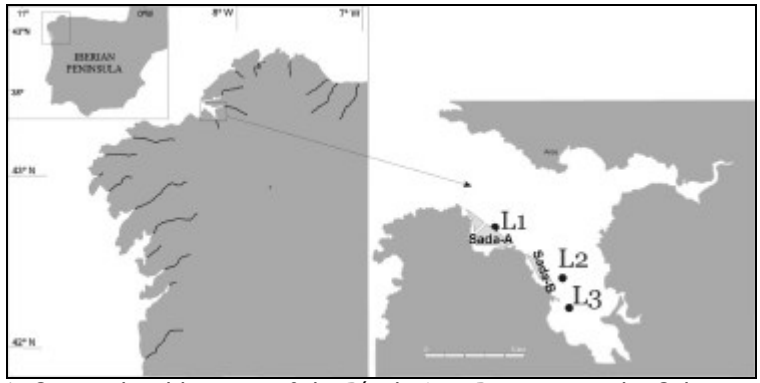

Figure 1. Geographical location of the Ría de Ares-Betanzos on the Galician coast (NW, Spain) and sampling stations chosen in this Ría for the study. The grey geometric figures are groups of rafts.

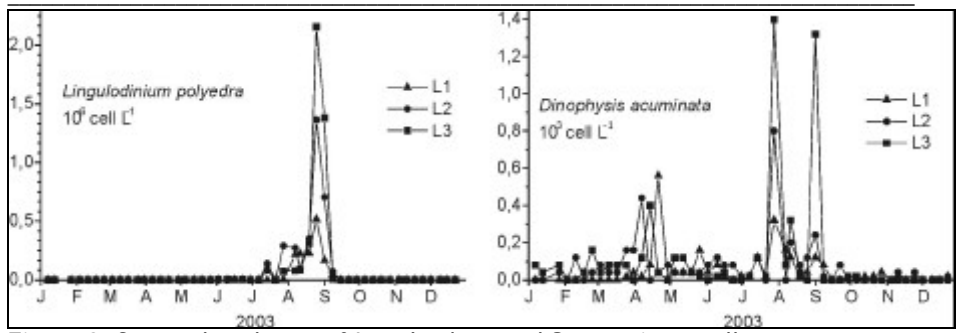

Figure 2. Seasonal evolution of *L. polyedrum* and *D. acuminata* cell concentrations in the oceanographic stations of Ría de Ares-Betanzos.

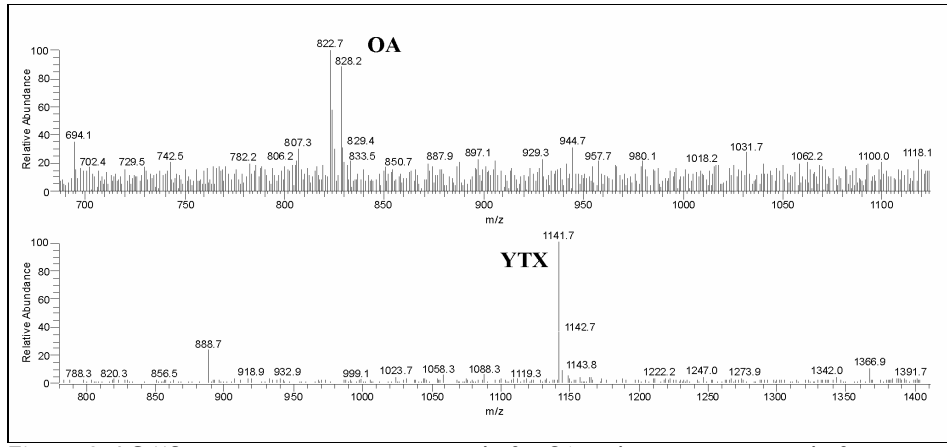

Figure 3. LC-MS spectra, in positive ion mode for OA and negative ion mode for YTXs, of a mussel sample collected from Sada-B on August 5th 2003.

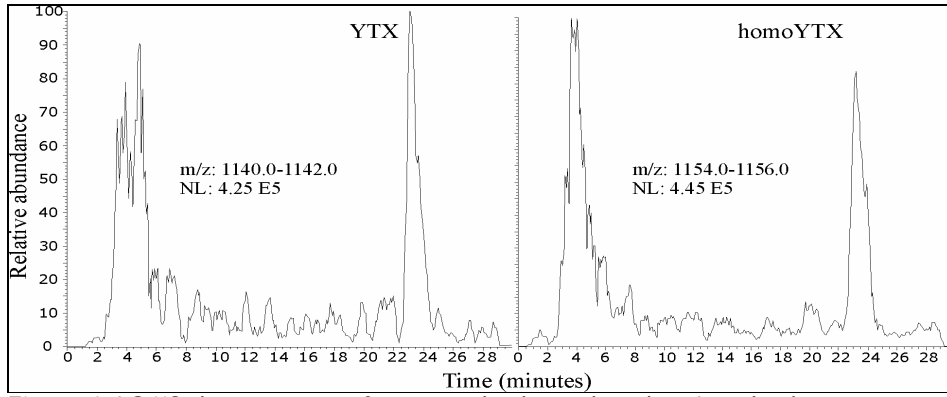

Figure 4. LC-MS chromatograms from a net haul sample rich in *L. polyedrum* collected in L2 station (1st September 2003). Extracted ion chromatograms from YTX (m/z 1140.0-1142.0) and homoYTX (m/z 1154.0-1156.0).

Discussion

This is the first reported detection of YTX's in shellfish in the Iberian Peninsula. The higher levels of OA over YTX detected in this study, were the result of a longer accumulation process, since *D. acuminata* reached measurable concentrations in April and maintained significant levels from mid-July onwards, while the first cells of *L. polyedrum* were detected from 31st July onwards.

The bloom concentrations of *L. polyedrum* and the absence of *P. reticulatum*, a species confirmed as a YTX producer (Satake *et al.*, 1997), would seem to indicate that the YTX producer was, in this case, *L. polyedrum*. Paz *et al.* (2004) have recently confirmed the presence of YTX in cultures of *L. polyedrum* obtained from the same geographic area (Ría de Ares-Betanzos). In the present study, it was not possible to estimate the toxin content per *L. polyedrum* cell, although the concentration of cells for analyses was one order of magnitude higher than that used by Tubaro *et al.* (1998) in order to obtain an estimation ranging from 1.5 to 1.1 $pg \cdot cell^{-1}$. Probably these types of differences are a result of the variability of toxin content per cell, in fact, Paz *et al.* (2004) have observed that the toxin content of *L. polyedrum* cultures is 10 times lower than that of *P. reticulatum*. However, an important factor in this case could be the amount of toxin released to the medium (Paz *et al.*, 2004).

The detection of a *L. polyedrum* bloom leads us to suspect the presence of YTX's allowing us to focus on the analytical search for toxins. These results underline the potentially of a systematic monitoring not only of known HAB species but also the phytoplankton community as a whole. An operative coupling between both phytoplankton and biotoxins monitoring allows us to study the biogenetic origin of the toxins. Moreover the detailed study of the historical data base series of phytoplankton and environmental variables will allow us to forecast the intensity and extension of any harmful phenomenon in the area.

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TOXIC ALGAE IN AQUACULTURE SITES IN THE PHILIPPINES AND OTHER SOUTHEAST ASIAN COUNTRIES

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Abstract
There has been an increase in the occurrence of Harmful Algal Bloom (HAB's) in the past two decades in the Southeast Asian region. Most of these blooms have led to the contamination of shellfish in harvest and aquaculture sites thus causing economic havoc and negative public health impacts in many coastal areas in the region. To date, *Pyrodinium bahamense* var. *compressum* has been a major Paralytic Shellfish Poisoning (PSP) causative organism and has tolled about 2,709 cases in the region. In 2003, *Alexandrium minutum* was recorded in an aquaculture site in Northern Philippines and has caused 6 PSP cases and 2 deaths in the area at a very high PSP toxin concentration in *Perna viridis,* the agent of poisoning. In several Southeast Asian countries, *A. minutum*, *A. tamiyavanichii* and *Gymnodinium catenatum* have also been reported and have caused PSP cases in several occasions and places. Other toxic algae like *Dinophysis spp*. and *Pseudo-nitzschia spp*. that can cause Diarrhetic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP) respectively, have also been reported in the region. No poisoning has been associated with these species but their health risks have been considered.

Introduction
The Southeast Asian region is currently beset with HAB problems emanating mostly from Paralytic Shellfish Poisoning (PSP) causing organisms. Other harmful algal species that have the potential to cause Diarrhetic Shellfish Poisoning (DSP), Amnesic Shellfish Poisoning (ASP), Neurotoxic Shellfish Poisoning (NSP) and Ciguatera Fish Poisoning (CFP) have not caused these types of poisoning in areas where they have been detected. Reviews made by Azanza (1999), Azanza and Taylor (2001) and the summary presented at the harmful algal bloom of Southeast Asia (HABSEA Portal - http://portal.unesco.org/habsea) also show that problems from harmful algae during more recent years include fish kills.

This paper is limited to the presentation of data and information on recorded toxic algal blooms and poisonings at or near aquaculture sites in the Philippines and Southeast Asia (i.e. Brunei, Malaysia, Indonesia, Singapore and Vietnam) from 1908 to the present.

Recorded History of Harmful Algal Blooms/ Red Tides in Southeast Asia (1908-1998)
The earliest sighting of a sea discoloration in Southeast Asia was in 1908 and was apparently not harmful. *Peridinium* sp. (O.F. Müller) was reported as the causative organism (Smith 1908). Published reports in the region show only 3 types of harmful algal blooms. In 1959, the first PSP event in the region was experienced in Indonesia with 11 cases. The causative organism of said poisoning, however, has remained unidentified up to now (Adnan 1993). The worst event occurred in 1976 when one harmful algal bloom after another befell the Indo-Malayan region: Malaysia, Brunei, Indonesia, Singapore and Philippines with Brunei and Malaysia reporting PSP cases (Azanza and Taylor 2001). From 1976 to 1998 the primary causative organism of PSP in the Philippines, Malaysia, Brunei and Indonesia was *Pyrodinium bahamense* var. *compressum* (Böhm) (Azanza and Taylor 2001). In 1983, 63 PSP cases were also reported in Pranburi, Southern Thailand (Suvapepun 1984) which was their first and

so far only reported poisoning. Low cell concentrations of *Alexandrium tamarense* (Lebour) (formerly *Protogonyaulax tamarensis*) were detected. *Alexandrium tamiyavanichii* (Balech) was observed in Sebatu, Malacca in 1997 and suspected of causing the poisoning of 3 people in 1991. *Alexandrium affine* was found to bloom in Ambon Bay, Indonesia in 1997. In 1985 then 1989, cases of fish poisoning co-occurring with a bloom event was reported in Indonesia although the causative algal organism was not identified (Adnan, 1983). In 1995 Vietnam also reported food poisoning relating to a bloom of *Noctiluca scintillans* although this could be blamed on decaying fish which the people may have eaten during the fish kill (Nguyen Ngoc *et al*., 1997; Nguyen and Doan, 1996).

Recent Reports of Toxic Algal Blooms in Southeast Asia (1999 to Present)
Paralytic Shellfish Poisoning has remained a major problem comprising 55 % of the total reported types followed by fish kills (35 %) and poisoning from fish during a harmful algal bloom (10 %) (Figure 1). There have been no other poisonings due to other harmful algae reported in the region during this period although causative species (Table 1) have been detected during monitoring (HABSEA Portal, 2004). Aside from *Pyrodinium*, other PSP causing organisms have recently been recorded in Southeast Asia. Toxic algal species that have been identified in Vietnam with no confirmed poisoning reports are *Alexandrium* spp. in Binthuanh waters and *Pseudo-nitzschia* spp. (Peragallo) in Cam Ranh Bay (Lam and Larsen 2003; Dao and Kodama 2003). *Pyorodinium bahamense* var. *compressum* have been reported in Indonesian waters (Widiarti *et al*. 2003). No recent poisoning, however, has been reported which can be attributed partly to Indonesians' non-preference for mussels as part of their diet. In Singapore, no poisoning has been recorded yet and the present monitoring system has revealed potentially harmful microalgae in their waters. These include causative organisms of PSP: *Gymnodinium catenatum* (Graham); DSP: *Dinophysis caudata* (Saville-Kent), *D. ovum* (Schütt), *D. sacculus* (Claperède and Lachmann) and *D. rotundata* (Claperède and Lachmann) and CFP: *Gambierdiscus toxicus* (Adachi and Fukuyo), *G. belizeanus* (Faust) and *G. yasumotoi* (Holmes) although cultures of *Gambierdiscus* spp. were not found to produce ciguatoxins (Holmes and Teo 2002).

In Malaysia, *Pyrodinium bahamense* var. *compressum* has been re-dected in Sabah in 2000, and *A. minutum* (Halim) in the East Coast of Peninsula Malaysia has been detected in 2001. The latter species has been linked to 6 PSP cases, the victims consumed *Polymesoda* sp., a benthic bivalve (Usup *et al*. 2002). Like Singapore, there were no recorded DSP and CFP cases, only potentially harmful microalgae: *Dinophysis* spp. (Ehrenberg), *Prorocentrum* spp. (Ehrenberg), *G. toxicus*, *Ostreopsis ovata* (Fukuyo), *O. lenticularis* (Fukuyo) and *Coolia* spp. (Meunier) (Lim *et al*. 2003).

In the Philippines, *Pyrodinium bahamense* var. *compressum* which in the past bloomed in Manila Bay has been reported only in some other bay areas (Table 2 and Figure 2). Other toxic species recently identified in Manila Bay include *Alexandrium tamiyavanichii* and *Gymnodinium catenatum* (Montojo *et al*. 2003). The observation has caused the imposition of a shellfish ban in 2002 but there has been no report of poisoning associated with this incidence. In 2003, a new PSP causing organism, *A. minutum* was detected at low concentrations in Bolinao, Pangasinan, Northern Philippines where there is intensive aquaculture (Azanza *et al*. 2003). High levels of PSP toxins that ranged from 930 to 2,894 STXeq/100g green mussel (*Perna viridis*) have been detected and 6 poisoning cases have been reported (Bajarias *et al*. 2003).

Countries in Southeast Asia share common bodies of water such as the South China Sea, Sulu Sea, Celebes Sea, Strait of Malacca and Gulf of Thailand hence, it is highly

possible that a harmful microalgal species threat to one country is a threat to another. The most recent example of this is the *Cochlodinium* bloom along Brunei Bay that reached Malaysia, then the Philippines last February to May, 2005 (Azanza *et al.*, 2005; Azanza and Baula, 2005). Due to the East Asian monsoon-controlled anticyclonic circulation of the South China Sea at that time, *Cochlodinium* cells were easily advected from Brunei to Philippines and maintained for a long period of time. By strengthening linkages among these countries, a possible warning system can be devised. In enclosed bay areas, however blooms are possibly consequences of germinated cysts. Paralytic Shellfish Poisoning incidences have been known to occur at the beginning of the monsoon seasons as well as towards its end (Azanza and Taylor, 2001). Due to this shift in monsoons wherein a significant amount of wind forcing and tidal current is present, the seabed becomes highly turbulent enabling cysts in the sediments to be resuspended and potentially start a bloom (Villanoy *et al.*, in press). A substantial number of researche projects are on-going in Southeast Asia that look into possible causes of blooms in the region. Collaborative projects such as those of the HABSEA Portal (UNESCO-IOC) and Dinoflagellate Cyst Mapping in SEA (JSPS) among others address HAB challenges that plague the region. By actively involving researchers from all over the region, HAB problems will be narrowed down and effectively managed in the future.

Table 1. List of toxic microalgae found recently (1999 to present) in SEA waters*

	PSP	DSP	CFP	ASP	References
Singapore	*Gymnodinium catenatum*	*Dinophysis caudata* *D. ovum* *D. sacculus*	*Gambierdiscus toxicus* *G.* cf. *belizeanus* *G. yasumotoi*		Holmes and Teo 2002
Malaysia	*Alexandrium tamiyavanichii* A. minutum *Pyrodinium bahamense* var. *compressum*	*Dinophysis* spp. *Prorocentrum* spp.	*G. toxicus* *Ostreopsis ovata* *O. lenticularis* *Coolia* sp.		Usup 2002; Lim, 2003
Vietnam	*Alexandrium* spp.	*Dinophysis* spp.		*Pseudo-nitzschia* spp.	Lam and Larsen 2003
Indonesia	*Pyrodinium bahamense* var. *compressum*				Widiarti *et al*. 2003
Philippines	*A. minutum* *A. tamiyavanichii* *G. catenatum* *Pyoridinium bahamense* var. *compressum*			*Pseudo-nitzschia* spp.	Azanza *et al*. 2003; Montojo *et al*. 2003
Brunei	-	-	-	-	
Thailand	-	-	-	-	

- no report

* Poisonings were reported only for PSP

Table 2. Areas / Bays in the Philippines with reports of *Pyrodinium bahamense* var. *compressum* (1983 to present) *

Province	Bay/Water Area	Year
Zambales	Masinloc waters	1987 to present
	Palauig Bay	1987 to present
Metro Manila	Manila Bay	1988 to 1998
Sorsogon	Sorsogon Bay	1983 to 1999
	Juag Lagoon, Matnog	1994 to present
	Casiguran	2004
Masbate	Mandaon waters	1983 to present
	Milagros waters	1983 to present
Leyte and Samar	Calbayog waters	1983 to 1993
	Maqueda Bay	1983 to 1993
	Villareal Bay	1983 to 1993
	Carigara Bay	1983 to 1993
	San Pedro Bay	1989 to present
	Cancabato Bay	1994 to 2003
Capiz	Capiz waters	1983 to 1988
Palawan	Honda Bay	1998 to present
	Malampaya Sound	1998 to present
Negros Oriental	Bais Bay	2003
Surigao del Sur	Borobo Bay	1997 to 1999
	Lianga Bay	1997 to 1999
	Hinatuan	1997 to 1999
Zamboanga del Sur	Dumanquilas Bay	1994 to present
	Sibuguey Bay	1994 to present
	Illana Bay	1994 to present
Davao Oriental	Balite Bay, Mati	1983 to present
	Pujada Bay	1983 to 1999

*Red Tide Update 1998 to 2004; Relox and Bajarias 2003

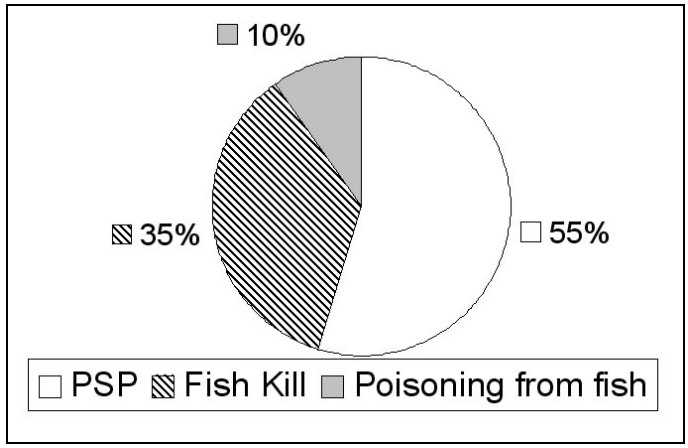

Figure 1. HAB types in South East Aisa from 1959 to 2004 (from: HABSEA Portal, 2004)

Figure 2. Bays/areas in the Philippines where *Pyrodinium bahamense var. compressum* blooms have been recorded (1983- present). Table 1 presents years when blooms were reported.

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THE MONITORING PROGRAMME FOR MARINE TOXINS AND HARMFUL PHYTOPLANKTON IN THE CATALAN COASTLINE, NORTH WESTERN MEDITERRANEAN, SPAIN.

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Abstract
The Regional Government of Catalonia is responsible for the monitoring of marine toxins and harmful phytoplankton in the Catalan coastline in Spain. In an attempt to protect public health widely, the monitoring programme covers all coastal waters including recreational waters and shellfish production areas. The design and implementation of the monitoring programme takes into consideration the geographic and oceanographic characteristics of the Catalan coastline in order to efficiently distribute sampling stations for phytoplankton along the approximately 580 km of coastline and to optimise toxin analysis in shellfish. This strategy has proven to be efficient for prediction and prevention of illnesses from unsafe levels of phycotoxins from harmful algal blooms (HABs) in shellfish from recreational and harvest areas. This paper gives a brief description of the two existing Catalan Monitoring Programmes for HABs and puts emphasis on the importance of studying carefully the local information previous to the design of monitoring programmes.

Introduction
The Government of Catalonia has two distinct HAB-related monitoring programmes that are strongly linked for an efficient implementation. The Catalan coast in Spain (NW-Mediterranean) is monitored for the presence of marine toxins and harmful phytoplankton in shellfish harvesting areas by the Institut de Recerca i Tecnologia Agroalimentaries (IRTA) as a mandate for the fisheries department of the Regional Government of Catalonia (Direcció General de Pesca i Afers Maritims, DGPiAM). The presence of potentially harmful phytoplankton along the Catalan coast is monitored by the Consejo Superior de Investigaciones Cientificas (CSIC) as a contract of the Agencia Catalana de l'Aigua (ACA) in agreement with the Water Framework Directive (WFD). Both CSIC and IRTA communicate constantly and share a complementary monitoring strategy for HABs in the 580 km coastline. This strategy is adapted to the characteristics of the coastal geography and to the oceanographic conditions along the Catalan coastline. The monitoring programmes take into account the locations of harvest areas and the most probable conditions for the development and distribution of HABs. This paper gives a description of two co-existing and collaborative monitoring programmes in Catalonia. It also intends to put the emphasis on the importance of the conception and design of a monitoring programme and to the necessity of conducting additional updates according to results obtained through linked research and through constant review of historical data within the monotoring programmes.

Material and methods

Major production areas (representing 90-95 % of the total production of bivalves) within semi-enclosed coastal embayments such as Alfacs and Fangar Bay within the Ebre Delta, are monitored weekly for phytoplankton. The presence of toxins in bivalves in these bays is also monitored weekly for PSP and DSP toxins, and every 15 days for ASP. Toxin determination in shellfish is conducted according to the mouse bioassay for DSP described by Yasumoto (1978), the mouse bioassay for PSP (AOAC) and HPLC determination for Domoic Acid (ASP) based on the method described by Lawrence *et al*. (1991).

When toxins are detected in the shellfish or when an increase in the harmful phytoplankton concentration is reported, more intense sampling of both phytoplankton and bivalves is conducted to evaluate the presence of toxins. For example values arbitrarily defined above 500 cells·L^{-1} for *Dinophysis* spp., 1000 cells·L^{-1} for *Alexandrium* spp. and 200000 cells·L^{-1} for *Pseudo-nitzschia* spp. trigger increased monitoring frequencies for phytoplankton. By far, the dominant cause for shellfish area closures in the Ebre Delta embayments has been DSP. From 1989 until 2005, 16 out of 21 closures were due to DSP.

Sixteen harbours, distributed along the coast, are sampled weekly during the warm season (from May to September) and twice a month from October to April. In the summer, 6-10 additional stations situated on beaches are sampled twice a week. Also, once in midsummer, all the ACA environmental stations that regularly are monitored for environmental factors that condition swimming suitability are sampled for harmful phytoplankton (about 250 beaches).

When potentially toxic species are identified in semi-enclosed areas (harbours and bays) close to harvest areas in open coastal waters, increased monitoring both for phytoplankton and shellfish toxins is conducted within the production areas. PSP toxins are analysed in field samples during monospecific blooms and algal cultures. Algal material is concentrated on GF/F filters by vacuum filtration, and toxins are subsequently extracted in acetic acid. HPLC analyses are performed with an Thermo Separation Products SpectraSystem chromatograph (see Van Lenning *et al*. 2003 for details on set-up) using the methods of Oshima *et al*. (1995). The equipment is calibrated with standards obtained from NRC (Hallifax, Canada). Relevant details on these procedures are summarised in Table II. DSP toxin profiles are first determined by means of fluorescence derivatization, 9-anthryldiazomethane (ADAM) (Lee 1987) is used as a fluorescent label for the determination of okadaic and dinophysistoxins (DTX1, DTX2, DTX3), and the fluorescent dienophile (4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl) ethyl]-1,2,4-triazoline-3,5-dione) (DMEQ-TAD) (Yasumoto 1997) is employed for the detection of yessotoxins. The introduction of the liquid chromatography coupled to a mass spectrometric detector has highly improved and enlarged the capacity to analyse toxins in marine samples. LC-MS-MS methods for multitoxin detection has been developed lately by several research groups, leading to the determination of yessotoxin, hydroxyyessotoxin, okadaic acid, and the pectenotoxins, PTX2, PTX1i, PTX2-SA, PTX1i-SA in mussel and phytoplankton samples in one chromatographic analysis (McKenzie 2002 and 2005, Fernández 2004, Madigan 2005).

Applicable regulations

The Monitoring Programme for Marine Toxins and Harmful Phytoplankton in Catalonia has been designed to respond to the European Union, Spanish and Catalan legislation. The main European Union legislation that relates to shellfish monitoring is included in (but not exclusively): EU Directive 91/492/EEC, EU Directive 97/61/EC and EU Decision

2002/225/EC. Harmful phytoplankton is monitored according to the European Water Framework EU Directive (WFD) 2000/60/EC.

Marine toxins and harmful algal species of concern along the Catalan Coast

The primary syndromes associated with harmful marine toxins in the Mediterranean include mainly PSP, DSP and ASP. On the Catalan coast, closures of shellfish production areas have resulted from the presence of DSP and PSP toxins measured above regulatory levels and occasionally preventive closures have been dictated when the PSP occurring species *Alexandrium catenella* has been at high concentrations. Regarding DSP, several species of the genus *Dinophysis* are recurrent in Catalan waters, and *D. sacculus* is the dominant one. Although no episodes of ASP have been reported on the Catalan coast, monitoring for ASP toxicity is important due to the occurrences of blooms of *Pseudo-nitzschia* spp. in the area as well as ASP events on the Mediterranean coast of France (Ifremer, 2003). Other toxins of concern include the yessotoxins which have been detected elsewhere in the Mediterranean and in the Ebro Delta bay of Alfacs. Until now, *Protoceratium reticulatum* has been isolated in the area, cultures have been established and produce yessotoxins.

Monitoring for harmful phytoplankton focuses on several species (Table 1) implicated in shellfish poisoning: *Alexandrium spp.* (principally *A. catenella* and *A. minutum)* responsible for PSP toxin production, *Dinophysis* spp. (principally *D. sacculus, D. caudata* and *D. rotundata)* for DSP, and *Pseudo-nitzschia* spp. for ASP. Additionally, screening for other species and genera of ichthyotoxic species (such as *Karlodinium* sp., *Chattonella* spp.), benthic toxin producing microalgae (such as *Prorocentrum lima, Ostreopsis* spp.) and bloom forming non toxic species (such as *Prorocentrum micans, P. triestinum, Heterocapsa* spp., *Scrippsiella* spp., *A. taylori, A. pseudogonyaulax*, among others) are also considered in all coastal waters, especially both in recreational waters and shellfish and fish production areas. Regarding these non toxic species, whenever possible, the follow-up of their blooms is carried out in order to predict eventual nuisance situations, such as anoxia conditions, and also obtain data regarding phytoplankton population dynamics that could add information to understand their ecology. Regarding benthic species, recently, blooms of the genus *Ostreopsis* spp., have been described in Italy and Spain (Mediterranean Sea). They have been related to human respiratory difficulties in people on beaches when they inhale marine aerosols (Sansoni *et al.* 2003, Masó *et al.*, 2005). In August 2004 around 200 people on the Llavaneres beach (Catalonia) or living in close proximity to the beach were affected by respiratory difficulties (rhinorrea, nose and throat irritation, coughing, expectoration, eye irritation and migraine). An epidemiologic study was carried out by the Serveis Territorials del Departament de Salut de Barcelona as advised by ACA. Moderate *Ostreopsis* spp. concentrations (maximum detected: 23,000 cells l^{-1}) were recorded in the water column. Palytoxin analogs have been detected in all the Mediterranean isolates of *Ostreopsis* spp. strains that have been analysed (Riobó *et al.,* 2004; Penna *et al,* 2005). Palytoxin is the most toxic natural occurring organic substance known, and it causes fatal human intoxications from the ingestion of fish (Taniyama *et al.,* 2003). Thus, an hypothesis for breathing irritation could be related to the inhalation of marine aerosols produced by *Ostreopsis* spp. present in the water. Consequently, benthic dinoflagellates constitute an interesting community related with human respiratory problems and governmental monitoring programmes should also focus on them.

Commercial shellfish production on the Catalan coast

Shellfish harvesting areas along the Catalan coast are located both in open waters along the coast and also in inshore waters in coastal embayments such as Alfacs and Fangar bays (Ebre Delta). Production areas include natural shellfish beds such as *Donax trunculus*

(clam), *Cerastoderma edule* (Cockle), *Tapes semidecussata* (Manila clam), *Callista chione* (Italian clam), *Bolinus brandaris* (Purple Dye Murex) but also shellfish farming in semi-enclosed coastal embayments for mussels *(Mytilus galloprovincialis)* and oysters *(Cassostrea gigas)*. In order to ensure safe marketable products, the Catalan shoreline is divided into geographical sectors where, according to the species of shellfish harvested, key shellfish species are monitored for toxins.

Design of the monitoring programme according to the characteristics of the Catalan coast and the shellfish sectors
The Catalan coast is formed by sharp rocky and flat sediment areas exposed to open waters (with currents going primarily in a south-westerly direction). The coast holds few coastal embayments and numerous fisheries, commercial and recreational harbours with more or less confined water areas, which constitute optimal locations for microalgal growth and proliferations (Vila and Masó 2005). The monitoring programme includes phytoplankton identification and quantification, toxin determination and recording of environmental conditions.

The objective of the monitoring programmes is to develop a warning of HABs not only associated with areas with aquaculture activities, but also within confined areas with a high risk of HAB occurrences. This strategy has been implemented and improved as a result of historical data analysis that suggests that the main occurrence for HABs in production areas in Catalonia arise from at least two different situations: (1) the development of HABs in confined waters (bays and harbours) with the possibility for expansion to the open nearshore waters and shellfish production areas along the coast if the oceanographic conditions are favorable (mainly southward along the Catalan coast) and (2) advection of harmful algae from offshore Mediterranean waters.

Thus, the monitoring stations are located in harbours, coastal embayments and other areas with active shellfish production. On the Catalan coast, confined areas can act as resonance boxes (Vila *et al*. 2001 a; Vila and Masó, 2005) where environmental conditions (physical confinement, nutrients and light) may favour microalgal growth and the development of HABs, that might spread to production areas in open waters if oceanographic conditions are favourable (Garcés *et al.*, 1998; Vila *et al* 2001b). Additionally, the confinement may increase the accumulation of resistance forms (cysts), which may initiate future episodes (Garcés *et al.*, 2004). Thus, monitoring in confined waters could be used as an early warning detection system for these algal blooms.

Sampling at strategic locations such as confined waters in important commercial harbours and coastal bays may additionally give further indications of the possible introduction of new harmful species via routes such as ballast-water or mollusc translocation.

The advection of HA from outer waters is more difficult to monitor and predict. In order to improve the awareness on possible implications of advective processes from outer waters, whenever it is possible, intense sampling is performed in open waters close to areas where blooms occur.

Communication, data transfer and administrative decisions:
CSIC and IRTA teams are responsible for the design and implementation of the monitoring in coastal water and shellfish production areas and maintain constant communication with one another in order to share information and optimise the reaction capabilities regarding phytoplankton monitoring. In warning situations (presence of harmful species at important densities or presence of toxins), communication is opened between all participating institutions. DGPiAM in Catalonia is the institution responsible for the

eventual closures and openings of shellfish production areas and ACA is responsible for decisions involved with water quality management in relation to the WFD.

Research programmes associated with the monitoring programme in Catalonia:
In an attempt to encourage the implementation of innovative approaches within the monitoring programme, CSIC and IRTA institutions actively participate in national and international research programmes that include ecological studies and the development and validation of field and laboratory methods for toxin and toxicity detection in microalgae and shellfish (e.g. EU STRATEGY project (EVK3-CT-2001-00046); EU SEED project (GOCE-CT-2005-003875); LIFEHAB (EVK3-2001-00080); NRC-CSIC-IRTA. In the same way, information regarding the detection and quantification of harmful algae, marine toxins and environmental parameters obtained within the monitoring programme (Delgado *et al.*, 2004; Fernanndez-Tejedor *et al.*, 2002; Fernandez *et al.*, 2004; Vila *et al.*, 2001 c), are also very valuable for the design and implementation of ongoing research projects.

Table I. HAB species that cause or may cause problems in Catalan coastal waters.

Species	Harmful effect	References
DIATOMS		
Pseudo-nitzschia delicatissima	Domoic acid	Vila (2001)
P. pungens	Domoic acid	Vila (2001)
P. pseudodelicatissima	Domoic acid	Vila (2001)
P. brasiliania	Bloom forming	Quijano-Scheggia *et al*. 2005
P. multiestriata	Domoic acid	Quijano-Scheggia *et al*. 2005
DINOFLAGELLATES	(Table 1. contd.)	
Alexandrium catenella	PSP	Vila *et al*. 2001a, Vila *et al*. 2001b, Vila *et al*. 2001d, c
A. margalefi	ichthyotoxic	Bravo *et al*. submited
A. minutum	PSP	Delgado *et al*. 1990, Delgado *et al*. 1997a, Delgado *et al*. 1998, Garcés 1998, Garcés *et al*. 1998b, Garcés and Masó 2001, Vila *et al*. 2001a, Galluzzi *et al*. 2004, Garcés *et al*. 2004, Vila *et al*. 2004a, Vila *et al*. 2004b, Galluzzi *et al*. 2005, Vila *et al*. 2005, Van Lenning *et al*. 2004
A. tamarense	PSP	Vila *et al*. 2001c
A. taylori	Bloom forming and discolouration	Delgado *et al*. 1997b, Garcés 1998, Garcés *et al*. 1998a, Garcés *et al*. 1999b, Garcés *et al*. 1999c, Garcés and Masó 2001, Vila *et al*. 2001a, Garcés *et al*. 2002, Masó *et al*. 2003, Basterretxea *et al*. 2004

Table 1 contd.

A. pseudogonyaulax	Bloom forming and discolouration	Delgado *et al*. 1999
Amphidinium spp.	Ichthyotoxic haemolytic	Monitoring
Cochlodinium polykrikoides	ichthyotoxic	Garcés *et al*. 2005, Vila *et al*. 2005
Coolia monotis	Cooliatoxin ?	Masó *et al*. 2003, Penna *et al*. 2005, Vila *et al*. 2001c, d
Dinophysis acuta	DSP	Monitoring
D. caudata	DSP	Vila *et al*. 2001a, c
D. rotundata	DSP	Vila *et al*. 2001a, c
D. sacculus (incl. D. pavillardi)	DSP	Delgado *et al*. 1996, Garcés *et al*. 1997, Garcés and Masó 2001, Vila *et al*. 2001a, c
D. tripos	DSP	Monitoring
Gymnodinium pulchellum	ichthyotoxic	Monitoring
K. veneficum (=*Gymnodinium veneficum*, *Gyrodinium corsicum)*	ichthyotoxic	Garces *et al* (in prep), Garcés 1998, Delgado and Alcaraz 1999, Garcés *et al*. 1999a, Garcés and Masó 2001, Fernández-Tejedor *et al*. 2004, Vila *et al*. 2001a, c
Noctiluca scintillans	discolouration anoxia	Monitoring
Ostreopsis ovata	toxic compound	*Penna et al. 2005*
O. cf. *siamensis*	ostreocine/ palytoxin	*Vila et al. 2001d, Penna et al. 2005*
Prorocentrum emarginatum	DSP ? haemolytic ?	*Vila et al. 2001d*
P. rhathymum (= P. mexicanum)	haemolytic toxins	*Vila et al. 2001a*
P. lima	DSP	*Monitoring*
P. minimum	ichthyotoxic	Monitoring
Protoceratium reticulatum	yessotoxin	Monitoring
RAPHIDOPHYTE		
Chattonella spp.	ichthyotoxic	Monitoring

Conclusions

The present Catalan Monitoring Programme for HABs takes into account social factors such as EU directives and regulations, consummer habits and producer activities. Its' design relies though on a careful and continuous analysis of the biological, ecological, oceanographical and geographical conditions that may influence HABs and shellfish along the Catalan coastline. Our monitoring programme is the result or a compromise between the conclusions of this analysis and the financial means available for its implementation. We want to put emphasis on the importance of the analytical work previous to the design of any monitoring programme in order to have an effective tool to protect public health and assure the development of the shellfish and tourist industry.

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PLANKTON TOXICITY AND SHELLFISH CONTAMINATION BY PHYCOTOXINS IN A NEW MEDITERRANEAN LOCALITY

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Abstract
Harmful Algal Blooms were studied between 2000-2005 in a Mediterranean locality (Sicily, Italy), recurrently subject to outbreaks of toxic dinoflagellates, mostly *Alexandrium minutum* and *Dinophysis* spp., whose identity was confirmed by molecular tools (genus and species-specific PCR assays). Evidence for toxicity of phytoplankton natural assemblages was gained through HPLC analysis, indicating the occurrence of either Paralytic Shellfish Toxins (gonyautoxins and saxitoxin) or Diarrhetic Shellfish Toxins (okadaic acid and dinophysistoxin-1) in bloom samples from various Sicilian areas. On some occasions, shellfish (*Mytilus galloprovincialis*) from Ionian and Tyrrhenian farming areas were also analysed for toxicity by mouse bioassay and HPLC, since no previous information on possible shellfish contamination by phycotoxins is available from the Sicilian coast. Gonyautoxins (mostly GTX4) were detected in 2001 in mussels, although their concentration was below the safety threshold for human consumption. In some cases (2000 and 2004), mouse assays gave positive results and HPLC analyses of farmed mussels indicated the presence of yessotoxins (2000), as well as spirolides and gymnodimine (2004), although at trace levels. These first records of phycotoxins both in Tyrrhenian and Ionian localities, suggest a risk for future seafood contaminations, and the need for an intensive shellfish monitoring in target areas of Sicily particularly susceptible to Harmful Algal Blooms.

Introduction
Harmful Algal Blooms (HABs) are outbreaks of microalgae toxic to humans, as well as microalgae having a negative impact on the ecosystem through production of ichtyotoxins or causing conditions noxious to the habitat, such as hypoxia or anoxia. Toxic dinoflagellate blooms have long been recognised to have a significant impact on the utilization of shellfish resources, in relation to the filter-feeding ability of bivalve molluscs and accumulation of toxins in their tissues (Shumway and Cucci, 1987). The human consumption of the contaminated product can lead to various syndromes, including Paralytic and Diarrhetic Shellfish Poisonings (PSP and DSP). Thus, the consequences of HABs are especially severe on public health, although their impact on aquaculture operations and seafood marketing cannot be understimated. In recent years, observations of shellfish contamination by phycotoxins, e.g. PSP toxins, have been reported from various Mediterranean localities, such as the northern Adriatic Sea (Italy) (Honsell *et al.* 1996; Poletti *et al.*, 1998), the Catalan coast (Delgado *et al.*, 1990), Balearic islands (Forteza *et al.*, 1998), Eastern Aegean Sea (Koray and Buyukisik, 1988). Paralytic Shellfish Poisonings shellfish contamination were frequently associated with the toxic dinoflagellate *Alexandrium minutum* Halim, a species widely distributed in the Mediterranean Sea - see e.g. Vila *et al.* (2005). These authors also indicated the occurrence in some Mediterranean areas, such as the Sicilian coast, of high biomass blooms of *Lingulodinium polyedrum* (Stein) Dodge – a species reported also from the Adriatic Sea and producing yessotoxin-like compounds (Tubaro *et al.*, 1998).

The most important organism responsible for DSP in humans belongs to *Dinophysis* Ehrenberg - a dinoflagellate genus widespread in the Mediterranean as well (see Maestrini 1998 for a review). Among the DSP toxins, okadaic acid (OA) and dinophysistoxin-1 (DTX-1) can cause intestinal disorder and both are considered as tumor promotors and inhibitors of protein phosphatases (e.g. Yasumoto *et al.*, 1985). Despite different records of toxic species in the Mediterranean, including southern Italy (e.g. Giacobbe and Maimone, 1994; Giacobbe *et al.*, 2000), there are no studies in this last region on the possible contamination of shellfish in sites subject to recurrent blooms of HAB species. Here, a first approach was undertaken on HAB problematics in Sicilian areas of shellfish farming, using both conventional and molecular tools (genus and species-specific PCR assays). Evidence for toxicity of phytoplankton natural assemblages and first data on shellfish contamination by phycotoxins, although at low levels, are provided.

Materials and Methods

In the context of programmes for the monitoring and management of HABs (Projects MiPA 5C8 - Fisheries and Aquaculture, and Strategy EVK3-CT-2001-00046) water samples were taken for 5 years (2001-2005) in the Syracuse bay (37° 3'Lat N, 15° 17'Long E), a shallow area located on the Ionian coast of Sicily (Figure.1). Sampling frequency varied from monthly to weekly, starting from February-March to late summer/early autumn. Routine samples were collected at the subsurface (-0.5m) in 2-4 sampling points, except for some occasions when a higher number of stations (10-15) was sampled, including vertical points through the water column (upper 5m). The area is subject to freshwater inputs, favouring outbreaks of harmful phytoplankton (Vila *et al.*, 2005). A sector of the bay is used for productive activities such as shellfish farming, with suspended cultures of *Mytilus galloprovincialis* Lamarck. Shellfish samples were taken from Syracuse for toxicity analysis in April and June 2001, and monthly in 2004 and 2005 (spring-summer). Mussels and/or natural phytoplankton assemblages were collected from other sites located on the Tyrrhenian coast of Sicily (Figure 1): Faro Lagoon (9 sampling occasions between 2000 and 2001), Verde Pond and Portorosa (7 sampling occasions in each area from March to June 2003). HAB species were identified in microscopy,using a Axiovert 200 Zeiss microscope, equipped with epifluorescence, Axiocam and Axiovision software for photography, cell measurements and thecal plates analysis (see Vila *et al.*, 2005 for detailed methodologies). To confirm the species identities, *A. minutum* and other toxic dinoflagellates were also tested on field mixed samples, by using genus and species-specific PCR assays. The PCR primers were developed versus the 5.8S rDNA-ITS regions (Penna and Magnani, 1999; Galluzzi *et al.* 2004). The PCR amplified products were visualised on 2.0 % agarose gel.

Mixed phytoplankton samples collected in various areas of Sicily and containing *Alexandrium* species were sonicated with 0.1M acetic acid. Extracts, after centrifugation, were treated for cleanup according to Lawrence and Ménard (1991) and eluates were injected into a HPLC system. The LC determination of PSP toxins was carried out using ion-pair elution with sodium hexanesulphonate and sodium heptanesulphonate, post-column oxidation with periodic acid and fluorescence detection (Sullivan and Wekell, 1984). Paralytic Shellfish Poisoning toxicity in blue mussels was tested by mouse bioassays (AOAC, 1990) and HPLC, as described above.

Mussel samples were also analysed in order to detect the presence of lipophilic toxins by mouse-test (Yasumoto *et al.*, 1984) and HPLC (for okaidates and yessotoxins). A precolumn derivatization of okadaic acid and dinophysistoxin-1 was employed, using 9-anthryldiazomethane as fluorescent-labeling reagent, reversed

phase HPLC with fluorescence detection and acetonitrile: methanol:water (8:1:1) as mobile phase (Lee *et al.*, 1987 - modified). The presence of yessotoxins was tested following the method of Yasumoto and Takizawa (1997) that involves a derivatization with a dienophile reagent, DMEQ-TAD and fluorescence detection.

In 2004, mouse biossays of mussel samples were performed according to the new Italian legislation (Health Ministry D.M. 16/05/2002, G.U. N.1655 – 16/07/2002), while the chemical analyses were carried out by using LC-MS techniques (McNabb *et al.*, in press).

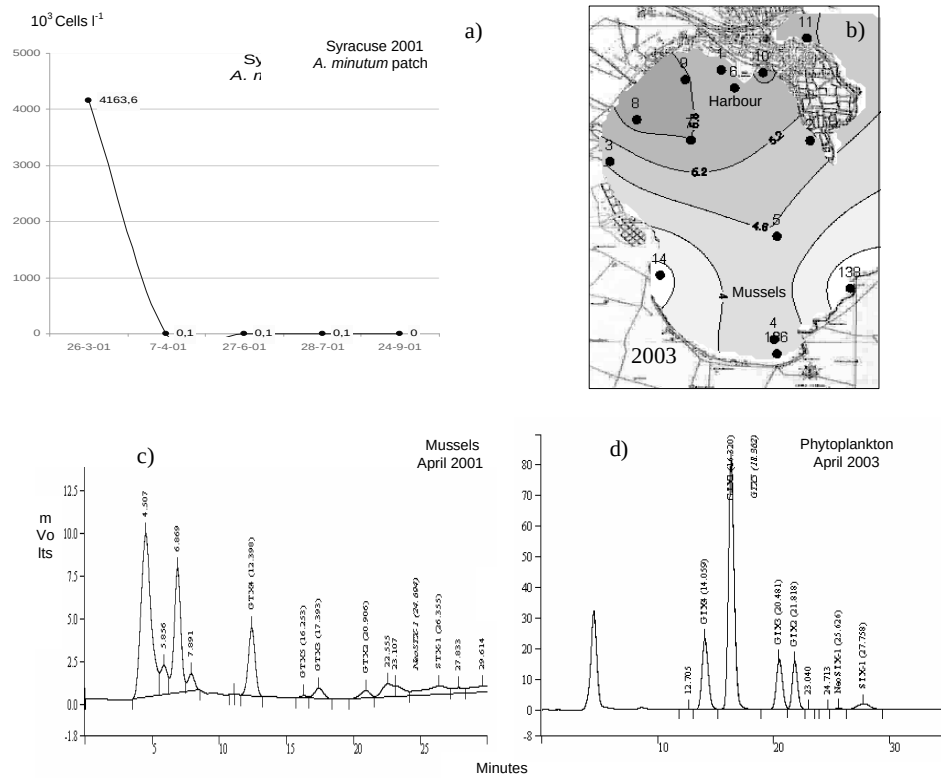

Figure 1. (a-b) Densities and distribution of *Alexandrium minutum* in the Syracuse bay (Ionian Sea, Sicily). Numbers at the isolines refer to cell density (Log cells l^{-1}). (c-d) HPLC profiles of PSP toxins in shellfish and field phytoplankton assemblages.

Results and Discussion

Phytoplankton toxicity

The most significant data on toxicity of phytoplankton assemblages from Syracuse, as well as from other Sicilian areas, are reported in Table 1. The first site is subject to outbreaks of various dinoflagellate species, among which *Alexandrium minutum*, producing in early spring mixed blooms and greenish-brown patches in the waters. In March 2001, a bloom of $4.6x10^6$ cells l^{-1} was observed near the area where mussels (*Mytilus galloprovincialis*) are present in suspended culture. Similar events also occurred in springs 2002-2005, although in slightly smaller proportions, with maximum peaks of 1.2×10^6 cells l^{-1} in April 2003, as shown in Figure2a (see also

Vila *et al.*, 2005 - for further bloom details). In 2003, as in most of the study years, the spatial distribution of *A. minutum* over the bay, showed cell densities higher in the harbour area than in the mussel area and were mostly influenced by freshwater inputs, (Figure.2b). Maxima were found at the subsurface. HPLC analyses of mixed phytoplankton assemblages taken during *A. minutum* blooms, indicated the presence of saxitoxin (April 2, 2003 – 8 % of PST) in addition to gonyautoxins (Figure 2c), both in Syracuse and in a Tyrrhenian area (March 19, Verde Pond in Table 1). In Tyrrhenian phytoplankton samples, STX (75 % of PST) and GTXs coincided with the presence of *A. minutum* and *A. tamarense* (Lebour) Balech. Apparently, in both areas the most important associated phytoplankters were not PSP-producers, e.g. *Prorocentrum* spp. Ehrenberg, other small-sized dinoflagellates, *Akashiwo sanguinea* (Hirasaka) Hansen and Moestrup. However, further studies focused to the identification in these areas of the exact source of saxitoxin are in progress.

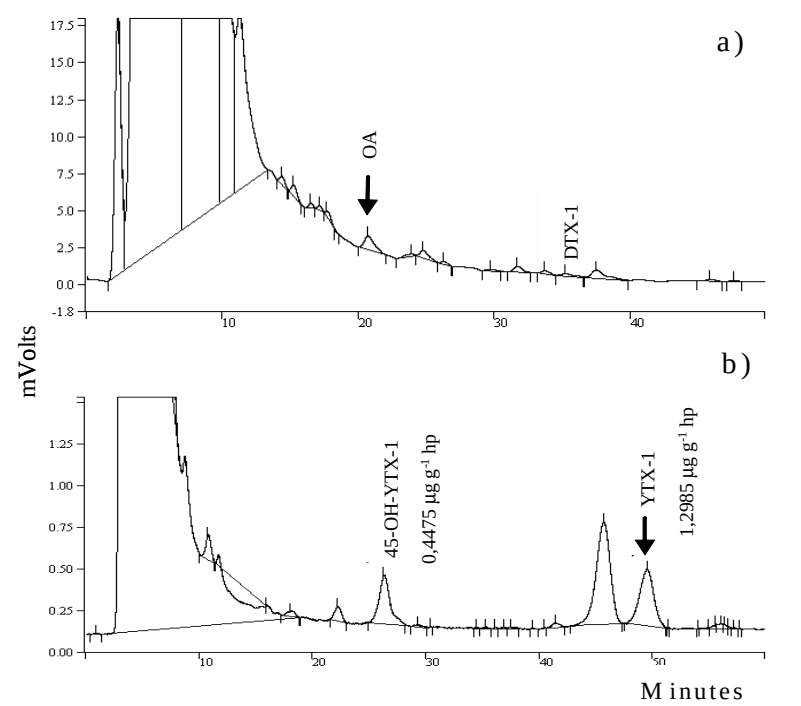

Figure 2. HPLC chromatograms of DSP toxins from *Mytilus galloprovincialis* hepatopancreas. (a) samples from Syracuse, June 2001 containing okadaic acid; (b) samples from the Faro Lagoon – Sicily, October 2000, containing yessotoxins.

DSP toxins (99 % okadaic acid), were also detected by HPLC in surface net samples (-1m) from Portorosa (Tyrrhenian), dominated by *Dinophysis sacculus* Stein. The highest amounts of OA, 7420 ng/total cells - reported in Table 1, resulted on March 28 in a quasi-monospecific sample. Cell counts in the net sample processed by HPLC (879648 *Dinophysis* cells) gave a toxin cellular content of 8.4 pg OA per cell. This value was comparable to those reported by Graneli *et al.* (1998) for *D. acuta* Ehrenberg and *D. acuminata* Claparède and Lachmann, when exposed to balanced or limited nutrient conditions, respectively. The detection of different toxins in phytoplankton natural assemblages from Sicily containing *Alexandrium* or *Dinophysis* species, suggests that a major attention must be addressed to the study of Harmful Algal Blooms in this Mediterranean region, as well as to the development of

appropriate control and prevention strategies to minimise their possible future impact.

Shellfish contamination

Results on phycotoxins obtained by mouse biossays and HPLC analyses of mussel extracts from Sicily are presented in Table 2. A slight shellfish contamination by PSP toxins was found in the Syracuse bay on April 30, 2001. This coincided with a limited bloom of *A. minutum*, the only PSP organism present in the water (see Table 1 for associated species). The dominant toxin was GTX4, followed in minor proportions by GTX3 and GTX2). The highest amount of GTXs was 19 μg STX equivalent $100g^{-1}$ of edible tissue (HPLC data, Table 2). Values of total PSP toxins were well below the safety threshold for human consumption (80 μg STXeq 100 g^{-1} e.t.), and PSP was undetectable by mouse biossay. PSP levels in the edible tissue were also much lower than the relatively moderate toxicities reported for European waters (≤ $4x10^3$ μg STX eq $100g^{-1}$ e.t.) and the high maxima of the Atlantic and Pacific coasts of North America (Bricelj and Shumway, 1998). Our data indicated however, that in the Syracuse bay - subject to successive HAB events by different species - there is a potential for future shellfish contaminations by phycotoxins. Differences in gonyautoxins composition between shellfish and natural phytoplankton samples were evidenced, with a dominance of GTX4 in mussels and GTX1 in phytoplankton (Table 1-2, Syracuse samples). This could be explained by the possibile epimerization of ingested toxins, chemical or enzymatic transformation and/or selective retention of individual toxins (see e.g. Bricelj and Shumway, 1998). Thus, changes in toxin profiles between phytoplankton and bivalves are suggested to limit the ability to predict shellfish toxicity from the phytoplankton measures only.

As regards lipophilic toxins, such as okadaic acid and dinophysistoxin-1, the presence in June 2001 of OA in mussels from Syracuse was detected only at trace levels, due to the scarce presence in the water of *Dinophysis* species (*D. sacculus* and *D. caudata*). In May 2004, two mussel samples were positive to mouse biossay. HPLC indicated only the presence, again at trace levels, of a relatively new class of toxin (Fast Acting Toxins): spirolides – pharmacologically active macrociclyc compounds - and/or gymnodimine, a bioactive spiroimine (Tab.2). The toxin source could has been gonyaulacoid and gymnodinioids dinoflagellates, respectively, as also previously reported in literature (e.g. Cembella *et al.*, 2001; Stirling, 2001). Our phytoplankton data indicated the presence of mostly *Alexandrium* cysts, *Lingulodinium polyedrum* and *Gymnodinium* cf. *nollerii* in the water (Figueroa, pers. comm.). Further work will be necessary to follow future shellfish contaminations by this toxin class, exact source-organisms, and other possible lipophilic toxins.

Table 1. Concentrations of PSP and DSP toxins (ng/total cells) in phytoplankton net samples from Sicily containing *Alexandrium minutum*, *Dinophysis sacculus* and/or other dinoflagellates. Sampling dates: 30 April 2001, 2 April 2003, 19 and 28 March 2003, respectively. Numbers in brackets indicate the species density in the water during sampling and the year maxima are reported below (cells l^{-1}). Data in bold refer to the dominant toxin.

	IONIAN Syracuse 2001		IONIAN Syracuse 2003		TYRRHENIAN Verde Pond 2003		TYRRHENIAN Portorosa 2003	
PSP TOXINS	ng	%	ng	%	ng	%		
GTX4	89.4	26.8	638.8	25.7	16.5	0.5	Samples not tested for PST	
GTX1	195.5	**58.5**	1267.6	**51.1**	34.6	1.0		
GTX3	12.9	3,9	87.8	3.5	247.1	6.9		
GTX2	36.2	10.8	294.5	11.9	601.3	17.0		
STX	0	0	192.8	7.8	2642.8	**74.6**		
Total	334.0	100	2481.5	100	3542.2	100		
DSP TOXINS	Samples not tested for DST						ng	%
OA							7420	**99.4**
DTX1							43	0.6
Total							7463	100
DINOFLAGELLATES	%		%		%		%	
Akashiwo sanguinea	0		0		0.4		0	
Alexandrium minutum	0.4 ($6x10^4$) $4x10^6$ in March		5.8 ($1.4x10^5$) $1.2x10^6$ in April		95.6 ($1.3x10^5$)* $6.7x10^5$ in April		0	
Dinophysis sacculus	0		0		0,8 (10^3)		93 (520) 10^5 on March 12	
Lingulodinium polyedrum	2.8		0		0		0	
Prorocentrum micans	0		2.8		0		0	
P. triestinum	96.4		87.6		0		0	
P. minimum	0		0		0.8		0	
P. scutellum	0		0		1.6		0	
Protoperidinium spp.	0.2		0		0		0	
Scrippsiella spp.	0.1		0		0.4		0	
Other small dinos	0.1		3.8		0.4		7	
Total	100		100		100		100	

* In the Verde Pond, *A. tamarense* cells were associated to *A. minutum*

Finally, first records of yessotoxins (YTX and homoYTXnd, 45-OHYTX and 45-OH-homoYTX) were obtained by HPLC only in mussels from a brackish site in Sicily (Faro, October 2000). This corresponded to the presence in the water of *Lingulodinium polyedrum* and *Akashiwo sanguinea*, indicating the first species may have been responsible for the slight mussel contamination. Mouse bioassay gave a positive result (3h 55' death time, Tab.2).

Table 2. Summary of phycotoxins found in mussels from Sicily (data from mouse assays and HPLC). OA: okadaic acid; YTXs: yessotoxins (μg/g^{-1} hepatopancreas); GTXs: total gonyautoxins (μg STX eq. 100g^{-1} edible tissue); nd: not detected; dt: death time.

		LIPOPHILIC TOXINS			PSP TOXINS	
Mussel samples	**Date**	**Mouse test**	**OA**	**YTXs**	**GTXs**	**Dominant toxins**
Faro	16/10/00	+ 3h 55'dt	nd	1.7	nd	YTX 74 % 45-OHYTX 26 %
Syracuse StnA	30/04/01	-	nd	nd	9.8	GTX4 95,3 %
B	30/04/01	-	nd	nd	16.6	GTX4 95,9 %
C	30/04/01	-	nd	nd	18.8	GTX4 96,4 %
D	30/04/01	-	nd	nd	17.0	GTX4 95.4 %
E	30/04/01	-	nd	nd	nd	-
B	07/06/01	-	trace levels	nd	nd	OA 100 %
C	07/06/01	-	trace levels	nd	nd	OA 100 %
D	07/06/01	-	nd	nd	nd	-
E	07/06/01	-	trace levels	nd	nd	OA 100 %
B	04/05/04	+ 16h 30'dt	nd	nd	n.d.	Spirolides and gymnodimine (trace levels)
C	04/05/04	+ 15h 35'dt	nd	nd	n.d.	Gymnodimine (trace levels)

As a whole, the data reported here on shellfish toxicity, although indicate a low level of contamination – not representing up to now a risk for human health - underlines the importance to include intensive shellfish monitorings in HAB surveys, as a complementary information to toxic or noxious species occurrence.

PCR assays

By using species-specific PCR primers, designed for the genus *Alexandrium*, PCR amplification of the 5.8S rDNA-ITS regions yielded products of 111 base pair size for the field samples, containing *Alexandrium* spp. cells. Species-specific PCR amplifications for *A. minutum* resulted positive for the presence of this species in the Verde Pond and Syracuse field samples, giving a PCR species-specific fragment of 212 bp. The application of other species-specific PCR primers to field samples, showed the presence of *A. tamarense* only in the Verde Pond sample, in agreement with the microscopic examination.

The ITS regions and 5.8S rDNA gene revealed to be useful genetic markers at genus and species level for *Alexandrium* and offered a high resolution level for the *A. minutum* and *A. tamarense* identification, thus representing a technological improvement in our ability to accurately identify HAB species.

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SHELLFISH TOXICITY RECORDED DURING MONITORING PROGRAMME ON CROATIAN SHELLFISH FARMS DURING 2001

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Abstract
The monitoring programme was conceived as an effort to control shellfish breeding farms on the Eastern Adriatic coast. Mussels were collected from February 2001 to February 2002 and their tissue tested for biotoxins, such as Diarrhetic Shellfish Poisoning (DSP) and Paralytic Shellfish Poisoning (PSP). Information was collected on environmental parameters (oxygen saturation, salinity, temperature) and on the state of the phytoplankton community in respective marine areas. The samples of *Mytilus galloprovnicialis* were subjected to the mouse bioassay on a monthly basis during the colder part of the year (from November to March) and on a fortnightly basis (from April to November). The test was not positive for PSP but gave two positive results for the presence of DSP toxins. Some additional analysis (Protein Phosphatase Assay) showed the presence of okadaic acid (OA) in those samples, even though their concentrations were very low. The studies demonstrated the widespread (in all shellfish-breeding areas) but occasional presences of potentially toxic phytoplankton organisms (*Dinophysis fortii, D. acuminata, D. acuta, D. sacculus, D. caudata, Prorocentrum minimum, Lingulodinium polyedrum, Pseudo-nitzschia complex*). Their abundance was higher in the breeding areas of the northern part of the study area than in its southern part. Undoubtedly, the highest number of potentially toxic species and their higher abundance were recorded in the warmer period of the year (from April to November).

Introduction
Diarrhetic toxins are secondary metabolites produced by toxigenic dinoflagellates of *Dinophysis* and *Prorocentrum* species provoking a severe gastrointerstinal disease named Diarrhetic Shellfish Poisoning (DSP) from consumption of contaminated shellfish. The number of toxins, out of which most common are okadaic acid (OA), dinophysitoxin 1 (DTX 1) and dinophysitoxin 2 (DTX 2) are causative agents of DSP outbreaks. In the Adriatic Sea, DSP (Diarrhetic Shellfish Poisoning) outbreaks have been known since 1989, when a case of human gastroenteritis was recorded for the first time (Viviani *et al.*, 1990; Boni *et al.*, 1992; 1993). The shellfish intoxication with DSP was due to the presence of dinoflagellates of the genera *Dinophysis* and *Prorocentrum* in the Northen Adriatic. Shellfish toxicity has been regularly occurring in the Northern Adriatic Sea ever since 1989.

In summer 1994, DSP toxin was for the first time established, by the HPLC analysis (Orhanovic *et al.*, 1996), in a sample of wild mussels population from Kaštela Bay on the Eastern Adriatic coast

During the summer of 1995 and 1996, OA and 7-epi-PTX-2SA toxins were identified in mussels from the central Adriatic Sea. The shellfish toxicity was associated with the occurrence of *Dinophysis* species (Marasovic *et al.*, 1998, Pavela-Vrancic *et al.*, 2001). In 2000, the Ministry of Agriculture, Water Resources and Forestry of the Republic of Croatia established control mechanisms for preventing the risk of DSP-contaminated-seafood consumption. This included: the sea water, marine phytoplankton and shellfish analyses to detect DSP and PSP toxins in the shellfish digestive glands.

Four shellfish breeding regions were included in monitoring programme; four shellfish areas in the North-Eastern Adriatic area (Peruzula, Solina, Solaris, Rt Zub =**NA**), one in Novigrad sea area (Prdelj =**NS**), and a number in Šibenik Channel (Šibenik 1, Šibenik 2 , Šibenik 3 =**ŠC**) and Mali Ston Bay (Bistrina, Mali Ston, Usko and Sutvid =**MB**) (Figure 1).

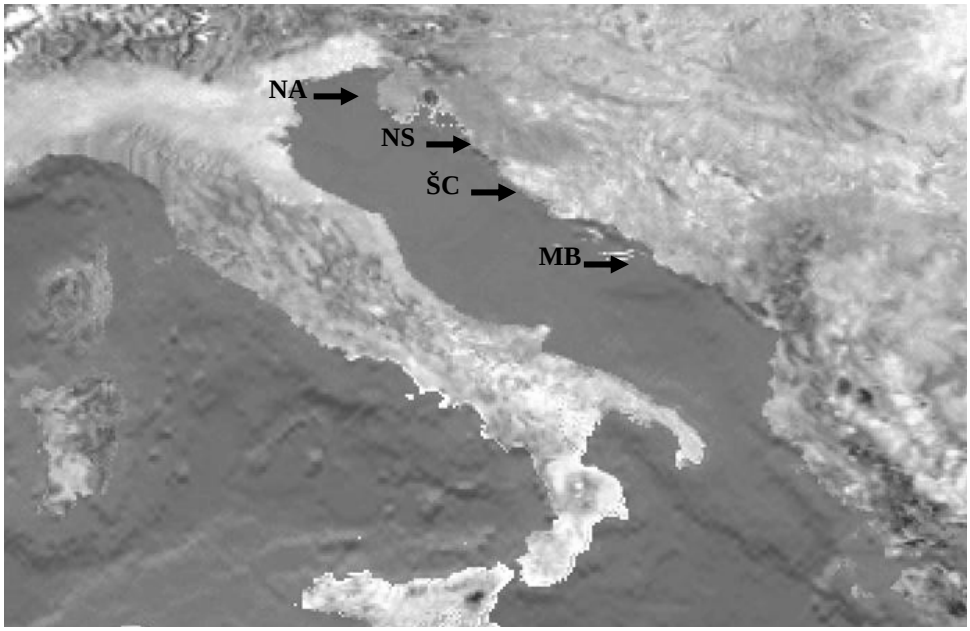

Figure. 1. Map showing the locations of studied shellfish breeding farms NA=Northern Adriatic, NS=Novigrad Sea, ŠC=Šibenik Channel, MB=Mali Ston Bay

Materials and Methods

Samples of mussel *M. galloprovincialis* from the farms along the eastern Adriatic coast were collected on a monthly basis during the colder part of the year (from February 2001 to February 2002) and on a fortnight basis from April to November 2002.

The DSP toxicity was analysed after the method developed by Yasumoto *et al.* (1978; 1984). The Yasumoto method includes extractions of DSP from 20 g of the hepatopancreas of mussels. The acetone phase and diethylether were evaporated and the residue resuspended in 4 ml of 1 % Tween 60. Aliquots of 1 ml of this solution were intraperitonealy injected into 18-20g mice. Three parallel tests were performed, and the reaction of the mice was observed for 24 hours or until mice death, if any. The mouse bioassay was regarded as positive and shellfish as toxic for humans if at least two of three mice died within five hours. Mean time of death shorter than 24 hours and longer than 5 hours indicated the presence of the toxins, even though they were below the level harmful for human health.

Positive mouse bioassay results and those suspected to be indicative of DSP toxin presence were additionally subjected to the Protein – Phosphatase Assay (PP2A) (Holmes, 1991; Tubaro *et al.*, 1996) to establish the okadaic acid concentrations.

PSP analysis was conducted by the mouse bioassay according to the method proposed by the Official Analytical Chemists (AOAC, 1990).

The species composition of the phytoplankton community on shellfish farms was also analysed. Samples were collected with a plankton net (mesh size 20 μm) from the entire water column and by Nansen bottles from the surface layer. All the samples were preserved in 5 % glutaraldehyde solution. Species composition and quantity of phytoplankton samples were determined by counting aliquots of 25 ml after the Utermöhl method (Utermöhl, 1958). Physical-chemical parameters (temperature, salinity and dissolved oxygen) were determined by standard oceanographic methods (Strickland and Parsons, 1972).

Results and Discussion

Mean annual temperature and oxygen saturation values were relatively uniform for all the study areas for the research period (Figures 2, 3 and 4). However, annual mean salinity values were found to vary significantly from one study area to another. Salinity was significantly lower in Šibenik (mouth of river Krka) and Novigrad Sea areas (mouth of river Zrmanja), in relation to other study areas (Figure 5) due to considerable freshwater inflows. In the Šibenik area the highest values of mean phytoplankton abundances were recorded, which are up to five times higher in comparison to other localities (Figure 6). Although, the abundance is an insufficient indicator of phytoplankton density, because of the variety of phytoplankton cell size, this area is also characterised by a high dinoflagellate portion in phytoplankton community. The ratio between diatom and dinoflagellates cell abundance (N L^{-1}) could be a very good indicator of eutrophication of certain area, which was further confirmed with the results of this paper (Figure 7). The bulk of the results of our studies of rather enclose bays points to the fact that phytoplankton communities are changed under eutrophication conditions, evidenced as an increase in the proportion of flagellates (particularly dinoflagellates) in relation to diatoms. Various organic compounds play important role in promoting the growth of some dinoflagellates species (Okaichi and Yagyu, 1969).

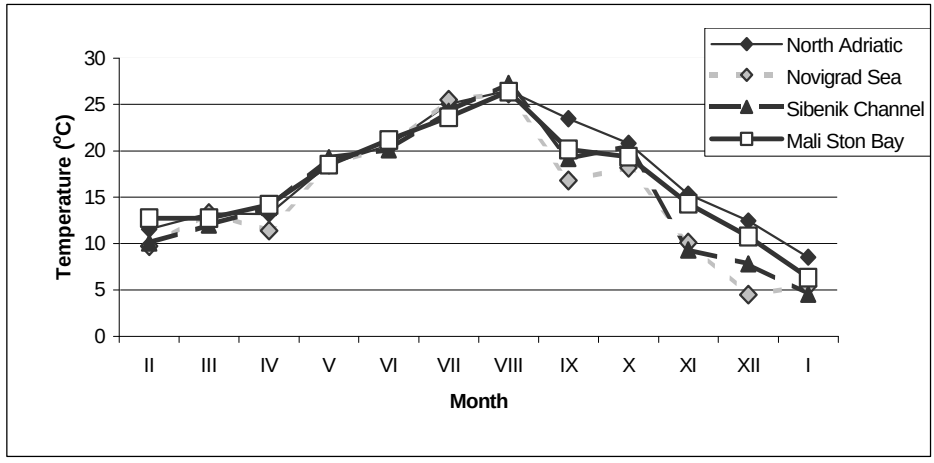

Figure 2. Mean values of temperatures on four breading areas from February 2001 to February 2002

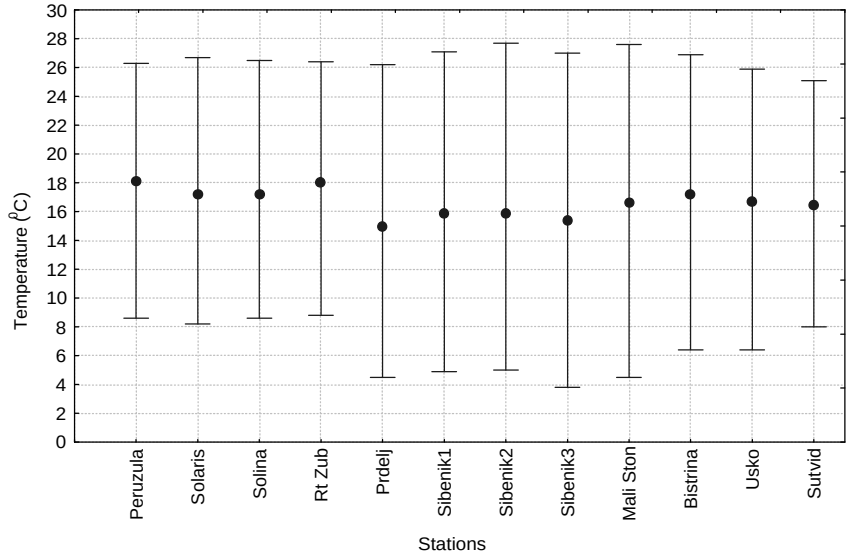

Figure 3. Range and mean annual surface temperature values at studied stations from February 2001 to February 2002.

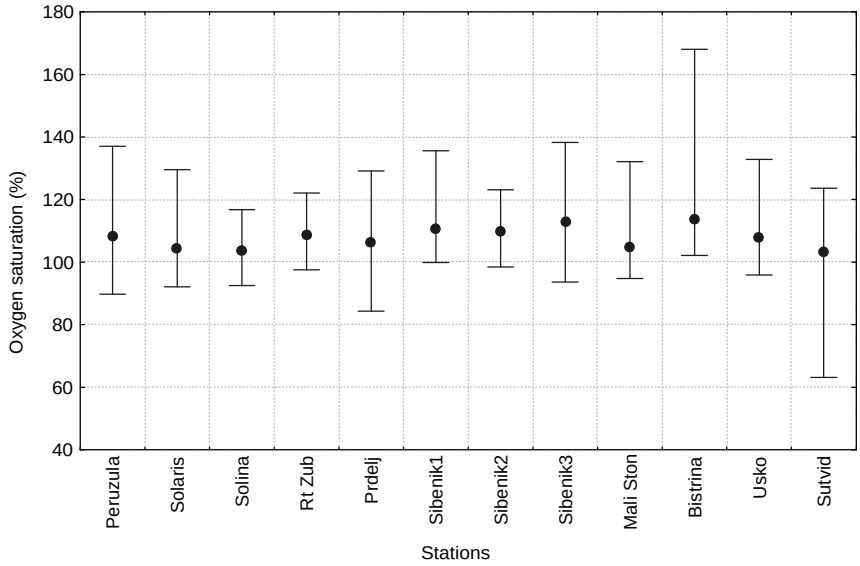

Figure 4. Range and mean annual surface oxygen saturation values at studied stations from February 2001 to February 2002

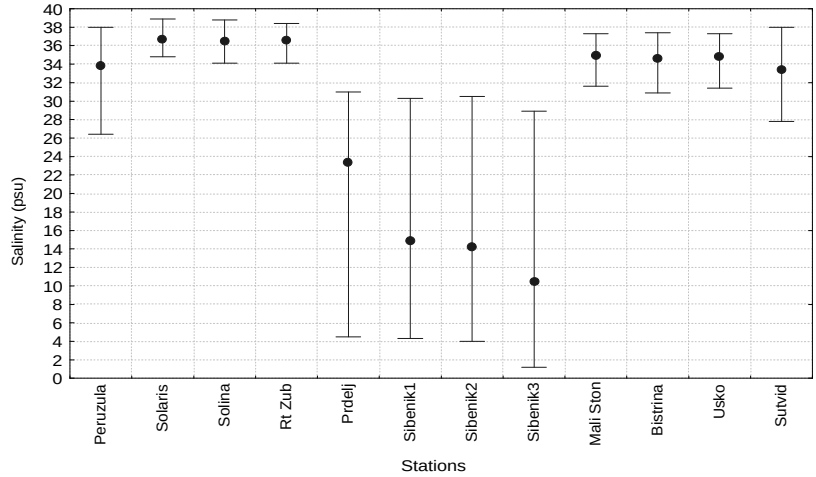

Figure 5. Range and mean annual surface salinity values at studied stations from February 2001 to February 2002

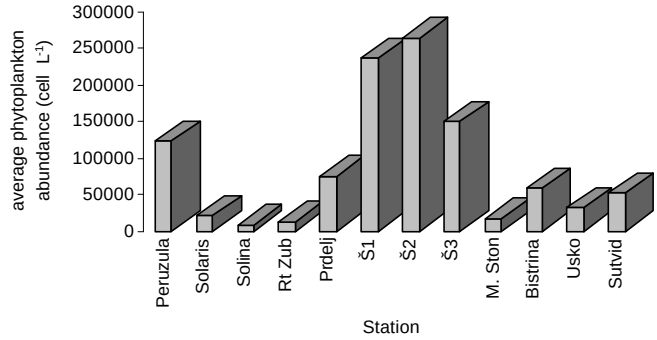

Figure 6. Average phytoplankton surface abundance (Cell l^{-1}) at studied stations from February 2001 to February 2002

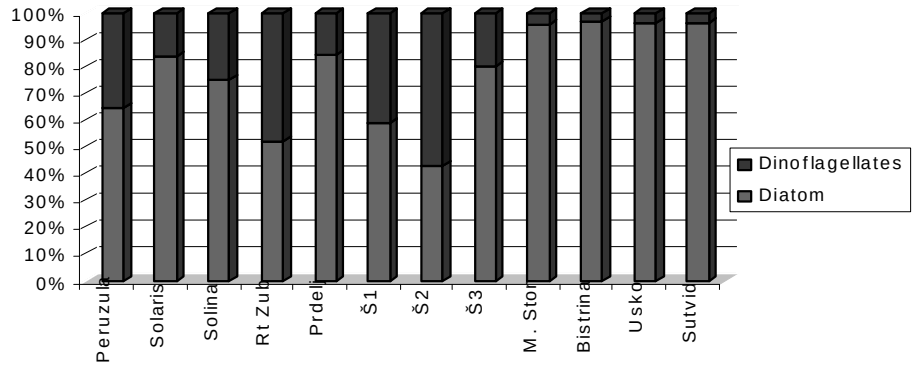

Figure 7. Ratio of diatom and dinoflagellates phytoplankton cells at studied stations from February 2001 to February 2002

The highest ratio of the mean number of diatom cells to that of dinoflagellates was recorded from the area of Mali Ston Bay, and the lowest from the Šibenik area. Diversity index values (Shannon and Weaver, 1963) ranged from 0.18 (Šibenik 2) and 4 (Solaris). The highest diversity index value and the lowest range of value oscillations were both recorded from Mali Ston Bay. On the contrary, the lowest values with much higher range of their oscillation were recorded from the Šibenik area (Figure 8).

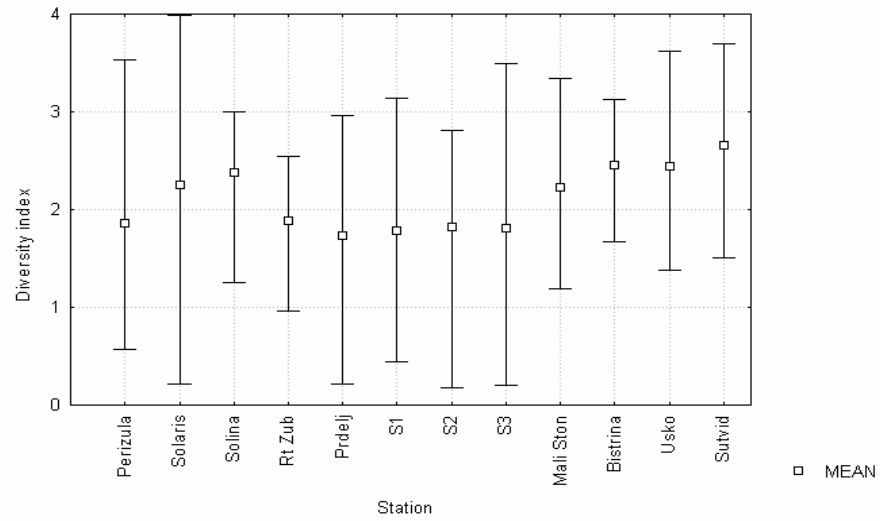

Figure 8. Range and mean annual diversity index values at studied stations from February 2001 to February 2002.

Positive results of the mouse bioassay for DSP presence were recorded in two samples of mussels from Solina and Solaris in the Northern Adriatic (Figure 10). Additional analyses of the same samples (Protein Phosphatase Assay) showed the presence of okadaic acid (OA), even though its concentrations were very low, between 0.5 and 0.01 µg/g hepatopancreas. Considering the fact that the lethal dose for mice is 4 µg/g of hepatopancreas the question is whether there exist some other toxin compounds from the DSP group exist that we are not able to detect by available methods and prove by available analyses (pectenotoxin and yessotoxin)

The results on the phytoplankton composition analysis coincided in time with the positive mouse bioassay. This may be indicative of the fact that the occurrence of DSP mussel toxicity is related to a higher number of *Dinophysis* species (Figure 10). *Dinophysis fortii, D. caudata, D. rotundata, D. sacculus and D. acuminata* were recorded from the northern Adriatic, while *D. sacculus* was the only species recorded from Bay of Mali Ston.

The highest number of *Dinophysis* species was recorded from the breeding farms in the Northern Adriatic area (**NA**), and the lowest from the **MB** area in the south (Figure 10). On the basis of the time of death of mice and the PP2A assay we found that the mussels contained very low toxin concentrations, at the detection levels, in the areas of Šibenik 1 and Šibenik 2. However, *Dinophysis* species were not recorded from Šibenik farms in the period of the positive mouse bioassay (March 2001) (Figure 10). Records of *Dinophysis* species in the Northern Adriatic contrasts with those at stations Šibenik 1 and Šibenik 2 where a low quantity of the species *Prorocentrum*

minimum was found. This species could have been the cause of toxicity. Due to the long-term regular occurrence of rather high density of *P. minimum* in that area (particularly in spring and summer), ant the fact that shellfish poisoning has not occurred by this time, we do not believe it to be the cause of toxicity in this case.

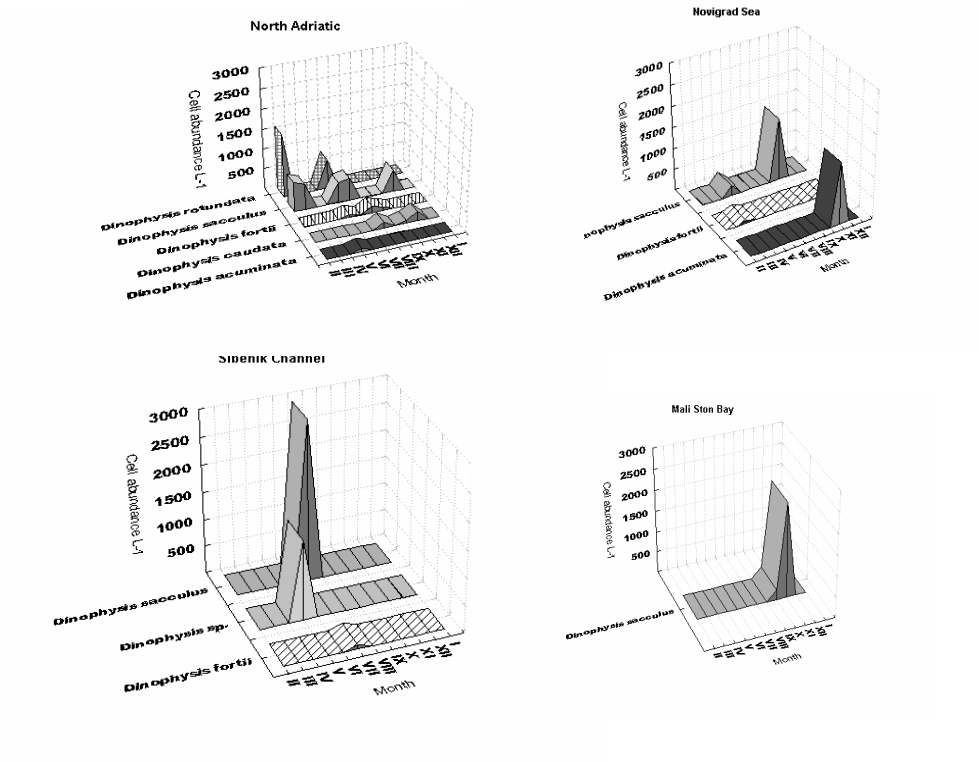

Figure 9.Temporal and spatial distribution of the genus *Dinophysis* in the investigated area from February 2001 to February 2002

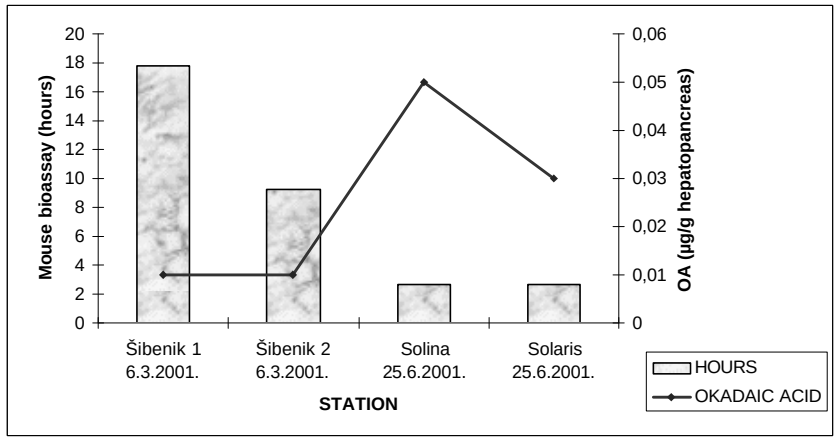

Figure10. Comparison of positive results of the mouse bioassay and Protein Phosphatase Assay (PP2A) during monitoring period from February 2001 to February 2002.

The cause of the death of mice in our experiment may be related to a higher quantity of species of the genus *Dinophysis* (*D. fortii, D. caudata, D. sacculus, D. acuta)* several months earlier that is from September to December 2000. This leads us to assume that mussels accumulated the toxin during the autumn 2000 when sea temperatures were still relatively high. Due to low sea temperatures during winter, the metabolic activity of mussels decreases and the depuration process slows down, so low toxin concentrations remain in mussels until the early spring. PSP toxicity was not recorded during the research period.

The results obtained so far have shown the presence of DSP toxins along the Eastern Adriatic Coast, pointing to the necessity of further investigation of still unresolved issues concerning the environmental factors impact on toxic phytoplankton species further affecting toxicity development in shellfish.

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PARALYTIC SHELLFISH POISONING TOXIN ACCUMULATION IN THE CRAB (*TELMESSUS ACUTIDENS*)AND GASTROPOD (*RAPANA VENOSA*) IN JAPANESE COASRAL WATERS

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Abstract
The commercially harvested species of crab *Telmessus acutidens* and predatory gastropod *Rapana venosa* were collected with bivalve molluscs during the blooming seasons of causative dinoflagellates of paralytic shellfish poisoning (PSP), and their toxicities were analysed by mouse bioassay. The crab was collected at Onahama, Fukushima Prefecture, during 2001 to 2003, and the toxicity level of the crab was highest in 2003 when mussels *Mytilus galloprovincialis* also showed high toxicity. The highest toxin concentration in the crab hepatopancreas was 85.3 MU/g, and the highest total toxin amount in an individual crab corresponded to approximately 1/3 of the human minimum lethal dose. The predatory gastropod *R. venosa* and several species of bivalves were collected at Mikawa bay, Aichi Prefecture in 2001. Although the toxicity of *R. venosa* was relatively lower than those of the bivalves, the toxicity (3.5 MU/g in whole soft tissue) was almost the same level as the regulation limit for molluscan shellfish (4.0 MU/g). These results suggest that the crab and gastropod should be monitored as PSP toxin vectors. In April 2004, the Ministry of Health, Labour and Welfare in Japan set the regulation limit for the carnivorous or scavenging species at 4 MU/g, taking into consideration our monitoring results.

Introduction
Paralytic shellfish poisoning toxin accumulation in bivalve molluscs is a serious problem for the aquaculture industry and public health all over the world (Shumway 1990, Hallegraeff 1993). In Japan, PSP toxins in bivalves have been monitored since 1979 to prevent human intoxication (Yamamoto and Yamasaki 1996). Besides toxin accumulation in bivalves, carnivorous and scavenging shellfish have been also reported as PSP toxin vectors (Shumway 1995). The toxicity levels of the lobster in eastern Canada (Desbiens and Cembella 1995) and gastropods in southern Chile (Compagnon *et al*. 1998) was high enough for human intoxication. We also found the crab *Telmessus acutidens* (Stimpson, 1858), a commercially harvested species, to have high concentration of PSP toxin (80 MU/g, which is 20 times the regulation limit of molluscan shellfish) at Onahama, in the north-eastern part of Japan, during the blooming season of the causative dinoflagellate of PSP, *Alexandrium tamarense* (Oikawa *et al*. 2002, Oikawa *et al*. 2004). This was the first observation of the toxin in this crab species. Similarly, little attention has been given to the toxin accumulation in gastropods, though many species of gastropods are commercially harvested in Japan.

In this study, the crab *T. acutidens* was collected using crab pots during the blooming season of toxic dinoflagellates *A. tamarense* from 2001 to 2003 at Onahama, Fukushima Prefecture, in order to monitor the toxicity of the crab in the natural environment. In addition, the gastropod *Rapana venosa* (Valenciennes, 1846), which is also a commercially harvested species, was collected at Mikawa Bay, Aichi Prefecture, to investigate the possibility of toxin accumulation.

Materials and Methods

Shellfish

The crab *T. acutidens* was collected using crab pots at Onahama, Fukushima Prefecture in the north-eastern part of Japan (Figure 1) in spring from 2001 to 2003, because *A. tamarense,* has been reported to occurr in Onahama mainly in spring (Oikawa *et al*. 2004, Personal communication from Fukushima Prefectural Fisheries Experimental Station). The mussel *Mytilus galloprovincialis* Lamarck, 1819 was also collected during the same period at the same point to confirm the occurence of PSP.

The gastropod *Rapana venosa* and four species of bivalves, *Ruditapes philippinarum* (Adams and Reeve, 1850), *Mactra chinensis* Philippi, 1846, *Phacosoma japonicum* (Reeve, 1850) and *Scapharca kagoshimensis* (Tokunaga, 1906) were collected at Mikawa bay, Aichi Prefecture (Figure 1), during and after toxic dinoflagellates blooms.

Toxicity assay

In the crab samples, the hepatopancreas, thoracic muscle and appendicular muscle were individually analysed. The gastropods were shucked, and the foot muscle and viscera were separated. Six specimens for each tissue were thencombined for analysis. In the bivalves, 5-10 specimens of whole soft tissue were combined for analysis. Toxicity was determined by mouse bioassay described by Kawabata (1978) as the Japanese official method of PSP toxin analysis.

Results and Discussion

The crab, *T. acutidens,* was found for the first time to be highly toxic by accumulation of PSP toxin at Onaham in 1998(Oikawa *et al*. 2002). Since only three specimens were collected and analysed, a more intensive investigation was necessary to clarify the toxin accumulation in the crab.

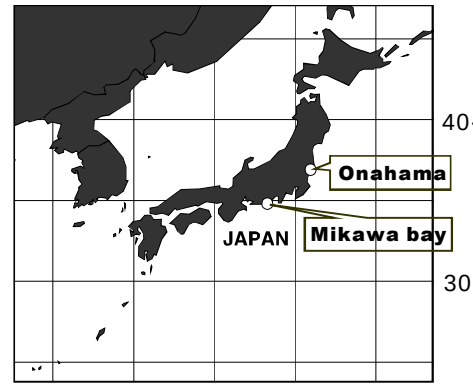

Figure 1. Location map of the sampling areas. The crab *T. actutidens* was collected at Onahama, Fukushima Prefecture. The gastropod *R. venosa* was collected at Mikawa Bay, Aichi Prefecture.

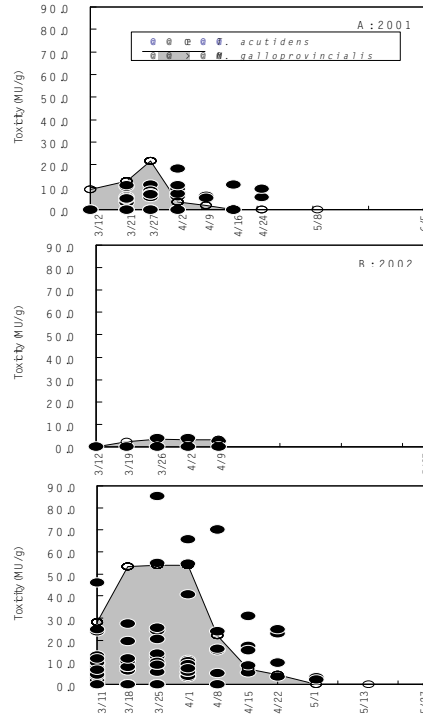

Figure 2 Toxicity in the crab *T. acutidens* (hepatopancreas) and mussel *M. galloprovincialis* (whole soft tissue) collected at Onahama from 2001 to 2003. The toxicity of the crab was individually analyzed, and ten specimens of mussels were combined for analysis.

In this study, a large number of the crab specimens were collected using crab pots at Onahama from 2001-2003. In three years sampling, the concentration of PSP toxin detected in the crabs and mussels were highest in 2003 (Figure 2C). Of the 75 specimens collected, PSP toxins were detected in 68 and the highest toxin concentration was 85.3 MU/g. Since 80 µg STX/100g was estimated to 235 MU/100g by Kawabata (1978), the highest concentration in the crab hepatopancreas was estimated to be 2,904 µg STX/100g. The total toxin amount contained in an individual crab hepatopancreas was calculated by multiplication of the toxin concentration and the weight of hepatopancreas, and the most toxic specimens contained 1,120 MU in the hepatopancreas. The human minimum lethal dose was reported as 3,000 MU (Hashimoto 1988), therefore the crab contained the toxin corresponded to approximately 1/3 of the human minimum lethal dose in the hepatopancreas. In contrast, 14 crab specimens collected before the mussel became toxic showed no toxicity (data not shown). Additionally, both crabs and mussels collected in 2001 and 2002 showed relatively lower toxicity compared to those in 2003. In 2001 (Figure 2A), the maximum toxicity in the mussels was 21.5 MU/g, and the highest toxicity of the crab hepatopancreas was 18.2 MU/g. In 2002 (Figure 2B), the toxicity of the mussels was the lowest in the three years, and only 3 of 38 specimens showed a low toxicity in the hepatopancreas. These results suggest that PSP toxicity in the hepatopancreas of the crab *T. acutidens* can vary depending on the toxicity level observed in the bivalves inhabiting in same coastal waters.

Table 1 Toxicity distribution in the crab *T. acutidens* collected on 25 March 2003

No.	Body weight	Toxicity (MU/g)		
	g	Hepato-pancreas	Thoracic muscle	Appendicular muscle
1	116.4	85.3	2.8	N.D.
2	117.3	N.D.	N.D.	N.D.
3	219.4	24.3	N.D.	N.D.
4	94.1	25.6	N.D.	N.D.
5	226.8	5.6	N.D.	N.D.
6	148.9	12.7	N.D.	N.D.
7	126.3	9.5	N.D.	N.D.
8	168.0	N.D.	N.D.	N.D.
9	164.2	8.7	N.D.	N.D.
10	162.4	14.0	N.D.	N.D.
11	138.2	10.0	N.D.	N.D.
12	339.1	54.9	3.4	N.D.
13	190.0	8.9	N.D.	N.D.
14	183.2	20.5	N.D.	N.D.
15	170.7	N.D.	N.D.	N.D.

N.D.: Not detected.

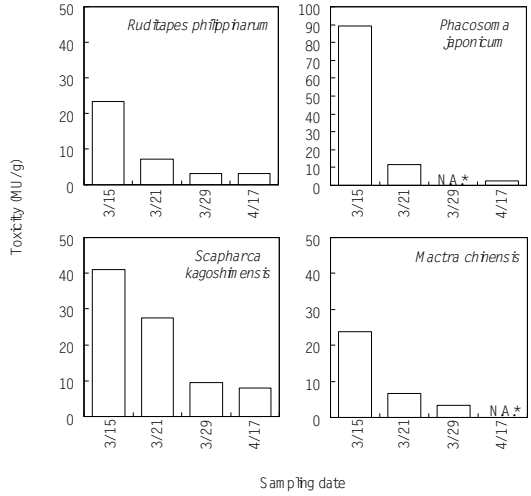

Figure 3. Toxicities in the four species of bivalves collected together with the gastropod at Mikawa bay in 2001. Five to ten specimens of whole soft tissues were combined for analysis. N.A.*: Not analysed.

The toxicities of the appendicular muscle and thoracic muscle on 25 March, 2003 were also investigated (Table 1). Low toxicities were detected only in two specimens of the thoracic muscle, when the

specimens had high toxicity in the hepatopancreas. But in the appendicular muscle, no specimens were toxic in the 15 samples tested. The results indicate that the white muscle was safe to eat even if the hepatopancreas showed high toxicity. However, people in Japan prefer to eat the hepatopancreas, and so the toxicity in the hepatopancreas should be monitored to prevent intoxication from crab. In April 2004, the Ministry of Health, Labour and Welfare in Japan set the regulation limit for the carnivorous or scavenging crabs, taking into consideration our 2003 monitoring results of the crab. Consequently, the dealing of crabs over 4 MU/g in hepatopancreas was prohibited by the Food Sanitation Law in Japan.

In 2001, edible species of gastropod and bivalves were collected at Mikawa bay during and after the toxic dinoflagellate blooms. Maximum PSP toxicities varied among bivalve species, and *P. japonicum* was found to be the most toxic species (89.1 MU/g in whole soft tissue), showing four times more toxicity than *R. philippinarum* and *M. chinensis* and twice the toxicity of *S. kagoshimensis* (Figure 3). The gastropod *R. venosa* showed toxicity in the viscera, and the maximum toxicity during the sampling was 7.1 MU/g (Figure. 4). Since no toxicity was found in the foot muscle (data not shown), the toxicities based on the whole soft tissue of the gastropod was calculated to be 3.5 MU/g. The toxicity of *R. venosa* was relatively lower than those of the bivalves, however, the toxicity of the gastropod changed in different patterns from those of the bivalves. The toxicity of the gastropod increased, whereas those of the bivalves decreased during the same sampling period. These results suggested that the toxicity in the gastropod would have become more toxic. We could not collect shellfish after 17 April 2001, and thus we could not clarify the maximum toxicity level in the gastropod. Although the gastropod *R. venosa* was found to be a possible vector species of PSP toxins in this study, further investigation is required to clarify the toxin accumulation properties in the gastropod.

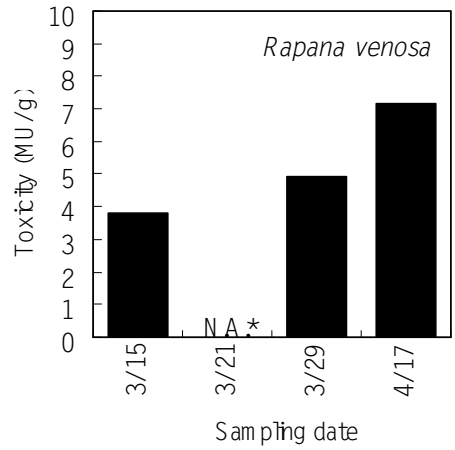

Figure 4. Toxicity of the gastropod *R. venosa* collected at Mikawa bay in 2001. Six specimens of the gastropod viscera were combined for analysis. N.A.*: Not analyzed.

Acknowledgements
This study was partially supported by grants from Research Project for Utilising Advanced Technologies in Agriculture, Forestry and Fisheries of AFFRC. We thank Dr. Yuichi Kotani in NRIFS and Aichi Fisheries Research Institute for collecting the shellfish samples. We also thank Ms. Hatano in NRIFS for her great assistance.

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TEMPORAL AND SPATIAL DISTRIBUTION OF DIARRHEIC SHELLFISHH TOXINS IN BLUE MUSSELS, *Mytilus edulis*, ALONG THE SWEDISH SKAGERRAK COAST

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Abstract
Bivalves are ancient animals that feed by filtering large volumes of water. In this way, phytoplankton in the water column are concentrated in the mussels. The hazards associated with the consumption of mussels are thus dependent on the occurrence and composition of toxic algae in the areas where shellfish are grown. Diarrheic shellfish toxins have occurred regularly in Sweden since 1987. A rapid intoxication versus slow detoxification of mussels is a common phenomenon in Swedish waters. Concentrations of DST in mussels are normally low from March to August (<160 µg/kg mussel meat) and high from October to December (>160 µg/kg mussel meat). Some years minor peaks above the limit can be observed from mid June to mid July. Peaks of toxins in mussels are mostly recorded from October to December, but the pattern can, however, differ significantly due to location and year. In 1997 mussel farmers in the Tångesund/Nösund region experienced very low levels of DST. However, the following year, 1998, high levels were recorded 27 weeks in a row. A particularly interesting area is the Kolje Fjord. This region had low levels of toxins until 1999, despite regular recordings of potentially DST producing algae. Today mussels grown and harvested in this area have similar toxin levels as mussels from other fjords in the Skagerrak region.

Introduction
Marine plankton are an important food for filter-feeding shellfish. This means that the proliferation of phytoplankton is beneficial for cultured and wild shellfish. However, about 10 % of all planktonicplankton algae have the capacity of producing substances that may be harmful. The most potent toxin producers are the dinoflagellates and several that cause harmful effects seldom reach magnitudes cell abundances which make them visible as blooms. This means that some of the toxin-producers are harmful even in very dilute concentrations and can kill directly or travel high up in the food chain (Anderson and White 1992, Shumway, 1995).

Blue mussels (*Mytilus edulis*) are the most important consumer of phytoplankton in Swedish coastal waters. The aquaculture industry produces mussels which are mostly grown on longlines. The high filter-feeding capacity of these bivalve shellfish makes them act as "biological filters", ideal as controllers of eutrophication. Thus, beside their importance as a food resource they are also of significant value for the environment, leading to clear water and increased biodiversity. Longline blue mussel culturing systems have been used in Skagerrak since the early 1980s (figure 1.). The gross production capacity of an average Swedish long-line unit is about 150 to 200 tons of mussels in two years. Each long-line unit occupies a water surface

area of about 2000 m^2 and acts as a large three-dimensional biofilter (10 x 200 m, ~8 m deep). An optimal site at the Swedish West Coast produce up to 40 kg of fresh mussels per m^2 and year, filtering off the phytoplankton biomass produced by about 25 m^2 of sea surface (Haamer, 1995).

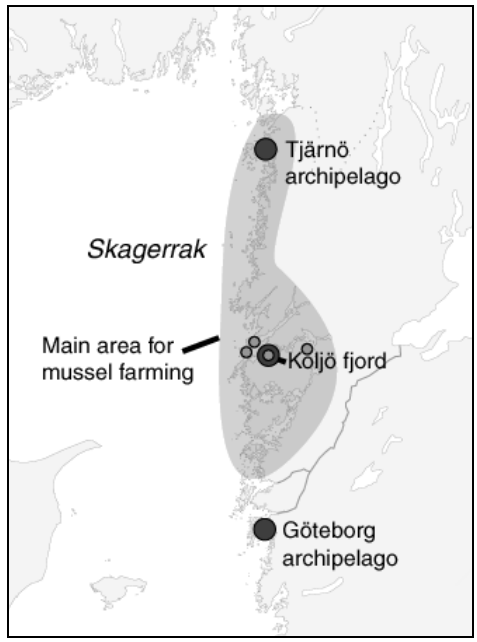

Figure 1. Major mussel culturing area along the Skagerrak coast.

Diarrhoeic shellfish poisoning (DSP) is a gastrointestinal illness that was first reported in Japan in 1976 (Yasumoto *et al* 1978). Since then, DSP or occurrences of Diarrhoeic Shellfish Toxins (DST) in shellfish have been reported from almost all regions of the world (Hallegraeff 1995). Today DSP has become the predominant threat in Sweden (Haamer *et al*. 1990a, Lindahl *et al* 2005). The species in Sweden that has been linked to DSP can be found within the *Dinophysis* genera *D. acuminata* Claparède *et* Lachmann, *D.acuta* Ehrenberg, and to a minor extent *D. norvegica* Claparède *et* Lachmann and *D. rotundata* Claparède *et* Lachmann. The last species is heterotrophic and synonymous with *Phalacroma rotundatum* (Claparède et Lachmann) Kofoid et Michener 1911 while the other are mixotrophic organisms.

The major toxin is okadaic acid (OA) but the group also includes a number of related polyether toxins, i.e. dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2), dinophysistoxin-3 (DTX3), 7-*O*-acylDTX2 (acylDTX2), and 7-*O*-acylOA (acylOA). OA, DTX1 and DTX2 are toxins produced by the algae. The others are toxic derivates that have been metabolised within the mussels. All the toxins share the same backbone structure of OA and are thus collectively referred to as the OA group or DTXs (Yasumoto *et al*. 1985, Kumagai *et al*. 1986, Carmody *et al*. 1996). OA strongly inhibits protein phosphatase 2A (PP2A) and to less extent protein phosphatase 1 (PP1). These enzymes regulate various eukaryotic cellular events and inhibition of PP2A leads to cell-signalling disorders and leakage of water from the cells.

The major distribution of OAs in shellfish is in the hepatopancreas (HP or digestive gland). Normal preparation of mussels for consumption, i.e. cooking or steaming, cannot reduce the toxin level due to the chemical stability and lipophilic properties of DTX.

The maximum level of toxin that does not cause a health risk is estimated to be 160 µg/kg. This level is now the upper limit for DST content in mussels allowed on the market in all EU (Report of the meeting of the workgroup of toxicity of DSP and AZP 21th-23th May 2001).

Monitoring of DST in Sweden
The EC regulation for controlling DSTs in bivalves is based on a mouse bioassay. This test has the advantage of being a protection against unknown toxins that might appear in the area. However, the mouse test is not always satisfactory in terms of detectability, specificity, and reliability. Use of animals in shellfish monitoring programmes has also become increasingly unacceptable in several EU countries for ethical reasons. Analyses by chemical methods have however, other disadvantages. These include a low probability of detecting new toxins and problems associated with the supply of pure toxin standards needed in the tests. In Sweden, the surveillance of DST-concentrations in mussels has been performed by HPLC (High Performance Liquid Chromatography) for 14 years (Haamer *et al*. 1999). From the middle of the year 2000, the analyses were transferred to a commercial company, AnalyCen Nordic AB, and the method of choice was subsequently changed to HPLC-MS (HPLC with Mass Spectrometrical detection).

Materials and methods
Sampling
For routine monitoring of DSP, 15 mussels, five from the upper (1m), five from the middle part (5m), and five from the bottom level (8m) of the mussel culturing band, are collected and weighed after removal of the shells. The depths are ca. 1 m, 5 m and 8 m. The hepatopancreas is dissected and weighed separately for determination of the relative proportions, which can vary from 10-25 % and result in a total of 10-30 g HP. No boiling of mussel meat is performed.

Analytical procedure
A modified instrumental method using HPLC with fluorescence detection according to Lee *et al*. was used (Lee *et al*, 1987). The hepatopancreas sample was homogenised, extracted and analysed as previously described (Haamer *et al.*, 1990, Svensson *et al.*, 2000, Godhe *et al.*, 2002).
From the middle of year 2000 toxin analyses has been performed by a commercial company, AnalyCen Nordic AB by using HPLC-MS. The analytical procedure follows that of Aase and Rogstad (Aase and Rogstad, 1997).

Results and Discussion
The long Swedish regular analysis of DST in mussels is a unique quantitative dataset. Okadaic acid has been the dominating DST, but dinophysis toxin-1 has occurred occasionally. Other DSTs have usually not been analysed. In general, concentrations of DST in mussels are normally low from March to August and high from October to December (Figure 2A). Minor peaks can be observed some years in mid-June to mid-July (Figure 3).. However, the toxin pattern can differ significantly both due to location and year. In the major mussel farming area, the Tångesund/Nösund region, farmers have experienced nearly toxin free years, i.e. 1997. However up to 34 consecutive weeks of high toxin levels have also been recorded. Fortunately for the industry, there are usually some regions with toxin concentrations below the limit

for consumption and the mussel farmers can usually manage to supply the market with mussels by harvesting at different locations. In the Tjärnö archipelago, toxin levels fluctuate significantly and mussels can become toxic within days (Figure 2C3). This was clearly shown in a study by Godhe at el (2002). During this study three distinct water masses passed through the vicinity of the mussel farm. The second water mass contained a high abundance of *Dinophysis* spp., high concentrations of DST in the phytoplankton population, and a subsequent rapid increase in the toxicity of mussels was observed. After 8 days, the water mass containing *Dinophysis* was replaced and cell numbers again returned to low levels. However, the toxicity of mussels continued to be high for the remainder of the study. This rapid intoxication vs. slow detoxification of mussels is a common phenomenon in Swedish waters (Svensson *et al.*, 2000). In the Koljö Fjord area previous studies have shown low toxin levels, despite regular recordings of DST producing algae (Lindahl and Andersson, 1996). A dramatic change of this pattern has been observed since year 1999 (Figure 2D). Today mussels grown and harvested in the Kolje Fjord have similar toxin levels as mussels from other fjords in the Skagerrak region. We can only speculate about the cause of this changing pattern. One striking observation is that the increase in toxicity occurred just after a large translocation of mussel farming units from toxic cultivating areas into the Koljö Fjord area.

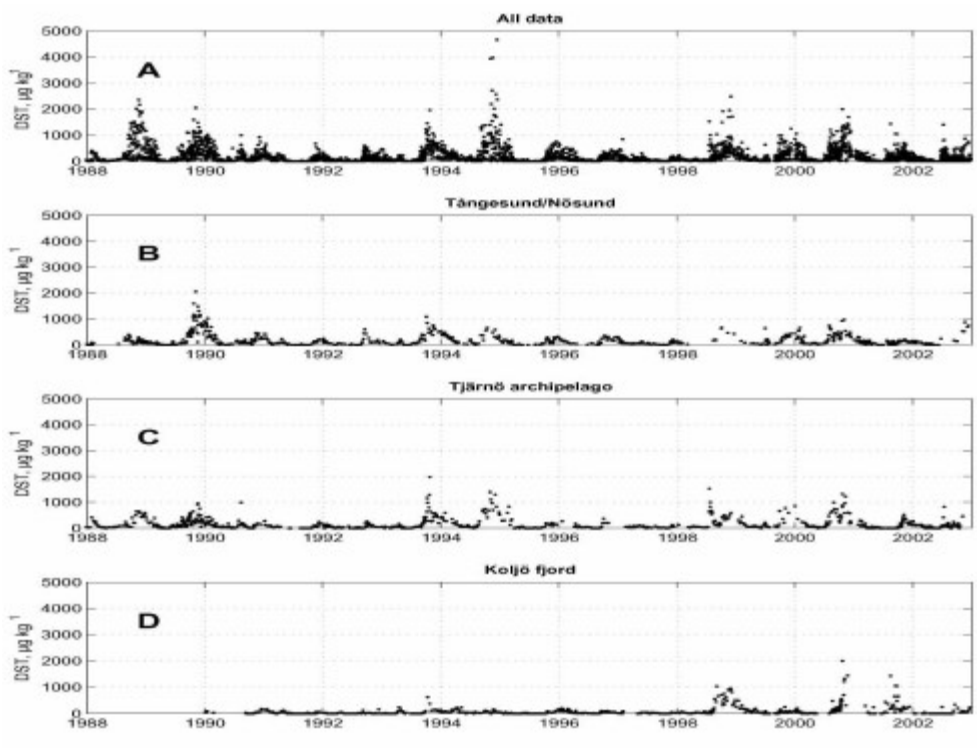

Figure 2. Diarrhoetic Shellfish Toxin concentrations in blue mussels along the Swedish Skagerrak/Kattegat coast 1988-2002. A. All data, B Tjärnö Archipelago, C Tångesund/Nösund and D Koljö fjord.

Bivalves are a globally important food resource. Marine bivalve farming combats eutrophication and could be an important tool for restoring coastal areas (Lindahl *et al.*, 2005) However, the feeding mode of bivalves entails special risks for consumers and can affect public health. To set up a cost effective monitoring programme an understanding of the underlying causes is necessary. These include but are not restricted to: (1) The ecology of the harmful plankton species, (2) the physical oceanography of the area and (3) the ecology and physiology of the bivalves, e.g. depuration time. A monitoring programme may include phytoplankton sampling and analysis, a numerical model of the hydrodynamic situation and analysis of toxins in both plankton and bivalves. Thus, risks should be analysed with respect to seasonal and geographical differences in occurrence of algal toxins, hydrodynamic situations and the species-specific bivalve being cultivated, etc. With such an approach, the design of control programs will differ between areas with priority given to public health rather than trade marketing. With proper food-safety controls, bivalve culture could be an important tool, in both providing increasing harvests and mitigate eutrophication problems.

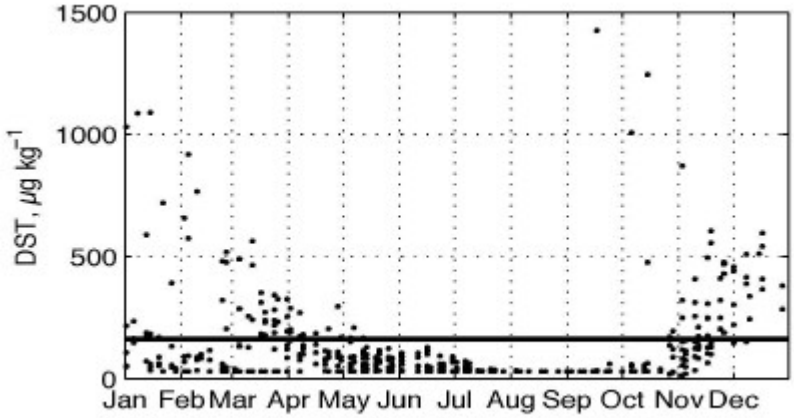

Figure 3. DST toxin concentrations during year 2003.The limit for marketing, 160 µg per 100g is shown as a solid line.

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PROROCENTRUM LIMA STRAINS FROM NEW ZEALAND: DSP TOXIN PROFILES AND UPTAKE AND CONVERSION IN GREENSHELL MUSSEL™

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Abstract
Prorocentrum lima strains from New Zealand were studied to determine toxin profiles in pellets and culture fluids. Profiles of DSP toxins in *P. lima* cell extracts were determined using LC-MS following centrifugation of cultures with and without boiling. Total production rates ranged from 5 to 50 pg/cell^{-1}. Okadaic acid (OA) and OA-diol esters were found at approximately equal levels in the different strains. The predominant OA-diol esters identified in all strains were: OA-C8:2 (39 %), OA-C9:2 (two isomers, 9 %, 7 %) and a novel compound OA-C10:3 (45 %). DTX1 was detected at low levels in the free form (=8 % free OA), but not in ester forms at these low levels. A novel nitrogen-containing polyether was detected in the extracts by LC-MS. The concentration of toxins found free in growth media during growth was studied using solid phase extraction (SPE). DTX4 forms of the esters were determined by LC-MS following boiling of the harvested cells. Feeding trials, using Greenshell™ mussel (*Perna canaliculus*), were carried out to determine the distribution of toxins from *P. lima* cultures within mussel tissues. Results of this study will be integrated into New Zealand's routine shellfish biotoxin monitoring programmes using LC-MS.

Introduction
The dinoflagellate *Prorocentrum lima* is a common mat-forming inhabitant of harbour sediments in Northland, New Zealand (Rhodes and Syhre 1995), and has been found on oyster farm infrastructure and, less commonly, throughout New Zealand growing epiphytically on seaweeds and in the plankton community. Previous analyses using sample hydrolysis led to the determination of esterified okadaic acid (OA) in some New Zealand *P. lima* strains (Rhodes *et al*. 2001). Quilliam *et al*. (1996) used modified extraction methods and LC-MS to confirm the presence of sulphated ester forms (DTX4) and diol esters (Figure 1). Boiling was used in order to stop enzymatic bioconversions of sulphated DTX4 type compounds to diol esters. For this study samples were either boiled or, when full bioconversion to diol esters was desired, harvested cells were left to stand at 34^{0}C. Proportions of free and esterified OA were determined in cells and in supernatants from *P. lima* cultures. Shellfish feeding trials were carried out to determine the extent of uptake and bioconversion of free and esterified forms of OA.

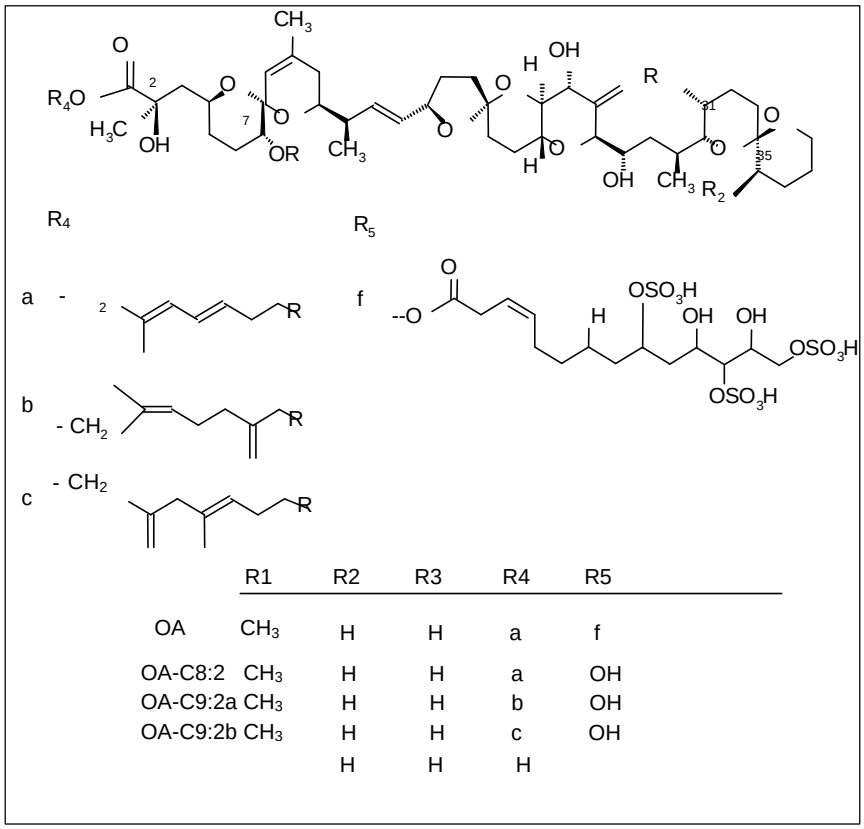

Figure 1 Structures of known okadaic acid toxins.

Materials and Methods

Prorocentrum lima strains used in this study included isolates from Northland, New Zealand (CAWD33, 67, 70, 94), and one isolate, for comparative purposes, from Spain (CAWD32; refer Table 1). Strains were maintained in the Cawthron Institute Culture Collection of Microalgae. Batch cultures were grown under standard conditions (100 µmol/m^{-2} s^{-1} photon flux; 14:10 h light:dark; 19°C) in f2 medium (Guillard 1975). Cells were harvested at stationary phase by centrifugation (1000 *g*).

Toxins were extracted from algal cells by sonicating (30 min. at 34°C) with MeOH/water (8:2 v/v). For the analysis of DTX-4 compounds, algal cells were boiled (100°C, 3min.) prior to methanol extraction (Quilliam *et al*. 1996). Hydrolysis was completed under basic conditions followed by acid neutralisation (Mountfort *et al*. 2001). Okadaic Acid and DTX1 were analysed in hydrolysed and unhydrolysed cell or tissue extracts by LC-MS/MS (Waters 2790; Micromass Quattro Ultima; 150x2mm Luna C18 column; acetonitrile/water gradient and acidic ammonium formate buffer; ESI-) (McNabb *et al* 2005). Extracts of *P. lima* cells (unboiled) were fractionated on a C18-SPE column using methanol/water mixtures (30 %, 60 %, 80 %, 100 % MeOH v/v). The 80 % MeOH fraction was analysed for diol esters of OA and other toxins by LC-MS and LC-MS/MS (C18 column, acetonitrile/water gradient with acidic ammonium formate buffer; ESI+). Methanol extracts of boiled *P. lima* cells were analysed for DTX4 using LC-MS (C18 column, acetonitrile/water gradient with neutral ammonium acetate buffer; ESI-).

Greenshell™ mussels (*Perna canaliculus*) were held in glass cylinders (10 L capacity) on wire mesh grids to raise them above their faeces, and fed stationary phase *P. lima* cells incrementally over 6 days (total cell number, 36 $x10^6$). Controls (unfed mussels) were frozen until analysed. Shellfish tissues were extracted by homogenisation with 90 % MeOH and analysed by LC-MS (ESI+, acidic ammonium formate buffer). LC-MS (ESI-, neutral ammonium acetate buffer) was used to detect 7-acyl esters of OA (DTX3 type compounds).

Results

Free and ester forms of okadaic acid in New Zealand strains of P. lima.

A time course of OA and DTX1 production in *P. lima* (CAWD70) was analysed at intervals over 34 days (Figure 2). DTX1 was determined as <1 % of OA. No esters of either toxin were detected in the supernatant. Cell counts increased from 0.94 $x10^6$ cells/L^{-1} to 10.9 $x10^6$ cells/L^{-1} during the course of this experiment. The culture reached stationary phase at day 18.

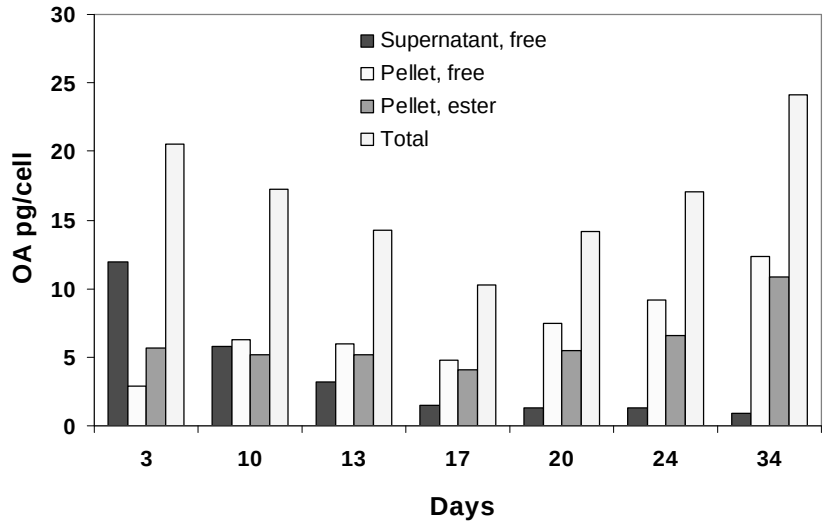

Figure 2 Changes in okadaic acid concentrations (pg per cell) in *Prorocentrum lima* (CAW D70) over time in culture: cell pellet and cell free supernatant.

Stationary phase cells (unboiled) harvested by centrifugation from cultures of four isolates of *P. lima* and one Spanish isolate were analysed by LC-MS (Table 1).

Table 1. Production of DSP toxins by strains of *P. lima*. Esterified DTX1 was <0.1 pg. cell^{-1}

Strain	Location	Free OA pg/cell	Free DTX1 pg/cell	OA-ester pg/cell	Total pg/cell
CAW-D32	Spain	3.6	0.3	1.4	5.3
CAW-D33	Rangaunu	10.4	0.1	13.2	23.7
CAW-D67	Rangiputa-1	8.0	<0.1	4.8	12.8
CAW-D70	Whatawhiwhi	28.8	0.1	20.4	49.3
CAW-D94	Rangiputa-2	10.5	0.6	10.2	21.3

Diol esters from P.lima

Several diol esters of OA were identified in the *P. lima* cultures by LC-MS. Based on a standard of OA-C8:2 and their characteristic MS/MS spectra, the following esters were characterised in strain CAW-D67 (MNH_4^+, proportion). OA-C8:2 (946, 39 %), OA-C9:2 (two isomers, 960 ca 9 %, 7 %) and a novel compound OA-C10:3 (972, 45 %). The structure of an OA-C10:3 diol ester isolated from *Prorocentrum belizeanum* has recently been reported (Suárez-Gómez *et al* 2005). The proportions of the diol esters from *P. lima* strains CAW-D33 and CAW-D95 were similar to CAW-D67. A novel N-compound (MH^+ 856) was also found both in the diol ester fractions of these three *P. lima* strains and, in lower concentrations in comparison with OA, in the hepatopancreas of the Greenshell™ mussels fed *P. lima* (Figure 3). The same N-compound was a minor component of the Spanish *P. lima* isolate (CAWD32).

Greenshell™ mussel feeding study

Twelve shellfish Greenshell™ mussels were fed *P. lima* (CAWD70) incrementally over a period of 6 days. The harvested shellfish were dissected and the tissues analysed by LC-MS for free and esterified OA. The total OA detected was 2.0 mg/kg whole flesh and 87 % was present in ester forms (Table 2). Over 98 % of the total OA was in the hepato-pancreas. No diol esters were detected in the shellfish but acyl forms of OA (DTX3-type compounds) were tentatively identified by LC-MS (to be confirmed). DTX4 was not analysed as the labile nature of these compounds means it is unlikely they would be accumulated intact in shellfish. The mussels recovered ca. 22 % of the toxins fed to them (calculated from data in tables 1 and 2) based on the assumption that the micro-algal isolate produced 50 pg per cell total OA. The calculations included free OA in the added culture fluids but faeces and pseudo-faeces were not analysed.

Table 2. Toxin accumulation in 12 Greenshell™ mussels fed 36 x10^6 *Prorocentrum lima* cells (CAWD70) over a 6 day period.

	OA mg/kg flesh		OA µg per shellfish	
Shellfish portion	Free	Ester	Free	Ester (% ester)
Hepatopancreas	1.4	14	2.4	24 (91)
Rest of flesh	0.12	0.34	1.8	5.1 (74)
Total			4.2	29.1 (87)

Mean of 5 replicates (RSD<10 %).

Av. Shellfish flesh weight = 16.7 g (av. Hepatopancreas weight = 1.7 g).

DTX4 in New Zealand strain of P. lima (CAWD70)

LC-MS (ESI-) detected DTX4 (sulphated C8-diol ester) in *P. lima* methanol extracts following boiling of harvested cells to prevent enzymatic desulphation. Also present were a range of DTX4 analogues, many of which appeared unique to the NZ strains (to be confirmed).

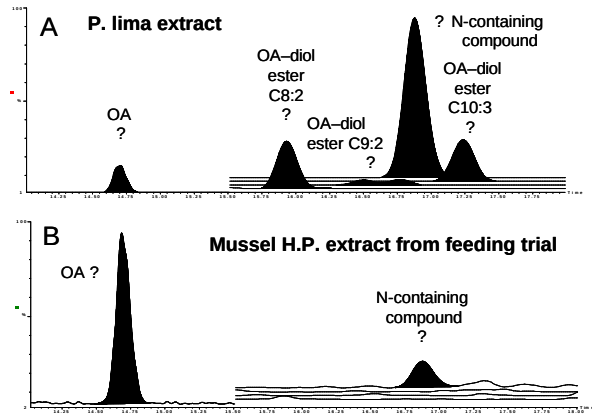

Figure 3. LC-MS of: **(A)** Stationary phase culture of New Zealand *Prorocentrum lima* culture (CAWD70; unboiled) showing OA, four diol esters and a novel N-compound; **(B)** Extract of Greenshell™ mussels fed *P. lima* (CAWD70) showing OA and N-compound, but no diol esters. H.P.: hepatopancreas.

Discussion

The four New Zealand *P. lima* strains studied were prolific producers of OA with ˜50 % being present in ester forms. Little DTX1 was detected from these four strains or from the one Spanish strain. The algal cells initially released free OA to the culture medium, but the proportion of OA to ester reduced as cultures neared stationary phase. The diol ester fraction (unboiled cells) contained mainly OA-C8:2, two isomers of OA-C9:2 and OA-C10:3. A novel N-compound was detected in the diol ester fraction which is being characterised and investigated further. A complex mixture of sulphated esters (DTX4s) was detected in the *P. lima* extracts when cells were boiled to prevent enzymatic conversions of the toxin post-harvest. Greenshell™ mussels fed *P. lima* accumulated high levels of ester forms of OA and some free OA and recovered ca. 22 % of the toxins fed to them. Direct uptake by shellfish of toxins from solution needs further determinations using cell free supernatant. 87 % of the total OA toxin in the mussel tissues was present in ester forms. Most of the toxin was detected in the hepatopancreas and the shellfish appear to convert OA, DTX4 and OA-diol esters to 7-acyl esters (DTX3-type compounds). Figure 4 presents schematically the intoxication process of mussel by *P. lima*.

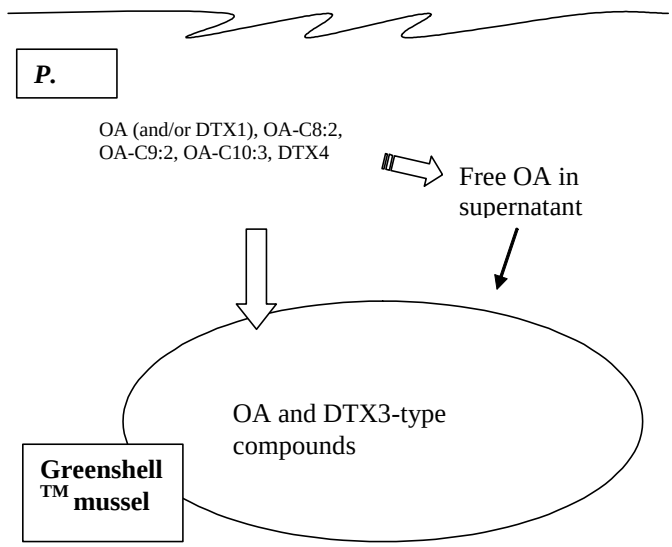

Figure 4. Passage and bioconversion of DSP Toxins from micro-alga to shellfish

Conclusions

New Zealand strains of *P. lima* were shown to be prolific producers of okadaic acid with ca 50 % being present in ester forms, probably mainly as DTX4 type sulphated esters. Extraction of these labile compounds from cells without inactivation of enzymes resulted in formation of a mixture of diol esters of okadaic acid with the unusual C10:3 form predominating. The high percentage of esters accumulated in intoxicated Greenshell™ mussel confirms the need to closely monitor for esters during algae blooms. For regulatory purposes (analytical and toxicological) it is concluded that the focus should be on acyl, rather than diol, esters of okadaic acid, because the diol esters detected in the micro-algae cultures were not detected in the shellfish fed with *P. lima*.

Acknowledgements

Dr Chris Miles, AgResearch, Hamilton supplied an okadaic acid diol ester standard. This research was largely funded by the Foundation for Research Science and Technology (Contract No. CAWX0301).

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PROFILE OF YESSOTOXIN ANALOGUES IN A NORWEGIAN STRAIN OF *PROTOCERATIUM RETICULATUM*

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Abstract
A large culture (110 L) of a Norwegian strain of *Protoceratium reticulatum* (strain AP2) was established. The culture was harvested with a specially designed pump and column system and the yessotoxins (YTXs) extracted by solid phase extraction. Fractions from before and after harvesting were analyzed by a recently developed YTX-ELISA with a broad specificity for YTXs, and by LC-MS. The culture contained 85 mg YTXs by ELISA and 43 mg YTX by LC-MS. LC-MS^3 analysis revealed the presence of YTX, trinorYTX, heptanor-41-oxoYTX, heptanor-39-methyl-39,40-ene-41-oxoYTX, YTX 32-*O*-monoglycoside, two dihydroxyYTXs, what may be two hydroxy-trinorYTXs, as well as traces of other YTX analogues.

Introduction
Shellfish accumulate marine biotoxins from algae, and this is an important obstacle to the shellfish industry. Further knowledge of the wide variety of biotoxins present and their importance to human consumer safety are needed. This work focuses on *Protoceratium reticulatum*, a species known to produce yessotoxin (YTX) throughout the world (Ciminiello *et al.*, 2003; Samdal *et al.*, 2004; Satake *et al.*, 1997a; Satake *et al.*, 1999). YTX is a disulfated polyether first isolated from scallops in Japan (Murata *et al.*, 1987) and was originally associated with the diarrhetic shellfish poisoning. Since then, many analogues of YTX have been described (Ciminiello *et al.*, 1998; Ciminiello *et al.*, 1999; Ciminiello *et al.*, 2000a; Ciminiello *et al.*, 2000b; Ciminiello *et al.*, 2001; Ciminiello *et al.*, 2002; Daiguji *et al.*, 1998; Konishi *et al.*, 2004; Miles *et al.*, 2004a; Miles *et al.*, 2004b; Miles *et al.*, 2004c; Miles *et al.*, 2004d; Satake *et al.*, 1997b; Satake *et al.*, 2002). The toxicity of YTXs towards humans is questionable, because although YTX is toxic by i.p. dosing in the mouse bioassay, it is of low toxicity when administered orally (Aune *et al.*, 2002; Ogino *et al.*, 1997; Tubaro *et al.*, 2003). Investigation of a New Zealand culture showed production of a wide range of known and unknown analogues of YTX (Miles *et al.*, 2004a; Miles *et al.*, 2004b; Miles *et al.*, 2004c). This finding prompted us to investigate a Norwegian strain of *P. reticulatum*.

Materials and Methods

Reagents

ELISA analysis (Briggs *et al.*, 2004) was performed with a quantitative YTX standard obtained from Dr. M. Satake, Tohoku University, Japan. Reagents for the YTX-ELISA were prepared according to Samdal *et al.* (2004). Reagents for LC-MS were prepared according to Aasen *et al.* (2004).

Algal culture

A *P. reticulatum* culture was established from net-haul AP2 (Samdal *et al.*, 2004) by single-cell capillary isolation (Guillard, 1973) and cultured in modified Erd-Schreiber medium (Throndsen, 1978) at 15 ± 1°C, a light intensity of 50 $\mu E/m^2s^1$, a light:dark interval of 12:12 hr, and a salinity 30 psu in four tanks with a mean cell density of 7950 cells/L.

Sample extraction

The culture (110 L) was harvested with a portable system containing a pump, filter and column (Figure 1). Yessotoxins (YTXs) were extracted by pumping the culture of *P. reticulatum* at 1.3 L/min through a glazed polypropylene felt filter (USFilter, BP-420, 5 µ, Plymouth Products, USA) and then on to a column of HP20 (10 × 21.5 cm, Supelco, USA) with a filter pad of cotton wool on top. The system was rinsed with 4 L growth medium and 20 L distilled water, and the filter and cotton wool frozen. The column effluent was retained for analysis. The HP20-resin was transferred to a glass column and washed with 800 mL water. Excess water was removed by applying a flow of N_2 (0.5 bar). YTXs were eluted with MeOH and 500-mL fractions collected. The algal filter and the cotton were extracted with MeOH (2 × 500 mL), the extracts filtered under vacuum, and the filter washed with MeOH (100 mL). The cell culture and effluent (1 mL aliquots) were extracted with Bond-Elut cartridges (C18, Varian). The cartridges were pre-equilibrated with methanol (3 mL), then with water (3 mL), the sample (1 mL) applied, washed with water (5 mL) to remove salts and eluted with MeOH (3 mL). All fractions were analysed by ELISA and by LC-MS.

ELISA analyses

The concentration of YTXs in each extract was determined by indirect competitive ELISA (Briggs *et al.*, 2004) with minor adjustments to plate coater and antibody concentrations (Aasen *et al.*, 2004; Samdal *et al.*, 2004).

LC-MS analyses

YTX was quantitated by LC-MS using an accredited method at the Norwegian School of Veterinary Science, based on modification to the method by Goto *et al.* (2001). Analogues of YTX were analysed by $LC\text{-}MS^3$ on an LCQ Deca ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, USA) coupled to a Surveyor™ HPLC using a Prodigy ODS column (150 × 2 mm; 5µm; 100Å; Phenomenex, USA) and gradient elution with (A) methanol–0.1 % aqueous ammonium formate (3:17) containing 0.1 % formic acid and (B) 100 % methanol as described in Miles *et al.* (2004b).

Results and Discussion

Analysis showed that the culture contained 85 mg YTXs by ELISA and 43 mg YTX by LC-MS. From this, 22 mg YTXs by ELISA and 13 mg YTX by LC-MS was recovered from the HP20 column, 7 mg YTXs by ELISA and 6 mg YTX by LC-MS from the algal filter, and 6 mg YTXs by ELISA and 3 mg YTX by LC-MS from the cotton wool. This corresponds to a recovery of 41 % of YTXs by ELISA and 51 % of the YTX by LC-MS. The remainder of the toxin and some cells were found in the column effluent, indicating a leak in the filter and column system. Examination of algal cells on the filter by a microscope revealed that the cells were broken.

LC-MS^3 analysis (Table 1, Figure 2) revealed the presence of YTX, trinorYTX, heptanor-41-oxoYTX, heptanor-39-methyl-39,40-ene-41-oxoYTX, YTX 32-*O*-monoglycoside, two dihydroxyYTXs, two probable hydroxy-trinorYTXs, as well as traces of other YTX analogues including YTX 32-*O*-diglycoside and a hydroxyYTX (not 45-OHYTX). There were no signs of the 41a-homo- and 9-methyl-41a-homo-YTXs (Miles *et al.*, 2004d) or their polyhydroxyamido (Miles *et al.*, 2004c) derivatives seen in strain CAWD40 from New Zealand. These results indicate similarities and differences in toxin profiles between *P. reticulatum* strains. Examination of the toxin profile of other strains of *P. reticulatum* is under way. Interestingly, traces of trinorYTX have been detected in only one sample of Norwegian mussels (John Aasen, pers. commun.) despite this being a significant minor component of strain AP2.

Table 1. Relative abundance of YTX analogues in the *P. reticulatum*-culture (AP2).

m/z	Analogue	Abundance (%)
1141	YTX	100
1101	trinorYTX	3.8
1047	ketoYTXs	1.1
1175	dihydroxyYTX	2.0
1177	Possibly dihydroxyYTX	1.5

Figure 1. System for collection of YTXs from the *P. reticulatum* culture: Pump, filter and column with HP20-resin.

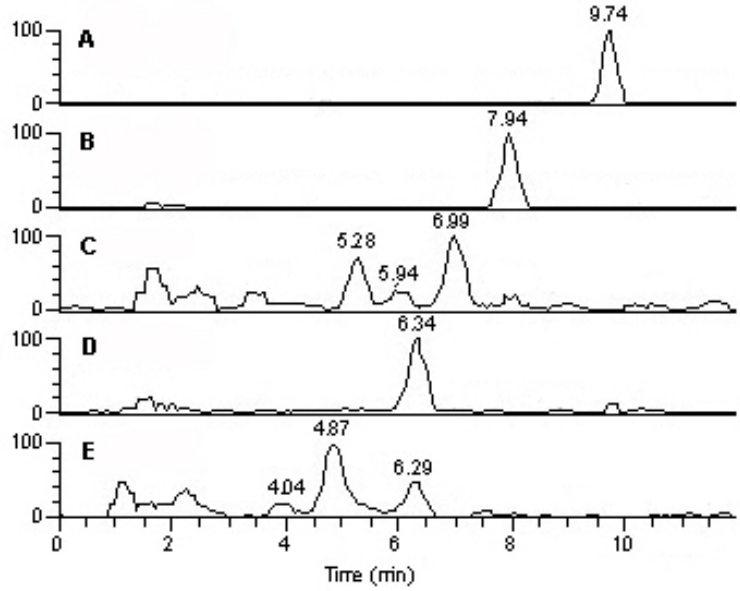

Figure 2. Selected ion chromatograms from LC-MS^3 analysis of the extract of *P. reticulatum* ; (**A)** YTX at *m/z* 1141, 9.74 min; (**B**) trinorYTX at *m/z* 1101, 7.94 min; (**C**) ketoYTXs at *m/z* 1047; 5.28, 5.94, and 6.99 min; (**D**) dihydroxyYTX at *m/z* 1175; 6.34 min; (**E**) possible dihydroxyYTX at *m/z* 1177, 4.87 min.

Conclusion

The profile of YTXs in the Norwegian strain differed from that in New Zealand strain CAWD40, with YTX and trinorYTX as major constituents by LC-MS. As with CAWD40, however, YTX constituted only about half of the total YTXs as measured by ELISA. This observation suggests that production of a complex array of minor YTX analogs may be a common feature of *P. reticulatum*.

Acknowledgements

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MORPHOLOGY, PHYLOGENY AND PSP TOXIN COMPOSITION OF *ALEXANDRIUM SPP*. ISOLATED FROM IRISH COASTAL WATERS.

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Abstract
The dinoflagellate genus *Alexandrium* (Halim) has received considerable attention in recent years. In part, this has been due to recent taxonomic advances within the Dinophyceae. Many species from this genus however, produce paralytic shellfish poisoning (PSP) toxins that have had serious economic impacts within the aquaculture industry worldwide. The occurrence of PSP toxins in shellfish from the south coast of Ireland has been linked to the suspected presence of an *Alexandrium* population. This study describes what is currently understood of the distribution of *Alexandrium* spp. around Ireland, in terms of both species diversity and toxicity. *Alexandrium* cysts and vegetative cells were collected from sediment and water column samples from Irish coastal waters. These were used to establish a suite of mono-specific cultures. Morphological identification of the vegetative cells using the fluorescent stain Calcofluor White was confirmed by partial sequencing of amplified LSU rDNA. Toxicity testing showed that of all the species isolated only *Alexandrium minutum* from the south coast produced PSP toxins. This species had both a toxin profile (GTX-2 and GTX-3) and a distribution which confirmed that it has been responsible for historical occurrences of contamination of shellfish with PSP toxins.

Introduction
The genus *Alexandrium* has a world-wide distribution and consists of several species, some of which are localised to particular regions whilst others have a cosmopolitan distribution (Balech, 1995). Their morphological identification is very complex and requires the observation of their size, shape, ability to form chains and, in particular, small details of their thecal plate structure. The techniques used to reveal these fine details are usually Scanning Electron Microscopy or epifluorescence microscopy on the theca stained with Calcofluor White (Yoshida, 2001). The *Alexandrium* life cycle includes both motile and non-motile stages. Under specific environmental conditions, *Alexandrium* populations can actively grow and reach high cell densities that can lead to discoloured waters (i.e. red tides). Many species within the genus *Alexandrium* produce toxins that can cause severe illness in human after the consumption of shellfish contaminated by PSP toxins (Kao, 1993). These 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). About 20 different forms of PSP toxins have so far been isolated and these are classified into three groups: a) Saxitoxin (STX) and Neo-saxitoxin b) Gonyautoxins (GTX) and c) Carbamoyl-toxins (C-toxins) (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, and when respiratory muscles are affected death sometimes occurs from 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 *A. minutum* observed along the west coast of Ireland in 2001 (Hansen *et al.*, 2003) was not associated with contamination of shellfish, despite increased monitoring at the time (Irish Marine Institute, unpublished records). An objective of

current research carried out within the Martin Ryan Institute is to investigate the diversity of *Alexandrium* spp. around Ireland and their toxin composition. This paper describes what is currently understood of their distribution and toxicity.

Materials and Methods

The diversity of *Alexandrium* species along the south and west coasts of Ireland (Figure1) was investigated through the establishment of mono-specific cultures derived either from the isolation of resting cysts or vegetative cells. Batch cultures were maintained in illuminated incubators in *f*/2 medium at 15°C and with a 14:10 (LD) light cycle. Species identification was performed by epifluorescence microscopy with Calcofluor white stain (Fritz and Triemer, 1985) and was confirmed by sequencing the D1-D2 domain of the LSU rDNA. Sequences obtained were compiled with other *Alexandrium* sequences extracted from databases and used for phylogenetic analysis with the software PAUP version 4.0b2. Paralytic shellfish poisoning toxin analysis of the cultures was carried out using the HPLC-FD method described by Franco and Fernández-Vila (1993).

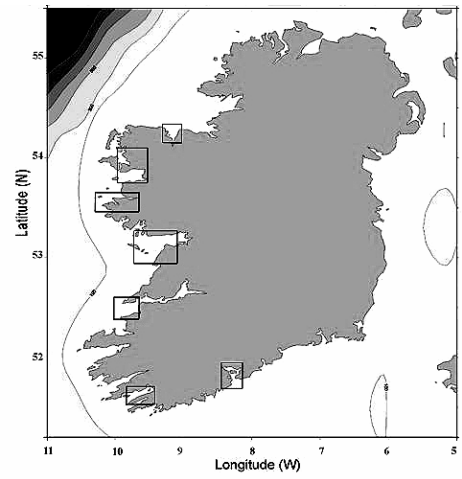

Figure1. Map of Ireland showing locations from which sediment or water column samples were collected.

Results

Morphological Identification.

Alexandrium tamarense and *A. minutum*, which were both present along the south and west coasts, largely conformed to Yoshida's (1993) emended descriptions regarding the shape of the posterior sulcal plate of the vegetative cells (Figure 2). Both *A. minutum* from the south and west coasts did not have any ventral pore on the first apical plate.

LSU Sequencing and Phylogeny.

The phylogenetical analysis gave similar results to previous investigations (Figure 3), clustering *Alexandrium* species into two major clades (Scholin *et al*. 1995, Usup *et al*. 2002, Hansen *et al*. 2003). Both *A. tamarense* and *A. minutum* were grouped as expected from the literature, the former (non-toxic), belonging to the Western European Rybotype while the latter clustered with other European strains of this type (Higman *et al*., 2001).

Toxin Composition. Out of all the cultures established, only the isolates of *A. minutum* from the south coast appeared to produce PSP toxins (Figure 4). The toxin analysis revealed, under our culture conditions, the presence of GTX-2 and GTX-3

with some traces of dcGTX2-3, which identifies *A. minutum* as the organism responsible for the PSP events recorded in this area in the past. Its localised distribution supports the hypothesis that this species prefers zones with low turbulence conditions, high residence time and high anthropogenic inputs such as harbours or rias (Hansen *et al.*, 2003).

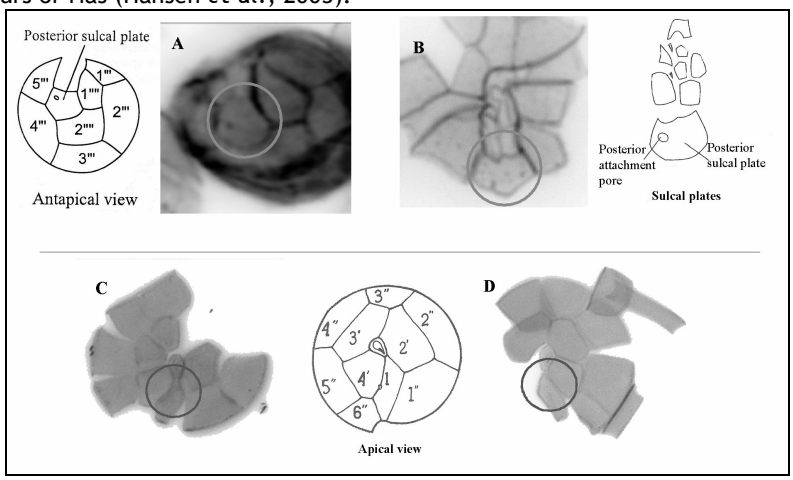

Figure 2. Details of the thecal plates of *Alexandrium* vegetative cells after treatment with Calcofluor White stain. The posterior sulcal plate is longer than broad for *A. tamarense* (A) and wider than long for *A. minutum* (B). The first apical plate of both *A. minutum* from the south (C) and the west coasts (D) did not have a ventral pore. (pictures not on scale)

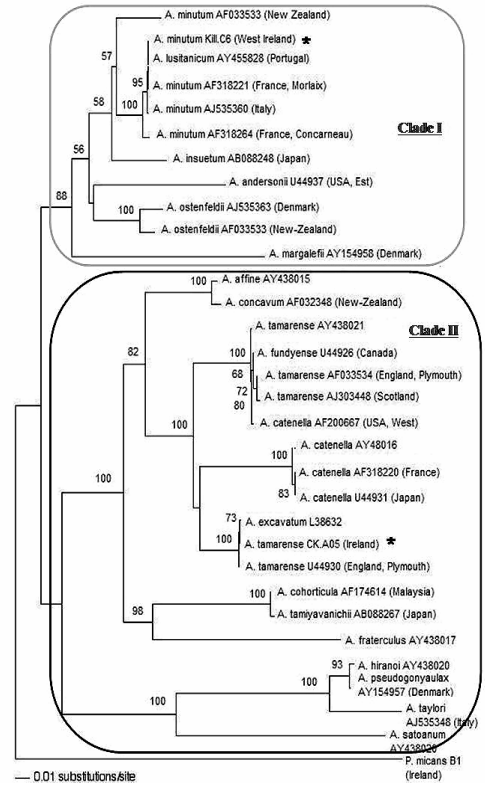

Figure 3. Phylogenetic tree based on nucleotide sequences (D1-D2 domains of LSU rDNA) and inferred by the neighbour-joining method from a matrix of Logdet distances. Numbers on the branches indicate branch frequency from 100 bootstrap samples.

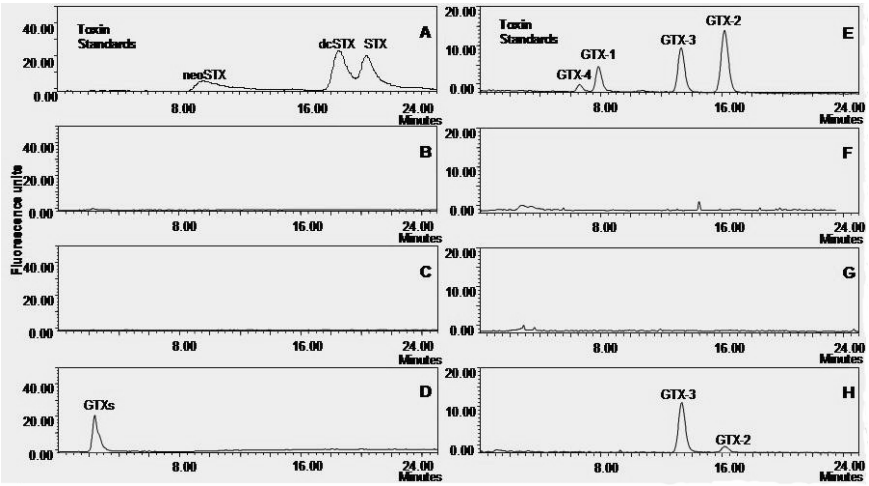

Figure 4. HPLC-FD chromatograms of PSP toxins of Irish *Alexandrium* spp. after extraction in CH_3COOH 0.05M: (A,E) Standards of toxins. (B,F) *A. tamarense* sample. (C,G) West coast *A. minutum* sample. (D,H) South coast *A. minutum* sample. Only the *A. minutum* strains from the south coast appeared to synthesise PSP toxins (GTX2-3) under our culture conditions.

Discussion

Both *A. tamarense* and *A. minutum* were detected on the south and west coasts. The morphology of *A. minutum* did not allow us to explain the divergence in the toxin profile among the toxic and non-toxic strains of this species. On the other hand, attempts to use toxins to establish taxonomic relationships does not appear to be reliable because too many external parameters such as bacterial activity, salinity or nutrient concentrations could influence both toxin synthesis and the toxin profile (Hwang and Lu, 2000 ; Lippemeier *et al.*, 2003). Nevertheless, the rDNA LSU sequences could not differentiate between our strains of *A. minutum* isolated from the south and the west coasts, although one is toxic (GTX2-3) and the other seems not to be. From other studies carried out with toxic dinoflagellates (*Alexandrium* spp. and *Gymnodinium catenatum*), the ITS and SSU regions are also not discriminant enough at the population level (Camino Ordas *et al.*, 2004). Other markers such as RAPD or microsatellites might be used to assess the genetic polymorphism of the strains. To conclude, this seems to be the first report of the occurrence of a non toxic *A. minutum* population on the Atlantic European coast. The current situation in Irish waters therefore offers the opportunity to investigate two distinct populations of the same species. Further research is needed to ensure the two phenotypes are not mixed, and an understanding of the oceanography of the area is essential in order to find out if physical barriers separate them.

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TOXIN PRODUCTION BY *Dinophysis* SPP. AT RIA DE AVEIRO, PORTUGAL

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Abstract
Plankton extracts from a coastal lagoon where diarrhetic shellfish poisoning (DSP) events are very recurrent and severe were analysed weekly over 5 months for the presence of dinophysistoxins and pectenotoxins. Okadaic acid data was correlated with the sum of *Dinophysis* cf. *acuminata* plus *D. acuta* enumeration; dinophysistoxin-2 (DTX2) and pectenotoxin-2 (PTX2) with *D. acuta* enumeration only. The cellular toxin content increased after blooming. Toxin cellular levels at maximum bloom were not equal in all bloom peaks, thus contributing to different toxin biomasses available as a food source for shellfish. Despite the variability of toxin production, the PTX2/DTX2 ratio was always greater than 1:1, reaching maximal levels of 5:1 immediately after maximal *D. acuta* growth. The ratio OA/DTX2 seemed to be constant at 3:2 for several weeks when *D. acuta* was the exclusive toxin producer detected in the plankton. The index of *D. acuta* over total toxic *Dinophysis* (*D.* cf. *acuminata* + *D. acuta*) was a good predictive estimate for the ratio of DTX2/OA found in the plankton. Knowledge simply from plankton enumeration of expected toxin profiles is an important tool for management of bivalve's contamination when using rapid immunoassays that are targeted at OA but have lower cross-reactivity towards other dinophysistoxins, such as DTX2.

Introduction
Diarrhetic shellfish poisoning (DSP) is the most frequent toxicity event on the Portuguese coast, particularly recurrent on the northern coast, being the costal lagoon, Ria de Aveiro (40º 30', 40º 50' N), one of the most affected sites (Moita, 1993; Vale and Sampayo, 2003). Ria de Aveiro is a site where several blooms of phytoplankton species belonging to the dinoflagellate genus, *Dinophysis*, occur every year producing DSP toxins. It is also a place where a great variety of natural bancs of different shellfish species exist, making it an important area to shellfish farmers for exploitation of halieutic resources (Sobral *et al.*, 2000). This exploitation takes place throughout the year, unless the harvest is closed due to the presence of biotoxins in the bivalve molluscs. *Dinophysis acuta* Ehrenberg and *Dinophysis* cf. *acuminata* Claparède and Lachmann are the toxic species more related to DSP episodes, and they can be found in the water column from late spring until mid autumn, associated with weak to moderate upwelling events, that occur many times in the northern Portuguese coast due to the prevailing northerly winds (Moita, 1993; Palma *et al.*, 1998). *Dinophysis. acuta* and *D. cf. acuminata* distributions are restrained by water salinity, temperature and stratification. Although these two species often coexist, blooms are not coincident in space or in time, but when there are widespread blooms of *D. cf. acuminata*, *D. acuta* is nearly absent along the coast and *vice versa* (Palma *et al.*, 1998). At different bloom episodes of *Dinophysis*, bivalves reach different toxin levels (Vale and Sampayo, 2000). The toxin levels are difficult to predict with precision from phytoplankton cell counts and one of the causes may reside in the proportion of toxic plankton in relation to non-toxic plankton and/or different cellular levels of toxins (Sampayo, 1990). Unlike other microalgae producer of biotoxins, *Dinophysis* has not been cultured with success (Sampayo, 1993), and scarce data exists on toxin production by this genus. Plankton extracts from Ria de Aveiro were analysed weekly over 5

months for DSP toxins and pectenotoxins in order to study variations in cellular concentrations of toxins in natural populations. Okadaic acid (OA) data were correlated with the sum of known OA producers: *Dinophysis*: *D.* cf. *acuminata* plus *D. acuta* (Vale and Sampayo, 2000). Dinophysistoxin-2 (DTX2) and pectenotoxin-2 (PTX2) were correlated only with *D. acuta* (the only producer occurring in the studied period (Vale and Sampayo, 2000 and 2002)).

Materials and Methods

At one selected station, Triângulo das Correntes, water samples were collected weekly for identification and phytoplankton counting. The samples were preserved in the field with acidified formaline solution. Phytoplankton counts were performed using a light microscope Axioskop 2 *Plus*, and a Palmer-Maloney chamber, after water concentration by centrifugation (3000 rpm for 20 min) and the lower limit of detection was 100 cells / Litre. Two-litre water samples were filtered onto 10 µm nylon membranes, and frozen immediately until extraction. After thawing, toxins were extracted with aqueous 80 % methanol, sonicated and left for 2 hours at room temperature for spontaneous hydrolysis of esters (Quilliam and Ross, 1996). The supernatant was washed with hexane and partitioned into dichloromethane. The dichloromethane fraction was analysed on a liquid chromatograph coupled to a mass-spectrometer (LC-MS) as described in detail in Vale (2004).

Results

In 2003, *D.* cf. *acuminata* was observed in the water column between June and August, and reached the maximum abundance in July with a concentration of 2150 cells/L. In the beginning of September the concentrations of *D.* cf. *acuminata* decreased and *D. acuta* appeared in a widespread bloom, with a maximum concentration of 4150 cells/L. Presence of toxins in plankton showed a strong positive linear correlation with toxic *Dinophysis* counting's: $r > 0.85$ for all toxins (graphics not shown). In different bloom episodes, concentrations of toxins in the water were not always tightly related to *Dinophysis* abundance (Figure 1a). The explanation resides in different cellular toxin concentrations found at maximum cell numbers during blooming (Fig. 1b). The cellular toxin concentrations increased during bloom decay, but maximum levels also varied between different blooms (Fig. 1b). Nevertheless, due to the rapid decrease in cell numbers, the higher cell toxin levels did not contribute further to shellfish contamination (Vale, 2004). In general, the ranges of toxins found during blooming periods were comprehended in an interval not overlapping the interval for the ranges found in decay periods (Table 1). Despite the variability of toxin production, the PTX2/DTX2 ratio was lowest during blooming phases and increased in decay phases (Fig. 2a, Table 1). The ratio DTX2/OA seemed to be constant for several weeks when *D. acuta* was the exclusive toxin producer detected in the plankton (Fig. 2b). The index of *D. acuta* over total toxic *Dinophysis* (*D.* cf *acuminata* + *D. acuta*) was a good predictive estimate for the ratio of DTX2/OA found in the plankton (Fig. 2b). More toxins were available to shellfish feeding when *D. acuta* bloomed (OA+DTX2 ranged from 16 – 38 pg/cell), than when *D.* cf *acuminata* bloomed (OA: 5 - 21 pg/cell).

Table 1. Ranges of cellular concentrations of toxins and PTX2/DTX2 ratio found during growth phase and latency phases, respectively.

	Cell growth	*Cell latency*
OA (pg/cell)	5 – 21	25 – 70
DTX2 (pg/cell)	2 – 17	12 – 48
PTX2 (pg/cell)	1 – 38	23 – 152
PTX2/DTX2 ratio	1.1 – 2.2	3.2 – 5.1

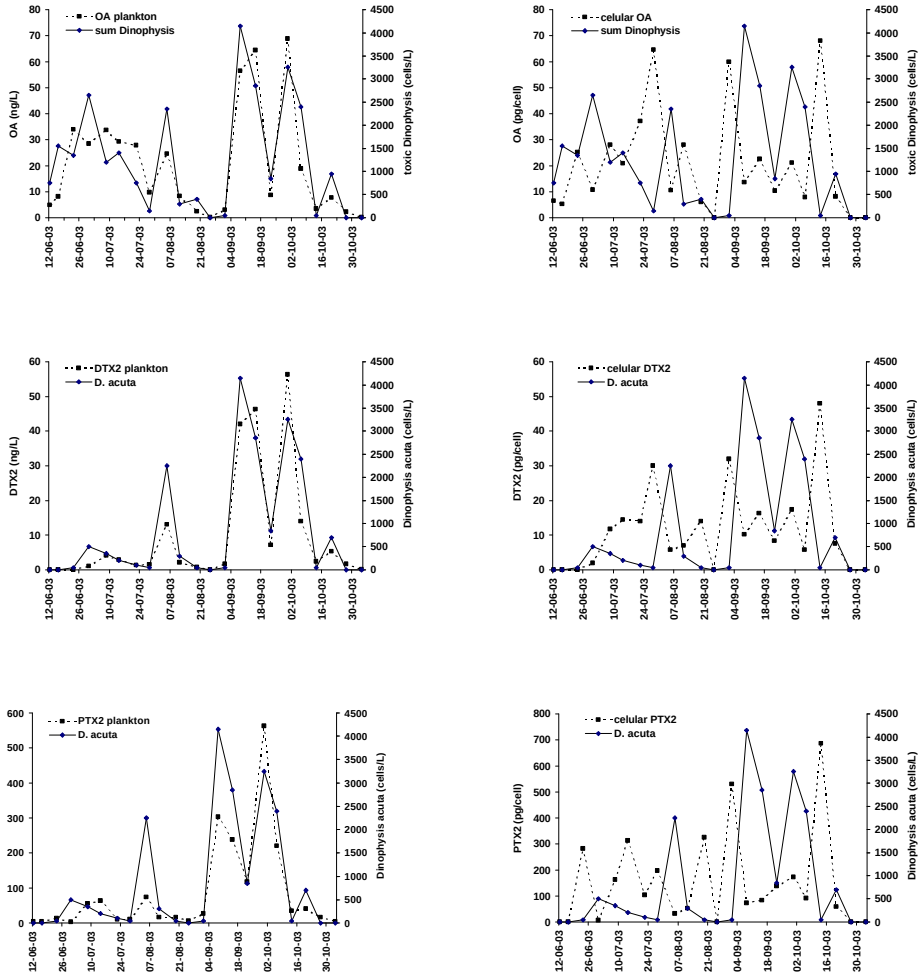

Figure 1. a) Relationship between toxins in plankton and toxic *Dinophysis* enumeration in the water column. b) Relationship between cellular content and toxic *Dinophysis* enumeration in the water column.

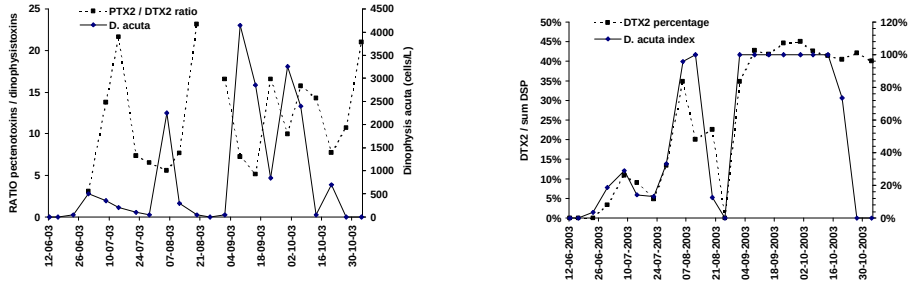

Figure 2. a) Evolution of ratio of PTX2 over DTX2 plotted against *Dinophysis acuta* counting's. b) Relation between DTX2 percentage in the plankton (over the sum OA+DTX2), and the *Dinophysis acuta* index.

Discussion

In 2003, *D. cf. acuminata* and *D. acuta* coexisted, but their maximum abundances did not coincide in space or time, as observed previously (Palma *et al.*, 1998). Production of dinophysistoxins (OA and DTX2) and pectenotoxins (PTX2) by *Dinophysis* was found to be constructive. Cellular concentrations of toxins increased when the species declined, which has also been observed in toxic *Prorocentrum* cultures, a benthic species and producer of DSP toxins (Quilliam *et al.*, 1996). Cellular ranges of dinophysistoxins (OA and DTX2) recorded during the bloom were similar to ranges found by other authors for *D.* cf. *acumimata* and *D. acut*a (Fernandez *et al.*, 2000). This data reinforced our previous results indicating that *D.* cf. *acuminata* contributes only produces OA, while *D. acuta* is responsible for OA, DTX2 and PTX2 contamination (Vale and Sampayo, 2000, 2002). When *D. acuta* was the dominant toxic species more DSP toxins where available for shellfish feeding than when *D.* cf. *acuminata* was dominant. Phytoplankton counting is an important tool for managing bivalve contamination when using commercial immunoassays that are targeted at OA but have lower cross-reactivity towards DTX2, in particular in shellfish species that have the tendency to build up DTX2 levels due to its slower elimination in relation to OA (Vale *et al.*, 2004 and Vale, 2004).

Acknowledgements

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MODELLING PHYCOTOXINS ACCUMULATION IN BIVALVES: A REVIEW.

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Introduction

Mathematical models are simplified mathematical representations of systems. Any biological system can be characterised by an input from the environment, a series more or less complex of processes inter-related and a response which is the result of applying the processes to the input and to the initial system state. This kind of modelling, therefore, requires: a) the conceptual simplification of the system by means of identifying the relevant processes and descriptive variables, and b) their description by means of sets of equations, in some cases, differential equations. Mathematical modelling is specially useful because of multiple reasons. Models allow to summarise complex information, to predict the system behaviour, to estimate flows or amount that are difficult to measure, to test hypothesis of the actual knowledge and, finally, to suggest lines for future research.

In order to model the accumulation of phycotoxins in bivalves there are several groups of processes that have to be taken into account. The first two, the most obvious, are those which represent the toxin gain and loss by the bivalve body: toxin ingestion and toxin depuration or elimination. Two other, which are specially important in many cases are the anatomical redistribution and toxin transformation. None of them involve gain or loss of toxin by the bivalves, and, in the case of toxin transformations the processes take place without any change in toxin amount (on a molar basis).

Three different kinds of models of accumulation are considered hereafter because three variables have been used frequently: toxicity, toxin concentration and toxin content. Even when the responses of the first two variables are affected by weight (in the two cases) and by transformations (in the first one), usually the remaining involved processes have enough importance to dominate the parameters and processes included in the models, and consequently, the three kinds of responses can be considered equally valid to show the basic types of accumulation models.

Models of Depuration

One-compartment models (Figure1)

The first developments arise form the need of summarising the depuration kinetics with one or a few parameters that simplify the comparisons. Initially, zero order reactions, in which the toxins loss did not depend on the toxin amount or concentration in the bivalve, were implemented, but probably in some cases, not being aware of that. In those cases, toxin decrease was described by a straight line of the type:

$$Tox=Tox0-k\cdot t$$

which is the solution of the differential equation

$$dTox/dt=-k$$

which, in turn, indicates that the velocity of toxin loss is constant and therefore independent from the toxin amount/concentration (zero order kinetics)

Notwithstanding, the most frequent way of simplifying the depuration kinetics has been to assume an exponential decrease of the toxin amount, concentration, with can be described by the equation:

$$Tox=Tox0-e-k\cdot t$$

This exponential decrease in the solution of the differential equation

$$dTox/dt=-k\cdot Tox$$

which assumes that the velocity of toxin loss at a particular time is directly proportional to the toxin concentration at that time. This corresponds, therefore to a first order kinetics of depuration.

Two-compartment models (Figures 2 and 3)

Frequently, the first order model of depuration does no correctly fit the data because, an apparently bi-phasic depuration takes place. In those cases, the initial steps of depuration seem to proceed fast and the final ones slowly. This situation has been modelled assuming that two compartment models, in which it is assumed that there are two different pools of toxins, instead of only one, and that each pool is differently bound to bivalve tissues, and consequently has a different depuration rate.

These two-compartment models have been usually described mathematically by a set of two differential equations describing each of them a first order depuration of the toxins corresponding to that compartment and also a first order transfer from the first (fast depurating) to the second (slowly depurating) compartments (Silvert and Cembella, 1995; Blanco et al 1997, Moroño et al 1998a,b, Blanco et al 1999):

$$dTox1/dt = -TR12 \cdot Tox1 - D1 \cdot Tox1$$

$$dTox2/dt = +TR12 \cdot Tox1 - D2 \cdot Tox2$$

where Toxn are the amount or concentration of toxin (or toxicity), Dn are the depuration rates and TR12 is the transfer rate between compartment 1 and 2. Sub-indices of Tox and D indicate the compartment.

Models with external variables

Environmental control or the effects of other external or internal variables have been included into models mainly by replacing some parameters of the above cited models by equations which compute them as a function, usually linear, of the new variables to be taken into account. Silvert and Subba Rao (1992) implemented the effect of temperature on domoic acid depuration in this way, and Blanco et al (1997, 1999) implemented the effects of temperature, salinity, fluorescence (as an index of itoplankton abundance), underwater light transmission (as an index of suspended matter) and body weight, also in the same way.

This kind of models can be useful in several ways: first, they can be used to simplify the description of the depuration process or they can be used as a tool to estimate the actual effect of the included variables, by allowing their coefficients to vary while optimising the model fitting to the data. In the last case is specially important to know precisely the kind of model to which the new variables are added, because, as shown by Moroño et al (1998a), an effect can be a attributed to the variables only because they are able to partially correct the response of an incorrectly chosen base model.

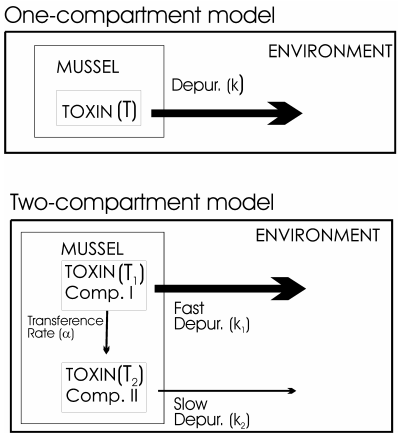

Figure 1.- Conceptual 1- and 2-compartment model of toxin depuration (from Blanco *et al* 1997)

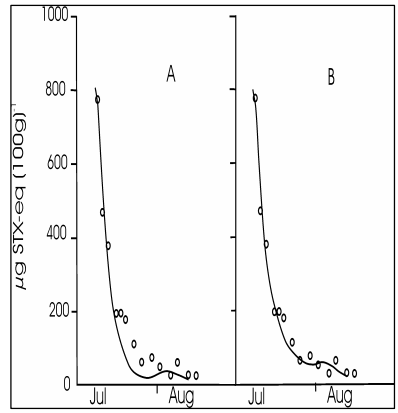

Figure 2.- Fitting of a 1.compartment and a 2-compartment model to the accumulation of PSP toxins (expressed as toxicity) in mussels (redrawn in part from Silvert and Cembella 1995)

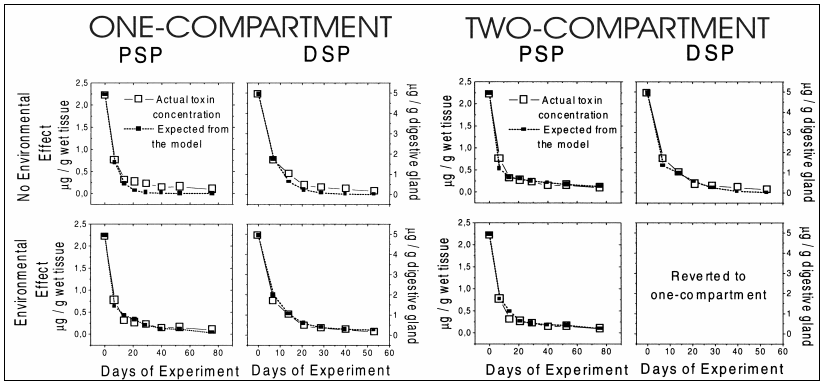

Figure 3. Fitting of one- and two- compartment models to PSP and DSP concentration in mussels (whole soft tissue and digestive gland, respectively) considering and not considering and effect of environmental conditions (salinity, temperature, seston concentration and phytoplankton concentration) and body weight, on the depuration rate (from Fernández *et al.*, 1998)

Multi-compartment models

Two compartment models are usually enough to model depuration with good precision. Notwithstanding, some species have organs or body fractions with special characteristics because of their commercial value, as is the case of the adductor muscle

and the gonad of the scallop Pecten maximus or because they retain strongly some toxins, as is the case with the digestive gland of P. maximus retaining domoic acid, or foot and siphon of the langostillo Acanthocardia turberculata, retaining PSP toxins. . To adequately manage the toxic populations of those species, more

complex –multi-compartmental- models are needed, because the depuration kinetics of each organ or main body fraction has to be understood.
This kind of models have been used to try to describe or predict the depuration/ accumulation of toxins from/in the organisms, but also to try to identify the functions of different organs in relation to toxin depuration or the processes involved in the anatomical redistribution of some toxins.

The implementations have been made in a way similar to that used for two-compartment models, using first order reactions to describe transfers between organs/ fractions and depuration of toxins from each of them, both, in the case of domoic acid in scallop *Pecten maximus* (Blanco *et al* 2002), and PSP toxins in the same species. In the case of PSP toxins three different conceptual models were translated to mathematical models the first one assuming that all organs are able to depurate the toxins, the second one assuming that only digestive gland and kidney are able to do that, and finally, a third one built to test if toxins can be directly lost with the biomass of the gonad during spawning (Figure 4).

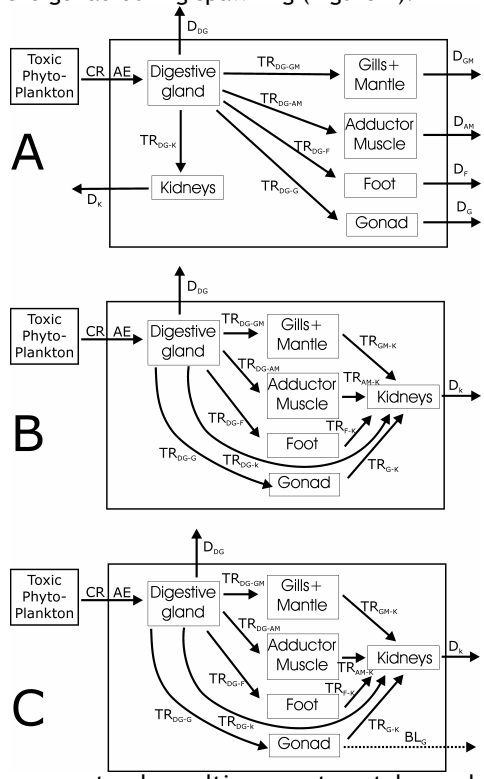

Figure 4. Three conceptural multi-compartmental models of PSP toxins accumulation in scallops. A, B anc C correspond to models 1, 2 and 3 in Figure 5.

The fit of the first and second models were very similar (Figure 5), but, taking into account that it is difficult to figure an efficient depuration mechanism from gonad, foot or adductor muscle, it seems clear that depuration takes place mainly through digestive gland and kidneys, making clear the actual cause of the high PSP toxin concentrations found in many cases in the scallop kidneys (Lassus *et al.*, 1989, 1992).

The third model was the same than the second one with the only difference that a toxin loss equivalent to the loss of gonadal mass was included. The fit was substantially improved strongly (Figure 5) suggesting that the implemented mechanism is acting. Nevertheless, in the first model of anatomical compartmentalisation of domoic acid in the same species (Blanco *et al.*, 2002), no conclusion was attained as no transfer seem to exist, in sight of the estimates obtained by fitting the model.

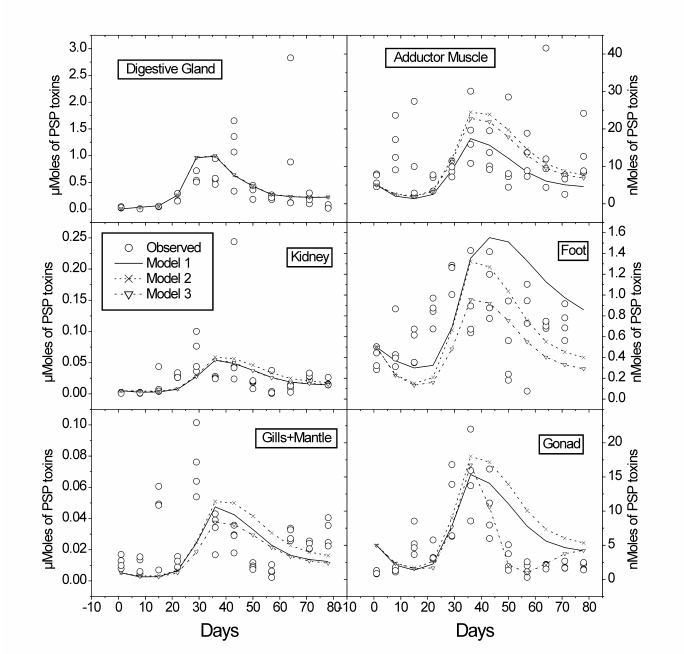

Figure 5. Fitting of three models, described in Figure 4, to the content in PSP toxins of several body fractions of the king scallop *Pecten maximus*.

Models of toxin incorporation

The incorporation of toxins into bivalves is usually modelled by assuming both, constant rates of toxic cells ingestion and constant cellular toxin contents. Usually under the mathematical form (Silvert and Subba Rao 1992; Silvert and Cembella, 1995):

$$dTox/dt = \text{Feeding rate}[cells]_{water} \cdot [toxins]_{cell}$$

in which Tox= Toxin amount; Feeding rate= the rate at which the bivalves withdraw particles from water; $[toxins]_{cell}$= cellular toxin contents; and $[cells]_{water}$= the concentration of cells in water that is usually variable with time. When the toxin amount that can be accumulated in the gut is considered to be negligible and there is only interest on the toxins that pass the gut walls, and additional factor to correct for the non-absorbed toxin can be added, as it is usually made with organic matter, (the absorption efficiency) (Blanco *et al* 1995; Moroño et at 1998b). Absorption efficiency is usually assumed to be constant even when in the case of organic matter it has been found to depend on the food quality (which determines the gut passage time) (Hawkins *et al* 1990), and also on an equivalent variable (toxin per unit of volume of seston) in the case of PSP toxins (Moroño *et al*. 2001).

This approach of modelling toxin incorporation give, in general, good results but is has some limitations which are important in some cases. The first limitation is a practical one: it is very difficult to correctly sample phytoplankton populations because they frequently are strongly heterogeneous both, in space and time. As an example, Silvert and Cembella (1995) found two typical situations while modelling PSP accumulation in mussels, derived from not detecting toxic phytoplankton and from assuming that the populations detected at a precise time are representative of a time span longer than they actually are (Figure 6).

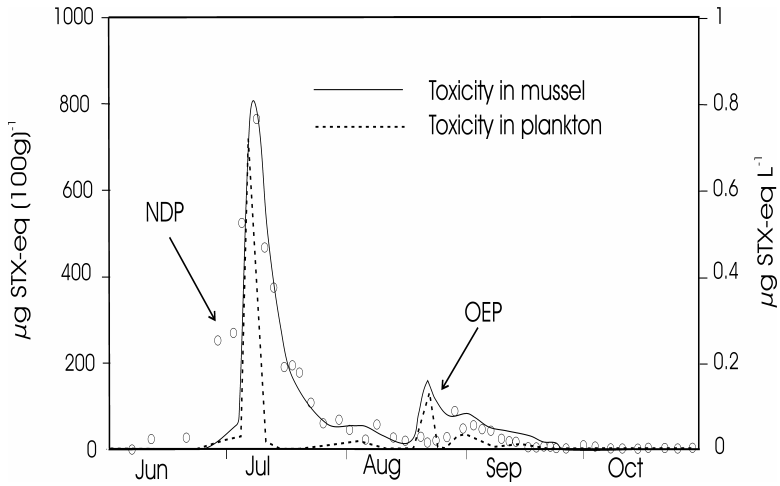

Figure 6. PSP toxicity in mussel digestive gland and suspended in seawater (mostly in plankton), as well as the output of a 2-compartment model of toxicity accumulation. Initial anomalous levels of toxicity in mussels (NDP) was probably due to non detected toxic planlton. A clear overestimation of mussel toxicity by the model (OEP) was due to a high estimation of plankton toxicity for that particular period of time (Redrawn from Silvert and Cembella 1995)

Toxin content/toxicity per toxic phytoplankton cell is also variable in many cases (Moroño *et al* 1998b), and the same is true for filtration/ clearance rate, which can be affected by the toxic population because the specific characteristics of the cells (see review in Blanco-Pérez 2001), because the amount/type of suspended matter (Bayne *et al*. 1987, Iglesias *et al*. 1992, Bayne *et al*. 1993, Navarro and Widdows 1997), or even because the environmental conditions associated to the toxic phytoplankton blooms. Silvert and Subba Rao (1992), as an example, suggested that the alteration of the filtration rate might have been the cause of the impossibility of fitting correctly one model to two consecutive *Pseudo-nitzschia* (domoic acid producer) blooms. Douglas *et al.*, (1997) were also unable to fit a model of accumulation of domoic acid in scallops without assuming that the filtration rate was substantially reduced during the first six days of feeding. Moroño *et al* (unpublished data) could only fit a model of PSP toxins accumulation in mussels after assuming an exceptionally low clearance rate, probably associated to the reduced salinity water in which the causative species *Alexandrium minutum* populations develop. Additional effort is needed to be able to predict correctly the incorporation of toxin if we are interested in obtaining good predictions of the attainable toxicity of the episodes.

Models of transformations between toxins

It is clear that the most important processes to be considered when modelling toxin accumulation are those which involve toxin gain and/or loss from a particular organism or body fraction, as the ones we have just dealt with. Notwithstanding, due to the metabolic activities of the organisms that accumulate the toxins, and also to the different physical and chemical environment inside those organism with relation to the producers, toxins undergo transformations. Toxin groups are usually made of different compounds that share a common base structure but differ in the spatial disposition of some radicals, or in the kind and number of the radicals. These chemical differences usually make the toxins to have different toxic power. Therefore, transformations become specially important because of two reasons: they produce changes in toxicity without any change in the toxin content (in molar basis), and they make impossible to trace the accumulation (or any process involved in it, as depuration for example) of a particular form when it can be inter-converted with other forms of the same group of toxins.

Several transformations have been modelled to date. In the case of PSP toxins (Figure 7) epimerization, reductions and decarbamoylation were modelled during the accumulation and depuration by the clam *Spisula solidissima* (Silvert *et al*. 1998), and the two first processes in mussels *Mytilus galloprovincialis* (Blanco *et al*. 2004), in both species, after the ingestion of *Alexandrium* cells. Some transformations were considered to be uni-directional (reductions and decarbamoylations) and the other (epimerization) bi-directional, but all of them were assumed to be first order reactions. In general, the two models can be described as:

$$
\begin{aligned}
dGTX_1/dt &= -K\cdot GTX_1 + E_{4\text{-}1}\cdot GTX_4 - E_{1\text{-}4}\cdot GTX_1 - R_{1\text{-}2}\cdot GTX_1 - DC_1\, GTX_1 \\
dGTX_2/dt &= -K\cdot GTX_2 + E_{3\text{-}2}\cdot GTX_3 - E_{2\text{-}3}\cdot GTX_2 + R_{1\text{-}2}\cdot GTX_1 - DC_2\, GTX_2 \\
dGTX_3/dt &= -K\cdot GTX_3 + E_{2\text{-}3}\cdot GTX_2 - E_{3\text{-}2}\cdot GTX_3 + R_{4\text{-}3}\cdot GTX_2 - DC_3\, GTX_3 \\
dGTX_4/dt &= -K\cdot GTX_4 + E_{1\text{-}4}\cdot GTX_1 - E_{4\text{-}1}\cdot GTX_4 - R_{4\text{-}3}\cdot GTX_2 - DC_4\, GTX_4 \\
dDcGTX_1/dt &= -K\cdot DcGTX_1 + DC_1\, GTX_1 \\
dDcGTX_2/dt &= -K\cdot DcGTX_2 + DC_2\, GTX_2 \\
dDcGTX_3/dt &= -K\cdot DcGTX_3 + DC_3\, GTX_3 \\
dDcGTX_4/dt &= -K\cdot DcGTX_4 + DC_4\, GTX_4
\end{aligned}
$$

In which, the equations that defined the basic depuration model were complemented with the different transformations that were assumed to be proportional to the amount of the toxin that undergoes them. Epimerizations were described by $E_{n\text{-}m}\cdot GTX_n$, reductions by $R_{n\text{-}m}\cdot GTX_n$, and decarbamoylations by $DC_n\, GTX_n$, were $E_{n\text{-}m}$ are the rates of epimerization between the toxins indicated in the sub-index, $R_{n\text{-}m}$, the reduction rates and DC_n. the decarbamoylation rates.

The models fit well the observed data with the exception of the a-epimers (GTX1-2) (Figure 8) which seem to be overestimated in the central portion of the incorporation. It seems that this strong trend in the case of GTX2 in *Spisula solidissima* led to Silvert *et al.,* (1998) to suggest the possibility that GTX2 were more strongly retained than other toxins of the same group. In the experiment with *M. galloprovincialis* (Figure 9), notwithstanding, the fit did not improve by allowing different depuration rates for the different toxins involved, and the estimated rates were very similar to each other (Blanco *et al.,* 2004). Also in this latter work, a Michaelis-Menten kinetics was tried, but the resulting fit was worse that that obtained using first order reactions. This inadequacy of the new model suggest that enzymes do not play an important role in the transformation of PSP toxins.

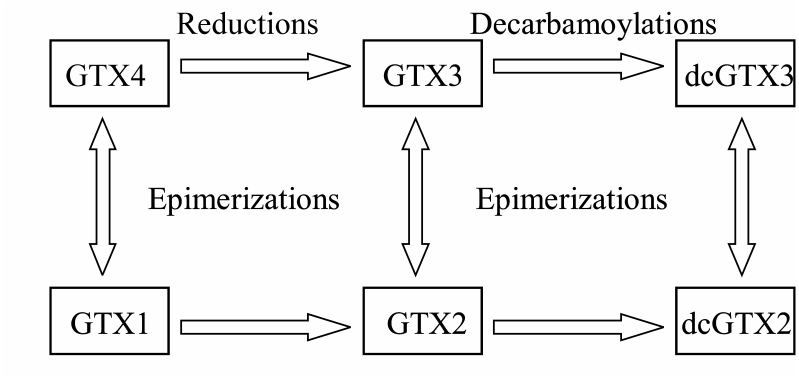

Figure 7. Some of the transformations of PSP toxins that have been modeled.

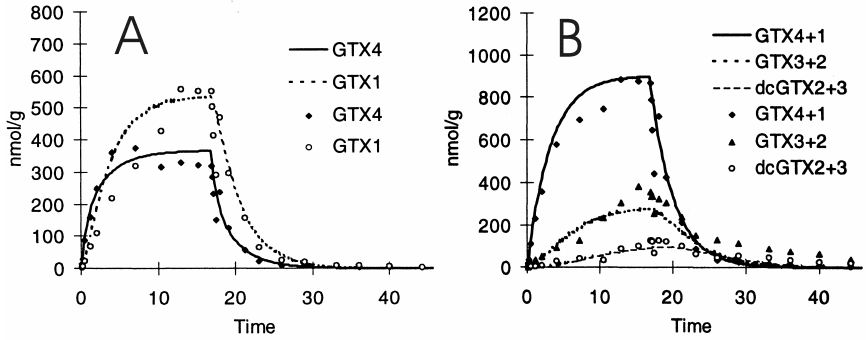

Figure 8. Fit of an accumulation model including transformations between PSP toxins in the clam *Spisula solidissima*, showing the influence of epimerization (A) and of reduction and decarbamoylation (B). (From Silvert *et al* 1998)

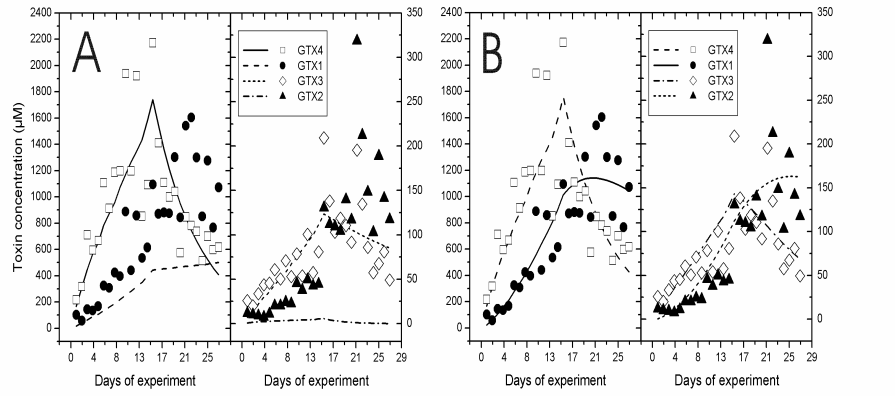

Figure 9. Fit of two models of accumulation of PSP toxins in mussels. The model represented in A assumed that the toxins depurate at different velocities, while the one in B assumes a common depuration rate but the existence of epimerizations and reductions (from Blanco *et al* 2004).

Transformations of DSP toxins have also been modelled. The first attempts tried to model the depuration kinetics of okadaic acid and DTX2, including the expected acylation of okadaic acid in bivalves to produce low polarity derivatives generically known as DTX3 (Fernández *et al* 1998). Again first order reactions were used to describe all processes. A two compartment model was needed. The model fit well the data in the study but some striking features were found (Figure 10). The estimated depuration rate of the low polarity derivatives was very high and the estimated acylation rates were very low, thus raising doubts about the real origin of the low polarity derivatives and consequently, about the mechanisms involved in the change. It is obvious that the low polarity derivatives at the beginning of the experiment were not produced by the mussel but ingested with plankton and quickly hydrolysed to their free acid forms. The high apparent depuration rate derived from the fact that two processes were involved, the toxin elimination from mussels and the hydrolysis to the free acid form. When a new experiment was modelled (Figure 11), including the acquisition of low polarity forms from plankton and their corresponding hydrolysis (Moroño *et al*. 2003), the high depuration rate was only partially corrected but some evidences of undetected compounds still persisted, showing the capability of models to reveal unknown processes or toxins pools involved in the accumulation or depuration of toxins.

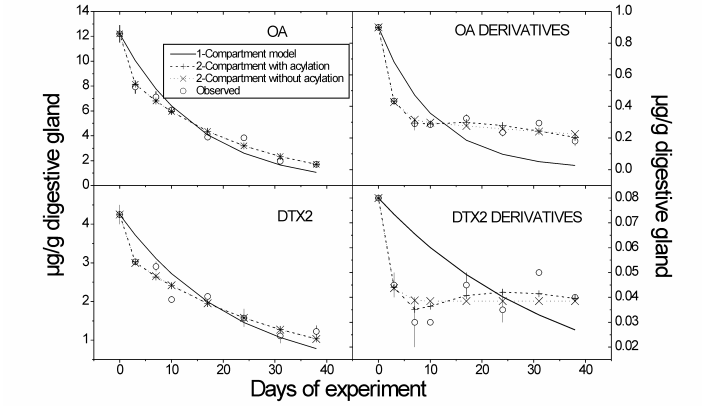

Figure 10.- Fit a 1-compartment model, and two 2-compartment models: one incuding acylation of okadaic acid (OA) and DTX2, to the observed concentrations of the free acid forms of the toxins and their correspondent low polarity derivatives. The high slope in the decay of the low polarity derivatives is most likely due to the hydrolysis of ingested low polarity derivatives (from Fernández *et al* 1998).

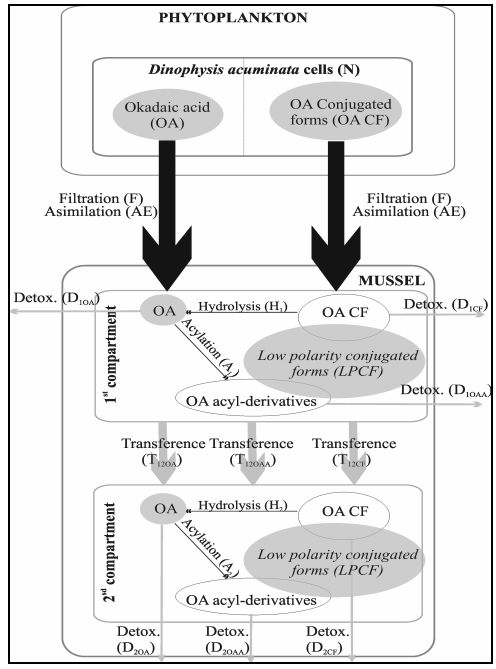

Figure 11. Conceptual model of DSP toxins accumulation, including ingestion and hydrolysis of low polarity derivatives (redrawn from Moroño *et al* 2003)

Some benefits of using models

From the previous sections it is clear how models are useful to describe the complex accumulation, ingestion, elimination, transfer to different organs and transformations, by means of a few parameters which, in most cases are reliable enough to allow comparisons between different species or situations. It was also shown how the rates of some processes difficult to measure, as the transfer between anatomical compartments, can estimated, and how the lack of fit of some models can contribute to identify new processes or toxin pools involved in toxin accumulation, as well as to test the actual knowledge about toxin accumulation.

Additionally to these uses, models can be useful to evaluate the relative importance of the parameters and processes which they include on different responses of the system. This can be carried out by means of the analysis of sensitivity and it allows to focus the research on the most relevant aspects of the toxin accumulation for a special goal. Moroño (2000) for example examined, by means of this kind of analysis, the possible effects of the parameters of a model of accumulation PSP toxicity in mussels, on two key aspects of the response: the maximum accumulated toxicity and the time needed to depurate the toxicity to the legal limit threshold (Figure 12). It made clear that, in the case that the main interest was the attained toxicity, the research should focus on the depuration of compartment 1, but if the interest is the depuration time, then the focus should be placed on the depuration of compartment 2.

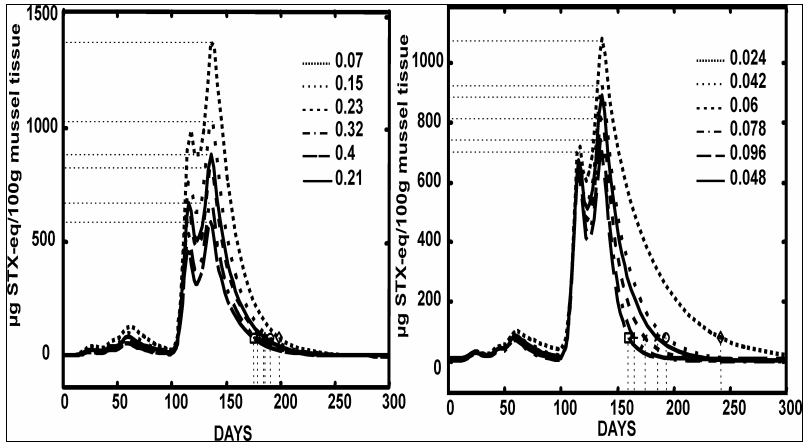

Figure 12. Some aspects of the sensitivity analysis of a 2-compartment model of PSP toxicity accumulation (redrawn from Moroño 2000).

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REDUCING THE TOXICITY OF HARMFUL ALGAE BY OZONE

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Abstract
A sample of harmful micro-algae, namely *Alexandrium tamarense* (strain ATCI03), was treated by continuous injection of ozone in laboratory. It was shown that ozone had effectively killed the cells of *Alexandrium tamarense* within 20 minutes. Moreover, the toxicity of *Alexandrium tamarense*, as indicated by the residual level of C2 toxins in cells and medium, reduced by 36% at the 20th minute of experiment. As a side effect of the depuration process, the levels of ammoniacal nitrogen (NH4-N) and total inorganic nitrogen (TIN) in cultivating media also decreased remarkably. Ozone is demonstrated an effective agent in mitigating harmful algal blooms.

Introduction
Harmful algal bloom (HAB) has raised increasing concern in the world. It results in discoloration of seawater and massive fishkill. The economic loss of HAB might be extremely high. To reduce the harms, numerous scientific researches on mitigation of HAB including the injection of ozone have been done (Ho, 1998; Ho et. al., 2001; Botes et. al., 2003). This paper discussed the effectiveness of reducing the toxicity of harmful algae by ozone.

Materials and Methods
A strain of Alexandrium tamarense i.e. strain ATCI03 was chosen for ozonation experiments. This strain was known produced mainly the C2 toxin that caused paralytic shellfish poisoning as well as ciguatera. During the experiments, ozone was injected into the culture medium that contained 3 liter of *Alexandrium tamarense*. Moreover, ozone was injected at a rate of 1 gm-O3 per cubic meter of seawater with the experimental duration being 20-40 minutes. The concentrations of living/burst algal cells in the culture medium were measured by taking samples at every 2-3 minutes. The cells of *Alexandrium tamarense* were counted under inverted microscopes with the use of Sedgewick-Rafter Chambers. For toxicity tests, the algal toxins were extracted from the cells by 50mM acetic acid. Then the cells were frozen in –80^{o}C and thawed under room temperature three times. After sonication on ice thirty times with the duration of 0.5 second each, the cells were centrifuged for 15 minutes under 13000 rpm. The extracts from the cells, together with the supernatant of medium after centrifugation were analyzed for concentration of C2 toxins by HPLC-FLD.

The changes of total inorganic nitrogen (TIN) in culture medium were tested according to standard method (APAH, 1992). The changes of dissolved oxygen in culture medium were measured by a dissolved-oxygen meter.

Results and Discussion

Measurement of inactive and dead algal cells

As shown in Fig.1, during O3 treatment, algal cells died and burst very quickly. There was no living (active moving) cell detected under microscope after 10 minutes of ozone treatment. Furthermore, only a few cells died but kept intact after 20 minutes of ozonation. The number of dead (inactive) cells increased greatly during the first 2-6 minutes.

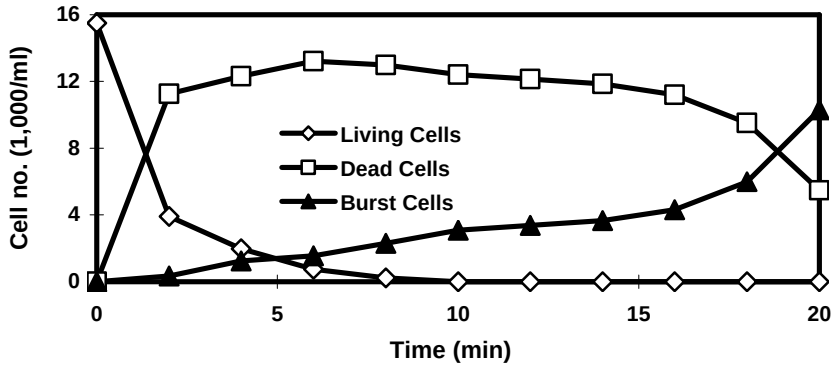

Figure.1 Changes of algal cell number during ozone treatment

Measurement of C2 toxin concentration

As shown in Fig. 2, C2 toxin concentration in sampled algal cells decreased greatly after 6 minutes of ozone treatment. Such decrease in C2 in algal cells was simultaneous with increase in C2 toxin in culture medium. This phenomenon indicated that burst cells released the C2 toxin into the medium after ozone treatment. We observed that the total concentration of C2 toxin in cultivating medium decreased by 36% at the 20th minutes of experiment.

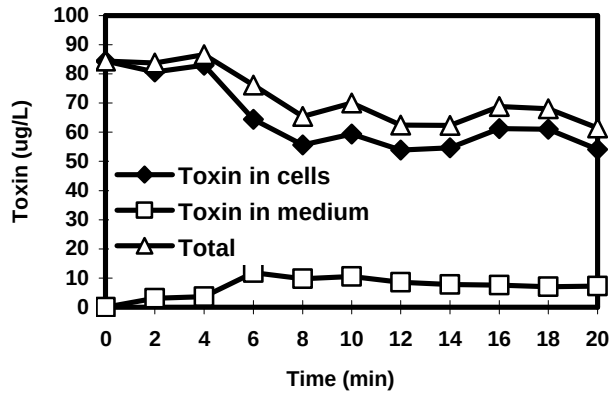

Figure 2. Changes of C2 toxin in algal culture due to ozone treatment

Changes in Dissolved Oxygen (DO) Levels

As shown in Fig.3, the dissolved oxygen concentration in cultivating medium began to increase after 4 minutes of ozone treatment. It increased significantly from 7th minutes to 40th minutes. When ozone injection was stopped after 40th minutes, dissolved oxygen dropped greatly but maintained at a steady level afterward. In this period, metabolic activities of aerobic bacteria in medium were assumed active that consumed oxygen dissolved in medium. Results coincided with those of past researches (Ho, 1998; Ho et. al., 2001; Botes et. al., 2003) that demonstrated ozone was effective in purifying water quality by raising the level of dissolved oxygen. Ozone is very unstable in seawater. Most of them may change into dissolved oxygen quickly. Therefore, we believed that with the increase in dissolved oxygen level in seawater, fishes could be saved from anoxia that is a common adverse impact of red tide (harmful algal bloom).

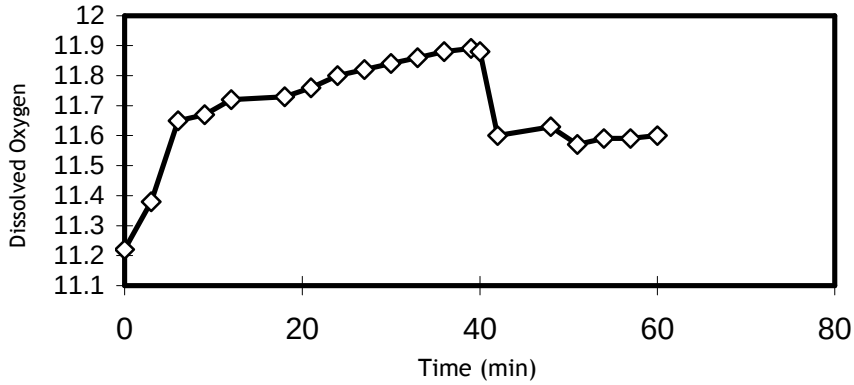

Figure 3. Changes of dissolved oxygen during experiment

Changes in Total Inorganic Nitrogen (TIN) concentrations

Figures 4a & 4b showed that there were steady but remarkably decreases of TIN and ammonical nitrogen in medium after ozonation. Results demonstrated that a side effect of ozonation was that residual oxygen increase could improve water quality by increased bacterial metabolism for degradation of organic components e.g. total inorganic nitrogen and ammonium in seawater.

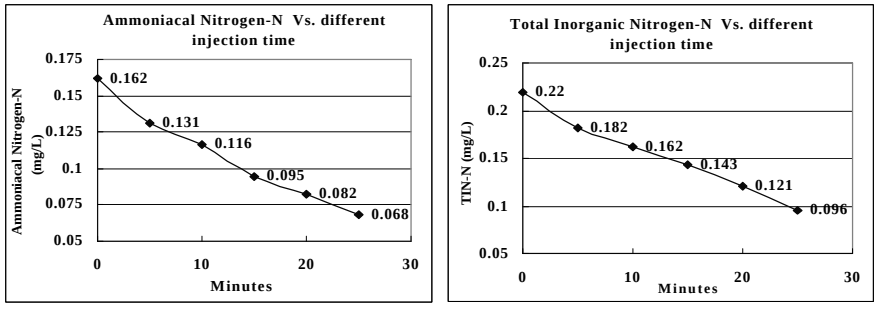

Figure 4. Changes in the levels of (a) NH_4-N and (b) TIN after ozonation

Conclusion

Experimental results showed that ozone was an effective agent in reducing the C2 toxins produced by *Alexandrium tamarense* (strain ATC103). The relevant ozone injection process also helped to reduce problems of eutrophication.

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DECONTAMINATION RATES OF PSP TOXINS IN MUSSEL (*Mytilus galloprovincialis*) AND OYSTER *(Crassostrea gigas*)

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Abstract
During PSP outbreaks which occurred along Moroccan shores, decontamination processes of different bivalve molluscs species monitored showed that mussels (*Mytilus galloprovicialis*) eliminated PSP toxins in three phases. In the first phase, 60 % of the PSP toxin content were quickly depurated after five day stage nick, corresponded to the evacuation of non assimilated toxins retained in digestive glands. The relatively slow second phase allowed the loss of 40 % of PSP toxin content after 40 days, corresponded to the elimination of assimilated toxins in different organs. The last phase could last over 40 days and allowed the elimination of the remaining 10 % of the initial quantity of PSP toxins, which were specifically retained in different tissues. The decontamination rate in oysters (*Crassostrea gigas*) collected from Oualidia lagoon was biphasic. 80 % of PSP toxins were depurated in only 5 days and the 20 % remaining were eliminated in 50 more days.

Introduction
The frequent occurrences of toxic events along the coastal waters of Morocco have led in 1992 the implementation of a monitoring network of harmful algae and phycotoxins, composed of 7 stations covering the Moroccan shore (Figure 1). Since then, most PSP toxic episodes were monitored in many areas with a high frequency (2 or 3 times per week). In 1994, the Atlantic coast of Morocco was slighted to enormous bloom of *Gymnodinium catenatum* which even reached the Iberian coast (Taleb *et al.,* 1995; 1998; 2003). As never seen before, very high PSP toxin levels (6000 µg STX eq./100g flesh) were recorded in shellfish, causing officially death of four persons. The appearance of PSP toxic events along the Mediterranean coast of Morocco is quite frequent and occurred periodically every year in spring and winter (Taleb, 1997; Taleb *et al*., 1998 ; 2001 ; Maman *et al.,* 2000). Reliable information regarding toxicity of shellfish was obtained from the outcomes of this regular surveillance of PSP outbreaks. The aim of the present paper consist to describe the decontamination process and determine the elimination rates of PSP toxins components of some bivalve molluscs species during the toxic events.

Material and Method
Mouse bioassay
Mytilus galloprovincialis Crassostrea gigas from samples were obtained as an integrant part of the Moroccan monitoring programme from Casablanca, Atalaâyoun and Oualidia (Figure 1). Shellfish samples were extracted according to AOAC method (1990) for the mouse bioassay. 100 g of homogenised tissues were mixed with 100 mL chlorydric acid 0,1 M and boiled for 5 min. After centrifugation (5 min, 3000 rpm), the pH was adjusted to 2-3 with chlorydric acid 5N or sodium hydroxid 5N. One mL of the supernatant was injected intraperitoneally into three 20 g albinos mice. Samples were diluted with distilled water when median survival time was below 5 minutes (AOAC, 1990). A conversion factor of 0.196 was applied using STX from National Research Council of Canada (NRC).

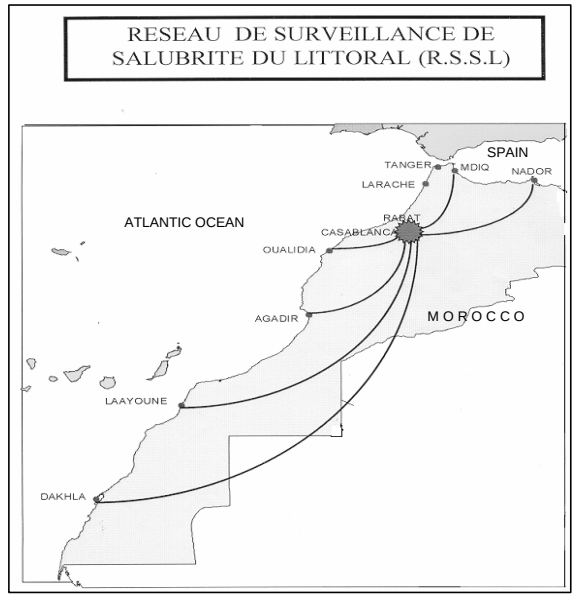

Figure 1. Monitoring network and localisation of sampling sites

Sample preparation for HPLC
Toxin profile analyses were conducted by HPLC/FD (Fluometric detection) on supernatants already assayed by MBA, using automated pre-column oxidation based on the method of Lawrence *et al* 1995 with minor modifications described in Vale and Taleb (2005). Supernatants already assayed by MBA were re-centrifuged in eppenddorf tubes (1000 µL) at 12,000 rpm. Extracts were filtered into screw-cap autosampler vials (1.5 mL) using a 0.22 µm nylon (∅ 11 mm) disposable syringe filter. Routinely, the equivalent of 1.25 mg extract (2.5 µL) was oxidised and injected on the column without clean up.

LC separation and detection
Liquid chromatography was performed on a Hewlett-Packard (HP) equipment consisting of: Model 1050 quaternary pump and autosampler, Model 1046-A fluorescence detector and Model 1100 in-line degasser. The HP Chemstation software performed data acquisition and peak integration. Toxins were separated on a 125 mm x 3 mm id column packed with 5 µm *Nucleosil* 100-5 C18 (Macherey-Nagel, Duren, Germany), and equipped with a 4 x 4 mm id guard column packed with 5 µm *Lichrospher* 100 RP-18 (Merck, Darmstadt, Germany). Column temperature was kept at 30 ºC. Detection wavelengths were set at 330 nm for excitation and 390 nm for emission, and gain set at 2^{14}.

Toxin determination
The set of four ampoules, PSP-1B, and recently the CRM-dcSTX, were purchased from National Research Council of Canada (NRC, 1997; 2002). All standard solutions were diluted with distilled water as required. Calculations were based on peak height due the incomplete separation of all toxins by the short gradient used. For routine determination a single-point calibration was run at the beginning and after each six consecutive samples with a working solution prepared by mixing equal volumes of dcSTX, GTX2+3, STX and toxin-free oyster extract, to reach a final concentration of: 0.46, 0.67 and 0.63 µg/mL, respectively. Recently, a calibration

curve was run routinely, consisting of 1/7 and 1/3 dilutions of the working solution above.

For toxicity calculation, each toxin concentration obtained was multiplied by the respective relative toxicity towards STX (reported by Oshima, 1995; updated in NRC, 2002). As pre-column oxidation methodology overlaps some toxins with different specific toxicities, we averaged some responses as follows: dcGTX2+3 0.38 x STX; C1+2 0.05; dcSTX 0.51; GTX1/4 0.68; B1 0.06; STX/Neo 1.00. Total toxicity obtained was multiplied by a factor of 1.16 to match with mouse bioassay data calibrated against the US-FDA saxitoxin dihydrochloride solution (NRC, 2002).

Results

The first PSP toxicity screening was conducted in the mussels collected near Casablanca in November 1994. This site was marked by the maximum PSP toxin level recorded in mussel (about 6000 µg STXéqui./100g flesh determined by mouse bioassay). The detoxification curve for the mussels was composed of three phases (Figure 2a). During the first phase 60 % of PSP toxins were evacuated in five days. The second phase was characterised by a less pronounced slope, involving the elimination of only 30 % in 40 days. Finally, the third phase required 40 additional days to complete the depuration process.

Durant the same period, similar PSP toxin screening was conducted with mussels and oysters collected in the Oualidia lagoon. The maximum PSP toxin level recorded in mussels at this site was 2600 µg STXeq./100g flesh. In this case too the PSP toxin elimination involved a triphasic pattern: fast elimination of 60 % of the toxicity content in 5 days, slower phase allowing the depuration of 30 % in 35 days and slow step elimination of the remaining 10 % in 50 days.

The PSP toxin decontamination mode in oysters collected from in Oualidia Lagoon involved two phases to eliminate 522 µg STXequi./100g flesh (Figure 2b). The first stage allowed the elimination of about 85 % at five days and the second phase required 40 days to complete the depuration process.

Furthermore, the close screening of *Alexandrium spp.* bloom raging in winter 2004 at Nador lagoon (Figure 1), leads carrying out the mussel decontamination mode as well as the variation of PSP toxin profile at the different stages. The reached PSP level by mussel at this time was just 840 µg STX equi/100g flesh. As well, the obtained results in this case confirm the triphasic mode of mussels PSP elimination process (Figure 2c) but the duration of the total evacuation of PSP toxins content is reduced, only 65 days.

On the other hand, the PSP toxin composition investigated in Nador mussels (Figure 1), is mainly composed of gonyautoxins (GTX1-4) and a mixture of saxitoxins (STX) and neosaxitoxin (neoSTX). The temporal evolution of those compounds during the decontamination process shows a significant variation and a triphasic tendency curve as well (Figure 3). The first phase is marked by the gut evacuation of both compounds with daily lost of 9 % per day. At the second phase, GTXs continue to decrease slowly with rates equivalent to 0,7 % per day, whereas the elimination of STX/NeoSTX mixture is not regular, as it decrease and increase with mean rate of 0,1 % per day. At the third phase, the GTXs are eliminated, whereas the proportion of STX remains more or less constant. The mean values show that GTXs were eliminated at a faster rate than STX/Neo (4,85 and 2,9 % per day respectively).

Table 1. Décontamination rates (% of lost PSP toxins per day)

	Mussels			Oysters	Nador lagoon mussels 2004	
	Casablanca 1994	Oualidia 1994	Nador 2004		GTXs	STX/ NeoSTX
1st phase	12	12	10	17	9	8,5
2nd phase	0,75	0,9	1	0,4	0,7	0,1
3rd phase	0,25	0,2	0,33	-	-	0,1

The table 1 illustrates the evaluated decontamination kinetics of studied bivalve molluscs species during the PSP toxic episodes. At the first phase the decontamination rates of mussel varied between 10 and 12 % per day, independently of the accumulated PSP toxin level. But, for oyster this phase is much faster than for mussel, 17 % per day. However, at the second phase, the decontamination rates of mussel and oyster are relatively lowers, between 0,75 and 1 % per day for mussel against only 0,4 % per for oyster. The third phase just for mussel is so much lower; only 0,2 to 0,33 % per day. Following the mean values, oyster presents faster decontamination rate; almost 9 % per day against 4 % per day for mussel.

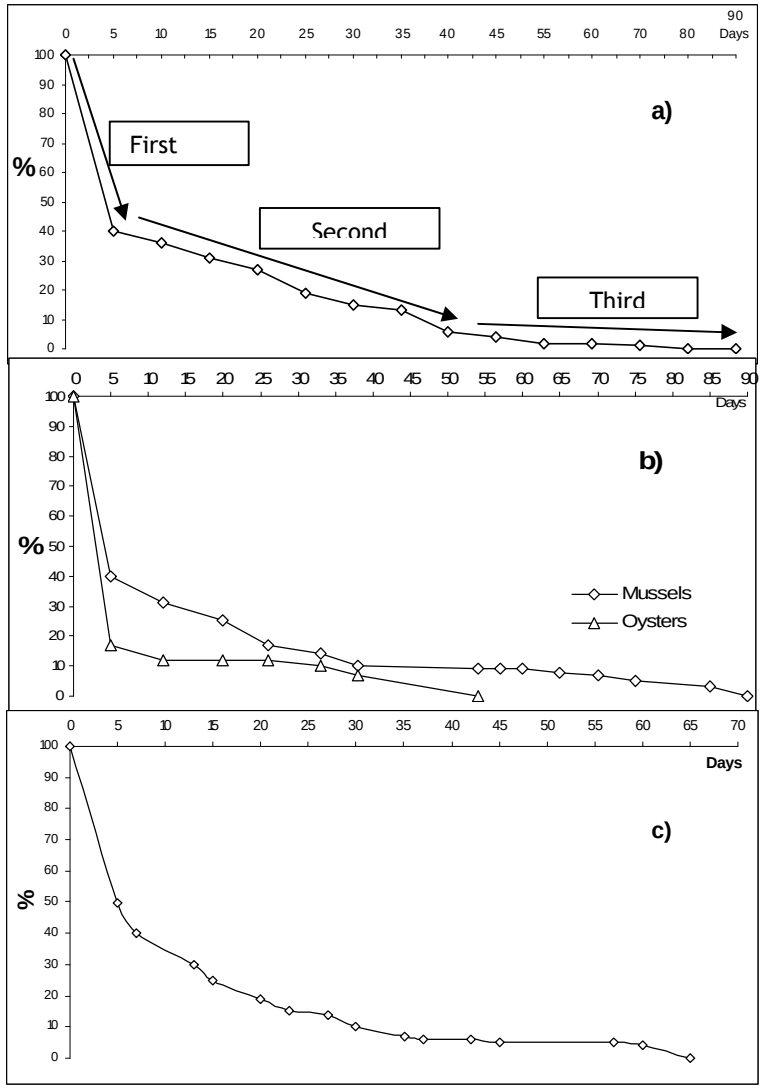

Figure 2. Elimination of PSPtoxins in a) mussels from Casablanca in november 1994 b) mussels and oysters from Oualidia lagoon in november 1994 and c) mussels from Nador lagoon in 2004

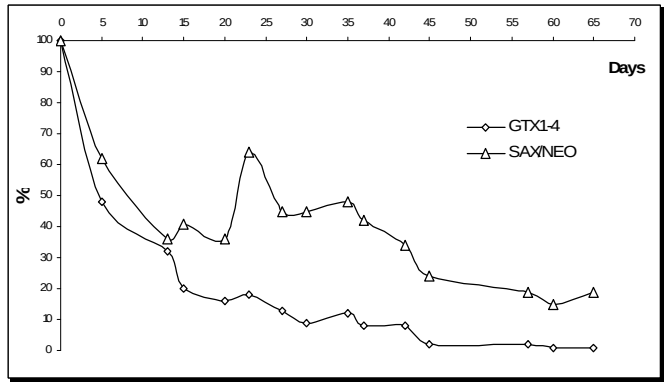

Figure 3. Variation of toxin profile during the decontamination process of mussels from Nador lagoon in 2004

Discussion

At the end of the present study it appears that the decontamination process is differential for studied bivalve molluscs species. The oysters eliminate PSP toxins more quickly than mussels. Moreover, the mussel detoxification process pattern involves three phases: one first rapid phase allowing the elimination of a great amount of PSP toxins for a few days, followed by a subsequent lowers second and third phases leading just lost of a few PSP toxin amount at longer period. The same mode has been observed for all studied mussel specimens independently of maximum PSP toxin reached. In short, the same total process length (three months) were necessary for the complete PSP toxins elimination in Oualidia and Casablanca mussels, despite the great difference of PSP toxins accumulated in both cases. Experiments conducted in mussel (*M edulis*) by Lassus *et al.* (1993) suggested the dependence of decontamination length at the initial toxicity. However, this relationship is not apparent in our case.

In contrast, the oysters decontamination process involves only two phases corresponding to the first and third phases observed in mussels.
The Nador mussels toxin profile is mainly dominated by GTX1-4 and STX/Neo mixture. The study of decontamination kinetic for individual toxins showed GTXs were eliminated at a faster rate than STX/Neo and the temporal evolution mussels toxin profile involves the same mode as mussel. The quick first phase leading the evacuation of both compounds with the same rates. The decrease of GTXs at the subsequent second phase, while STX/neoSTX increased. At the third phase GTXs continue to decrease, but STX/NeoSTX remains more or less constant.

As suggested, the rapidity of the first phase is likely due to the gut evacuation of unassimilated PSP toxins remaining in digestive gland as for GTXs and STX/neoSTX observed in Nador mussel. This fact was reported by several studies showing the prompt decontamination of digestive gland than remaining tissues (Bricelj *et al.*, 1991; Waiwood *et al.*, 1995; Bricelj and Cembella, 1995; Bricelj and Shumway, 1998). However, the second phase corresponds to the release of toxin assimilated and incorporated in different tissues, which are not specifically retained as GTXs in our case. The increase of STX/neoSTX proportion at the second phase is probably due to the biotransformation occurring inside the bivalve tissues which leads to the compensation of the loosed GTXs proportion. This was also observed by Lassus *et al.*, 1994. This fact could explain the slowing down of the second phase for mussel.

The earlier few studies on depuration kinetics suggesting the biphasic pattern of mussels decontamination have tackled the process just until the regulatory limit and didn't interest to the complete detoxification. This step beyond the safety threshold corresponds to our third phase which length is not negligible. This ultimate phase corresponds to the elimination of resistant PSP compounds (STX/neoSTX), that are likely specifically retained in mussels tissues (Blanco *et al.*, 2003 ; Bricelj and Shumway, 1998 ; Lassus *et al.*, 1989).

Acknowledgment
The HPLC analysis was run by Dr. Paulo Vale in Marine Biotoxin laboratory in Portugal.

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SOURCE SPECIES IDENTIFICATION OF *Escherichia coli* IN OYSTERS *Crassostrea virginica* AND OVERLYING WATERS

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Abstract
Contamination of shellfish with fecal-borne microorganisms is a public health concern throughout coastal New England (USA). Efforts to improve water quality are often limited because of lack of information on which contaminant sources are most significant. Ribotyping of *Escherichia coli* is a microbial source tracking method that has been used successfully in several studies in coastal New Hampshire. *E. coli* isolates were obtained from oysters and overlying waters and from potential source species in the Great Bay, New Hampshire watershed. Ribotyping was performed using a RiboPrinter®, regional and local source species databases, and restriction digestions using *Eco*RI or *Pvu*II. Using *Eco*R1 databases, livestock species (mostly cows) were the dominant source type for *E. coli* in oysters and water with human-borne isolates prominent at the site in prohibited waters. Minor differences between sources of *E. coli* probably reflected temporal differences in exposure to pollution events in oysters and water. Adding information from *Pvu*II digestions increased the number of successful source identifications for *E. coli* isolates. Although cost prohibitive for routine use, this approach provided greater insight into how shellfish are contaminated from multiple sources in the watershed.

Introduction
The contamination of shellfish with fecal-borne microorganisms is a public health concern throughout coastal New England (USA). Watersheds are subject to fecal contamination by a variety of sources, including pets, livestock, wild animals, birds and human sources like inadequately treated sewage effluent, septic systems, leaky sewage infrastructure and boats. Efforts to improve water quality are often limited because of lack of information on which contaminant sources are most significant. New Hampshire has small areas of wild oyster (*Crassostrea virginica*) resources harvested by recreational shellfishing and an emerging aquaculture industry that are threatened by poor water quality, and interest is keen to use best available technology to identify significant sources.

Numerous microbial source tracking (MST) methods have recently been developed to assess identify sources. Ribotyping of indicator bacteria is one method that has been used successfully in numerous areas in the US, including coastal areas of Maine and New Hampshire (Jones 2003, Jones *et al*. 2002). In many previous studies, digestion of DNA with a single restriction enzyme was successfully used to differentiate species-specific strains of targeted bacteria (Myoda *et al*. 2003, Hartel 2002, Hartel *et al*. 1999, Parveen *et al*. 1999). This approach can have limitations (Stewart *et al*. 2003), one of which is the occurrence of matching between the sample ribopattern and patterns from several different source species. Samadpour (2002) reported use of DNA digestion with two enzymes to lower the frequency of these "transient" strains that appear to be resident strains in multiple source species.

Microbial contaminants in shellfish accumulate and are depurated over time, depending on water quality conditions (Marino *et al*. 2003, Jones *et al*. 1991). The

classification of shellfish areas by the US National Shellfish Sanitation Program (NSSP) is based largely on bacterial levels in water, while the European Union uses a tissue contamination-based approach (Roughan 2004). Studies on the differences between these approaches relative to contamination source identification have not been reported, as most MST studies have focused on surface waters. The objective of this study was to explore differences in sources of *E. coli* between oysters and overlying water in approved and prohibited areas. This was done by first using a single DNA enzyme digestion then by using a different enzyme, with the information from both approaches used to maximise the identification of environmental isolates. An automated RiboPrinter® was used for ribotyping, but double enzyme digestions are not well suited to its use. Thus, successive enzyme digestions, instead of a single digestion with two enzymes, were necessary.

MATERIALS AND METHODS

The two sample sites were located in the Great Bay Estuary of New Hampshire, one in the Oyster River (OR) in a prohibited area and one near Nannie Island (NI) in approved shellfish harvesting waters (Figure 1). Oyster (20) and water samples were collected at the same time at each site, and within one hour at the two sites. Sampling occurred at low tide each month from May to October, 2003.

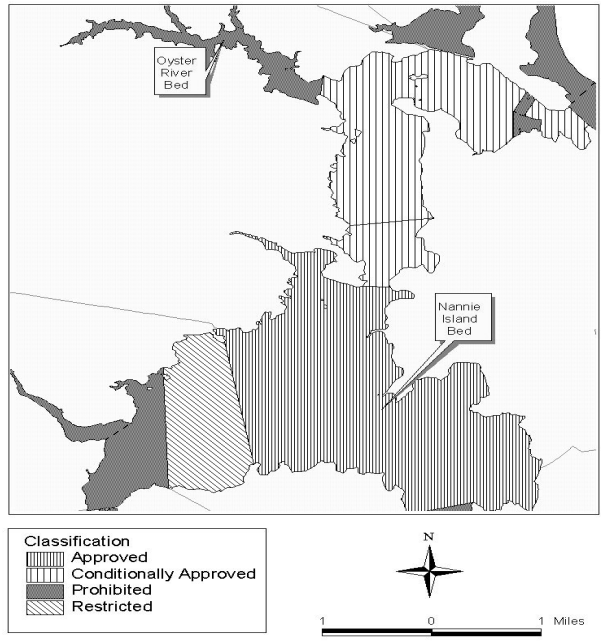

Figure 1. Oyster and water sampling sites in the Great Bay Estuary, New Hampshire, USA.

E. coli and fecal coliforms were enumerated from water samples using membrane filtration and mTEC media incubated at 44.5°C for 24 h, with confirmation of yellow/yellow-brown colonies as *E. coli* based on a negative urease test (USEPA 1986). *E. coli* in oysters were enumerated using multiple tube fermentation/MPN method. Oyster tissue was incubated in LT broth at 35°C for 24-48 h. Positive tube cultures were transferred to EC/MUG media and incubated for 24 h. *E. coli* strains

were isolated from positive tubes and plates and confirmed using biochemical tests (Jones and Bryant, 2002).

A RiboPrinter® was used to process *E. coli* isolate cultures for ribotype determinations. Between 1 and 10 isolates per sample were ribotyped, depending on *E.* coliconcentrations in samples. The automated RiboPrinter® process involved lysing cells and cutting the released DNA into fragments via the restriction enzyme *Eco*R1 or *Pvu*II. These fragments were separated by size through gel electrophoresis and then transferred to a membrane, where they were hybridised with a DNA probe and mixed with a chemiluminescent agent. The DNA probe targeted 5S, 16S and 23S ribosomal RNA genes. A digitizing camera captured the light emission as image data, from which the system extracted a RiboPrint® pattern. The images were transferred into GelComparII (Applied-Maths) analytical software. DNA bands in lanes containing the standard were labeled and entered into the memory for optimization of gel pattern images. The ribopattern data for each separate water sample isolate were then used for identification of source species. Three source species databases were used to analyze water sample ribopatterns (Table 1). The first was a local *Eco*R1 Great Bay database that included 231 ribopatterns from 16 source species, including septage and wastewater considered as "human" sources. The Regional *Eco*R1 database included 971 ribopatterns from 31 source species, and the *Pvu*II database from regional source species included 191 ribopatterns from 19 species. Ribopatterns from environmental samples were first analysed with the Great Bay database. Patterns for which a source species could not be identified were re-analysed with the Regional *Eco*R1 database. Isolates with patterns that remained unidentifiable and had 8 or 9 DNA band patterns were subject to digestion with the *Pvu*II restriction enzyme and the resulting patterns analysed using the *Pvu*II database.

Table 1. Number of *E. coli* isolates and source species in databases used for identifying source species from water and oyster tissue samples.

Source species type	Great Bay EcoR1		Regional EcoR1		Regional PvuII	
	Isolates	Species	Isolates	Species	Isolates	Species
Human	114	3	245	3	38	2
Pet	10	2	104	2	15	2
Bird	8	2	151	9	41	5
Wild animal	18	3	335	9	57	6
Livestock	81	6	136	8	40	4
TOTAL	231	16	971	31	191	19

Using GelComparII software, optimization was set at 1.56 % and band position tolerance was set at 1.00 %. Similarity indices were determined using Dice's coincidence index (Dice, 1945) and the distance among clusters calculated using cluster analysis. The source species profile with the best similarity coefficient was accepted as an indication of the possible source species for the water sample isolate. For this study, the predetermined threshold similarity index that was considered to be a minimum value for identifying source species was 90 %.

RESULTS

E. coli concentrations in water were consistent with the classifications at each site, but the levels in oyster tissue were similar and relatively low at both sites (Figure 2). The conditions during sampling were consistent with time in that no significant rain fell in the two days prior to the sampling date. Thus, temporal changes and spatial differences were probably not a result of storm water runoff-related sources.

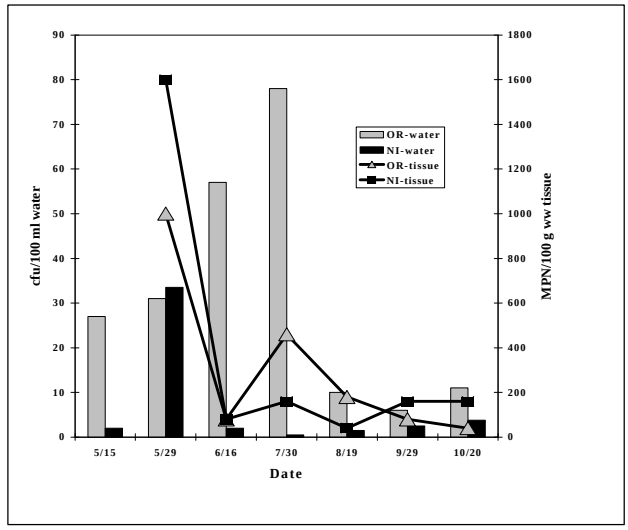

Figure 2. E. coli concentrations in oyster tissue and overlying water at 2 sites in New Hampshire, USA.

Ribotyping of 108 *E. coli* isolates included 57 from the OR site and 51 from NI, with relatively equal numbers divided between tissue and water samples. Livestock (mostly cows) were the most prevalent type of source species in both types of samples at both sites and birds were also common source types under all conditions (Table 2). Human sources were more commonly identified at the OR (prohibited) site, especially in oyster tissue. Other small differences in source species types were apparent between sites and sample type.

Table 2. Source species type identification using EcoR1 DNA digestion for water and oyster tissue samples from two sites in New Hampshire, USA.

Source sp. type	Total study		Overlying water		Oyster tissue	
	Isolates	%	Isolates	%	Isolates	%
			PROHIBITED AREA			
Human	10	18 %	3	12 %	7	22 %
Pet	2	4 %	1	4 %	1	3 %
Bird	9	16 %	4	16 %	5	16 %
Wild animal	2	4 %	2	8 %	0	0 %
Livestock	22	39 %	10	40 %	12	38 %
Unknown	12	21 %	5	20 %	7	22 %
			APPROVED AREA			
Human	4	8 %	2	7 %	2	9 %
Pet	1	2 %	0	0 %	1	5 %
Bird	6	12 %	4	14 %	2	9 %
Wild animal	4	8 %	3	10 %	1	5 %
Livestock	19	37 %	11	38 %	8	36 %
Unknown	17	33 %	9	31 %	8	36 %

Of the 48 isolates analysed using both *Eco*R1 and *Pvu*II data, statistical analysis suggested twelve new identifications, four instances in which the source species type did not change, four for which the source species changed for an isolate previously identified and 28 isolates for which no source species could be identified. This resulted in a greater degree of source species identification (fewer unidentified sources), an increase in bird sources and small increases in the other source types compared to using one restriction enzyme (*Eco*R1) alone (Figure 3).

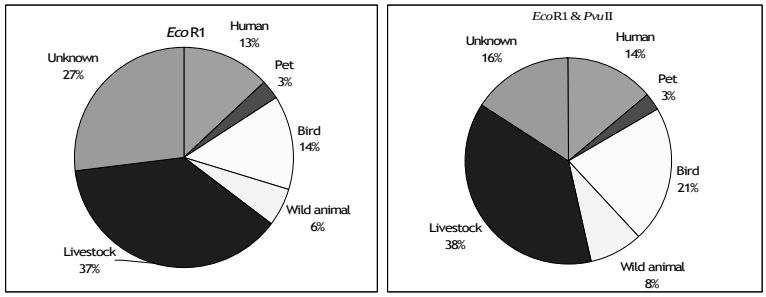

Figure 3. Source species types identified by ribotyping using one (EcoR1) or two (EcoR1 and PvuII) DNA enzyme digests.

Discussion

E. coli concentrations in water samples were not elevated at either site but were consistent with the NSSP-based classification of the two sites. However, bacterial levels in tissue from the two sites were not different. Tissue concentrations apparently reflected generally low levels of contamination and a balance between accumulation and depuration of bacteria between sampling dates during the spring-fall study period. The prevalence of livestock species as the most commonly identified source type under all conditions suggests bacterial transport from farms, grazing of animals in surface water, application of manure to residential lawns or other related sources along the shoreline. The study area has several diary and horse farms and one buffalo farm located along the shore. The greater influence of human sources at the OR site in prohibited waters is consistent with the surrounding land uses at both sites and the close proximity of the OR site to the Durham wastewater treatment facility. Even though *E. coli* concentrations in tissue at both sites were essentially equivalent, a greater level of human-borne indicator bacteria would be of greater concern because of the possible presence of human pathogens (Sinton *et al*. 1998).With the large number of *E. coli* isolates apparently from livestock sources, it was not possible to discern many substantial differences between oyster tissue and overlying water for other types of sources. The largest difference was for human sources at the OR site, where human-borne isolates were more prevalent in oyster tissue. Comparisons of oyster tissue contaminants that accumulate over time with water samples collected once a month might be expected to show greater differences than what was observed. This suggests that pollution sources at the two sites may be temporally consistent, at least during the time period of the study.The use of a single restriction enzyme provided useful information on source species, but there were numerous isolates for which a source species could not be clearly identified. Use of a second enzyme did increase the number of isolates for which a source species could be identified and thus provided further insight into the dynamics of pollution sources that contaminated the oysters. However, the two separate digestions made an already expensive analysis even more expensive, and this approach would not be recommended for routine microbial source tracking studies.

This study provides insight into how shellfish are contaminated from different pollution sources. Further experiments are underway to help interpret the data from this study and to further the understanding of the dynamics of shellfish contamination in estuaries with multiple sources.

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THE EFFECT OF GAMMA AND ELECTRON BEAM RADIATION ON THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

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Abstract
The objectives of this research were to determine the effects of gamma and e-beam irradiation on *Vibrio vulnificus* levels in raw oysters as well as the effect on shelflife of the oysters. Four groups of fresh shellstock oysters were subjected to gamma or e-beam irradiation dosages of 0, 1, 2 and 3 kilograys (kGy). After 7 days post irradiation using gamma or e-beam, statistical differences were determined to exist in survival between non-irradiated control oysters and irradiated oysters.
Specifically, after 10 days post irradiation, 93.3 % of the non-irradiated control oysters survived, were as both the gamma and e-beam irradiated oysters (1,2, and 3kGy) only survived at the following rates 78.2 %, 72.4 %, and 59.1 respectively.

There was an immediate statistical significant reduction in *Vibrio vulnificus* levels when oysters were irradiated at 2 and 3 kGy were compared to non-irradiated oyster controls. Non-statistical differences were detected between non-irradiated control oysters and oysters irradiated at 1 kGy. However, after 5 days post irradiation, all irradiated oysters regardless of dose, exhibited statistically significant reduction in *Vibrio vulnificus* content when compared to the non-irradiated control oysters.

Introduction
In recent years, raw consumption of the eastern oyster, *Crassostrea virginica,* during the warm seawater months (April-October) has been implicated in serious food borne illnesses (Rodrick, G.E., *et al*., 1994). The illness is associated with the Gram negative halophile, *Vibrio vulnificus* (Blake, P.A., 1983; DuPont, H.L., 1987). *V. vulnificus* infections result in primary septicaemia, hypotension, swelling and erythema around wounds and metastatic cutaneous lesions, and can be fatal (Blake, P.A. *et al*., 1979; Tacket, C.O. *et al*., 1984). An identifiable high risk group exists, consisting of individuals who are immunocompromised, diabetics, or suffering from liver disease and elevated iron levels (Blake, P.A. *et al*., 1979). Nearly 50 % of the people who develop primary septicaemia die (Morris, J.G. Jr. *et al*., 1985)

For these reasons, U.S. F.D.A. have mandated that all oyster processing states in the Gulf of Mexico have the potential to post-harvest treat 25 % of shellstock oysters during the month of April to October by 2004 and 50 % by 2006 if the number of *Vibrio vulnificus* case are not reduced by 15 %. All post harvest treatments must verified and validated and an end point of 60 CFU per gram or less of *Vibrio vulnificus* must be achieved using a FDA approved detection method.

Post harvest treatments have included heat and cold shock, freezing, pressure and radiation. The objectives of this research was to determine and compare the effects of gamma and electron beam radiation as a potential post harvest treatment and determine the effects on the total aerobic bacterial and *Vibrio vulnificus* content, and shelf-life of the live oysters exposed to 0, 1, 2 and 3 kilograys.

Materials and methods

Experimentation included analysis of the effects of gamma radiation on shellstock oysters in terms of reducing the bacterial numbers and extending the shelf-life of the eastern oyster, *Crassostrea virginica*. The oysters used for analysis were taken from Apalachicola Bay, Florida, United States of America.

Irradiation studies

Large-scale irradiation of shellstock oysters was conducted at a commercial oyster irradiation, ^{60}Co facility at Food Technology Services, Inc. in Mulberry. Florida. Ten bushels (about 600 lb) of shellstock oysters were transported by refrigerated truck (40-42 F) in boxes to radiation facility. The oysters were then unloaded and then placed in a holding cooler (4-6°C) located on the premises. Two bushels (about 150 shellstock oysters in each bushel) were irradiated with three doses, 0.82-0.89 (T_1), 1.63-1.79 (T_2) and 2.68-2.90 (T_3) kGy), and two bushels were set aside at the non-irradiated controls. The doses were determined by the technical staff of Food Technology Services, Inc. using cerium dosimeters. After irradiation, all ten bushels were transported in back to the University of Florida, refrigerated truck (40-42 F) and evaluated microbiological and for shelf-life.

Electron beam radiation of shellstock oysters was conducted at the State of Florida Department of Agriculture and Consumer Services facility at Gainesville, Florida.

Shelf-life studies

Upon completion of the irradiation procedures, the oysters were stored at 4-6°C and observed each day of death. They were separated according to dose (1 bushel pre dose) and stored in wax covered cardboard boxes. These boxes are identical to those used by the shellfish industry for the transport and dry storage of shellstock oysters. Each day, individual oysters were picked up by hand and examined for death, which is indicated by a gaping shell. All the dead oysters were removed from the boxes and the survivors returned t 4°C. The oysters were examined and counted everyday until the last oysters died.

Microbiological analyses

Oysters for the microbiological study were sampled before and immediately after irradiation. Those that were not used in the day 0 microbiological analysis were placed in dry storage at 4-6°C, and re-analysed using the same microbiological methods on days 2,,6,8, 10 and 14. Dead oysters were not used in these analyses. The oysters were washed, scrubbed, and subsequently shucked (about 10 oysters) under aseptic conditions into a Waring blender. The meat was homogenised for 1.5 minutes at high speed, and the homogenate serially diluted into peptone water.

Pour plates (using 1mL from each dilution bottle) were prepared, in duplicate, using plate count agar (PCA) and PCA + 2.5 % NaCl (PCAS) as media. The agars were allowed to solidify, and then the plates were incubated at 35°C for 24 hours. Plate counts were performed and recorded after 24 and 48 hours. PCA and PCAS were used as the media so that a distinction between halophiles and non-halophiles could be made.

Vibrio vulnificus was determined using an FDA approved method (F.D.A. ,2001) that employed a DNA probe. Specifically, oyster homogenates were serially diluted with phosphate buffer and the resulting diluted homogenates were hockey pucked onto Petri plates containing T1N1 media. The T1N1 plates were then incubated 20-24

hours at 37C. The T1N1 plates were then counted, blotted, fixed and probed for *V. vulnificus* using an alkaline phosphostase conjugated DNA probe.

Stastically test
A paired t-test was used to stastically compared the death rate and microbiological values of non-irradiated control oysters with the irradiated oysters

Results
Both electron beam and gamma irradiation live shell stock oysters at doses of 1, 2, and 3 kGy statistically reduced the number of total aerobic bacteria when compared to non-irradiated controls. Reduction of total aerobic bacteria ranged from 2.0 to 3.0 logs. Only a slight grow back of total aerobic bacteria was observed over 14 days of refrigerated storage (See Figures 1 and 2). In addition, both electron beam and gamma irradiation at 1, 2, and 3 kGy statistically reduced the number of *Vibrio vulnificus* in live shellstock oysters. Reductions ranged from 1.0-1.5 logs. The n umber of *V. vulnificus* increased slightly in the post irradiated oysters during the 14 days of refrigerated storage. This slight increase in numbers may have been a result of bacterial DNA repair. Moreover, there was a steady and significant decrease of *Vibrio vulnificus* in the non-irradiated controls over the 14 days of refrigeration storage (See Figures 3 and 4). Statistically significant differences in death rates were observed between non-irradiated controls and oysters irradiated at 1, 2, and 3 kGy were observed at days 4, 6, 8, 10, 12, and 14. Death rate was dose dependent; the higher the irradiation dose, more oysters died earlier.

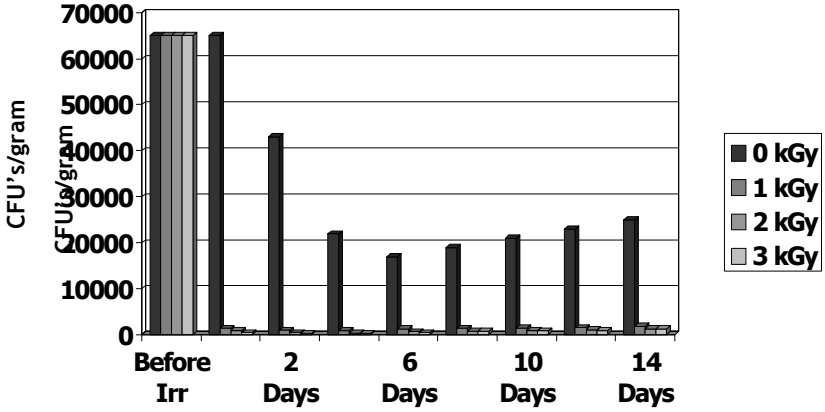

Figure 1. The effects of electron beam irradiation on total aerobic bacteria in the eastern oyster

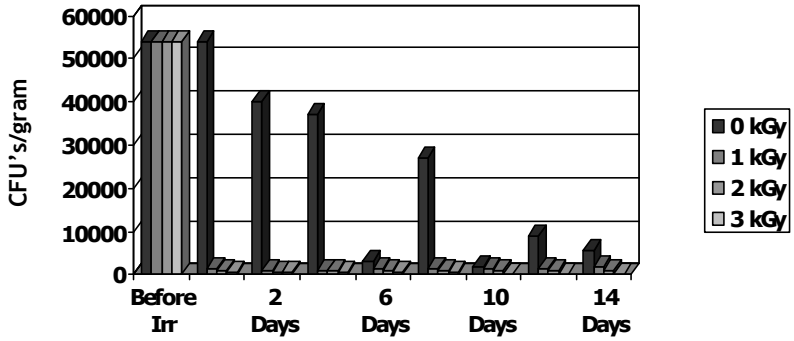

Figure 2. The effect of gamma irradiation on total aerobic bacteria in the eastern oyster

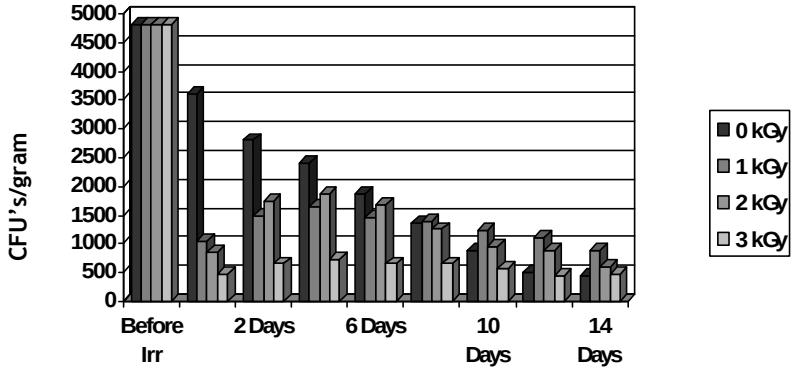

Figure 3. The effects of E beam irradiation on *Vibrio vulnificus* in the eastern oyster

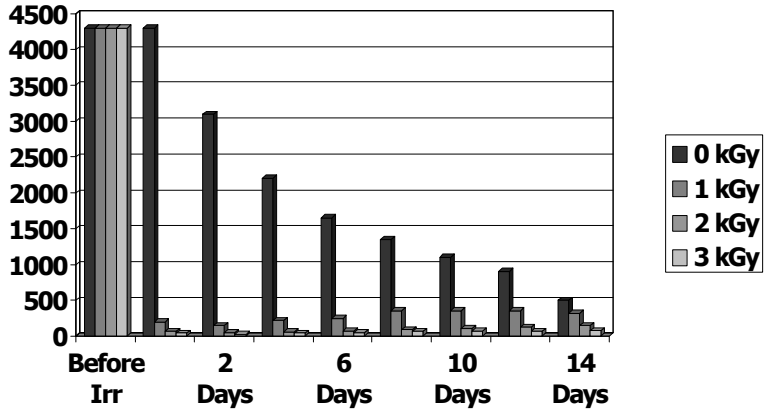

Figure 4. The effects of Gamma irradiation on Vibrio vulnificus in the eastern oyster.

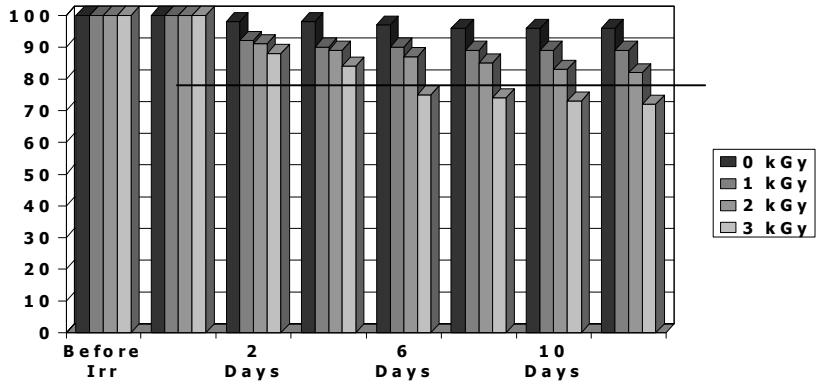

Figure 5. The effects of E beam irradiation on shelflife of the eastern oyster

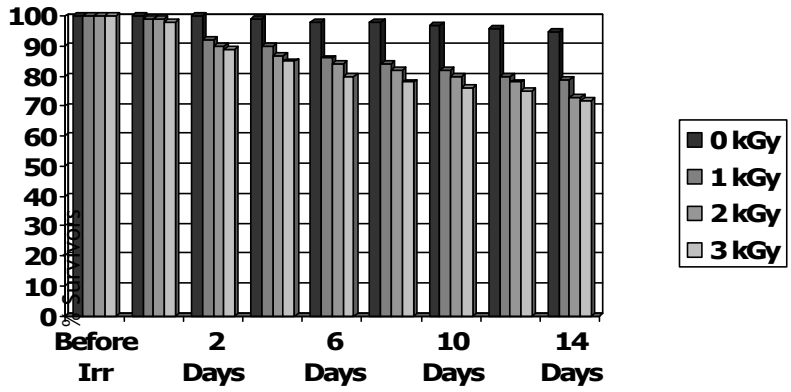

Figure 6. The effects of gamma irradiation on shelflife of the eastern oyster

Discussion

Shucked oyster and clam meats have been irradiated to reduce the bacterial numbers and to increase the shelf-life. However, very little research has been performed on live shellstock oysters and clams. Garner and Watts (1959), treated shucked oyster meats with 630, 830 and 3500 rad and experienced the development of an undesirable odour. [1] The odor was described as 'grassy' for the non-cooked, irradiated oyster meats and 'oxidised' for the cooked, irradiated oyster meats. These authors further concluded that ionizing radiation would not be effective in oyster meat preservation because enzyme action continues after 3500 rad and 5°C storage. No odor changes were detected in this study. These reported differences may by due to differences in the oysters diet.

Mallet *et al*. (1991), showed that alive Massachusetts shellstock oysters exhibited extreme radioresitance, with 2.5 kGy reducing bacteria and not adversely affecting the shell-life. These authors irradiated shellstock oysters at several different doses and found no significant difference in the 6-day survival times at doses of up to 2.5 kGy. In utilizing a professional taste panel, they also determined that oysters irradiated below 3 kGy were of fair and acceptable quality. However, these studies were limited because they did not look specifically at the levels of *V. vulnificus*, which is a pathogen of great concern. Furthermore, evaluation of the shelf-life of irradiated oysters were conducted for only 6 days. Our studies agree with Mallet's up to day 4. However, stastistical differences in shelf-life between the non-irradiated and irradiated oysters were observed after day 4 post irradiation at 2 and 3.0 kGy. In conclusion, post harvest treatments using electron beam and/or gamma irradiation have the ability to dramatically reduce total bacteria including *Vibrio vulnificus* in live shell stock oysters. The reduction of shelf life of the live oyster is minimal until after 5 days of refrigerated storage. This type of post harvest processing aid is one of few available to the oyster industry to allow for the sale of low bacterial containing live oysters.

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Vibrio parahaemolyticus, *V. cholerae* and *V. vulnificus* in Norwegian shellfish.

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Abstract
In order to assess the occurrence of *Vibrio parahaemolyticus*, *V. cholerae* and *V. vulnificus* in Norwegian shellfish, 486 samples were collected from sites all along the Norwegian coastline. In total 33, six and one sample(s) were positive for *Vibrio parahaemolyticus*, *V. cholerae* and *V. vulnificus*, respectively. The presence of the main virulence factor in *V. parahaemolyticus*, (thermostable direct haemolysin, *tdh*) and *V. cholerae* (cholera toxin subunit A, *ctx*A) was determined by PCR. No isolates were positive for these genes.

Introduction
Vibrio parahaemolyticus (*V. parahaemolyticus*), *V. cholerae* (*V. cholerae*) and *V. vulnificus* (*V. vulnificus*) are the main *Vibrio* species associated with food borne infections. The genera *Vibrio* belong to the family *Vibrionaceae*, and are normal inhabitants of estuarine and marine environments (Baumann and Schubert, 1984). *V. parahaemolyticus* and *V. cholerae* have multiple virulence factors that have been characterised, while the pathogenicity of *V. vulnificus* remains unclear (Oliver and Kaper, 2001). The shellfish industry is expanding due to increasing public demand, and the presence of potentially pathogenic *Vibrio* spp. in Norwegian shellfish is largely unknown. Here we present results from a surveillance program performed in 2003 that addressed the occurrence of potentially human pathogenic *Vibrio* spp. in Norwegian shellfish year round.

Materials and Methods
A total of 399 blue mussel (*Mytilus edulis*) samples were collected from 51 commercially approved sites from all along the Norwegian coastline. In addition, 49 blue mussel samples were collected from 'worst-case' locations along the south-coast. These sites were chosen because temperature and salinity were thought to favour *Vibrio* spp. Also, 15 great scallop (*Pecten maximus*) samples from the mid-coast, 14 crab (*Cancer pagerus*) samples from the south-coast, and 9 oyster (*Ostrea edulis*) samples from the mid-coast, were analysed. The samples were collected all year, except for crab, which were collected during summer.

The samples were analysed according to NMKL method no.156 (Anon, 1997) using two enrichments (alkaline peptone water with 2 % NaCl and salt polymyxin broth), and thiosulphate-citrate-bile-sucrose agar as the isolation medium. Presumptive *Vibrio* spp. were identified to species by Gram staining, glucose fermentation, motility, cytochrome oxidase, inhibition by vibriostatic agent O/129 (150 µg), halophilic characteristics (0, 3, 6, 8 and 12 % NaCl), colony morphology (e.g. hemolysis) on blood agar (bovine) and by API 20E (BioMérieux, France). To aid identification, species-specific primers were designed. These targeted the *tox*R gene, which encodes the transmembrane regulatory protein ToxR. The forward primer was universal for all three species, while the reverse primers were species-specific. All *V. parahaemolyticus* strains isolated were investigated by PCR for the presence of thermostable direct hemolysin (*tdh)*, while the *V. cholerae* isolates were analysed for the presence of cholera toxin (*ctx*A) with primers designed by Fields *et al*. (1992). All *V. cholerae* isolates were serotyped by the National Institute of Public health.

Results and Discussion

The sample results are presented in table 1. Most positive samples were part of the 'worst-case' sample group. The screening material also included samples collected from the south-coast, which constituted 37.5 % of the *V. parahaemolyticus* positive samples, and 50.0 % of the *V. cholerae* positive samples. The remaining positive samples in the blue mussel screening material were collected from the west-coast. No V. parahaemolyticus , *V. cholerae* or *V. vulnificus* were detected in the scallop samples. One of the oyster samples was positive for *V. cholerae* , and one crab sample was positive for *V. parahaemolyticus Vibrio* positive sites are indicated in Figure 2. The number of *Vibrio* positive samples increased during summer as illustrated in Figure 1a and 1b, and peaked in August. *V. parahaemolyticus* and *V. cholerae* was also detected during spring, late autumn and winter. The lowest water temperature recorded at sites positive for *V. parahaemolyticus* and *V. cholerae* was 5°C and 4°C. The species-specific *tox*R primers were highly specific and proved to be a useful supplement to the traditional analysis. All the *V. parahaemolyticus* isolates detected were negative for *tdh* by PCR. All *V. cholerae* isolates were of the non-O1/O139 serotype and negative for *ctxA* by PCR.

We show that *V. parahaemolyticus* , *V. cholerae* non-O1/O139 and *V. vulnificus* are present in Norway. To our knowledge this is the first time *V. cholerae* and *V. vulnificus* have been detected in Norwegian shellfish. The isolates were all negative by PCR for their main virulence factors, *tdh* (*V. parahaemolyticus*) and *ctx*A (*V. cholerae*). Several other virulence factors have been described (Oliver and Kaper, 2001) and the presence of these will be assessed in future studies.

Table 1. Results from the *Vibrio* surveillance program performed in 2003.

Sample material	No. samples	V. parahaemolyticus	V. cholerae	V. vulnificus
Blue mussel,				
- screening	399	8 (2.0%)	4 (1.0%)	0
- 'worst-case'	49	24 (49.0%)	1 (2.0%)	1 (2.0%)
Scallop	15	0	0	0
Crab	14	1 (7.1%)	0	0
Oyster	9	0	1 (11.1%)	0

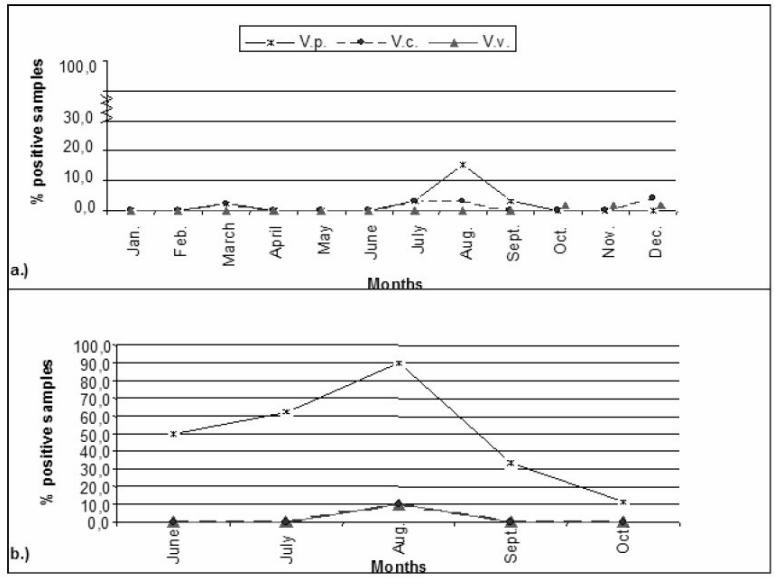

Figure 1. Percentage of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* detected in blue mussel over time, for samples collected from **a.)** commercially approved sites, and **b.)** 'worst-case' sites

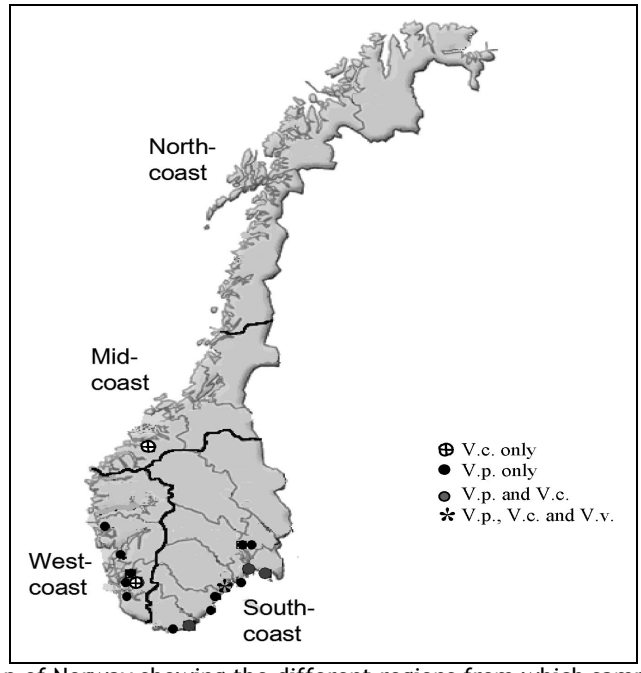

Figure 2. Map of Norway showing the different regions from which samples were collected. Sites positive for *Vibrio* spp. are indicated above.

Acknowledgements
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DETERMINATION OF *E. coli*, HAV, NOROVIRUSES AND VIBRIONACEAE IN SHELLFISH FROM ADRIATIC SEA

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Abstract
Outbreaks caused by shellfish consumption are an increasing problem. *E. coli* has been traditionally used as an indicator of sewage pollution for shellfish harvesting areas. However, no consistent correlation has been established between the presence of *E. coli* and the presence of other emerging pathogens such as viruses and *vibrios*. The aim of the present study was to evaluate the incidence of viruses and *Vibrionaceae* in shellfish in comparison to the indicator of faecal contamination *E. coli*. Sixty-three samples of shellfish (*Mytilus galloprovincialis* and *Tapes philippinarum*), collected during a 7 month period from three class B harvesting areas of the Adriatic Sea, were analysed to detect *E. coli*, HAV, noroviruses and *vibrios*. The results showed that the *E. coli* content was in accordance with the bacteriological limits established by Directive of Council 91/492/CEE for more than 70 % of samples. The virological analysis showed positive samples throughout the monitoring and most of the samples, positive either for HAV or noroviruses, were fully compliant with the bacteriological standard. *V. parahaemolyticus* was detected in 6 samples, either complying or not with the bacteriological standard adopted and showed a clear seasonal trend, being present predominantly during summer months.

Introduction
Human gastroenteritis from consumption of bivalve shellfish is an internationally recognised problem. Since the association of shellfish to the transmission of oral-faecal diseases, *E. coli* has been used as indicator of the sanitary quality of this product. European Community Directive 91/492/CEE requires that all shellfish sold for human consumption must comply with a standard of less than 230 *E. coli*/100 g of shellfish flesh. However, while current legislation appears to be effective for the control of bacterial illnesses, no consistent correlation has been established between the presence of *E. coli* and the presence of viral pathogens (enteric viruses, noroviruses and hepatitis A virus) (Croci *et al*, 2000, Walt *et al*, 2003) or pathogenic *Vibrio* (*V. parahaemolyticus* and *V. vulnificus*) (Koh *et al* 1994, Ripabelli *et al*. 1999). Gastroenteritis outbreaks associated to shellfish consumption are mainly of viral etiology with noroviruses (NVs) as the most important cause (Lees, 2000). Hepatitis A virus (HAV) has a lower incidence but represents an important health problem in endemic regions, such as Italy, where mollusk consumption is the major risk factor (Mele *et al*, 1997) and outbreaks are recurrent (e.g. spring 2004, Naples area) (Boccia, 2004). On the other hand, among bacterial gastroenteritis outbreaks, those related to autochthonous bacteria of marine environment, such as *V. parahaemoliticus*, are the majority (Lipp and Rose, 1997). It is also known that viral pathogens and *vibrios* are slowly released during the depuration processes (mandatory for products harvested in category B areas), persisting in shellfish after the removal of the microbiological indicator *E. coli* (Croci *et al*, 2002, De Medici *et al* 2001 and Lees, 2000).

The aim of this study was to evaluate the incidence of viruses (noroviruses and HAV) and Vibrionaceae in shellfish from class B harvesting areas of the Adriatic Sea, compared to the indicator adopted by legislation *E. coli*.

Materials and Methods

Sampling

The study was performed on shellfish of the species *Mytilus galloprovincialis* and *Tapes philippinarum*. Samples were collected each week from three class B harvesting areas in the Adriatic Sea over a period of 7 months (from June 2003 to December 2003) and shipped refrigerated to the laboratory.

Sample preparation

Shellfish were cleaned under running potable water to remove mud and opened with a sterile shucking knife. Body and shell liquor (approx 200 g) were collected and blended. Microbiological analysis (*E. coli* and *vibrios* detection) were performed immediately after opening, while shellfish homogenates for virus detection were stored at -20°C till analysis.

Analysis

E. coli detection

Enumeration of *E. coli* was performed with a 5 tube, 3 dilution MPN procedure, in accordance with Italian Legislation (Gazzetta Ufficiale della Repubblica Italiana, 1995). Briefly, tenfold dilutions of shellfish homogenate were inoculated in A1 medium and incubated at 37°C per 24 h. *E. coli* presence was confirmed by indole detection after subculture in tryptone water (44°C for 24 h). Assay sensitivity was 180 *E. coli* / 100 g of shellfish.

Virus detection

Viruses were concentrated through PEG precipitation and RNA extraction was performed according to Afzal and Minor (1994). HAV detection was performed through a RT-nested-PCR (Croci *et al.*, 1999) while noroviruses were detected through a previously developed RT-booster-PCR followed by Southern hybridization (De Medici *et al*, 2004).

Vibrios detection

Vibrios were isolated on TCBS medium after enrichment in alkaline peptone water (NaCl 3 %) and identification, carried out through API20NE modified by use of a 3 % NaCl inoculum medium, was confirmed by PCR for pathogenic species as *V. cholerae* (*ompW* gene) (Nandi *et al*, 2000) and *V. parahaemolyticus* (*toxR* gene) (Kim *et al*. 1999). Strains whose identification was confirmed were subjected to PCR for the detection of genes codifying for major virulence factors (*ctx* for *V. cholerae*, *tdh* and *trh* for *V. parahaemolyticus*) (Ripabelli *et al*, 1997 and Bej *et al* 1999).

Results

Data are summarised in Figure 1. Of the 63 samples analysed, 17 (27 %) samples were over the bacteriological limit of 230 *E. coli*/100 g; 32 (50,8 %) samples were at or below the detection limit of the method (180 *E. coli*/100 g).

Virological analysis showed the presence of either HAV or NVs in 18 samples (28,6 %): 5 (7,9 %) were found positive for HAV, 12 (19 %) for noroviruses, while in 1 sample (1,6 %) both viruses were detected. *V. parahaemolyticus* was detected in 6 samples (9,5 %), with one *tdh* + strain. No samples were found positive for *V. cholerae* O1 or *V. vulnificus*. The comparison between *E. coli* levels and the presence of other pathogens showed that of the 22 samples where HAV, NVs or *V. parahaemolyticus* were detected, 5 (22,7 %) were not compliant with *E. coli* standard; on the opposite, of the 17 samples exceeding *E. coli* limit, 12 (70,5 %) were negative for all the aforesaid pathogens.

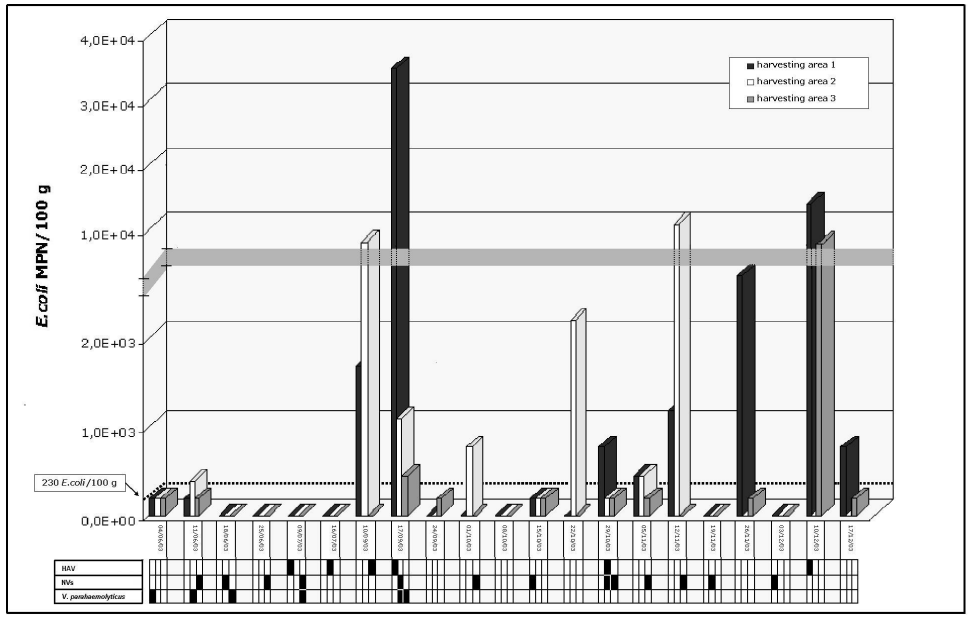

Figure 1. Results of the detection of *E. coli*, HAV, NVs and *V. parahaemolyticus* in samples. Histogram shows *E. coli* concentration; black squares in the table below show presence of HAV, NVs and *V. parahaemolyticus* in the samples

Conclusions

The results obtained showed that the efficiency of *E. coli* as a shellfish safety indicator was 54 % (34 of 63 samples showed a correspondence between *E. coli* levels and the presence/absence of viral and naturally occurring pathogens), confirming its inadequacy in predicting the presence of microorganisms transmitted through shellfish consumption. Enteric viruses, HAV and NVs were detected throughout the course of monitoring. In particular, NVs positive samples showed a regular distribution during the entire investigation period, regardless of the water temperature in the harvesting areas that, especially in early summer (June-July), reached values as high as 29°C. *V. parahaemolyticus*, as expected, showed a high seasonality, being detected only between June and September, but only one *tdh* + strain was detected during the sampling period.

In conclusion, considering the limited efficiency of the depuration processes on viruses and pathogenic *vibrios* (Croce *et al* 2002, De Medici *et al* 2001 and Lees 2000), the data obtained in this study on their incidence and on the reliability of *E. coli* as indicator suggest that shellfish of the species *Mytilus* and *Tapes* should be consumed only after adequate cooking.

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PRELIMINARY EVALUATION OF A SELECTIVE ISOLATION MEDIUM FOR *Vibrio hollisae*

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Abstract
Vibrio hollisae has been shown to produce gastro-enteritis after the consumption of seafood. Fresh clinical and environmental isolates show little or no growth on selective media commonly used for the isolation of human pathogenic *vibrios* from clinical or environmental samples. A new selective medium was developed which gave good recovery of two strains of *V. hollisae* together with differentiation of this species from other *vibrios*.

Introduction
Vibrio hollisae has been reported to cause diarrhoeal disease in humans (Hickman *et al*. 1982; Morris *et al*. 1982). Illness due to this organism has been associated with the consumption of oysters (Carnahan *et al*. 1994). Fresh isolates from clinical sources have been reported to show little or no growth on bile salts-containing media such as TCBS, the most widely used medium for the isolation of *vibrios* from clinical, food and environmental sources. The incidence of the organism in clinical samples and the environment is therefore not known. The present work was undertaken to develop a medium for the isolation of this organism.

Materials and Methods
Test strains
Bacterial strains (National Collection of Type Cultures, London, England) were propagated on marine agar and stored as described previously (Lee and Cole 1995).

Culture media
Marine Agar 2216 (Becton Dickinson UK Ltd, Oxford, England), TCBS and Isosensitest Agar (ISA; Oxoid Limited, Basingstoke, England) were prepared according to the manufacturers' instructions. After sterilisation of the ISA, the medium was cooled to 50°C and 5 % (v/v) laked horse blood added before pouring into plates. TCI medium was prepared as described previously (Beazley and Palmer 1992).

Vibrio hollisae agar (VHA) was prepared as follows: 55.1 g/l Marine Agar 2216 was suspended in 6.1 g/l Tris-HCl buffer pH 7.6 containing 15 g/l potassium iodide and 72 mg/l water soluble phenol red. The medium was brought to the boil and then autoclaved at 121°C for 15 minutes. The medium was cooled to 50°C and 38 ml of 20 % (w/v) D-mannitol and 38 ml of 20 % (w/v) maltose were added, the medium mixed and poured into plates.

Antimicrobial sensitivity testing
Inhibitory activity of antimicrobial agents was determined using a modified Stokes' disc diffusion test on ISA with laked blood using *Escherichia coli* NCTC 10418 as the control.

Determination of plating efficiency
A suspension of growth from marine agar was made in quarter strength Ringer's solution and further dilutions made in the same diluent. 0.1ml amounts of an appropriate dilution were spread over the surface of each of two tests plates and two Marine Agar plates. The plating efficiency were estimated as the percentage of the mean count on the selective agar (incubated for 24 h under the stated conditions) relative to the count obtained on Marine Agar at 30°C.

RESULTS

Sensitivity to antimicrobial agents

V. hollisae NCTC 11640 and NCTC 11641 were both sensitive to the following antibiotics (disc content):

Spectinomycin (10 μg)	Amikacin (30 μg)	Gentamicin (10 μg)	Netilmycin (30 μg)
Streptomycin (20 μg)	Tobramycin (10 μg)	Imipenem (10 μg)	Cefaclor (30 μg)
Cefuroxime (30 μg)	Ceftazidime (30 μg)	Cephradine (30 μg)	Chloramphenicol (10 μg)
Erythomycin (5 μg)	Aztreonam (30 μg)	Amoxycillin (2 μg)	Augmentin (30 μg)
Methicillin (10 μg)	Tetracycline (10 μg)	Piperacillin (75 μg)	Colistin (10 μg)
Ciprofloxacin (5 μg)	Nalidixic Acid (30 μg)	Cotrimoxazole (25 μg)	

Both strains showed intermediate sensitivity to Pencillin G (1 unit) and Oxolinic acid (2 μg). Both strains were resistant to Fusidic Acid (10 μg),Teicoplanin (30 μg) and Vancomycin (30 μg).

Growth on selective media

V. hollisae did not show growth on TCI medium if blood was omitted to facilitate the detection of fermentation. *V. hollisae* grew on VHA as pink, non-fermenting colonies, 1.5 to 2 mm in diameter while other *vibrios* grew as yellow, fermenting colonies, 1.5 to 3 mm in diameter, depending on the species (see Figure 1). Members of the *Enterobacteriaceae* showed weak or no growth on VHA. *Pseudomonas aeruginosa* grew on VHA as small non-fermenting colonies (<0.5 mm). *Enterococcus faecium* colonies grew on VHA as pale yellow (weakly fermenting) colonies 1 mm in diameter. Incubation under anaerobic conditions inhibited the growth of *P. aeruginosa* and increased the yellow colouration of fermenting organisms. Plating efficiencies on the selective media are shown in Table 1.

Table 1. Plating efficiency of *Vibrio hollisae* and other test species on selective media

		Plating efficiency[1] on		
Strain	Species	TCBS	VHA 37°C aerobic	VHA 37°C anaerobic
NCTC 11640	*Vibrio hollisae*	5	63	55
NCTC 11641	*Vibrio hollisae*	32	79	85
NCTC 10675	*Vibrio alginolyticus*	145	75	48
NCTC 4716	*Vibrio cholerae*	69	101	86
NCTC 10885	*Vibrio parahaemolyticus*	12	44	ND[2]
NCTC 12205	*Vibrio parahaemolyticus*	29	102	61
NCTC 7171	*Enterococcus faecium*	0	105	88
NCTC 9001	*Escherichia coli*	0	0	ND
NCTC 10332	*Pseudomonas aeruginosa*	0	97	0

1. Relative to the count on Marine Agar incubated aerobically at 30°C
2. Not done

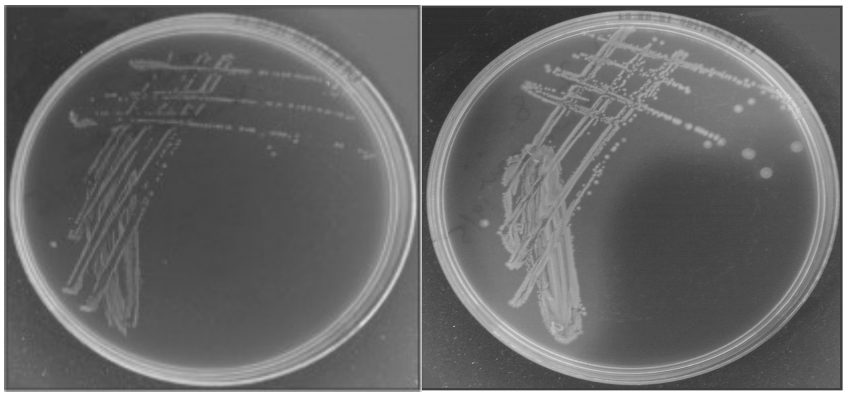

Figure 1. Appearance of *Vibrio hollisae* (left) and *Vibrio cholerae* (right) on VHA Colonies of *V. hollisae* are pink while those of *V. cholerae* (together with the surrounding medium) are yellow due to acid production

Discussion

None of the antibiotics to which the *V. hollisae* strains showed resistance was deemed suitable for use in a selective agar for the isolation of this species as many other bacteria expected to be present in clinical and environmental samples would also be resistant. Modification of TCI to produce a marine-agar based blood-free medium (VHA) allowed reasonable growth of *V. hollisae* strains together with differentiation from other *vibrios* which fermented D-mannitol and/or mannose. Anaerobic incubation of VHA may be used to suppress the growth of *P. aeruginosa* while vancomycin could be added to the medium to suppress the growth of enterococci.

The medium shows potential for use in the isolation of *V. hollisae* from clinical, food and environmental samples. However, there is a need to confirm its performance using a wider range of target and non-target strains prior to use with such samples. If the efficacy of the medium is confirmed, its use would help to clarify the role of *V. hollisae* in oyster-associated gastroenteritis.

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HEPATITIS A VIRUS: NEW INSIGHTS ON A WELL KNOWNSHELLFISH-BORNE VIRAL PATHOGEN

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Abstract
Hepatitis A virus (HAV) is responsible for around half of the total number of hepatitis diagnosed worldwide and is transmitted by the faecal-oral route. As a consequence of globalization, transnational outbreaks of foodborne infections are reported with increasing frequency. Some of these outbreaks are caused by shellfish complying with legal shellfish standards. One added concern is the evidence of the extremely high persistence of HAV in the environment, since it is able to maintain its stability in very adverse situations. Molecular procedures are now available and should be employed for the direct surveillance of HAV in shellfish tissues.

Introduction
Hepatitis A virus (HAV), the prototype of genus *Hepatovirus*, within the *Picornaviridae* family (van Regenmortel *et al.*, 2000), is composed of an icosahedral capsid that contains a positive-sense, single-stranded RNA genome of approximately 7.5 Kb in length. HAV is a hepatotropic virus, which represents a significant problem for human health. It is responsible for around half of the total number of hepatitis diagnosed worldwide and is mainly transmitted by the faecal-oral route, either by person-to-person contact or by ingestion of contaminated food or water, resulting in the widespread of endemic asymptomatic infection of infants and children in developing countries. Even though HAV transmission is well known, the way how it reaches the liver is not yet elucidated. Recently, two cellular receptors have been described, one of them has been isolated from human liver and kidney cells (Feigelstock *et al.*, 1998) and the other one from erythrocytes (Sanchez *et al.*, 2004). This latter receptor has been characterised as glycophorin A and it has been suggested to act as a decoy receptor, attracting pathogens to the erythrocyte and keeping them away from the liver (Gagneux, 1999; Sanchez 2004,). This finding opens new perspectives in the biological cycle of HAV.

The fatality rate in HAV infections is reported to be lower than 0.1 % (Hollinger and Emerson, 2001), although clinical data and manifestations show that HAV outbreaks are more severe in recent outbreaks than in the past (Kanda *et al.*, 2002), and in an outbreak caused in 2003 by ingestion of contaminated green onions in Pennsylvania, three deaths occurred among a total of 555 cases, representing a mortality rate of 0.5 % (CDC, 2003).In the last decades, the improvement of public health and hygiene has reduced the incidence of hepatitis A in children and adolescents worldwide. Under these circumstances, a massive outbreak of hepatitis A becomes possible among the general population who neither has suffered the infection nor has any protective antibodies (Armigliato *et al.*, 1986; Koff, 1995). HAV outbreaks have been related with shellfish consumption with increasing frequency, since are eaten raw or insufficiently cooked (Table 1). The occurrence of shellfishborne outbreaks result in a lack of public confidence over this marketable product and, therefore, in high economic losses by the seafood industry (Morse *et al.*, 1986). Additionally, hepatitis A must be considered the most severe viral infection related to molluskan shellfish consumption.

Table 1. Examples of HAV outbreaks related to shellfish consumption

Number of affected people	Implicated food	Location, year	Reference
278	Oysters	Texas (USA), 1973	(Mackowiak *et al.*, 1976)
132	Cockles	(UK), 1981	(O'Mahony *et al.*, 1983)
300.000	Raw clams	Shangai (China), 1988	(Halliday *et al.*, 1991)
5889	Mussels and clams	Puglia (Italy), 1996	(Lucioni *et al.*, 1998)
467	Oysters	New South Wales (Australia), 1997	(Conaty *et al.*, 2000)
184	Coquina clams	Valencia (Spain), 1999	(Sanchez *et al.*, 2002)

Nowadays, molecular biology techniques are applied to the study of environmental and food samples, including shellfish, and allow advances in epidemiological and clinical studies. Molecular characterization of an outbreak affecting 184 people in Valencia in 1999 (Bosch *et al.*, 2001b; Sanchez *et al.*, 2002), caused by the consumption of imported coquina clams complying with European Union shellfish standards, confirmed the implication of shellfish, after the detection of HAV RNA by reverse transcription-PCR in shellfish samples. The complete capsid region of virus isolates from clinical samples related to the mentioned outbreak was characterised and, in spite of the recognised low variability of HAV as reflected by the existence of a single serotype, two antigenic variants were detected: one in a discontinuous epitope defined by monoclonal antibody K3-4C8 and a second one in a linear VP1 epitope of the virus (Sánchez *et al.*, 1999). Later on, a new HAV variant with a 15 amino acid deletion located on the VP1 region was found (Costa-Mattioli *et al.*, 2002). Despite these mentioned examples, the frequency of nonsynonymous mutations observed in HAV is significantly lower than those found in other picornaviruses, such as PV-1 and FMDV. This high degree of conservation of the amino acid sequences of the capsid proteins suggests the operation of severe structural constraints in the HAV capsid (Sanchez *et al.*, 2003b).HAV shows a high codon usage bias, with the repeated occurrence of 22 rare codons for 14 amino acids (Sanchez *et al.*, 2003b). Most of the conserved rare codons of the P1 region were strategically located at the carboxy borders of β-barrels and α-helices and their function is likely to be the assurance of proper folding of the capsid proteins through a decrease in the translation speed. This proper folding gives rise to a highly compact capsid that ensures a high level of environmental persistence (Sanchez *et al.*, 2003b). Despite this antigenic conservation, it has been demonstrated that HAV replicates following a quasispecies distribution, and the high proportion of rare codons has been confirmed in the mutant spectrum, empathizing their importance and their specific location (Sanchez *et al.*, 2003a).

HAV is highly infectious and extremely resistant to degradation by environmental conditions (Abad *et al.*, 1994; Hollinger and Emerson, 2001). This is an important issue in the case of shellfish consumption, since this type of mollusks is typically cooked until their valves are open, which may occur at temperatures as low as 70°C, which is not enough to ensure HAV inactivation (Abad *et al.*, 1997; Croci *et al.*, 1999; Millard *et al.*, 1987). An additional matter of concern is that HAV may be transmitted through the consumption of shellfish complying with bacterial end-product standards, which once again shows that these standards do not provide a guarantee of virus absence (Bosch *et al.*, 1994; Mele *et al.*, 1989; Romalde *et al.*, 2002), and that bacterial depuration rates can not accurately predict the efficiency

of virus removal. For this reason standardised and validated protocols are necessary to produce reliable and comparable results, that can be accepted by regulatory authorities. Currently, several protocols for HAV detection on shellfish are available (Atmar *et al.*, 1995; Goswami *et al.*, 2002; Kingsley and Richards, 2001; Romalde *et al.*, 2001; Sanchez *et al.*, 2002), although more data are required on the performance and robustness of such protocols. Reliable automated standardised procedures are necessary for the routine monitoring of HAV in shellfish to ensure the safety of the consumption of bivalve mollusks. . In the meantime, standard molecular procedures should be employed for the direct surveillance of HAV in shellfish tissues, since have been shown to be useful to determine the source of some shellfishborne HAV outbreaks (Bosch *et al.*, 2001a; Furuta *et al.*, 2003)

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EVALUATION OF F-SPECIFIC RNA BACTERIOPHAGE AS INDICATOR OF VIRAL CONTAMINATION CLEARANCE DURING THE DEPURATION PROCESS

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Abstract
Escherichia coli is a widely utilised indicator of the sanitary quality of bivalve molluscan shellfish sold for human consumption. However, it is now well documented that shellfish that meet the *E. coli* standards for human consumption may contain human enteric viruses that cause gastroenteritis and hepatitis. On the basis of this fact, a new parameter based on FRNA bacteriophage charge in shellfish meat after the depuration process has been proposed, taken into account that these bacteriophages behave in a more similar way to enteric viruses than *E. coli*. The aim of this work was to evaluate FRNA bacteriophage elimination during depuration and its potential use as an indicator of virus removal during this process. Three Spanish commercial depuration centres were selected as being representative of a range of system types, and with different water treatments. Sampling was performed on a weekly basis from June to December 2003. FRNA bacteriophage and *E. coli* contents were measured so a comparison can be made with existing control measures. RT-PCR analysis for detection of Hepatitis A virus (HAV) and Norovirus (NV) were carried out in all samples prior to and after the depuration period. In addition, a number of randomly selected samples from the three sites were analysed for NV presence using a TaqMan real-time RT-PCR protocol.In general, depuration was more effective in removing *E. coli* than FRNA bacteriophages from shellfish. In fact, in some cases an increase in the phage numbers was observed at the end of the depuration process. In addition, seasonality was observed for the FRNA bacteriophages, being difficult to detect in August when temperatures were higher than 21ºC. Both HAV and NV could be detected by classic RT-PCR and/or real-time RT-PCR in samples meeting the *E. coli* and/or FRNA phage end product standards.

Introduction

Bivalve shellfish are readily contaminated with viruses present in water because of the concentrating effect of the filter feeding and, as a consequence of that these marine organisms have been implicated as vectors in the transmission of enteric diseases for many decades (Richards, 1987). Among the strategies employed to reduced this public health risk, the most extensively used throughout the world are to harvest shellfish from areas receiving minimal sewage contamination and purifying shellfish in tanks of clean seawater by a process called depuration (Doré *et al.*, 1998; Lees, 2000; Richards, 2001).

Current methods to determine the sanitary quality of marketable shellfish are based on bacterial indicators, namely fecal coliform bacteria and/or *Escherichia coli* (Anonymous, 1991, 1993), in both harvesting areas and end products. Regarding the classification of harvesting areas, those shellfish from zones A (<230 *E. coli*/100 g shellfish meet) can be placed directly in the market, whereas those harvested in zones B (< 4600 *E. coli*/100 g shellfish meet) have to be subjected to a purification process (depuration) before go the market. In addition, depurated shellfish has to comply with an end product standard similar to that of zones A (<230 *E. coli*/100 g shellfish meet). However, it is well known that these bacterial indicators may fail to detect viral contamination (Power and Collins, 1989). In fact, the periodic occurrence of illness outbreaks, mainly of hepatitis A and gastroenteritis with viral aetiology, associated to shellfish meeting the bacterial standards (Morse *et al.*, 1986; Chalmers and McMillan, 1995; Perret and Kudesia, 1995; Bosch *et al.*, 2001; Le Guyader *et al.*, 2003), has contributed to a public confidence problem over shellfish safety and resulted in important economic losses by the seafood industry.Male-specific (F) RNA bacteriophages are a group of single-stranded RNA viruses with a simple cubic capsid measuring 24-27 nm, which belong to the family *Leviviridae* (Van Meerten and Van Duin, 2002). They are commonly found at high levels in sewage and faecally contaminated waters and their physical and genomic properties are similar to those of hepatitis A virus (HAV) and Norovirus (NV), the most two important enteric viruses from a public health standpoint. On the basis of this fact, a new parameter based on FRNA bacteriophage charge in shellfish meat after the depuration process has been proposed, taken into account that these bacteriophages behave in a more similar way to enteric viruses than *E. coli* (Doré *et al.*, 2000; Lees, 2000). The aim of this work was to evaluate the FRNA bacteriophage elimination during depuration and its potential use as an indicator of virus removal during this commercial process.

Materials and Methods

Depuration plants

Three depuration plants located in Galicia (NW Spain) were selected for this study. Plant 1 is a flow-through system of concrete tanks with 1300 m^3 capacity and ozone-based water treatment. Plant 2 is a flow-through system of bins (1 m^3 each) with 900 m^3 capacity and chlorine-based water treatment. Finally, plant 3 is a flow-through system of concrete tanks with 2400 m^3 capacity and chlorine-based water treatment.

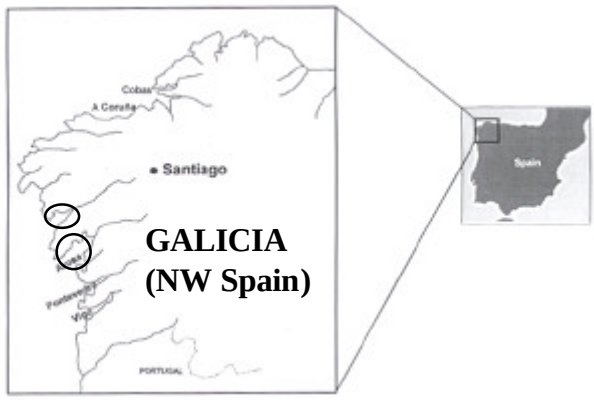

Figure. 1. Location of the depuration centers studies.

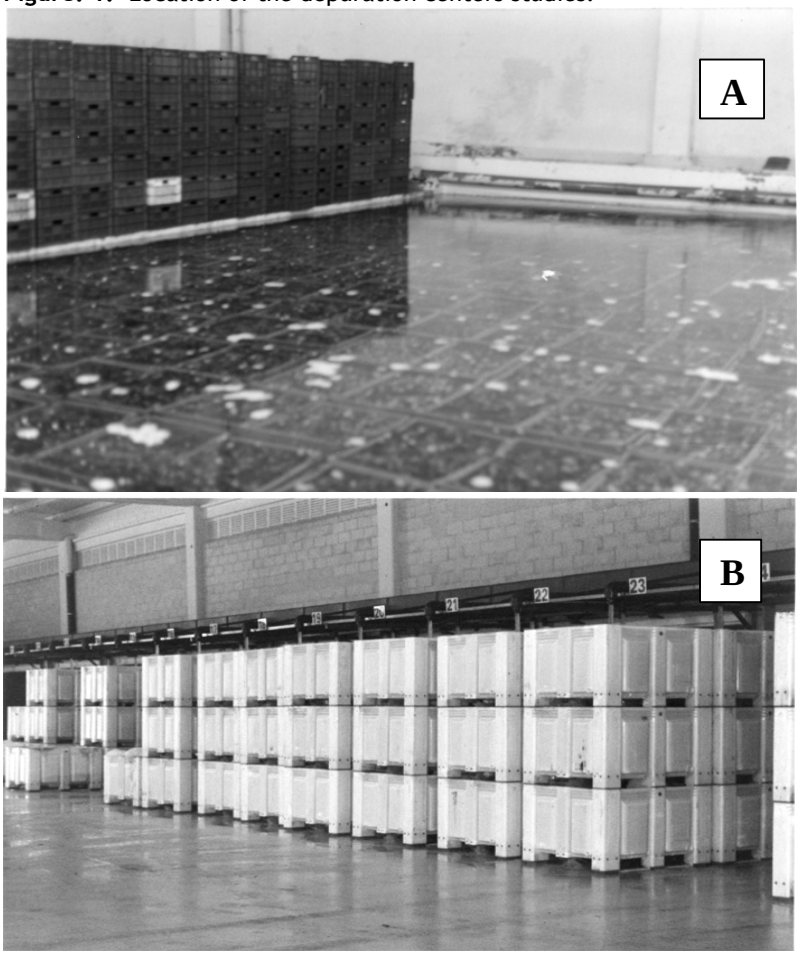

Figure. 2. Depuration systems investigated. (A) Flow-through systems with concrete tanks; (B) Flow-through system in bins (1 m^3 each).

Sampling
A total of 66 cycles of depuration, from the three different depuration plants were studied in this work from June to December 2003. Sampling was performed before and after the depuration process, following the procedures recommended by EU directives (Anonymous, 1991) on the basis of the size of mollusc stocks. Mollusc species included mussel (*Mytilus galloprovincialis*)(45 cycles), oyster (*Ostrea edulis*)(11 cycles), cockle (*Cerastoderma edule*)(8 cycles) and clam (*Ruditapes phillipinarum* and *Tapes* sp. (2 cycles). Whole shellfish or dissected tissues (stomach and hepatopancreas) were mechanically homogenised in an equal volume of sterile artificial seawater and distributed into pools for the respective subsequent bacteriological and virological analyses.

Bacteriological analysis
Enumeration of *E. coli* was performed following a standard most-probable-number (MPN) procedure (Anonymous, 1989; 1991). Briefly, the method used was a 5 tubes-3 dilutions test involving inoculation of tubes containing brilliant green bile broth (BGBB)(Oxoid Ltd., Basingstoke, UK) with Durham tubes, followed by incubation at 37ºC for up to 48 h. EC presence was confirmed by subculturing the tubes positive for gas production in BGBB and in 1 % tryptone water (Oxoid Ltd.) for 18 h. Tubes containing tryptone water were tested for indole production by using Kovács reagent, and subcultures that produced both indole and gas were considered as EC positive.

FRNA bacteriophage counts
Shellfish samples were assayed for FRNA bacteriophages by using the host bacterium *Salmonella typhimurium* WG49 in a standard double agar overlay method as previously described (Doré *et al.*, 2000). Briefly, to 2,5-ml portions of molten 1 % tryptone-yeast extract agar at 45ºC, we added replicate 1-ml portions of undiluted or diluted shellfish homogenates and 1-ml portions of a WG49 host culture. The molten agar and sample were mixed by inversion and poured onto previously prepared 2 % tryptone-yeast extract-glucose agar base plates. The overlays were incubated overnight prior to obtaining the FRNA bacteriophage counts that were expressed as number of plaque forming units (PFU) per 100 g of shellfish tissue.

Virological analysis.
Mussel homogenates were diluted 1:1 (wt/vol) in 0.1 % tryptone water (pH 7.5)(Oxoid, Ltd.) and incubated at 37ºC with shaking at 320 rpm for 1 h. Virus extraction and purification were then performed using the commercial kit Total Quick RNA Cells and Tissues-Mini (Talent) following the manufacturer's recommendations. According to a previous work (Ribao *et al.*, 2004), this kit possesses a good effectiveness for viral RNA extraction from shellfish meat. One µl of each extracted RNA was subjected to RT-PCR amplification using the Superscript One-step RT-PCR kit (Invitrogen) for both HAV and NV following the manufacturer's instructions. Primers employed for HAV RNA amplification were HAV240 (5'-GGAGAGCCCTGGAAGAAAGA-3') and HAV68 (5'-TCACCGCCG TTTGCCTAG-3')(Bosch *et al.*, 2001). For NV, primers employed were NVp35 (5'-CCTGTTGGTTTGAGGCCATAT-3') and NVp36 (5'-ATAAAAGTTGGCATGAACA-3') (Atmar *et al.*, 1995). Amplification programs consisted in a first RT step at 43ºC for 1 h, followed by a denaturation cycle at 94ºC for 4 min and 40 cycles of template denaturation at 94ºC for 1 min; primer annealing at 55ºC for 1 min (HAV) or 90 s (for NV); and primer extension at 72ºC for 90 s (HAV) or 1 min (for NV). A final extension at 72ºC for 15 min was performed in all cases. Specific amplified products were 174 and 470 bp in length for HAV and NV respectively, and were detected by agarose gel

electrophoresis (FMC BioProducts, Rockland, USA), and ethidium bromide staining. A negative control consisting in sterile water was included in each RT-PCR set-up.

Results were confirmed by southern blot using specific probes for each virus as previously described (Romalde *et al.*, 2002). Briefly, DNA separated by electrophoresis was transferred to nylon membranes (0.45 µm; Roche Diagnostics, Barcelona, Spain) after denaturation by treatment for 15 min in a 150 mM NaOH solution and neutralization with 0.5 x TBE. After transfer, DNA was fixed with UV light for 3 min and membranes were kept desssicated at 4ºC until use. Prehybridization (3-4 h) and hybridisation (overnight) were performed at 40ºC for HAV and 55 for NV, in hybridisation solution containing 5 x SSC, blocking reagent (1 % wt/vol; Roche), 0.1 % (wt/vol) sarcosyl (Sigme Química, Madrid, Spain), and 0.02 % (wt/vol) SDS. The digoxigenin-labeled probes employed were complementary to internal fragments of the correspondent amplicons, having sequences of 5'-TTAATTCCTGCAGGTTCAGG-3' for HAV (Bosch *et al.*, 2001) and 5'-GGCCTGCCATCTGGATTGCC-3' (NVp69) for NV (Atmar *et al.*, 1995). After hybridisation, the membranes were washed once at room temperature in 2 x SSC, 0.1 % (wt/vol) SDS, and once in 0.5 x SSC, 0.1 % SDS at room temperature of 45ºC for HAV and NV respectively. Then, membranes were blocked and incubated with the anti-digoxigenin-alkaline phosphatase conjugate, and the bands were colorimetrically visualised using the DIG DNA labelling and detection kit (Roche) following the manufacturer's instructions.

In addition, 38 samples, corresponding to 19 cycles of depuration, were randomly selected for detection of NV by real time RT-PCR using TaqMan analysis (Henshilwood *et al.*, 2003). cDNA for TaqMan assay was synthesised from extracted RNA using random primers as previously described (Green *et al.*, 1998). Primer-probe sets for genogroup I strains (Vinje *et al.*, manuscript in preparation) and Grimsby (a genogroup II strain) were designed using Primer Express™ software (Applied Biosystems). For each primer-probe set used, duplicate 2 µl aliquots of cDNA were added to adjacent wells of a 96-well optical reaction plate and made up to 25 µl with TaqMan reaction mix (100 mM Tris (pH 8.3), 50 mM KCl, 75 mM passive reference dye ROX, 5 mM $MgCl_2$, 200 µM dNTPs, 0.025 U/ µl Taq polymerase) (Applied Biosystems). Optimal primer-probe concentrations were determined according to the Applied Biosystems protocol. The wells were capped and the reaction was placed in an Applied Biosystems GeneAmp SDS 5700 real-time PCR machine and subjected to the following amplification cycle: 50ºC for 2 min, then 95ºC for 10 min, followed by 50 cycles of 95ºC for 15 s and 60ºC for 1 min. Threshold cycles (C_t values) were determined using the GeneAmp system software. Samples giving a single positive duplicate reaction were counted as an overall positive.

Statistical analysis.
A statistical test of dependence (chi-square test) among the numbers of *E. coli*, FRNA bacteriophage and the presence of virus was performed employing the software Chidos for Windows (Rossi, 2002).

Results
The numbers of *E. coli* cells per 100 g of shellfish were determined for all the samples by the MPN method (Tables 1-3), both prior and after the depuration process. Before depuration (shellfish collected from the harvesting areas), levels of *E. coli* in shellfish meat ranged from <20 to 5400 MNP 100 g^{-1}, which were consistent with their designation as category B areas following the classification of the EU legislation. In addition, 75.75 % of these samples were within the end product standard required for bivalve mollusc in the legislation (Table 4). This percentage increased to 89.39 % after depuration, which implies that 10.61 % of the samples sent to the market failed to comply with the existing end product standard (Table 4).
Variable reductions in *E. coli* levels and the ability to meet the end product standard were observed among the different systems studied. Thus, plant 2 (system using bins and chlorine) managed to clear *E. coli* levels to below 230 MPN 100 g^{-1} in 95.93 %, plant 1 (tanks and ozone) in 80.95 %, and plant 3 (tanks and chlorine) in 91.66 % of the samples analysed (Table 4). It is interesting to point out that the percentage of samples complying the end product standard after depuration could not be correlated with the bacterial contamination levels prior depuration. In this sense, plant 3 despite having the highest average of *E. coli* counts (1300 MPN 100 g^{-1}) prior to depuration failed to meet the end product standard in 8.34 % of samples, whereas plant 1, with a lower average *E. coli* numbers (540 MPN 100 g^{-1}), failed in 19.05 % of occasions. On the other hand, in some cases an increase in the *E. coli* levels were detected after depuration (Tables 1-3).

As for *E. coli*, numbers of FRNA bacteriophage were determined prior and after depuration for all samples. FRNA phage levels ranged from <30 to 5 x 10^5 PFU 100 g^{-1} prior depuration and from <30 to 3767 PFU 100 g^{-1} after depuration (Tables 1-3). Before depuration, 54.54 % of the samples already comply with the proposed end product standard based on FRNA phage (Table 4). After depuration, this percentage increased to 63.63 % and, therefore 36.36 % of the samples showed no compliance with the proposed standard (Table 4). Again variable results were obtained for reduction in FRNA levels depending on the depuration system analysed. Whereas plant 2 achieved the highest number of samples in compliance with the propo sed FRNA phage end product standard (71.42 %), plants 1 and 3 only managed to reduce the phage numbers to levels within the proposed standard in 58.33 % and 61.9 % of the cases, respectively (Table 4). Moreover, in 34.8 % of depuration cycles studied, numbers of FRNA bacteriophage were found to have increased following depuration compared with levels observed prior to purification (Tables 1-3).

No statistical correlation could be established by the chi-square test between the contamination levels of the shellfish samples deduced from the *E. coli* numbers and those detected on the basis of FRNA bacteriophage numbers, neither prior nor after the depuration process, since values obtained with both Pearson index or likelihood ratio were not significant at a confidence level of 95 %.

HAV was detected in 5 (7.57 %) of the shellfish samples harvested from the areas studied, and persisted after depuration on 3 occasions (60 %) where it was present

before this process (Tables 1-4). In addition, on two cycles HAV was detected in depurated samples where it was not detected prior to depuration (Tables 1-3).

Only one sample (1.5 %) was confirmed as positive for NV using the classical RT-PCR method employed. In this sample NV remained present after depuration (Tables 1-3). When the TaqMan analysis was performed on a group of 38 randomly selected samples, corresponding to 19 cycles of depuration in the three plants, a total of 3 samples (7.8 %), from 3 different cycles, were positive for NV genogroup I, and 9 samples (23.6 %), from 8 different cycles, were positive for NV genogroup II (Table 5). However, results indicated a low viral charge in the samples, since Ct values were high, ranging from 38.99 to 48.24. On the other hand, although in four cycles clearance of the viral charge was obtained through the depuration process, in one case NV persisted following depuration and in 6 cases samples were positive for NV after depuration but not before this process (Table 5).

No link could be demonstrated by statistical analysis between presence of virus and high levels of *E. coli* or FRNA bacteriophage.

Table 1.- *E. coli* and FRNA phage counts and viral detection in Depuration center 1 (flow through shallow concrete tanks with ozone disinfection).

Sample	Species	Pre/ Post	Date	Depuration[a]		Results[b]		Viral detection[c]	
				time	T[a]	*E. coli*	Phage	HAV	NV
P1.1A	*M. galloprovincialis*	Pre	17.06.03			70	2100	-	-
P1.1B		Post	19.06.03	48 h	16	50	<30	-	-
P1.2A	*M. galloprovincialis*	Pre	24.06.03			500	<30	-	-
P1.2B		Post	26.06.03	48 h	16.5	20	700	-	-
P1.3A	*M. galloprovincialis*	Pre	01.07.03			40	<30	-	-
P1.3B		Post	03.07.03	48 h	17	<20	<30	-	-
P1.4A	*M. galloprovincialis*	Pre	08.07.03			20	<30	-	-
P1.4B		Post	10.07.03	48 h	17	<20	100	-	-
P1.5A	*M. galloprovincialis*	Pre	15.07.03			130	180	-	-
P1.5B		Post	17.07.03	48 h	17	90	<30	-	-
P1.6A	*M. galloprovincialis*	Pre	22.07.03			20	<30	-	-
P1.6B		Post	24.07.03	48 h	17	110	60	-	-
P1.7A	*M. galloprovincialis*	Pre	29.07.03			310	180	-	-
P1.7B		Post	31.07.03	48 h	17.6	70	<30	-	-
P1.8A	*M. galloprovincialis*	Pre	06.08.03			310	60	-	-
P1.8B		Post	07.08.03	48 h	19.3	40	1770	-	-
P1.9A	*M. galloprovincialis*	Pre	13.08.03			5400	360	-	-
P1.9B		Post	14.08.03	48 h	20.5	160	770	-	-
P1.10A	*M. galloprovincialis*	Pre	19.08.03			20	<30	-	-
P1.10B		Post	21.08.03	48 h	20.5	50	860	-	-
P1.11A	*M. galloprovincialis*	Pre	26.08.03			20	60	-	-
P1.11B		Post	28.08.03	48 h	19	200	590	-	-
P1.12A	*M. galloprovincialis*	Pre	02.09.03			40	<30	-	-
P1.12B		Post	04.09.03	48 h	18	250	1060	-	-
P1.13A	*M. galloprovincialis*	Pre	09.09.03			<20	<30	-	-
P1.13B		Post	11.09.03	48 h	17.6	20	120	+	-
P1.14A	*M. galloprovincialis*	Pre	17.09.03			<20	60	-	-
P1.14B		Post	18.09.03	48 h	17	250	<30	-	-
P1.15A	*M. galloprovincialis*	Pre	24.09.03			1750	60	-	-
P1.15B		Post	25.09.03	48 h	16.5	160	<30	-	-
P1.16A	*M. galloprovincialis*	Pre	30.09.03			70	360	-	-
P1.16B		Post	02.10.03	48 h	16	110	3767	-	-
P1.17A	*M. galloprovincialis*	Pre	07.10.03			1300	<30	-	-
P1.17B		Post	10.10.03	48 h	16.2	70	<30	-	-
P1.18A	*M. galloprovincialis*	Pre	14.10.03			40	<30	-	-
P1.18B		Post	16.10.03	48 h	16	1300	<30	-	-
P1.19A	*M. galloprovincialis*	Pre	21.10.03			<20	<30	-	-
P1.19B		Post	23.10.03	48 h	14.5	2800	60	-	-
P1.20A	*M. galloprovincialis*	Pre	28.10.03			110	120	-	-
P1.20B		Post	30.10.03	48 h	14.3	40	<30	-	-
P1.21A	*M. galloprovincialis*	Pre	04.11.03			<20	<30	-	-
P1.21B		Post	06.11.03	48 h	14.4	70	<30	-	-

[a] Temperature data are the average of the temperature during depuration.

[b] *E. coli* and phage numbers expressed as MPN/100g^{-1} and PFU/100g^{-1} shellfish meat respectively.

[c] HAV and NV detection by RT-PCR.

Table 2.- *E. coli* and FRNA phage counts and viral detection in Depuration center 2 (flow through bin system with chlorine disinfection).

Sample	Species	Pre / Post	Date	Depuration		Counts of		Viral detection	
				time	T[a]	*E. Coli*	Phage	HAV	NV
P2.1A	*M. galloprovincialis*	Pre	11.06.03			310	1x10 ^{5}	-	-
P2.1B		Post	13.06.03	48 h	17.5	130	500	-	-
P2.2A	*M. galloprovincialis*	Pre	17.06.03			220	<30	-	-
P2.2B		Post	19.06.03	48 h	16.5	50	<30	-	-
P2.3A	*M. galloprovincialis*	Pre	24.06.03			130	<30	-	-
P2.3B		Post	26.06.03	48 h	17.3	90	550	-	-
P2.4A	*M. galloprovincialis*	Pre	02.07.03			40	<30	-	-
P2.4B		Post	03.07.03	48 h	16.5	70	<30	-	-
P2.5A	*M. galloprovincialis*	Pre	08.07.03			40	<30	-	-
P2.5B		Post	10.07.03	48 h	17	20	<30	-	-
P2.6A	*M. galloprovincialis*	Pre	15.07.03			40	120	-	-
P2.6B		Post	17.07.03	48 h	17	220	<30	-	-
P2.7A	*M. galloprovincialis*	Pre	22.07.03			50	<30	-	-
P2.7B		Post	24.07.03	48 h	18.3	<20	120	-	-
P2.8A	*M. galloprovincialis*	Pre	29.07.03			130	120	-	-
P2.8B		Post	31.07.03	48 h	18.7	70	60	-	-
P2.9A	*M. galloprovincialis*	Pre	05.08.03			<20	60	-	-
P2.9B		Post	07.08.03	48 h	19.5	40	<30	-	-
P2.10A	*M. galloprovincialis*	Pre	12.08.03			20	<30	-	-
P2.10B		Post	14.08.03	48 h	22	20	<30	-	-
P2.11A	*M. galloprovincialis*	Pre	19.08.03			<20	<30	-	-
P2.11B		Post	21.08.03	48 h	22	<20	<30	-	-
P2.12A	*M. galloprovincialis*	Pre	26.08.03			40	<30	-	-
P2.12B		Post	28.08.03	48 h	20	40	60	-	-
P2.13A	*M. galloprovincialis*	Pre	02.09.03			<20	<30	-	-
P2.13B		Post	04.09.03	48 h	18.5	20	<30	-	-
P2.14A	*M. galloprovincialis*	Pre	09.09.03			<20	<30	-	-
P2.14B		Post	11.09.03	48 h	17.5	310	180	-	-
P2.15A	*M. galloprovincialis*	Pre	16.09.03			40	<30	+	-
P2.15B		Post	18.09.03	48 h	16.8	<20	60	+	-
P2.16A	*M. galloprovincialis*	Pre	24.09.03			40	1980	+	-
P2.16B		Post	25.09.03	48 h	16.4	20	240	+	-
P2.17A	*M. galloprovincialis*	Pre	07.10.03			3500	<30	-	-
P2.17B		Post	10.10.03	48 h	16.5	<20	<30	-	-
P2.18A	*M. galloprovincialis*	Pre	14.10.03			<20	<30	-	-
P2.18B		Post	16.10.03	48 h	16	<20	<30	-	-
P2.19A	*M. galloprovincialis*	Pre	21.10.03			40	<30	-	-
P2.19B		Post	23.10.03	48 h	14.5	<20	60	-	-
P2.20A	*M. galloprovincialis*	Pre	28.10.03			40	120	-	-
P2.20B		Post	30.10.03	48 h	14.3	<20	180	+	-
P2.21A	*M. galloprovincialis*	Pre	04.11.03			<20	120	-	-
P2.21B		Post	06.11.03	48 h	14.5	20	120	-	-

[a] Temperature data are the average of the temperature during depuration.

[b] *E. coli* and phage numbers expressed as MPN/100g^{-1} and PFU/100g^{-1} shellfish meat respectively.

[c] HAV and NV detection by RT-PCR.

Table 3.- *E. coli* and FRNA phage counts and viral detection in Depuration center 3 (flow through shallow concrete tanks with chlorine disinfection).

Sample	Species	Pre / Post	Date	Depuration		Counts of		Viral detection	
				time	T[a]	*E. Coli*	Phage	HAV	NV
P3.1A	*Ostrea edulis*	Pre	11.06.03			40	$5x10^5$	-	+
P3.1B		Post	13.06.03	47 h	17.3	<20	1000	-	+
P3.2A	*Cerastoderma edule*	Pre	17.06.03			520	5000	-	-
P3.2B		Post	19.06.03	44 h	16.5	70	1700	-	-
P3.3A	*C. edule*	Pre	24.06.03			50	200	-	-
P3.3B		Post	26.06.03	42 h	17	<20	<30	-	-
P3.4A	*O. edulis*	Pre	02.07.03			700	700	-	-
P3.4B		Post	03.07.03	42 h	17.5	70	<30	-	-
P3.5A	*O. edulis*	Pre	08.07.03			90	<30	-	-
P3.5B		Post	10.07.03	41 h	17.3	20	<30	-	-
P3.6A	*Ruditapes philipinarum*	Pre	15.07.03			1700	180	-	-
P3.6B		Post	17.07.03	42 h	16.8	950	<30	-	-
P3.7A	*O. edulis*	Pre	22.07.03			<20	420	-	-
P3.7B		Post	24.07.03	41 h	18.3	<20	<30	-	-
P3.8A	*O. edulis*	Pre	29.07.03			40	120	-	-
P3.8B		Post	31.07.03	41 h	18.5	40	<30	-	-
P3.9A	*C. edule*	Pre	05.08.03			40	120	-	-
P3.9B		Post	07.08.03	41 h	20	40	600	-	-
P3.10A	*Tapes* sp.	Pre	12.08.03			1700	590	-	-
P3.10B		Post	14.08.03	42 h	21.9	20	840	-	-
P3.11A	*C. edule*	Pre	19.08.03			40	60	-	-
P3.11B		Post	21.08.03	50 h	22	40	60	-	-
P3.12A	*O. edulis*	Pre	26.08.03			70	180	-	-
P3.12B		Post	28.08.03	40 h	21	50	120	-	-
P3.13A	*C. edule*	Pre	02.09.03			20	420	-	-
P3.13B		Post	04.09.03	47 h	18.5	70	120	-	-
P3.14A	*C. edule*	Pre	09.09.03			250	120	-	-
P3.14B		Post	11.09.03	44 h	17.5	50	1860	-	-
P3.15A	*O. edulis*	Pre	16.09.03			40	1160	+	-
P3.15B		Post	18.09.03	42 h	17.2	20	180	-	-
P3.16A	*C. edule*	Pre	24.09.03			750	<30	+	-
P3.16B		Post	25.09.03	42 h	16.5	130	<30	-	-
P3.17A	*M. galloprovincialis*	Pre	30.09.03			<20	<30	-	-
P3.17B		Post	02.10.03	41 h	17	160	<30	-	-
P3.18A	*M. galloprovincialis*	Pre	07.10.03			2400	<30	-	-
P3.18B		Post	10.10.03	42 h	16.9	40	<30	-	-
P3.19A	*O. edulis*	Pre	14.10.03			90	240	-	-
P3.19B		Post	16.10.03	41 h	16.1	40	<30	-	-
P3.20A	*M. galloprovincialis*	Pre	21.10.03			40	<30	-	-
P3.20B		Post	23.10.03	43 h	14.3	<20	<30	-	-
P3.21A	*O. edulis*	Pre	28.10.03			220	480	-	-
P3.21B		Post	30.10.03	42 h	14.5	160	<30	-	-
P3.22A	*C. edule*	Pre	04.11.03			2400	300	-	-
P3.22B		Post	06.11.03	42 h	14.5	430	1180	-	-
P3.23A	*O. edulis*	Pre	11.11.03			<20	120	-	-
P3.23B		Post	13.11.03	41 h	14.3	<20	180	-	-
P3.24A	*O. edulis*	Pre	02.12.03			20	1090	+	-
P3.24B		Post	04.12.03	42h	14	<20	120	+	-

[a] Temperature data are the average of the temperature during depuration.

[b] *E. coli* and phage numbers expressed as MPN/100g^{-1} and PFU/100g^{-1} shellfish meat respectively.
[c] HAV and NV detection by RT-PCR.

Table 4.-Percentage of samples in compliance with the *E. coli* and FRNA phage end product standards and detection of HAV and NVprior and after depuration[a].

	Prior to depuration				After depuration			
	E. coli	**Phage**	**HAV**	**NV**	***E. coli***	**Phage**	**HAV**	**NV**
Plant 1	71.42	71.42	0	0	80.95	57.14	4.76	0
Plant 2	90.47	71.42	9.52	0	89.39	71.42	14.28	0
Plant 3	66.66	25.00	12.50	4.16	91.66	58.33	4.16	4.16
Total	75.75	54.54	7.57	1.51	89.39	63.63	7.57	1.51

[a] End product standard for *E. coli* is <230 MPN/100 g^{-1} shellfish meat, and end product for FRNA bacteriophage is <100 PFU/100 g^{-1} shellfish meat, or a 95 % reduction in phage content after depuration.

Table 5.- Detection of Norovirus in randomly selected samples by real time PCR.

Sample	Species	Pre / Post	Date	TaqMan[a]	
				NV GI	NV GII
P1.1A	*M. galloprovincialis*	Pre	17.06.03	-	-
P1.1B		Post	19.06.03	-	-
P1.2A	*M. galloprovincialis*	Pre	24.06.03	-	+ (41.44)
P1.2B		Post	26.06.03	-	-
P1.3A	*M. galloprovincialis*	Pre	01.07.03	-	+ (48,24)
P1.3B		Post	03.07.03	-	-
P1.4A	*M. galloprovincialis*	Pre	08.07.03	-	-
P1.4B		Post	10.07.03	-	-
P1.5A	*M. galloprovincialis*	Pre	15.07.03	-	-
P1.5B		Post	17.07.03	+ (40.58)	-
P1.14A	*M. galloprovincialis*	Pre	17.09.03	+ (42.53)	-
P1.14B		Post	18.09.03	-	+ (42.38)
P1.15A	*M. galloprovincialis*	Pre	24.09.03	-	-
P1.15B		Post	25.09.03	-	-
P2.1A	*M. galloprovincialis*	Pre	11.06.03	-	-
P2.1B		Post	13.06.03	-	+ (40.29)
P2.1A	*M. galloprovincialis*	Pre	17.06.03	-	-
P2.2B		Post	19.06.03	-	-
P2.3A	*M. galloprovincialis*	Pre	24.06.03	-	-
P2.3B		Post	26.06.03	-	-
P2.4A	*M. galloprovincialis*	Pre	02.07.03	-	-
P2.4B		Post	04.07.03	-	+ (40,41)
P2.5A	*M. galloprovincialis*	Pre	08.07.03	-	+ (38.99)
P2.5B		Post	10.07.03	-	-
P2.8A	*M. galloprovincialis*	Pre	29.07.03	-	+ (41.43)
P2.8B		Post	31.07.03	-	+ (40.34)
P3.1A	*O. edulis*	Pre	11.06.03	-	-
P3.1B		Post	13.06.03	+ (44.49)	-
P3.2A	*C. edule*	Pre	17.06.03	-	-
P3.2B		Post	19.06.03	-	-
P3.3A	*C. edule*	Pre	24.06.03	-	-
P3.3B		Post	26.06.03	-	+ (40.13)
P3.4A	*O. edulis*	Pre	02.07.03	-	-
P3.4B		Post	04.07.03	-	-
P3.9A	*C. edule*	Pre	05.08.03	-	-
P3.9B		Post	07.08.03	-	-
P3.17A	*M. galloprovincialis*	Pre	30.09.03	-	-
P3.17B		Post	02.10.03	-	-

[a] Numbers in parenthesis are the Ct values obtained in the respective real time PCR reaction.

Discussion

The inadequacy of *E. coli* as an indicator of the viral risk associated with shellfish consumption is well documented (Power and Collins, 1989; Morse *et al.*, 1986; Chalmers and McMillan, 1995; Perret and Kudesia, 1995; Bosch *et al.*, 2001; Romalde *et al.*, 2002; Le Guyader *et al.*, 2003) and has prompted calls for investigations of alternative viral indicators (Doré *et al.*, 2000; Lees, 2000). The inadequacy of the *E. coli* standards was confirmed in this study, since samples meeting the bacterial criteria (< 230 MNP/100 g^{-1} shellfish meat) were positive for HAV or NV. If this finding can be a point of concern in samples harvested from the areas investigated, it is even more worrying when occurring in samples after undergoing a depuration process. Depuration has proved to effectively remove bacterial contamination, however, it seems clear that this process, as currently undertaken, is considerable less effective for virus elimination. In fact, no statistical correlation could be found in the present work between viral presence and high levels of *E. coli*. These findings support those obtained by other authors in numerous laboratory studies which indicated that viruses are eliminated from bivalve shellfish at a slower rate than faecal coliforms (Richards, 1988; Power and Collins, 1989; Jaykus *et al.*, 1994; Doré and Lees, 1995). A number of alternative indicators have been proposed during the last years for a better assessment of viral contamination in shellfish, including bacteria, bacteriophage and human viruses (Lees, 2000). Among them, one of the most promising candidates is FRNA bacteriophage (Havelaar, 1987; Chung *et al.*, 1998; Doré *et al.*, 2000), since its elimination kinetic during depuration reflects those of enteric viruses (Power and Collins, 1989; Doré and Lees, 1995). However, more experimental data are needed since it has been reported that different enteric viruses may purge from shellfish at different rates (Bosch *et al.*, 1995; Kingsley and Richards, 2003). Results obtained in this work, although preliminary, seem to indicate that FRNA bacteriophage may be a better indicator than *E. coli*, since the majority of the depurated samples positive for HAV or NV do not comply with the proposed FRNA phage-based end product standard (<100 PFU/100 g^{-1} shellfish meat or a reduction greater than 95 % during the depuration process; Doré *et al.*, 1998). However, as in the case of *E. coli*, a statistical correlation between levels of FRNA phage and presence of these viruses could not be established. One of the reasons that can explain this lack of correlation is the clear seasonal variation in levels of FRNA bacteriophage in shellfish prior to depuration, as previously reported by some authors (Doré *et al.*, 2000). An interesting finding of the present study was that in 6 % of depuration cycles investigated, levels of *E. coli* increased, in samples with low charge of this indicator prior to depuration, to values exceeding the end-product standard after depuration. An increase in the levels of FRNA bacteriophage was also observed in 15.15 % of the depuration cycles studied. The reasons for this fact of major concern are unclear, but it can be due to recontamination during the depuration process as a result of resuspension of sediments in the depuration tanks. Also it may indicates the existence of poor operational practices at the depuration plants studied, although we can not rule out the possibility of variability in *E. coli* and FRNA bacteriophage concentrations across the batches of shellfish.

In summary, results presented here demonstrated the poor removal of viruses during commercial depuration. Further studies are needed in order to determine the responsible factors (system design, species of shellfish, time, temperature, etc) with the aim of improving the depuration effectiveness and, therefore, the sanitary quality of shellfish for human consumption.

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COMPARATIVE RING-TRIALS FOR DETECTION OF HEPATITIS A AND NOROVIRUS IN BIVALVE MOLLUSCAN SHELLFISH AMONG EUROPEAN NATIONAL REFERENCE LABORATORYS (NRL) AND THEIR METHODS EMPLOYED.

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Abstract
The principal illnesses associated with the consumption of raw or lightly cooked shellfish produce, are viral gastroenteritis caused by norovirus and hepatitis caused by Hepatitis A virus. As the European Community Reference Laboratory (CRL) for microbial contamination in bivalve molluscan shellfish, CEFAS is charged with the responsibility of organising comparative testing/ring-trials for the assessment of the proficiency of laboratory detection for microbial contaminants including viruses, among European National Reference Laboratories (NRLs). In 2002 a pilot HAV ring-trial was organised among member states, followed by a further NV ring-trial in 2003. In addition to proficiency testing among laboratories, details of methodology was also collected. Methodology varied greatly among the NRL participants with diverse extraction methods in use. However, all procedures were based on Reverse Transcription - (RT) PCR Protocols varied with Single Round, Boosted and Nested PCR protocols being described.

In summary the results demonstrated both good and poor laboratory performance with all laboratories reporting results detecting the presence of NV and HAV in a least one of the samples to which virus had been added. However over interpretation of a single ring-trial series should be avoided. A further programme of ring-trials is planed to determine more reliably the effectiveness of laboratory techniques for the detection of enteric viruses.

Introduction
Sewage contaminated bivalve molluscan shellfish can present a significant risk if consumed raw or lightly cooked (Rippley 1994, Cliver 1997, Dore *et al*. 2004). To minimise these health risks most countries operate legislative controls on the harvesting and placing on the market of live bivalve shellfish (Lees 2004). Under the current European Shellfish Hygiene Directive (91/492/EEC), the microbiological requirements for bivalve molluscs being placed on the market are based on testing for bacterial indicators. Scientific progress has shown faecal bacteria are unreliable indicators of the presence of viruses in live bivalve molluscan shellfish. Despite such sanitary measures viral illness associated with shellfish consumption still regularly occurs.

Numerous PCR-based procedures have been reported for the detection of enteric virus contamination of mollusks from harvesting areas (Torrado *et al.*, 2003) and remain the only available procedures for the direct detection of non culturable enteric viruses in shellfish. The principle illnesses being gastroenteritis caused by *Norovirus* (NV) and infectious hepatitis caused by *hepatitis A virus* (HAV). Consequently EU Directive (1999/313/EC) makes reference to the necessity of introducing virus-testing procedures for bivalve shellfish. CEFAS, as the European Community Reference Laboratory (CRL) is responsible for ensuring a standardised

system throughout the European Community for the health checks required and for developing new procedures for more effective monitoring and control of viral contamination. An aspect of this is the responsibility of organising comparative testing/ring-trials for viruses in shellfish among the EU National Reference Laboratories (NRLs). Ring-trials have now been completed for HAV and NV detection in shellfish, which are reported here. The pilot ring-trial was conducted for HAV and consisted of three distributions among NRLs with the capability to carry out the relevant molecular assays required. The first two distributions required the analysis of various titres of tissue grown Hepatitis A virus, followed by the distribution of shellfish artificially contaminated with HAV in the laboratory. All analysis was performed according to the usual molecular methods applied in each participating laboratory. Following the completion of the hepatitis A ring-trial, the CRL developed and organised a further ring-trial for norovirus (NV). This followed the same approach as HAV with distributions of NV clinical (faecal) material followed by shellfish contaminated by NV in the laboratory.

This paper presents the results from both ring-trials together with an assessment of methods currently used for the detection of HAV and NV in polluted bivalve molluscan shellfish in NRLs in the European Community.

Materials and Methods

Hepatitis A (HAV) 2002/3

The pilot HAV ring-trial consisted of three distributions among NRLs who had agreed to participate, requiring the analysis of various titres of tissue grown Hepatitis A virus, followed by the assaying of shellfish contaminated with HAV. All analysis was performed according to the usual molecular methods employed in each laboratory.

Tissue grown Hepatitis A virus (strain HM 175).

HAV, strain HM175 was cultured on FRHK-4 cells, frozen, thawed and briefly spun. Supernatants were titrated and assayed by the CRL nested PCR method (Green *et al.*, 1998) for norovirus but utilising HAV 1st and 2nd round primers (Formiga-Cruz *et al.*, 2002) (Table 1). Supernatants were serially diluted in tissue culture media to create high and low titre test material. Negative test material was derived from control FRHK cell cultures.

Shellfish contaminated with HAV by bio-accumulation:

Pacific oysters (*C. giggas*) were sourced from a Grade 'A' harvesting area. Two hundred shellfish were placed in a 500 litre re-circulation system at 15^{o}C. 50 ml of inoculate (Hep A HM175, at a concentration 1.5 x 10^{6} pfu/ml) was added to the system. Shellfish were allowed to bio-accumulate for a 48h period. On harvesting a test extraction and titration was undertaken to assess the level of contamination.

Norovirus (NV) 2003/4

The CRL developed and organised a ring-trial for norovirus (NV). This followed the same approach as for HAV, with distributions of NV clinical (faecal) material followed by norovirus contaminated shellfish (Table 2). Two NV Containing faecal extracts were used as test material in the NV ring-trial. 10 % faecal extracts, one containing a NV genogroup one (GI) strain and the other containing and NV genogroup two (GII) strain, were prepared by mixing a 1:10 dilution (wt/vol.) of faecal stool in PBSa followed by centrifugation at 3000 g for 5 minutes. The supernatants were serially diluted in PBSa and analysed for NV using the CRL nested PCR method (Green and Henshilwood *et al.*, 1998) to determine the virus titre. Stock dilutions of a high and low concentration of each NV genogroup were made. A

number of 1 ml aliquots of each stock dilution were prepared and stored frozen at -20°C for distribution in the ring-trial. A representative vial of each stock dilution was then defrosted and assayed to ensure NV was still detectable. Similar vials containing buffer only were also prepared and frozen (NV negative samples). Both the GI and GII strains were confirmed as NV sequences, with the GI sharing >95 % homology with strain Pelelui (EMBL AY149297) and the GII strain sharing > 95 % homology with the common Grimsby (Lordsdale) strain.

Shellfish contaminated with NV by bio-accumulation::
Pacific oysters (*C. gigas*) were sourced from Grade 'A' harvesting area. Two hundred shellfish were placed in a 500 litre re-circulation system at 15°C. 50 ml of NV inoculate was then added (1:10 dilution wt/vol of Grimsby faecal material). Shellfish were allowed to bio-accumulate for a 48 h period. On harvesting a test extraction and assessment was conducted using a newly developed NV real-time assay (Jothikumar *et al.*, 2005).

Shipping and Analysis
Test material for ring trials was shipped to participants frozen on dry ice in accordance with IATA packing instruction 650 for UN3373 'Diagnostic Specimens'. Laboratories were instructed to assess the contents of each vial and shellfish in duplicate by assaying a stipulated volume and employing their 'in-house' PCR techniques. Ring trials for HAV and NV were conducted on separate occasions during 2002 to 2004.

Results
Laboratory Results for the 2002-3 HAV Ring-trial
Results for part 1 of the ring-trial (vials A-D) are shown in Table 4.
All NRL's reporting results detected the presence in at least one of the samples analysed to which HAV had been added. All NRL's with the exception of NRL007 reported the absence of HAV in vial A and C. NRL007 reported the presence of virus in vial C only. All NRL's detected the presence of HAV in vial B that contained the high titre preparation. All participants with the exception of NRL003 detected the presence of the low titre HAV in Vial D. However NRL002 only detected the presence of HAV in one out of the two replicates analysed.

Results for part 2 of the ring (vials E-H) trial are detailed in Table 5.
All NRL's consistently detected the presence of HAV virus in the neat construct of vial H. With the exception of NRL001 and NRL007, all NRL's detected the presence of HAV in vial E to which low titre HAV had been added. NRL002 detected the presence of virus in one out of two replicates tested for vial E, whilst NRL007 failed to detect the presence of the virus in either replicate assayed.

Results for part 2 of the ring (vials E-H) trial are detailed in Table 6.
NRL002 and 009 failed to return results for part three of the HAV ring-trial. Those participating NRL's consistently identified package 'Q' as being negative for the presence of HAV and Package 'R' (Bio-accumulated Shellfish) as being +ve for the presence of HAV.

Laboratory Results for the 2003-4 NV Ring-trial
Analytical methods used
A summary of analytical methods reported by participants for ring trial parts 1 and 2 are shown in Table 3a. Table 3b display methods employed by participants for part 3

of the ring trial. Participants are coded to permit comparison with results of analysis.

Results for ring-trial part 1(vials E, B, F and A (faecal sample)) Table 7.
All NRLs reporting results detected the presence of NV in at least one of the samples to which NV had been added. All NRLs except NRL 07 reported the absence of NV in vials to which NV had not been added. NRL 07 reported the presence of NV in vials to which NV had not been added. All NRLs except NRL 15 reported the presence of NV in vial B, which contained a high titre of NV G2. NRLs 05, 07, 08, 09, 13, and 15 reported the presence of NV in vial A, which contained a low titre of NV GI. NRL 08 and 15 reported this in only 1 of 2 replicates. NRLs 01, 03, 04, 14 did not report the presence of NV in vial A (low titre GI strain). NRLs 04, 05, 08, and 09, reported a genogroup I and correctly distinguished between NV GI and GII strains. NRL 14 reported genogroup but did not correctly distinguished between NV GI and GII strains. NRLs 03, 07, and 15 did not report NV genogroup.

Results for ring-trial part 2 (vials C, G, D and H (faecal sample)) Table 8.
NRLs 03, 07, 15 reported results for part 1 but not part 2 of the ring-trial. The NRLs participating (except NRL 14) correctly reported the presence of NV in vials to which NV had been added. NRL 14 did not report the presence of NV in any of the vials. All NRLs reported the absence of NV in vials to which NV had not been added. All NRLs (except NRL 14) reported the presence of NV in both the high titre GI sample (vial D) and the low titre GII sample (vial C). All NRLs correctly distinguished between NV genogroups.

Results for ring-trial part 3 (packages J and K (shellfish samples)) Table 9.
NRLs 07, 14 and 15 reported results for part 1 and/or part 2 but not for part 3 of the ring-trial. Only 7 NRLs of the 12 agreeing to participate reported results for part 3 of the ring-trial. Of the NRLs reporting results all correctly reported the presence of NV in package K (shellfish contaminated in the laboratory with a NV GII strain). Of those NRLs reporting results all correctly reported the genogroup as NV GII. Of those NRLs reporting results all reported the anticipated result for package J (grade A shellfish found not to contain NV by the CRL method) which was the absence of NV.

Discussion

Laboratory performance HAV

All participants detected the presence of HAV in one or more samples assayed. Four out of the nine participating NRLs correctly identified the presence of virus both in the concentrated and lower titre samples. Part I of the ring-trial was correctly identified by all participants with the exception of NRL 003 and NRL 007. NRL 003 failed to detected virus in the low titre vial D. This may have been a problem with sensitivity, whereas NRL007 who correctly identified the presence of low titre virus appeared to have a contamination problem, as virus was detected in negative vial C. In the second part of the ring-trial however, four out of nine participants detected the presence of virus in negative culture samples. Incorrect analysis was due to a combination of lack of sensitivity in the lower dilution sample (vial E, NRL007) and/or cross contamination of negative samples (NRL001, NRL002; NRL007 and NRL008). Although all participants correctly detected the presence of HAV in the laboratory bio accumulated shellfish (Package R), the levels of virus in these artificially contaminated samples analysed were extremely high. Analysis of the shellfish using nested PCR showed that virus could be detected down to a level of 10^{-3}. However, this is not representative of the low levels of virus normally found in the environmentally contaminated shellfish.

Laboratory performance NV

Most NRLs consistently reporting results performed very well in the ring-trial. These NRLs were clearly able to detect NV in faecal sample dilutions both high and low titre. These NRLs were also able to detect NV in shellfish samples contaminated in the laboratory and those which had not. These NRLs were also able to correctly distinguish between genogroups of NV. The most difficult sample to analysis was the low titre NV GI faecal sample (vial A) with only 6 of 10 participants successfully reporting the presence of NV. Only weak bands were visualised by gel electrophoresis by the CRL prior to dispatch, and, this was duly noted by other NRL participants. Thus assay sensitivity may not be equivalent in all laboratories. One NRL reported presence of NV in vials to which no NV had been added, indicating a problem with cross contamination. Four NRLs were only able to detect the presence of NV in the high titre GII sample and not the low titre GI sample. This probably indicates a problem with assay sensitivity, sample processing or broadness of reactivity for NV strains.

All remaining 7 NRLs reporting results for part 3 of the ring-trial correctly reported the anticipated result of presence of NV GII in one shellfish sample and absence of NV in the other shellfish sample. This is a good result for a first ring-trial distribution of shellfish material contaminated with NV. However, it should be noted that the CRL has analysed the NV containing shellfish sample by the recently developed NV real-time assay - which can give quantitative information – and the NV titre was found to be very high. The level of NV template found in the laboratory bio-accumulated shellfish was in excess of 10^6 times the limit of sensitivity of the real-time assay (CT value 26.2). This contrasts with environmentally contaminated shellfish samples, which typically contain no more than 10-100 times the limit of assay sensitivity (CT value 37.5 – 41). Clearly it would be advantageous to repeat this exercise with shellfish containing lower titres of NV template or with naturally contaminated samples.

.

Table 1. HAV Ring-trial material

Package	Sample	Quantity	Content
1	VIAL A	1 ml	-ve
	VIAL B	1 ml	+ve neat (high titre)
	VIAL C	1 ml	-ve
	VIAL D	1 ml	+ve –4 (low titre)
2	VIAL E	1 ml	+ve –4 (low titre)
	VIAL F	1 ml	-ve
	VIAL G	1 ml	-ve
	VIAL H	1 ml	+ve neat (High titre)
3	PACK Q	<50g	-ve shellfish
	PACK R	<50g	HAV contaminated shellfish

Table 2. HAV Ring-trial material

Package	Sample	Quantity	Content
1	VIAL A	1 ml	-ve
	VIAL B	1 ml	+ve neat (high titre)
	VIAL E	1 ml	-ve
	VIAL F	1 ml	+ve –4 (low titre)
2	VIAL C	1 ml	+ve –4 (low titre)
	VIAL D	1 ml	-ve
	VIAL G	1 ml	-ve
	VIAL H	1 ml	+ve neat (High titre)
3	PACK J	<50g	-ve shellfish
	PACK K	<50g	NV GII contaminated shellfish

Table 3a. Summary of methods reported by NRLs employed for the analysis of materials for parts 1 and 2 of NV ring trial.

NRL ID.	Nucleic Acid Ext.	RT – Protocol	PCR Protocol	PCR Cycling Parameters	Primers Used	Published Method	Additional Info
NRL 01	QIAamp Viral RNA Minikit (Qiagen)	OneStep RT-PCR Kit (Qiagen)	Titanium™ TaqDNA Polymerase (Clontech) Nested PCR	50ºC 30mins, 95ºC-15 mins and 35 cycles(94ºC-30sec, 42ºC-30sec, 72ºC-45sec) then 72ºC-10mins. Nested- 95ºC-1mins and 35 cycles(95ºC- 30 secs, 42ºC-60sec, 68ºC-60 sec) then 68ºC-3mins	Schreir *et al.*, 2000 ORF1: RNA polymerase gene (GI and G2) ORF 3: small basic protein region (G2) Vinje *et al.*, 2003.Primer mixture: ORF1 and ORF1 with modifications	YES	
NRL 03	NucleoSpin RNA II Kit (Macherey-Nagel, Durren, Germany)	Booster Protocol	Booster - PCR	94ºC-2mins and 40 cycles(94ºC-1min, 37oC-90sec, 68ºC-2min) then 68ºC-7mins	Vinje Primers:JV-12 and JV-13	No	
NRL 04	QIAamp Viral RNA Minikit (Qiagen)	Two Phase, Random Hexamers (PdN6)	Nested	94ºC-3 mins and 40 cycles(94ºC-60sec, 50ºC-30sec, 72ºC-2mins) then 72ºC-15mins. Nested- 94ºC-3mins and 40 cycles(94ºC- 30 secs, 50oC-30sec, 72ºC-60 sec) then 72ºC-7mins	PCR: Nishida *et al* 2003 GG1: COG1F +GISKR GG2:COG2F +G2SKR Nishida *et al.*, 2003 + Kojima *et al.*,2002 GG1: G1SKF +G1SKR GG2: G2SKF + G2SKR	Yes/no	
NRL 05	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green and Henshilwood *et al.*, (1998)	96ºC-10mins and 35 cycles(94ºC- 60 secs, 37ºC-60sec, 72ºC-60 sec) then 72ºC-10mins. Nested Same	SM31, Ando, NI ,E3	Yes/No	

NRL 07	Vertrel, viral precipitation with PEG Viral RNA extraction with Trizol. RNA adsorption in an affinity RNA matrix	OneStep RT-PCR Kit	Single Round	-	-	-	-
NRL 08	Boom *et al*, (1990)	JV131 specific primers	Single Round	94°C-2mins and 40 cycles(94°C- 60 secs, 37°C-90sec, 74oC-60 sec) then 74°C-7mins	Vinje Primers: JV-12Y and JV-13I	Yes	GI and G2 determination of sample using hybridisation.
NRL 09	QIAamp Viral RNA Minikit (Qiagen)	MuLV RT Protocol (Applied Biosystems)	Single roundTaq Polymerase Protocol (Applied Biosystems)	40 cycles (94°C-30sec, 50°C-30sec, 72°C-30sec) then 72°C – 7mins		YES	Hybridisation using Chemiluminesence
NRL 13	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green and Henshilwood *et al.*, (1998)	96°C-10mins and 35 cycles(94°C- 60 secs, 37°C-60sec, 72°C-60 sec) then 72°C-10mins. Nested Same	SM31, Ando, NI ,E3	Yes/No	
NRL 14	QIAamp Viral RNA Minikit (Qiagen)	OneStep RT-PCR Kit (Promega)		48°C-45mins, then 94°C-2mins, then 40 cycles (94°C-30sec, 50°C-1min, 68°C-min) and 68°C-7mins.	JV12 and JV13 – Vinje, *et al* (1996)	YES	
NRL 15	Boom (1990)	OneStep RT-PCR Kit (Qiagen)	QuantiTect SYBRGreen PCR Kit (Qiagen)	95°C–15mins then 40 cycles (94°C–20sec, 49°C-90sec) and 72°C-30sec.	RT/1st Rnd - *MJV12, RegA* 2nd Rnd PCR – *p290, Mp290*	NO	Smartcycler real-time PCR machine. (Cepheid)

(**Table 3a** Continued)

Table 3b. Summary of methods reported by NRLs employed for the analysis of materials for part 3 NV ring trial.

NRL ID.	Virus elution from tissue	Nucleic Acid Ext. and Purification	RT – Protocol	PCR Protocol	PCR Cycling Parameters	Primers Used
NRL 001	Whole animal homogenised in glycine buffer. Precipitation using PEG. Resuspension of pellet in Tri-reagent (Sigma).	Addition of chloroform followed by incubation and centrifugation. Aqueous layer precipitated in isopropanol. Pellets washed in ice-cold ethanol and resuspended in RNase free water. Addition of RNA binding buffer + Dynabeads-oligo$(dT)_{25}$, followed by washing protocols. Final resuspension in Rnase-free water and heating to liberate RNA from Dynabeads.	OneStep RT-PCR Kit (Qiagen)	Nested PCR	50°C 30mins, 95°C-15 mins and 35 cycles(94°C-30sec, 42°C-30sec, 72°C-45sec) then 72°C-10mins. Nested- 95°C-1mins and 35 cycles(95°C- 30 secs, 42°C-60sec, 68°C-60 sec) then 68°C-3mins	SM31, GI, GII Ando, NI ,E3
NRL 003	Whole animal Homogenised in glycine buffer. PEG precipitation (twice). Pellets resuspended in PBSA. RNA extraction using chloroform. Aqueous phase for analysis.	NucleoSpin RNA II Kit (Macherey-Nagel, Durren, Germany)	Two Phase JV13 primer, AMV reverse transcript-ase	Booster – PCR + Southern Blotting (Bergman *et al.*,1995)	94°C-2mins and 40 cycles(94°C-1min, 37°C-90sec, 68°C-2min) then 68°C-7mins	Vinje Primers: JV-12 and JV-13 Polymerase region
NRL 004	Stomach and Digestive diverticula removed via dissection. Homogenised in PBSa + antifoam B + chloroform-butanol. Homogenate added to Cat-Floc T. Following centrifugation aqueous phase removed for PEG precipitation.	Digestion with proteinase k. Extraction using Phenol-Chloroform-Water. Aqueous phase precipitated in ethanol. Pellets resuspended in water + CTAB. Resuspension of pellets in water and cetyltrimethyl-ammoniam bromide + NACl. Following incubation and centrifugation pellets resuspended in saline and precipitated in a solution containing ethanol and sodium acetate.	Two Phase, Random Hexamers (PdN6)	Nested	94°C-3 mins and 40 cycles (94°C-60sec, 50°C-30sec, 72°C-2mins) then 72°C-15mins. Nested- 94°C-3mins and 40 cycles (94°C- 30 secs, 50°C-30sec, 72°C-60 sec) then 72°C-7mins	PCR: Nishida *et al* 2003 GG1: COG1F +GISKR GG2:COG2F +G2SKR Nishida *et al.*, 2003 + Kojima *et al.*,2002 GG1: G1SKF +G1SKR GG2: G2SKF + G2SKR

NRL 005	Dissection of Glands (Stomach and Digestive diverticula). Homogenisation and addition of equal w/v Proteinase K. Incubation followed by inactivation of enzyme. Centrifugation and removal of supernatant for storage and analysis.	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green and Henshilwood *et al.*, (1998)	96°C-10mins and 35 cycles(94°C- 60 secs, 37°C-60sec, 72°C-60 sec) then 72°C-10mins. Nested Same	SM31, GI, GII Ando, NI ,E3
NRL 008	150 mg of digestive diverticula and 850 µl lysis buffer RLT (Qiagen; Plant and fungi kit, Maryland, USA) were added to a 2 ml eppendorf tube filled for 50 % with Zirconia beads (1.0mm Zirconia beads, BioSpec Products, Inc., Bartlesville, Canada) and homogenised for 40 seconds at 4.0 m/sec using the Hybaid ribolyserTM Cell Disrupter (Hybaid, Milford, USA).	RNeasy® Mini Kit (Qiagen, Plant and Fungi protocol).	Two phase JV13I primer AMV rev. transcriptase	Single round	94C-2min and 40 cycles (94C- 60 secs37°C- 90 sec, 74°C-60 sec) then 74°C-7min	Vennema *et al.*, (2002) J Clin Virol 25:233-235 (modified Vinje primers)-
NRL 009	Stomach and Digestive diverticula removed via dissection. Homogenised in PBSa + antifoam B + chloroform-butanol. Homogenate added to Cat-Floc T. Following centrifugation aqueous phase removed for PEG precipitation.	Following digestion with proteinase K, Samples extracted with phenol-chloroform-water and Aqueous phase precipitated in ethanol. Pellets resuspended in water + CTAB. Resuspension of pellets in water and cetyltrimethyl-ammoniam bromide + NACl. Following incubation and centrifugation. Pellets resuspended in saline and precipitated in a solution containing ethanol and sodium acetate.	MuLV RT (Perkin Elmar) Specific Primers	Single Round + Hybridisation	40 cycles (94°C-30sec, 50oC-30sec, 72°C-30sec) then 72°C – 7mins	Polymerase: P110/NI, P110/36, P110/4562. Capsid: G1SKF/SKR and G2SKF/SKR
NRL 013	Dissection of Glands (Stomach and Digestive diverticula). Homogenisation and addition of equal w/v Proteinase K. Incubation followed by inactivation of enzyme. Centrifugation and removal of supernatant for storage and analysis.	Modified Boom	Two Phase, Random Hexamers	Nested PCR, Green and Henshilwood *et al.*, (1998)	96oC-10mins and 35 cycles (94oC- 60 secs, 37oC-60sec, 72oC-60 sec) then 72oC-10mins. Nested Same	SM31, GI, GII Ando, NI ,E3

Table 3b (Continued)

Table 4. Tabulated Results reported by participating NRL's for the 2002/3 Hepatitis A Ring-trial, Part 1.

VIAL	A -	B neat	C -	D -4	A -	B neat	C -	D -4
NRL001	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
NRL002	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
NRL003	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve
NRL004	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
NRL005	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
NRL007	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
NRL008	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
NRL009	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
NRL014	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve

Table 5. Tabulated Results reported by participating NRL's for the 2002/3 Hepatitis A Ring-trial, Part 2.

VIAL	E -4	F -	G -	H neat	E -4	F -	G -	H neat
NRL001	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
NRL002	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve
NRL003	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
NRL004	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
NRL005	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
NRL007	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
NRL008	+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve
NRL009	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
NRL014	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve

Table 6. Tabulated Results reported by participating NRL's for the 2002/3 Hepatitis A Ring-trial, Part 3 (Shellfish).

VIAL	Q -	Q -	R +	R +
NRL001	-ve	-ve	+ve	+ve
NRL002*				
NRL003	-ve	-ve	+ve	+ve
NRL004	-ve	-ve	+ve	+ve
NRL005	-ve	-ve	+ve	+ve
NRL007	-ve	-ve	+ve	+ve
NRL008	-ve	-ve	+ve	+ve
NRL009*				
NRL014	-ve	-ve	+ve	+ve

*These laboratories did not return results for the ring-trial.

Table 7. Tabulated results reported by participating NRL's for the 2003/4 NV ring trial, part 1 (faecal samples). Results reported by NV genogroup are indicated in columns headed GI and GII. +/- indicates result as presence or absence of NV.

	E			B			F			A			E			B			F			A		
	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-
NRL001	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
NRL002 *																								
NRL003 [a]			-			+			-			-			-			+			-			-
NRL004	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
NRL005	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
NRL007						+			+			+			+			+			+			+
NRL008	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-
NRL009	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
NRL012 *																								
NRL013	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
NRL014	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
NRL015 [a]			-			-			-			-			-			-			-			+

* These laboratories did not return results for the ring trial.

[a] Results reported as just presence or absence of NV are reported only in columns headed +/-.

Table 8. Tabulated results reported by participating NRL's for the 2003/4 NV ring trial, part 2 (faecal samples). Results reported by NV genogroup are indicated in columns headed GI and GII. +/- indicates result as presence or absence of NV.

	C			G			D			H			C			G			D			H		
	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-
NRL001	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL002*																								
NRL003*																								
NRL004	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL005	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL007																								
NRL008	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL009	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL012*																								
NRL013	-	+	+	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+	-	-	-
NRL014	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NRL015*																								

*These laboratories did not return results for the ring trial.

Table 9. Tabulated results reported by participating NRL's for the 2003/4 NV ring trial,part 3 (shellfish). Results reported by NV genogroup are indicated in columns headed GI and GII. +/- indicates result as presence or absence of NV.

	J			K			J			K		
	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-	GI	G2	+/-
NRL001	-	-	-	-	+	+	-	-	-	-	+	+
NRL002*												
NRL003	-	-	-	-	+	+	-	-	-	-	+	+
NRL004	-	-	-	-	+	+	-	-	-	-	+	+
NRL005	-	-	-	-	+	+	-	-	-	-	+	+
NRL007*												
NRL008	-	-	-	-	+	+	-	-	-	-	+	+
NRL009	-	-	-	-	+	+	-	-	-	-	+	+
NRL012*												
NRL013	-	-	-	-	+	+	-	-	-	-	+	+
NRL014*												
NRL015*												

* These laboratories did not return results for the ring trial.

Conclusion

NRLs reported a variety of sample extraction protocols. Most common were silica based extraction procedure, namely the 'QIAmp Viral RNA Minikit' and 'Boom' based extraction methods. RT-PCR protocols also varied with both nested and single round protocols being described utilising a variety of different primers. It is however clear from ring-trial reports that the various methods currently in use cannot be interpreted as giving equivalent performances. This clearly points to the need to address the development of standard methods for detection of NV in shellfish.

Clearly these ring-trials have been a useful exercise. It has allowed participants to compare their routine methods of analysis. However, it is difficult to effectively assess the performance of a given method and participant from one trial. To truly gauge the efficacy of techniques for the detection of norovirus and HAV, we need to look at the results produced by participants from multiple ring-trials. Repeated failures or successes would offer clear evidence of a given technique suitability to the task.

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All European National Reference laboratories who participated in this study between 2002 –2004.

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DEVELOPING A STRATEGY TO LIMIT SHELLFISH VIRAL CONTAMINATION

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Abstract
Pathogenic microorganisms can be present in seawater anytime raw sewage or contaminated input is directly discharged along the coast. The complexity of the various parameters involved in the occurrence of faecal microorganisms in the environment has to be considered to determine the actions to address and solve this problem and to limit the viral risk.

For shellfish, viral contamination clearly occurs in the first stage of the production process, i.e. in growing and harvesting areas. During periods of epidemics, a large viral input can spread in the environment from sewage networks or inefficient sewage treatment plants. These sources of information could be applied for shellfish quality surveillance by defining the "weekly or monthly risk". Viruses persist in seawater and shellfish for a long time (several weeks or months). To limit the risk, the following actions should be proposed. They would mainly reduce viral input to harvesting areas and thus efficiently protect the environment. The first action consists in documenting all the critical points, which can affect the shellfish beds. By gathering data, it will be possible to identify the main viral sources and determine the corrective actions to be taken. The second action leads to the recommendation to implement an early warning system for shellfish production sites, since contamination can occur even when corrective actions reduce the risk. Different parameters could be recorded (rainfall, salinity, sewage network key-points, epidemics, etc.) and collected in a database. Specific events which could lower the water quality could be detected in this way and shellfish producers immediately informed. The early warning system could be a model with potential for use on all European production sites. However, if shellfish are contaminated despite efforts made to reduce viral input in harvesting areas, HACCP procedures during processing must be reinforced: shellfish traceability and increased depuration must be implemented and controlled.

Introduction
Contrary to other foodstuffs, in shellfish, viral contamination clearly occurs in the first step of the process, *i.e.* in growing and harvesting areas (Figure 1). There is no proof that other routes (food-handlers, aerosol) could be at the origin of the seafood contamination (Koopmans and Duizer, 2004). The shellfish trade's annual turnover represents 456 M Ecu per year in Europe: 775,000 T of shellfish are regularly shipped over EU countries for consumption in different places. This free exchange of shellfish on the European market is important for sustainable development of shellfish production. Nevertheless, risk assessment must take into account the hazards originating from the traditional consumption of raw or only lightly cooked bivalve shellfish. Shellfish, and especially oysters, are currently suspected of being implicated in gastro-enteritis outbreaks occurring in different European countries. The EU's "Rapid Alert System for Foodstuffs" indicates each year the implication of shellfish of different origins in outbreaks in Europe.

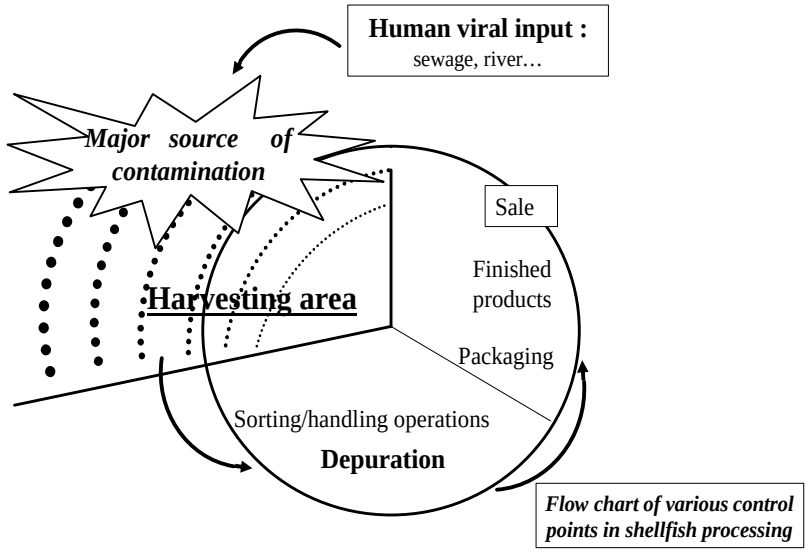

Figure 1. Proven route of shellfish contamination during the process and HACCP in shellfish processing.

Most of the time, the presence of viruses in shellfish is due to untreated sewage. However, direct contamination by people who are ill working in the growing area has also been reported (Berg *et al.*, 2000; Butt *et al.*, 2004). The presence of pathogens in the environment mainly depends on the density of coastal urban and animal populations. Seasonal outbreaks or rainfall inputs also contribute by modifying the load of pathogens released. During the epidemic period, a large viral input can spread in the environment from sewage networks or inefficient wastewater treatment plants (Miossec *et al.*, 1998). Some years, new epidemic strains give rise to winter epidemics in the population. For example, during the last main outbreak in France, 6 million people became ill over a 6-week period (January 2001- February 2001). The same observation was made around Europe (Lopman *et al.*, 2004). If we consider a rate of infection equivalent to 3 % of the population, and a viral concentration in faeces of about 30 million particles/patient, the viral flux from 15,000 inhabitants could be estimated at 600,000 viruses per minute (Pommepuy *et al.*, 2004). When combined with weather events (rainfall) or any failure in the sewage network, this could create viral shellfish contamination.

Epidemics are recorded in the human population by European Networks (Réseau Sentinelle – France (Sentiweb: www.b3jussieu.fr), RIVM - The Netherlands, PHLS – UK., etc.). These sources of information are interesting for shellfish quality monitoring and could help define "weekly or monthly risk" in a risk assessment procedure. Viruses persist in seawater and shellfish for a long time (weeks or months) (Wait and Sobsey, 2001; Bosch, 1995). Depuration, which is a procedure included in HACCP processing, cannot solve the problem with the present depuration systems using seawater at local water temperature: < 12°C during epidemic months (Lees, 2000; Doré and Lees, 1995; Sobsey and Jackus, 1991). However, depuration based on shellfish immersed in tanks of seawater at higher temperatures (20-25°C) for a long period, could - under specific conditions (low

contamination level, recent contamination) - partially solve the problem (Pommepuy *et al.*, 2004; Doré *et al.*, 2004). But in any case, this cannot provide a real guarantee of safe products because of the persistence of some viral species. Moreover, very little information exists regarding undesirable indirect effects at this temperature (*Vibrio* growth). Furthermore, this process is not suited to all species of shellfish (clams, mussels, cockles, etc.) and considerable mortality is observed for shellfish subjected to these temperatures. This process should only be used in exceptional circumstances, when there is a known enteric virus risk. For these reasons, alternative solutions including better knowledge about the faecal source of surface water from the catchment area, their limitation and their survival with an early warning system rather than focusing on shellfish depuration alone are proposed.

How to limit the risk?

1. Tracking the sources

Rivers are the main pathways for matter from natural and anthropogenic sources carried after weather events, from land to sea (Crowther *et al.*, 2001, 2002).

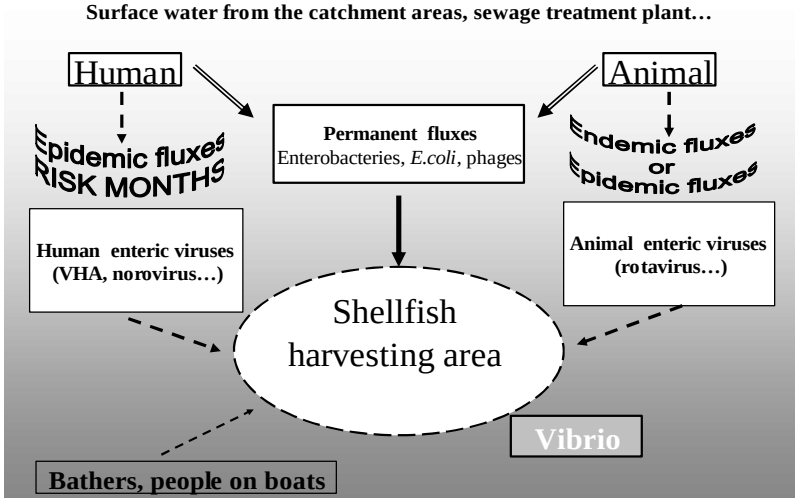

Figure 2. Major routes of faecal pollution in coastal areas.

Three different types of faecal fluxes exist (Figure 2). Permanent faecal fluxes from intestinal flora (estimated with *E. coli* and *Enterococcus)*, epidemic pathogenic fluxes resulting from human or animal outbreaks (ex: viruses) and endemic fluxes (ex: animal-borne bacteria) may reach estuaries and coastal waters. Among other sources of contamination, bathers, boats or vessels could also have a significant impact on water and sediment contamination (Gerba, 2000; Sobsey *et al.*, 2003; Dowel *et al.*, 1995) also demonstrating that overboard disposal of sewage, currently practiced in harvesting areas, could be at the origin of faecal oyster contamination.
Studies must be undertaken to document all the critical points (*i.e.* the sources of contamination, whether urban, industrial or agricultural), which can affect water quality in a harvesting area. Table 1 reports the main factors influencing the microbial quality of water in coastal areas and some examples of possible preventive actions, control or monitoring (Derolez, 2003).

Table 1. Determination of the potential sources which can affect coastal water quality.

	Hazard Analysis	**Parameters to gather**	**Preventive, control or monitoring procedure**
Sewage input (Document the sources: status quo)	*Human population*	Density Age of urban network and STP Population trends Harbour proximity	Establish the list of main outfall/sources, control network and survey
	Animal population	Density Density in the area 500 m from shoreline Animal population trends Farm effluents	Control agriculture regulations applications in the area and the compliance with laws (especially for slurry spreading)
	Network or existing survey	Open data bases Real-time data	Assess the trends in parameters for the latest period (ten years)
Factors influencing the microbial quality of the area	River fluxes STP (capacity, type, age) Wastewater network (age, combined or separate sewers) Marshy shore	Catchment area (urban or agriculture activities) Conformity Performance By-pass Lift station Sewage discharge Parasite water Drainage	Control the local urban planning and agricultural program. Control standards and enforcement of regulations Application of regulations Check the discharge
Aggravating factors	Outbreak in population Weather	Human gastro-enteritis Amplitude Period, length	Survey Survey

Gathering data will lead to identifying the main viral sources and determining of corrective actions to be taken: repairing the sewage network, building a new sewage treatment plant (STP) with a high capacity, biological treatment; eliminating illegal sewage outfalls in coastal areas, and so on.

Assessing pollution risks for harvesting areas is also linked to the shellfish and the influencing/aggravating factors of water quality. Table 2 reports the main critical points and the monitoring procedures to be undertaken in order to preserve and/or improve the quality of this activity.

Table 2. Critical points and seawater quality in harvesting areas.

Parameters	Hazard Analysis	Critical points	Preventive, control or monitoring procedure
Status quo	Activity	Shellfish species	Disease surveillance
		Reared on racks, ground, ropes	Silt removal
	Harvesting type	Growing	
		Stocking	Traceability
		Laying	
		Refining	
	Site description	Estuary, bay.	Description, Observation
		Water renewal	
		Tidal amplitude	
		Shore amplitude	
		Soil: silt, sand, etc.	
Influencing factors	STP and river input	Salinity variation, SM	Network
		Water stratification	
	Tide	Contaminants	
		Microbiological quality	
		Amplitude	*In situ* recording
	Rainfall	Percentage emersion	
		Salinity decrease	*In situ* recording
Aggravating factors	Inputs	Salinity decrease	*In situ* recording
	Season	Low temperature	*In situ* recording
	Urban input	Viral input	Population survey

To manage the risk, preventive and corrective actions must be taken in the event of a virus alert. Depending on the intended destination of potentially contaminated batches, different actions have to be recommended (Table 3).

Table 3. Preventive and corrective actions in the event of a virus alert.

Type of stock	Batches intended for	Preventive action	Corrective action
Spat	Transfer	/	/
18 months	Transfer	Traceability	More rigorous traceability
2 years	1. Sale to the public	Viral testing	More rigorous depuration
	2. Transfer	Traceability and Viral testing	Depuration prior to sale
More than 2 years	1. Sale to the public	Viral testing	More rigorous depuration
	2. Transfer	Traceability and Viral testing	More rigorous depuration prior to sale

2. Implementing an early warning system for shellfish production sites

Contamination may occur even when corrective actions have reduced the risk: a weather event, for example could cause sewage run-off, an STP could fail, etc... To avoid shellfish being contaminated, the required control points have to be monitored and an early warning system could be set up in coastal areas (Figure 3). Different parameters could be recorded (rainfall, salinity, sewage network key-points, epidemics, etc.) and gathered in a database. An automated monitoring network has already been proposed to survey toxic algal blooms in a fish farm in an estuary in south-eastern Tasmania (Butler *et al*, 2001). Specific events which could lower the water quality could thus be detected and shellfish producers immediately informed (ex: Figure 3, alarm from point 2).

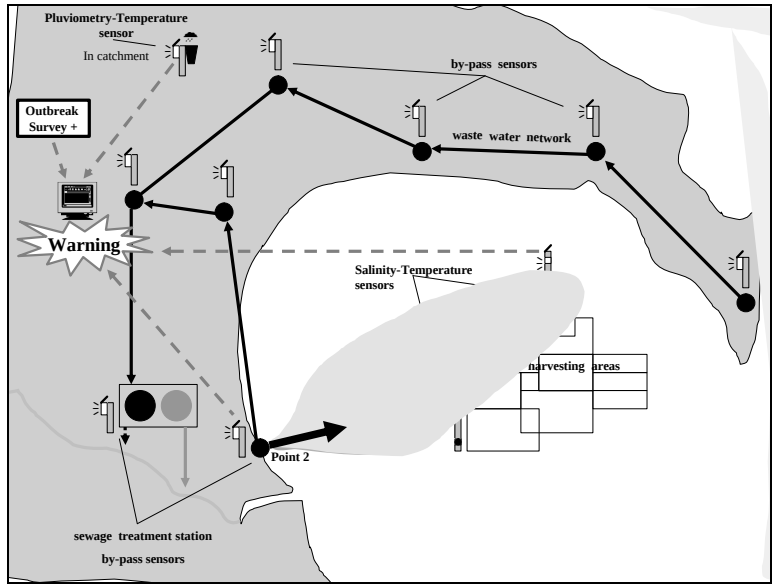

Figure 3. Early warning system implementation and operation

The *in situ* validation of selected parameters is required for such systems: a comparison of resulting water quality conditions has to be established on the different sites and can serve as the basis for the water quality advice. Recent development of such models based on a simple relationship between observed rainfall and pathogen concentrations or other systems based on complex models of the prevalent mixing and transport processes are already used for bathing waters risk management (EAP, 1999). A water quality model could be applied and used in the same way to manage the shellfish contamination risk. Once validated, the early warning system will produce a model which is potentially usable on all European production sites.

Conclusion

To limit the viral risk in shellfish, different propositions have been made, including the use of HACCP procedures and depuration (Jackson and Ogburn, 1997; NSSP, 1993; Furfari *et al.*, 1992; West, 1986). But because of the persistence of some species of viruses after depuration, a viral risk could persist even after reinforced purification. For that reason, further investigations in harvesting areas are

necessary to protect water quality from microbial contamination, and they should not only focus on shellfish depuration.

Critical points in harvesting areas and « high-risk months », epidemic outbreaks in population, must be taken into consideration to establish a warning system which can alert shellfish producers and administration when a contamination event could potentially occur. This warning system could prevent contaminated shellfish from being marketed and thus enhance consumer protection. When viral alerts occur, stringent depuration must be implemented. Traceability and HACCP procedures have to be reinforced to produce safe shellfish. If applied, these recommendations will be a real benefit for Europe, concerning economic development (shellfish trade), social stability (employment), safety and health protection and quality of life (reducing contamination in coastal areas).

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SANITARY QUALITY OF BIVALVES MARKETED IN PORTUGAL

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Abstract
In the European Union *Escherichia coli* is used to indicate the sanitary quality of bivalves sold for human direct consumption, although it is thought to present limited predictive value for viral pathogens. Male-specific RNA bacteriophages have been suggested as human enteric pathogenic viruses indicators for bivalves and proposed as additional sanitary control for depuration. The levels of FRNA bacteriophage contamination of oysters are documented to some extent, however little information is available regarding species of commercial interest in the South of Europe, such as clams. In this study the quality of 82 depurated bivalves was investigated using both the legislative and proposed indicators. *E. coli* and FRNA bacteriophages levels were determined over a 6-month period for purified bivalves marketed in Portugal, including clams, cockles, furrow shells and Portuguese oysters. Unexpectedly, only 60 % of the shellfish complied with the mandatory EC *E. coli* standard presenting 11 % levels higher than 4600 per 100 g. The additional application of the FRNA bacteriophage standard lowered by 20 % the overall compliance rate, particularly for oysters and clams. Findings suggest the urgent need to implement better purification practices and enforcement and indicate an impact on shellfish industry and product quality if adopting viral indicators for depuration.

Introduction
Bivalves are filter feeders and if the water in which they are grown is polluted, then shellfish may become contaminated and be injurious to consumers. Because bivalve molluscan shellfish are often consumed raw (or slightly cooked) and whole (including their gastrointestinal tract), they are generally classified as a high-risk food group. In order to reduce the risk associated with sewage-contaminated shellfish, live bivalve molluscs are purified in tanks of clean seawater by a process termed depuration. Controlled self-purification (depuration) of bivalves has been extensively used worldwide, constituting a major control point of their production. Historically, enteric bacteria have been adopted as surrogate indicator organisms to assess the quality of shellfish, and, consequently, to predict the risk of exposure to enteric pathogens. Nowadays, the legal standards for live bivalve molluscs official control purposes in the European Union countries are based exclusively in bacteriological parameters, such as faecal coliforms or *E. coli*. This latter criterion has been widely used to indicate the sanitary quality of bivalves sold for human direct consumption, which must comply with a standard of less than 230 *E. coli* per 100 g of shellfish (Anon., 1991). Nevertheless, this sanitary control is thought to have limited predictive value for viral pathogens and its reliability as a single indicator of depurated shellfish quality has been questioned. In fact, the occurrence of viral diseases, principally gastro-enteritis caused by Norovirus and infectious hepatitis caused by hepatitis A virus, associated with the consumption of shellfish complying with *E. coli* sanitary limits have been demonstrated, as referred in the review by Lees (2000) and Richards (2003). As a result, studies have been conducted with alternative indicator organisms, such as male-specific (F) RNA bacteriophages, to model virus removal during depuration (Doré *et al.*, 2000; Pommepuy *et al.*, 2003). Some of them indicated that the level of FRNA bacteriophages in oysters after depuration was related to the risk of virus infection. Consequently, a standard

for the control of shellfish purification, with limits of removal ≥ 95 % or to ≤ 100 pfu FRNA bacteriophages/100 g during the process, has been proposed by the European Commission. Although the levels of FRNA bacteriophage contamination of depurated oysters at the point of sale are documented to some extent (Doré *et al.*, 2000), little information is available regarding species of commercial interest in the South of Europe, such as clams. So, it is important to determine the possible impact on industry and bivalves sanitary quality of implementing a proposed European process criterion for depuration of such species. The aim of this work was to evaluate the compliance of purified bivalves placed on the market using the existing (*E. coli*) and proposed (FRNA bacteriophages) faecal indicators.

Material and methods

Samples of clams – *Venerupis* spp. (n=22), cockles – *Cerastoderma edule* (n=18), furrow shells – *Scrobicularia plana* (n=18) and Portuguese oysters – *Crassostrea angulata* (n=24), presenting a health mark from a purification centre and produced in more than one member state of Southern Europe, were collected in a supermarket in Lisbon area from May to October 2003. Shellfish were transported and stored at 2-6ºC and examined within 24 hours of collection. Duplicate pooled samples comprising a minimum of 10 individual animals were used. *E. coli* was assayed by a two-stage most probable number procedure of five tubes with three dilutions, incorporating a resuscitation step followed by confirmation in chromogenic agar (Donovan *et al.*, 1998), and F-RNA bacteriophages enumeration was performed by the double-agar-layer method, using at least 10 ml of supernatant, according to ISO 10705-1 (Anon., 1996).

Results and discussion

Unexpectedly, only 49/82 samples corresponding to 60 % of the tested shellfish presented levels of *E. coli* lower than 230 per 100 g, complying with the mandatory EC *E. coli* standard (Figure 1, A-D). Levels of contamination higher than 4600 *E. coli* per 100 g were registered for 11 % of samples (9/82), but all samples were *Salmonella*-free.

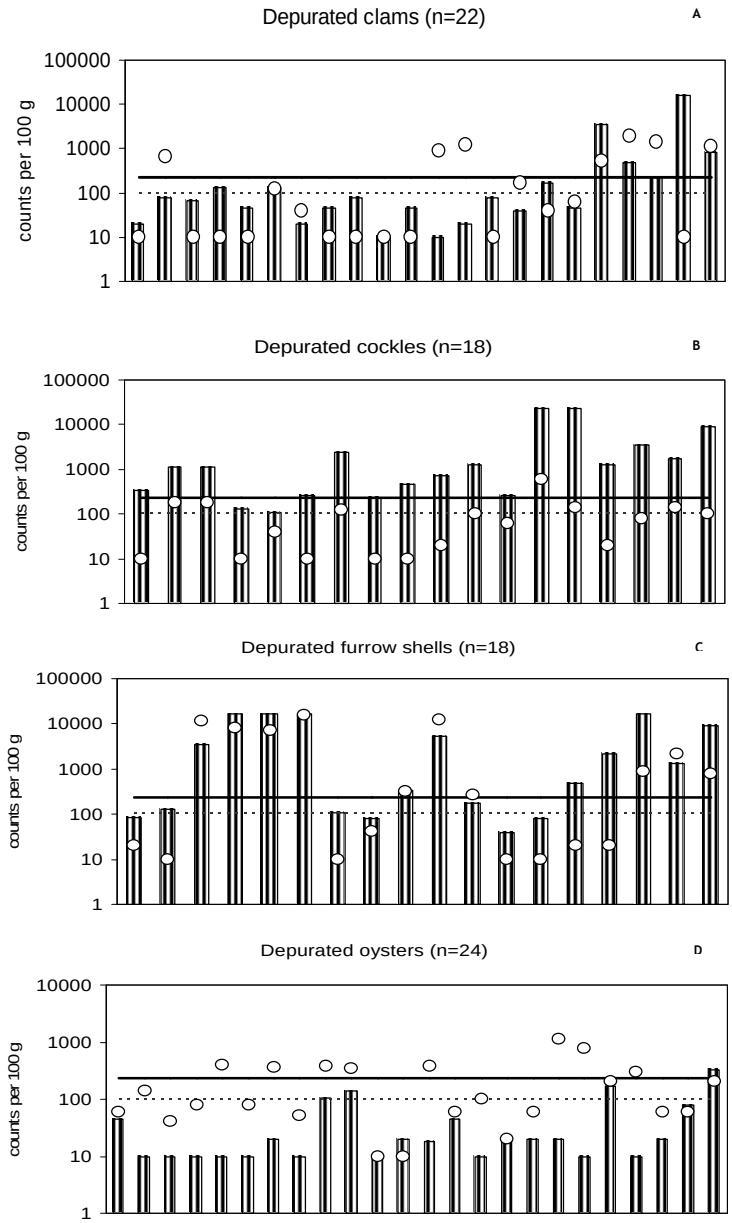

Figure 1 – Levels of *E. coli* ■ and F-RNA ⬭ in depurated bivalves, expressed respectively as mpn and pfu per 100 g of shellfish. Results are shown for clams (A); cockles (B); furrow shells (C) and oysters (D). The line and dotted line indicate, respectively, the mandatory limit for *E. coli* (<230 mpn 100 g^{-1}) and the proposed limit for FRNA (≤100 pfu 100 g^{-1}).

The failure to meet the existing criteria of <230 *E. coli* per 100 g of shellfish observed in the end products tested may be due to either high faecal pollution of shellfish growing areas, inadequate operation and/or design of the purification systems, direct contamination of product by food handlers, or storage in unhygienic conditions. In what concerns the first two reasons, quality improvements could be achieved by restricting the pollution loadings of shellfish entering the purification process and by stipulating legally minimum depuration times. Concerning species variation, results showed that 17/22 (77 %) of clams, 2/18 (11 %) of cockles, 7/18 (39 %) of furrow shells and 23/24 (96 %) of Portuguese oysters complied with the *E. coli* limit established in Directive 91/492 (Anon., 1991). The sanitary quality of the Portuguese oysters placed in the market was higher than that recorded for the other shellfish species examined, but not as good as that described in the UK for market-ready oysters (*Crassostrea gigas*), where all the shellfish complied with the mandatory EC *E. coli* standard and 90 % of those samples presented levels of contamination at or below the level of sensitivity of the assay (Doré *et al.*, 2000). Additionally, one clam, two cockles and four furrow shells had levels of *E. coli* higher than 10^4 per 100 g, suggesting that these products were never introduced in purification tanks. Unfortunately, no published study focusing on these species was found with which to compare the above results. Levels of FRNA bacteriophages lower than 100 pfu per 100 g were detected just in 56 % of shellfish samples, representing 13 clams, 12 cockles, 8 furrow shells and 13 Portuguese oysters (Figure 1, A-D). Some of the FRNA bacteriophages could possibly have been introduced in the products after depuration. No correlation was observed between the legal and proposed criteria. So, the simultaneous adoption of the *E. coli* limit (<230 mpn/100 g) and F-RNA bacteriophage standard (≤100 pfu/100 g) to the depurated market-bivalves originated still lower compliance rates (40 %). It was observed that the additional application of the proposed viral indicator criteria decreased by 20 % the overall compliance rate. This was particularly evident for oysters and clams, the species of commercial interest, which in the latter case exhibited only 54-55 % of samples complying with both criteria, representing a decrease in compliance of 42 % for oysters and 23 % for clams.

Conclusion

The introduction in the market of bivalve molluscan shellfish with high faecal contamination, as observed in this work for species other than oysters, constitutes a real health risk for consumers of shellfish, in particular coastal populations of Southern Europe that often eat raw clams and cockles. The frequent failure to meet the mandatory *E. coli* criteria, observed in the depurated market products tested, suggests the urgent need to implement better depuration practices and enforcement in some member states, including Portugal. Nevertheless, the adoption of more restrictive criteria for harvesting in contaminated areas can possibly improve consumer protection. The simultaneous application of the *E. coli* criteria and F-RNA bacteriophage standard to the depurated market-bivalves generally originated lower rates of compliant samples, particularly in species of commercial interest, such as oysters and clams, indicating a significant impact on shellfish industry and product quality if adopting viral indicators for depuration.

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SANITARY IMPACT OF SHELLFISH CONTAMINATION BY MeHg AND PAHs.

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Abstract
Sanitary risk assessments related to chemical contamination of seafood products, including shellfish, by Methylmercury (MeHg) and Polycyclic aromatic hydrocarbons (PAHs) have been evaluated by the French food safety agency. The methodology used is based on a deterministic calculation, in which French individual consumption data are crossed with mean French contamination level for each foodstuff. Results of these food exposure assessments show that the shellfish contribution to the global intake of MeHg is negligible whereas the contribution of PAHs can reach 25 % (adults). On the whole, shellfish accounts for 3 to 9 % of the adults' global intake and it doesn't exceed 5 % of the children's one. The Agency has also proposed two guide values for molluscs, crustaceans and cephalopods as indicative values in circumstances of accidental pollution like in the cases of Erika and Prestige. However, the quality of sanitary risk assessment related to consumption of contaminated shellfish would be improved by more knowledge on bioaccumulation kinetics of toxics by shellfish and on contamination data, a key element of food exposure assessment.

Introduction
AFSSA [Agence Française de Sécurité Sanitaire des Aliments], the French food safety agency is requested to evaluate sanitary risks in relation with occurrence of contaminants in foodstuffs. This was recently the case for Polycyclic aromatic hydrocarbons (PAHs) and Methylmercury (MeHg) in seafood products (AFSSA opinion 2003b, 2004). One step of the sanitary risk characterisation is the dietary exposure assessment. Only this step will be developed in the present paper as it deals with shellfish contribution to the whole dietary exposure.

Dietary exposure assessment
In the cases of MeHg and PAHs assessments realised by AFSSA the methodology is based on a deterministic calculation in which individual consumption data are crossed with the mean contamination level for each foodstuff.
Consumption data
Consumption data result from a national survey (INCA, Enquête individuelle nationale sur les consommations alimentaires), conducted in 1998/1999, for 7 days running, on 3003 persons (1985 adults and 1018 children). The mean dietary consumption per person of shellfish and crustacea (oysters, mussels, prawns, crabs) is 0.005 kg/day for adults (over 15 years) and 0.002 kg/day for children (3 to 14 years).
Contamination data
Contamination data are extracted from national plans of monitoring or control, realised between 1997 and 2002. For MeHg (table 1), shellfish represent 688 of 2500 data collected on sea products. MeHg concentrations were estimated from total mercury concentrations through correction with a corresponding factor for each product, according to literature (Claisse *et al.*, 2001; Cossa *et al.*, 1990) and not one common factor (43 %) as used in a previous assessment.

Table 1 : Statistical parameters for MeHg contamination in foodstuffs classified by sea products, in mg/kg fresh matter.

Category	Sample number	Mean concentration	Standard deviation	Mediane	P95	mini-maxi
Top predatory fish	650	0.259	0.348	0.163	0.774	0.002-3.698
Non predatory fish	1090	0.051	0.055	0.035	0.154	0.001-0.737
Molluscan bivalve	661	0.015	0.026	0.011	0.029	0.001-0.576
Crustacean	27	0.048	0.033	0.037	0.098	0.004-0.142
Cephalopod	22	0.039	0.025	0.036	0.082	0.002-0.095
Echinoderm and gasteropod	6	0.02	0.006	0.02	0.029	0.015-0.033

For PAHs, as the 16 PAHs[1] proposed by US-EPA for food contamination assessment were not systematically analysed in all the samples, the data retained for assessing exposure relate to the 6 PAHs common in the analysis results from 1999 and 2001: benz(a)anthracene, benzo(b+j)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i) perylene. Shellfish represent 155 of 978 data collected. Tables present contamination data with two hypothesis for not detected results: the value allocated is 0 (table 2a) or the value of the quantification limit divided by 2 (LOQ/2) (table 2b).

[1] Acenaphtene, acenaphtylene, anthracene, benz(a)anthracene, benzo(b+j)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, benzo(a)pyrene, chrysene, dibenz(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3,-c,d)pyrene, naphthalene, phenanthrene, pyrene. From US-EPA (1984). Guidelines establishing test procedures for the analysis of pollutants under Clean Water Act: Method 610 – polynuclear aromatic hydrocarbons. Fed. Reg. 49. 43344-43352 [CFR part 136].

Table 2a: Statistical parameters for contamination in foodstuffs classified by category and expressed for 6 PAH in µg TEQ/kg FM (fresh matter), for B(a)P in µg/kg FM and for 6 PAH in µg/kg FM **(not detected=0)**

		Concentration of 6 PAH (µg TEQ/kg FM)			**Concentration of B(a)P (µg/kg FM)**			**Concentration of 6 PAH (µg/kg FM)**		
Food category	**No. sam**	**Mean**	**Stan. Dev.**	**P95**	**Mean**	**Stan. Dev.**	**P95**	**Mean**	**Stan. Dev.**	**P95**
Cereals 1	56	0.18	0.17	0.52	0.14	0.14	0.40	0.57	0.63	1.81
Cereals 2	14	0.04	0.9	0.23	0.03	0.08	0.17	1.19	0.32	0.83
Cereals 3	15	0.20	0.24	0.52	0.15	0.20	0.41	0.76	0.78	2.01
Oils	24	1.40	1.60	4.70	0.52	0.76	1.45	3.96	4.38	11.87
Meat products	68	0.65	1.38	3.60	0.30	0.71	1.52	1.18	3.00	7.49
Fresh fish	424	0.08	0.15	0.27	0.05	0.11	0.19	0.32	0.65	1.20
Smoked fish and products	19	0.08	0.24	0.68	0.06	0.19	0.52	0.33	0.84	2.57
Shellfish and crustacea	155	1.05	1.50	3.84	0.45	0.93	1.91	6.15	7.70	20.78
Vegetables (ex. potatoes)	85	0.04	0.13	0.20	0.01	0.06	0.10	0.30	0.75	1.10
Potatoes and related	9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dried fruit, nuts, oleaginous seeds	37	0.01	0.60	0.25	0.00	0.03	0.00	0.60	0.36	0.00
Snacks	22	0.86	1.96	6.43	0.46	1.04	3.36	4.55	10.40	33.97
Pizzas, quiches and savoury pastries	22	0.54	1.04	1.98	0.43	0.85	1.69	1.60	3.17	4.57
Fruits	10	0.00	0.00	0.02	0.00	0.00	0.00	0.03	0.07	0.15
Sugar and related products	18	0.13	0.23	0.55	0.08	0.15	0.40	0.65	1.13	2.20

Table 2b: statistical parameters for contamination in foodstuffs classified by category and expressed for 6 PAH in µg TEQ/kg FM (fresh matter), for B(a)P in µg/kg FM and for 6 PAH in µg/kg FM **(not detected =LOQ/2)**

		Concentration of 6 PAH (µg TEQ/kg FM)			Concentration of B(a)P (µg/kg FM)			Concentration of 6 PAH (µg/kg FM)		
Food category	**No. sam**	**Mean**	**Stan. Dev.**	**P95**	**Mean**	**Stan. Dev.**	**P95**	**Mean**	**Stan. Dev.**	**P95**
Cereals 1	56	0.33	0.14	0.57	0.17	0.11	0.40	0.97	0.54	1.94
Cereals 2	14	0.15	0.08	0.29	0.07	0.07	0.17	0.45	0.25	0.93
Cereals 3	15	0.25	0.23	0.57	0.15	0.19	0.41	0.88	0.75	2.08
Oils	24	1.45	1.56	4.73	0.53	0.75	1.46	4.18	4.31	11.92
Meat products	68	0.93	1.27	3.61	0.33	0.70	1.53	2.62	2.76	7.65
Fresh fish	424	0.13	0.16	0.32	0.07	0.10	0.19	0.43	0.64	1.36
Smoked fish and products	19	0.53	0.18	0.94	0.20	0.15	0.52	1.54	0.60	2.87
Shellfish and crustacean	154	1.33	1.45	3.89	0.56	0.89	1.90	6.80	7.46	20.79
Vegetables (ex. potatoes)	85	0.20	0.12	0.31	0.06	0.06	0.10	0.67	0.70	1.40
Potatoes and related	9	0.17	0.00	017	0.05	0.00	0.05	0.40	0.00	0.40
Dried fruit, nuts, oleaginous seeds	37	0.43	0.06	0.56	0.12	0.05	0.25	1.25	0.25	1.30
Snacks	22	1.14	2.00	6.68	0.51	1.02	3.37	5.27	10.32	34.47
Pizzas, quiches and savoury pastries	22	1.05	1.01	2.23	0.56	0.79	1.69	2.72	2.97	5.08
Fruits	10	0.17	0.05	0.17	0.05	0.00	0.05	0.42	0.05	0.50
Sugar and related products	18	0.18	0.23	0.60	0.09	0.14	0.40	0.48	1.20	2.26

Approach to assess the risks from dietary exposure to a PAHs mixture
Exposure results to PAHs are expressed in 3 different units: TEQ (toxic equivalent quantity), mass or B(a)P.

The TEF (toxic equivalence factor) concept is based on the basic premise that, for the various molecules taken into account, the same effect for which there is a common origin (mechanism) is considered. The TEFs were developed to answer the question: how can one indicate the toxicity of a complex mixture of substances for which at least one toxic mechanism of action is common to them all? TEFs therefore represent values used to weight the respective mass of each of the components of a mixture in order to indicate their relative toxic efficacy with respect to a given effect. This value is defined in comparison with a reference substance for its teratogenic and carcinogenic effects observed in animals; in the case of PHAs, this is B(a)P. The product of "TEF x mass of the component" makes it possible to calculate a toxic equivalent quantity (TEQ) for each component. The toxic equivalents of all the components of the mixture are then added together to define, as the TEQ, the relative toxicity of the mixture. For its assessments, AFSSA used the TEF scale proposed by Nisbet and LaGoy (1992) except concerning diB(a,h,)A, which TEF value

was changed from 5 to 1, because 5 is very high compared with the factors used for this molecule by other authors (between 0.69 and 1).

Results

Results of these food exposure assessments (table 3) show that the shellfish contribution to the global intake of methylmercury is negligible. It's hardly 3 % of the most sensitive populations global intake (infant, pregnant and nursing women). Concerning PAHs, shellfish contribution to the global intake can reach 25 % (adults), when contamination is expressed in TEQ and when non detected values are set to ½ of the limit of quantification. On the whole, shellfish accounts for 3 to 9 % of the adults' global intake and it doesn't exceed 5 % of the children's one.

Table 3 : Mean exposure assessments of shellfish consumer to MeHg and PAHs

	MeHg µg/pers/week *(% of shellfish in the global intake)*	6 HAP		B(a)P alone µg/pers/d
		µg TEQ/pers/d	µg/pers/d	
Adults	0,36 - 0,44* *(2 to 3 %)*	0,093[a] - 0,239[b] *(5.1 - 25 %)*	0,319[a] - 0,597[b] *(8.7 - 5.1 %)*	0,060[a] - 0,089[b] *(3.3 - 2.8 %)*
Children (3-14 years)	0,45 - 0,83 *(1 to 2 %)*	0,072[a] - 0,180[b] *(2.6 - 1.4 %)*	0,245[a] - 0,454[b] *(4.7 - 2.8 %)*	0,047[a] - 0,069[b] *(1.0 - 1.4 %)*

* according to age , [a] <LOQ = 0 ; [b] <LOQ = LOQ/2.

Guide values in case of accidental pollution

In its reports on the pollution caused by the wreck of the tanker Erika off the coast of Brittany (France) in December 1999 and that of the Prestige off the coast of Galicia (Spain) in December 2002 (AFSSA opinion, 2000, 2003a), AFSSA recommended two guide values for molluscs, crustaceans and cephalopods, according to which PAHs are analysed (the 16 from US-EPA or the 6 used by AFSSA) : 0.5 mg/kg dry matter for the sum of 16 PAHs and 0.2 mg/kg dry matter for the sum of 6 PAHs. These values were selected on the basis of the high level of the background pollution (levels observed in organisms from areas near polluted factories) on the one hand, and on the other hand, according to analytical methods characteristics (HPLC and florescence, GC/MS). As a second result of this new assessment AFSSA recommends to analyse the following 11 PAHs: anthracene, benz(a)anthracene, benzo(b)fluor-anthene, benzo(j) fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, benzo(a) pyrene, chrysene, dibenz(a,h)anthracene, fluoranthene, indeno(1,2,3,c-d)pyrene, for risk assessment of PAHs in foodstuffs. These 11 PAHs were selected according to the US-EPA list (most commonly found in the environment) and the IARC classification (toxicological aspect). AFSSA also recommends a guide value for non-processed bivalve, molluscs, cephalopods and crustacean of 10 µg TEQ/kg fresh matter for 11 PAHs (with the TEQ unit to take into account the toxic potential). These guide values should be considered as indicative values in circumstances of accidental pollution and, in view of safety elements taken into account when producing them, they could be used as the basis for calculation of exclusion threshold (2 to 5 times the guide values) designed to limit risk of consumer exposure to this contaminant. In the case of two

pollution incidents, Erika and Prestige, the risk managers set the exclusion values at twice the recommended guide values.

Conclusion

The quality of the sanitary risk assessment related to consumption of contaminated shellfish would be improved by more knowledge on bioaccumulation kinetics of toxics, influence of their concentrations in water, influence of physiological variations, relations between concentrations of contaminants in water, in sediment and in shellfish. For exemple, AFSSA was required in 2002 to evaluate sanitary risk linked to consumption of shellfish exposed to sea water with high concentration of cadmium. Experts noticed that the risk was strongly dependant on the ratio between particulate and dissolved cadmium, itself influenced by climatic condition, salinity on the one hand and on the other hand, dependant on bioaccumulation and metabolism in shellfish, mainly because of the influence of metallothionin on cadmium accumulation kinetics.

Finally contamination data, a key element in food exposure assessment, need to be completed. Except for few metals, PCB and dioxins, regularly sought in the scope of European and French monitoring plans, these data are too incomplete for many contaminants, in such a way that their assessment is not reasonably feasible.

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INFLUENCE OF SEVERAL BIOLOGICAL PARAMETERS ON *PCBs* LEVELS IN GALICIA MUSSEL *Mytilus galloprovincialis* L. (NW, SPAIN)

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Abstract
Influence of some biological parameters on content of PCBs (IUPAC nº 31, 28, 52, 101, 118, 153, 105, 138, 156 and 180) in cultivated and wild mussels (*Mytilus galloprovincialis*) coming from 3 zones in Galician coast (Sada, Puebla and Santa Cruz) were investigated by using bivariate techniques. Only the concentration of CB105 was positively correlated with fat content, shell length, condition index and flesh weight. Multivariate techniques of data exploration (Principal Components Analysis (PCA) and Cluster Analysis (CA)) have been applied to know spatial and temporal trends of PCBs levels in investigated mussels. PCBs were extracted by using Soxhlet device and analysed by GC-ECD and GC-MS.

Introduction
Polychlorinated biphenyls are physically stable, hydrophobic, persistent and ubiquitous contaminants that have a high affinity for lipid content of biota, so, they are bioaccumulated along food chain (Erickson 1997). In addition to lipid content, other biological endogenous factors such as condition index or size can have influence on bioaccumulation of PCBs. Lipid content of mussel is strongly connected to gonad development cycle because mussel looses part of lipid content during spawning period (Hawkins and Bayne 1992). Allometric ratios and physiological conditions can also be strongly influenced by exogenous factors such as temperature, light and salinity. The objective of this paper is to establish the relationships between levels of PCBs and biological factors, that could affect bioaccumulation (condition index, shell length, flesh weight and lipid content), in mussels coming from three sites in Galician estuarine bays (Rías). Raft mussels from Sada A and Puebla E in Ría de Ares-Betanzos and Ría de Arousa, respectively, and wild mussels from Santa Cruz in Ría de A Coruña, were collected at intervals of two months over a year-long period from February 2002 to April 2003.

Materials and Methods

Samples
Raft cultured mussels (*Mytilus galloprovincialis*) coming from Puebla E (Ría de Arousa) and Sada A (Ría de Ares-Betanzos) polygons, wild mussels (*Mytilus galloprovincialis*) coming from Santa Cruz (Ría de A Coruña) were collected each two months in the period from February 2002 to April 2003 (see Figure 1).

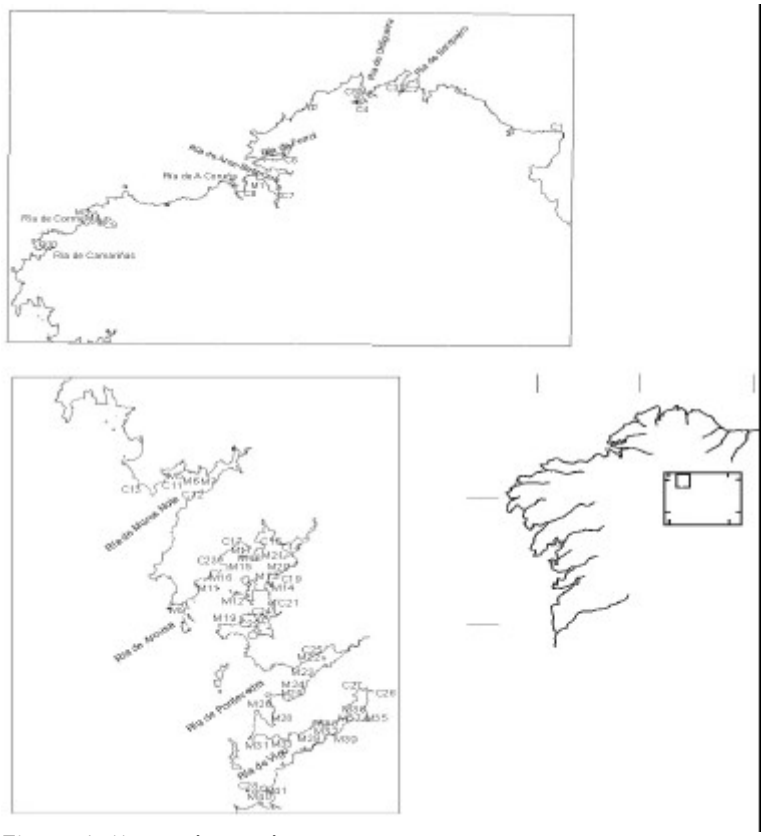

Figure 1. Map with sampling points.

Reagent and Apparatus

n-Pentane, dichloromethane, isooctane, aluminium oxide, silica gel and anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany). Analytical reagent grade PCB individual congener standards were supplied for Dr. Ehrenstorfer (Augsburg, Germany). A certified reference material, mussel tissue homogenate, supplied by National Institute of Standards and Technology (NIST 2977) was used for quality control. For quantitative gas chromatographic determinations, calibration was performed at five concentration levels for each congener spanning the range of 1-600 μg L^{-1} and using PCB 155 (700 μg L^{-1}) as internal standard. The concentrated extracts were analysed by high resolution gas chromatography using a Perkin-Elmer Autosystem Gas Chromatograph equipped with an electron capture detector (ECD). A TRB-5 (Teknokroma, Spain) 5 % diphenyldimethyl siloxane capillary column (60 m x 0,20 mm i.d. x 0,4 μm phase thickness) was used. The confirmation of polychlorinated biphenyl compounds was performed on a Varian Saturn 2000 Gas Chromatograph-Ion Trap Detector Mass Spectrometer (ITD). Data numerical analysis was performed by means of Minitab v.14 statistical software.

Quality assurance

Calibration curves have been carried out at five concentration levels using suitably diluted standards. The correlation coefficient was 0.999 for all target compounds. Experiments of method reproducibility were performed in five replicate samples

providing a mean relative standard deviation of 8.8 % for studied PCBs; the mean recoveries of target compounds were from 86.00 to 106.3 % . The detection limits were calculated as three times the standard deviation of the peak height for 30 determinations of the blank and were from 0.07 to 0.2 ng g^{-1} for studied PCBs.

Procedure

Sample preparation

Mussel flesh was frozen (-30 ºC), freeze-dried and Soxhlet extracted (5 g sample; 150 mL dichloromethane-pentane, 1-1; 8 hours) by duplicate. An aliquot of the extract was used to determine gravimetrically the lipid content. Interfering lipids were removed from a suitable portion of the extract by column chromatography over alumina (6 % deactivated), target compounds were eluted with n-pentane. PCBs fraction was separated using column chromatography on silica (1 % deactivated). Sample extract was concentrated under vacuum evaporation to near dryness and then redissolved in 1 mL of isooctane. PCB 155 was added as an internal standard prior to analysis by gas chromatography (González-Quijano and Fumega 1996).

Condition index

Condition indices were calculated taking account shell and body weights of each sample (thirty individual mussels). CI= [(body weight)/(body + shell weights)].

Results and Discussion

Statistical analysis

Statistical analysis considers 10 congeners of PCBs (IUPAC nº 31, 28, 52, 101, 118, 153, 105, 138, 156 and 180, recommended by the International Council for Exploration of the Seas, ICES, Duinker *et al*. 1988) and 4 biological parameters (fat content, shell length, condition index and flesh weight) as quantitative variables. Concentrations of PCBs reported as "not detected" were assigned of half the detection limit. Sampling zone and month are considered as qualitative variables.

Bivariate analysis.

In order to check the influence of biological variables on chemical variables, one-way ANOVA by using Fisher's method was performed. This analysis indicated a positive significant effect ($p<0.05$) of quantitative biological parameters on PCB 105 levels ($p=0.039$ for condition index, shell length and flesh weight and 0.002 for fat content). PCB 105 is a coplanar and mono-ortho Cl_5-congener whose particular structure could infer different chemical and physical properties. Even so, the relationship between PCB congeners and lipid content is very complex. Fat nature, extraction methods and difference between individual mussels have an important role which is difficult to know (Ewald *et al*. 1998). Spatial and temporal trends were studied by using one-way ANOVA, thus sampling zone and month were included as factors. Table 1 shows that when all mussel samples (wild and raft) were statistically analysed, both biological variables and higher chlorinated congeners levels (PCBs 101, 118, 153, 138, 156 and 180) established significant differences between zones. This fact can be explained by the great levels of the higher chlorinated compounds in investigated samples because they supply majority composition in commercial mixtures (Aroclors 1264 and 1260) and by their low mobility and water solubility in relation to the lower chlorinated compounds that means they remain closer to the source of the pollution. The lower chlorinated congeners are more water soluble and are diluted in marine environment reaching levels of concentration more homogenous (Wania and Mackay 1996). If only raft mussels (Sada A and Puebla E) were studied, the same variables than above, except

shell length (p=0.451) and flesh weight (p=0.103), showed a significant zone term in ANOVA, it means that all of raft mussels studied in this paper had similar sizes, but different lipid contents. In relation to temporal trends (sampling month variable), only the lower chlorinated biphenyls PCBs 31 and 28 presented a statistical significance (p=0.016 and p=0.010, respectively) occurring the highest levels in April. This can be explained because both congeners present high volatility, that allows to keep them during a long time in the atmosphere. In rainy periods they are deposited in sea and oceanic waters "humid deposition".

Table 1. Summaries of F ratios and sum of squares from analysis of variance (One Way) on differences in biological and chemical variables between mussels from studied zones..

One way	Sum of squares	F	P
	0.22	51.13	0**
Cond. Index			
	6327	97.40	0**
Shell length			
	289.57	52.22	0**
Flesh weight			
	50.54	14.30	0**
Lipid content			
CB31	0.289	1.29	0.287
CB28	0.3379	1.78	0.182
CB52	0.468	2.06	0.141
CB101	51.71	15.70	0**
CB118	30.49	11.11	0**
CB153	7759	26.30	0**
CB105	167	1.16	0.325
CB138	3033.1	25.20	0**
CB156	74.09	10.78	0**
CB180	249.65	35.71	0**

** Correlation is significant at the 0.01 level (2-tailed)

Multivariate analysis

Spatial trends

If ANOVA significant biological and chemical parameters (condition index, shell length, flesh weight, fat content, PCBs 101, 118, 153, 138, 156 and 180) were used in PCA (Principal Components Analysis) as variables (the type of matrix was correlation), the retained first three principal components explained the 88.2 % of the total data set. If the loading of the variables in the first three components was studied (see table 2), the contribution of biological and chemical variables to each principal components can be evaluated. The variables negatively contributing to the first component were the chemical parameters indicatives of contamination (PCBs 118, 153, 138, 156 and 180). The second component was mainly related with mussel size since the most contributing variables were shell length and flesh weight. The third principal component can be interpreted as lipid content. Three groups corresponding to the three studied zones (Sada A, Puebla E and Santa Cruz) appear in Figure 2 where the first two components are shown. The first group formed by samples from Puebla E was mainly influenced by the positive part of the first

component (negative score coefficients of chemical variables), mussels with the lowest PCBs levels. The second group, mainly related to the negative and positive part of the first and second component, respectively, (pollution indicative and mussel size), was formed by Sada A, samples with high levels of PCBs and good biological conditions, a great data dispersion appears in this group. The third group mainly influenced by the negative part of the two first principal components was formed by Santa Cruz, the highest polluted and smallest mussels.

Table 2. Loading of the variables in the first three components of the first PCA

Compound	Component		
	1	2	3
Condition Index	0.295	0.148	0.427
Shell Length	0.292	0.464	0.259
Flesh Weight	0.290	0.471	0.282
Lipid Content	0.223	0.470	-0.748
CB101	-0.280	0.298	-0.177
CB118	-0.316	0.266	0.175
CB153	-0.327	0.171	0.022
CB138	-0.322	0.290	0.032
CB156	-0.289	0.095	0.204
CB180	-0.331	0.041	0.070

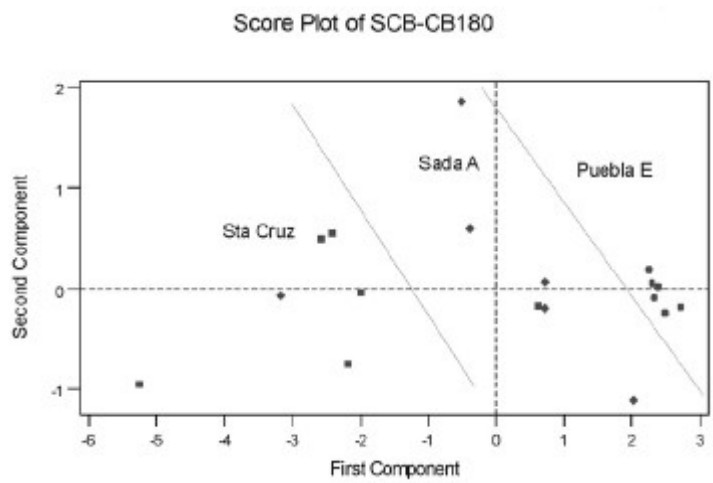

Figure 2. Distribution of mussels samples in the plane of the first and second components. Score plot of shell length, IC, lipid content, flesh weight and higher chlorinated compounds.

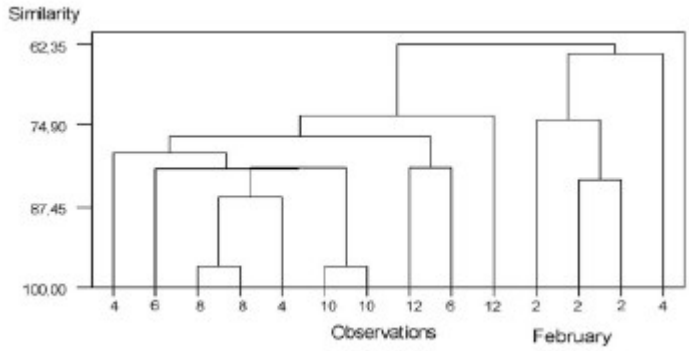

Figure 3. Dendrogram showing the results of a cluster analysis on chemical variables in Galicia mussels.

Temporal trends

In the second step of the multidimensional study, CA (Cluster Analysis; the distance measured was Euclidean) was carried out over sampling months, considering each zone by separate, in order to describe similarities on the basis of their biological and chemical characteristics. Clear separations have been found monthly for biological parameters in Santa Cruz. Samples collected in April and December were separated from the rest, it was mainly due to a fall of lipid content that in December could be due to last gametogenesis that uses all reserves stored during previous months. In relation to chemical parameters, Figure 3 shows the CA plot, samples collected in February in Sada A were separated from the rest due to high levels of PCBs. Examining it, at a similarity level of 75 % two main clusters can be identified: the first one was composed by mussels sampled in February, while the second cluster was made up of the remaining sampling months. In general, in rainy months the PCBs content was increased due to massive input of continental effluents and to high resuspension rates attributed to storm events.

Acknowledgements

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TRACE METAL AND CHLORINATED HYDROCARBONS IN SHELLFISH FROM IRISH WATERS 1993-2002

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Abstract
A 10 year review of trace metal and chlorinated hydrocarbon concentrations in shellfish from Irish waters was carried out by the Marine Institute. Data on contaminants in shellfish tissue were collected as part of the Marine Institute's annual monitoring programmes in accordance with the requirements of Council Directives 79/923/EEC and 91/492/EEC. Trace metals (Hg, Cd, Cr, Cu, Zn, Pb), polychlorinated biphenyls (PCBs) and various organochlorine pesticides (OCPs) were determined in mussels (*M. edulis)* and oysters (*C. gigas, O. edulis*) sampled at shellfish growing locations around the Irish coast between 1993 and 2002. Samples were collected in accordance with Oslo Paris Commission (OSPAR) monitoring guidelines. Trace metals were extracted by microwave acid digestion followed by determination using graphite furnace atomic absorption spectroscopy, flame atomic absorption spectroscopy (Zn), cold vapour atomic absorption spectroscopy (Hg 1993 - 1995) and cold vapour atomic fluorescence spectroscopy (Hg 1996 - 2002). PCBs and OCPs were determined by gas chromatography with electron capture detection. Data presented indicate that levels of contaminants were consistently low and well below available food safety maximum limits for shellfish

Introduction
Trace metals exist naturally in the environment, however, some trace metals such as mercury, lead and cadmium are not required for metabolic activity and are toxic at quite low concentrations. PCBs and OCPs are persistent anthropogenic pollutants that are ubiquitous in the marine environment, have a high affinity for lipids and bioaccumulate in fish tissue. In Ireland an annual monitoring programme for contaminants in shellfish tissue is carried out by the Marine Institute in part fulfillment of the monitoring requirements of various EU legislation, including:

- EU Council Directive 79/923/EEC on the quality required of shellfish growing waters
- EU Directive 91/492/EEC laying down the health conditions for the production and placing on the market of live bivalve molluscs,
- Commission Regulation 466/2001/EC (as amended by Regulation 221/2002/EC),
- This information also contributes to the Joint Assessment and Monitoring Programme (JAMP) as required by the 1992 OSPAR convention.

Between 1993 and 2002, trace metals (Hg, Cd, Cr, Cu, Zn, Pb), polychlorinated biphenyls (PCBs) and various organochlorine pesticides (OCPs) were determined in mussels (*M. edulis)* and oysters (*C. gigas, O. edulis*), sampled at shellfish growing locations around the Irish coast. Results of individual surveys have been previously published (Glynn *et al., 2004,* 2003a, 2003b; McGovern *et al.,* 2001; Bloxham *et al.,* 1998; Smyth *et al.,* 1997 and Nixon *et al.,* 1995, 1994, and 1991) and this paper presents a summary of this 10 year data set.

Materials and Methods

Mussels (50 individuals per sample, 4-6cm length) and oysters (25 individuals per sample) were collected from the main shellfish sites (Figure 1) around Ireland in accordance with OSPAR JAMP monitoring guidelines (total samples 173). Trace metals (Hg, Cd, Cr, Cu, Zn, Pb) were extracted by microwave, nitric acid digestion followed by determination using graphite furnace atomic absorption spectroscopy, flame atomic absorption spectroscopy (Zn), cold vapour atomic absorption spectroscopy (Hg 1993 - 1995) and cold vapour atomic fluorescence spectroscopy (Hg 1996 - 2002). Due to the lipophilic nature of PCBs and OCPs, lipid was extracted from tissue samples by either Soxhlet extraction (1993-1996) or by the Smedes (1997-2002) method (QUASH, 1998; QUASH, 1999). Chlorinated hydrocarbons were removed from the lipid by alumina column chromatography followed by separation of PCBs from OCPs using silica column chromatography. PCBs and OCP concentrations were determined by dual column gas chromatography with electron capture detection (GC-ECD).

Results and Discussion

A summary of results of the concentrations of mercury, trace metals and chlorinated hydrocarbon contaminants analysed in the shellfish tissue during the 10 year study period is presented in Table 1. Boxplots for each shellfish species for cadmium, chromium, copper, lead, mercury and zinc 1993-2002 are presented in Figure 2. Outlier and extreme values plotted are defined as having a value greater than 1.5 or 2 times the distance between the 25^{th} and 75^{th} quartiles.

The level of contaminants in shellfish is a good indicator of contaminant levels present in the water column and can provide valuable information on the quality of the shellfish and the waters in which they are grown. As such, Irish shellfish monitoring data has been used for environmental assessments as well as for the protection of consumers of Irish seafood products (Boelens *et al.*, 1999).

The EU has set maximum limits for Hg, Cd and Pb of 0.5, 1.0 and 1.5 mg kg^{-1} wet weight respectively for bivalve molluscs (Com. Reg. 466/2001/EC amended by Com. Reg. 221/2002/EC). As no other EU or national standards are available for other contaminants these were compared with the strictest Food Safety standards and guidance values applied by other OSPAR contracting parties.

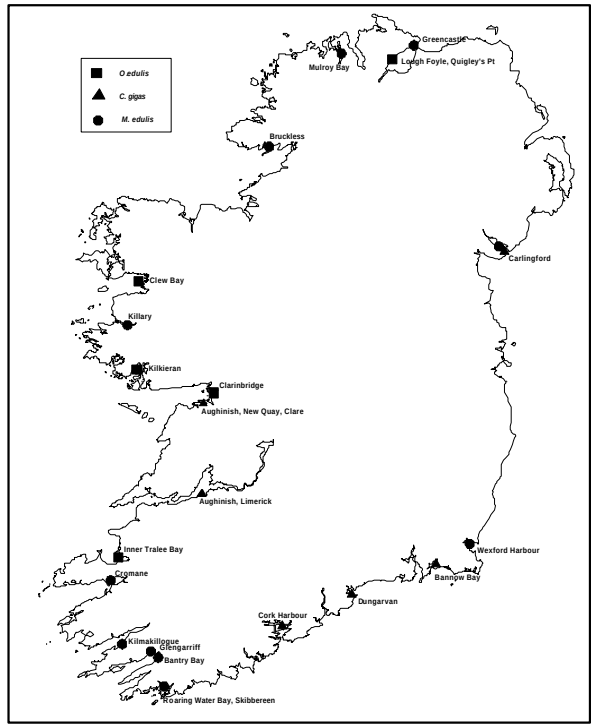

Figure 1: Shellfish sites 1993-2002

Table 1: Metals and chlorinated hydrocarbon concentrations (wet weight basis) [median (Range)] from Irish shellfish growing waters, 1993-2002.

	C.gigas	O.edulis	M.edulis
Cadmium (mg/kg^{-1})	0.30 (0.10 - 0.74)	0.45 (0.24 - 0.81)	0.14 (0.04 – 0.52)
Chromium (mg/kg^{-1})	<0.19 (nd - 0.31)	<0.19 (nd - 3.11)	<0.19 (nd - 0.86)
Copper (mg/kg^{-1})	11.8 (3.9 - 57.9)	4.90 (1.70 - 39.1)	1.50 (0.90 - 25.3)
Lead (mg/kg^{-1})	0.18 (nd – 0.45)	0.06 (nd - 0.24)	0.11 (nd - 1.82)
Mercury (mg/kg^{-1})	<0.03 (nd - 0.06)	<0.03 (0.02 - 0.12)	<0.03 (nd - 0.10)
Zinc (mg/kg^{-1})	225.90 (104 - 680)	303.05 (136 - 533)	16.96 (10.5 - 36.3)
åPCB7 (mg/kg^{-1})	2.62 (0.65 - 5.60)	1.48 (0.65 - 8.50)	2.19 (0.28 - 7.53)
Sum DDT's (mg/kg^{-1})	1.30 (0.15 - 2.64)	0.66 (0.37 - 3.81)	0.72 (0.16 – 2.99)
HCB (mg/kg^{-1})	0.06 (0.02 - 1.74)	0.05 (0.02 - 0.11)	0.06 (0.02 - 0.30)
?-HCH, (mg/kg^{-1})	0.33 (0.03 - 1.92)	0.24 (0.02 - 0.59)	0.13 (0.02 - 0.92)

Notes.
1. nd = not detected.
2. ∑PCB7 = sum of concentrations of PCBs 28,52,101,118,138,153 and 180.

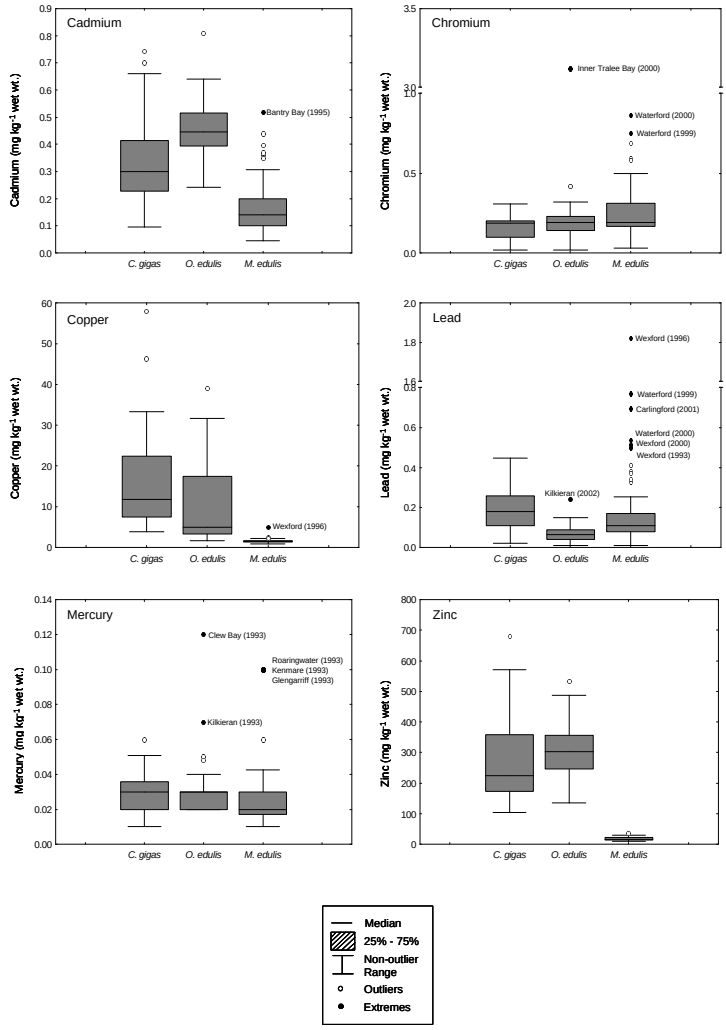

Figure 2: Boxplot for Pb, Cd, Cr, Hg and Zn

However these outliers values are still well within the strictest guidance and standard values apart from 1 sample exceeding the EU maximum limit for lead in mussel. This occurred in Wexford Harbour in 1996 for Pb (1.82 mg/kg^{-1}), however this points to an uncharacteristic result as results from this location since 1993 have been below the limit.

Levels of contaminants for Irish shellfish were well below food safety standard and guidance values, with organochlorines typically 1-2 orders of magnitude below guidance values. Though still low, PCB levels were typically highest in areas proximate to urban centres, such as in Cork harbour.

Conclusions

This 10-year review of data indicates that the water quality monitored in shellfish growing areas is good and conforms to the requirements of the Directives. It also shows that metal and chlorinated hydrocarbon levels in shellfish sampled are consistently low. Although levels are generally slightly higher in areas proximate to urban centres, there is no evidence of recent direct inputs of contaminants to Irish shellfish growing waters monitored.

The data presented in this report are indicative of the unpolluted nature of Irish waters and shellfish products with respect to environmental contaminants.

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SPATIAL AND TEMPORAL TRENDS OF CHEMICAL CONTAMINANTS IN TISSUES OF THE BLUE MUSSEL, *Mytilus edulis* L., IN THE GULF OF MAINE: 1993 - 2001

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Abstract
The Gulf of Maine is bordered by Canada and the USA, including three states and two provinces. Toxic chemical contamination is a trans-boundary issue and requires coordinated efforts from all jurisdictions. Gulfwatch is a mussel (*Mytilus edulis* L.) monitoring program that is measuring levels of 10 trace metals, 22 polychlorinated biphenyls (PCB) congeners, 24 polyaromatic hydrocarbons (PAH) and 16 chlorinated pesticides in mussel tissue from >60 sites around the Gulf, as part of a designed 9-year program from 1993-2001. Sampling and processing followed standardised methods and occurred between September and October each year. Four samples of 40 mussels, 20 each for metal and organic analysis, were collected from sites every three years in a rotational design. Five sites were sampled every year. The 9-year database was analysed to determine contaminant spatial and temporal trends. Where spatial trends were detectable, concentrations decreased from southwest to northeast, corresponding to human population levels. Some sites had much greater levels of silver or lead compared to other sites in the Gulf. All significant temporal trends observed were decreasing at annually sampled sites. Gulfwatch continues to illustrate the role of mussel tissue monitoring in coastal chemical exposure assessment and ecological risk assessment.

Introduction
The Gulf of Maine (GOM) is a highly productive ecosystem that supports many species and commercial activities (Sherman *et al*. 1996). Contamination of the GOM has become a significant concern in recent years as the effects of loading from a variety of industrial, commercial and municipal pollution sources have become better recognised and understood (Jones 2004, Wells *et al*. 1997). Regional issues such as toxic chemical contamination are trans-boundary in nature and their management requires coordinated efforts from the Canadian provinces and US states that border the GOM. Numerous bioindicators have been used to track environmental quality in the Gulf of Maine (Jones 2004; Chou *et al*. 2003). In 1991, a mussel watch approach called Gulfwatch was established by the Gulf of Maine Council on the Marine Environment (GOMCME) for monitoring chemical contaminants resulting from urban development and industrialization. The Gulfwatch program consists of a volunteer network of American and Canadian university and government scientists and environmental and resource managers from the three US states and two Canadian provinces that border on the Gulf. Since 1993, Gulfwatch participants have monitored the whole tissues of blue mussels (*Mytilus edulis*) to determine temporal and spatial trends for chemical contaminants in the GOM.

Chemical contaminant monitoring using tissue from a variety of bivalve mollusk species has been used in 52 countries from six continents around the world (Cantillo 1998, Monirith *et al*. 2003). Besides the GOM, *M. edulis* is the species of choice for monitoring programs in Scotland (McIntosh *et al*. 2004), Ireland (Glynn *et al*. 2004), the rest of the US (O'Connor 2002, Lauenstein and Cantillo 2002) and elsewhere. Long-term monitoring in these areas has provided useful insights into spatial and temporal trends in chemical pollution in coastal environments, helping to shape management strategies for reducing pollution levels. The Gulfwatch program has completed sampling and analysis of *M. eduilis* tissue from the GOM for a designed 9-year cycle of the program. The data have been compiled for each year and posted on the GOMCME web site (http://www.gulfofmaine.org/council/committees/eqmc/gulfwatch/default.asp). The objective of this paper is to present the results of initial analysis of temporal and spatial trends for chemical contaminant concentrations. This analysis is timely for informing managers in the region about the status of chemical exposure of the ecosystem and will help serve as a basis for modifying and expanding future chemical monitoring in the GOM.

Materials and Methods

The Gulfwatch program measured levels of trace metals and toxic organic chemicals in mussel tissue from >60 sites from 1993-2001. Each fall, intertidal blue mussels (*M. edulis*) were collected from coastal embayments around the GOM. Benchmark sites, located in each of the five jurisdictions (Massachusetts-MA, New Hampshire-NH, Maine-ME, New Brunswick-NB, Nova Scotia-NS) were sampled every year. The more numerous rotational stations were sampled every third year. The sampling design maximises spatial coverage of the GOM whilst generating contaminant concentration data for long-term temporal trends analysis.

Four discrete samples of 40 mussels, 20 each for metal and organic chemical analysis, were collected from each site. Standard operating procedures for the collection, preparation, chemical analysis and laboratory quality control have been described in Gulfwatch's Field and Laboratory Manual (Jones *et al*. 1998, Sowles *et al*., 1997). Chemical analytical procedures were as previously reported (Jones *et al*. 2001, Chase *et al*. 2001) and were conducted at the Environment Canada, ECB Laboratory in Moncton, NB (for organic chemicals) and at the State of Maine Health and Environmental Testing Laboratory in Augusta, ME (for trace metals). The targeted analytes included 10 trace metals, 22 PCB congeners, 24 PAHs and 16 chlorinated pesticides (Table 1). No discussion of PAH results are included in this paper because the data were not ready for analysis. No discussion of mercury results are included because of problems found with the analytical data.

Data analysis involved use of the Mann-Kendall test to detect both spatial and temporal trends. Temporal trends for contaminants were determined using data from benchmark sites. For spatial analyses, only sites with three or more years of data were used, resulting in analysis of data from 38 sites.

Table 1. Trace metals and organic contaminants determined by the Gulfwatch Program.

Trace metals	Pesticides	PCBs	PAHs
Silver	Hexachlorobenzene	PCB 8	Naphthalene
Cadmium	g-HCH (Lindane)	PCB 18	1-Methylnaphthalene
Lead	Heptachlor	PCB 28	2-Methylnaphthalene
Nickel	Aldrin	PCB 29	Biphenyl
Copper	Heptachlor	PCB 44	2,6-Dimethylnaphthalene
Chromium	Epoxide	PCB 50	Acenaphthylene
Zinc	Cis-Chlordane	PCB 52	Acenaphthene
Iron	trans-Nonachlor	PCB 66	Trimethylnaphthalene
Aluminum	Dieldrin	PCB 77	Fluorene
Mercury	Mirex	PCB 87	Phenanthrene
	a-Endosulfan	PCB 101 PCB 105	Anthracene
	b-Endosulfan	PCB 118	1-Methylphenanthrene
	o,p'-DDE	PCB 128	Fluoranthrene
	p,p'-DDE	PCB 138 PCB 153	Pyrene Benzo(a)anthracene
	o,p'-DDD	PCB 170	Chrysene
	p,p'-DDD	PCB 180 PCB 187	Benzo(b)fluoranthene
	o,p'-DDT	PCB 195 PCB 206	Benzo(k)fluoranthene
	p,p'-DDT	PCB 209	Benzo(e)pyrene
			Benzo(a)pyrene
			Perylene
			Indeno(123cd)pyrene
			Dibenzo(ah)anthracene
			Benzo(g,h,i)perylene

Results

Spatial Distribution of Contaminants in the Gulf of Maine

Silver was detected more frequently in the southwestern portion of the GOM, especially in Massachusetts (Figure 1). Sandwich, MA and Yarmouth, NS had mussel tissue silver concentrations which were consistently elevated compared to all other sites (Figure 2). Silver concentrations were also elevated at Boothbay Harbor, ME (Fig. 2). Elevated lead concentrations were consistently detected in mussels at the highly degraded Boston Inner Harbor site with concentrations that exceeded the US FDA guidance level of 11.5 µg/g DW (USFDA 1993) for the consumption of shellfish (Figure 3) and were markedly elevated compared to concentrations detected at other GOM sites. The range in concentrations for chromium was less than for lead and silver, and spatial trends were not found (e.g. Figure 4).

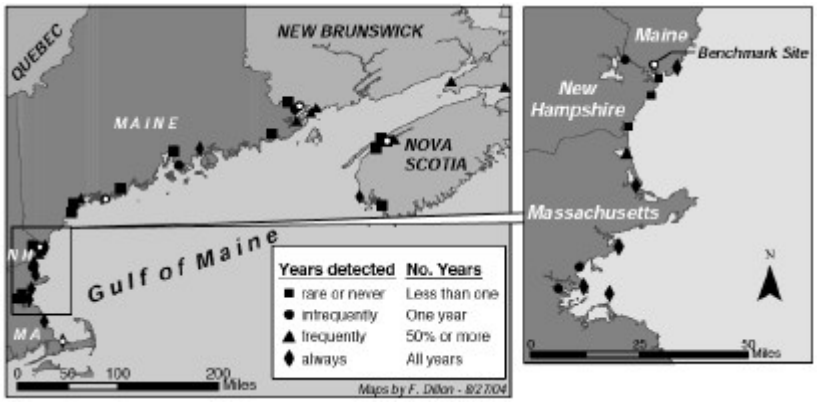

Figure 1. Frequency of detection of silver at Gulfwatch sites: 1993-2001.

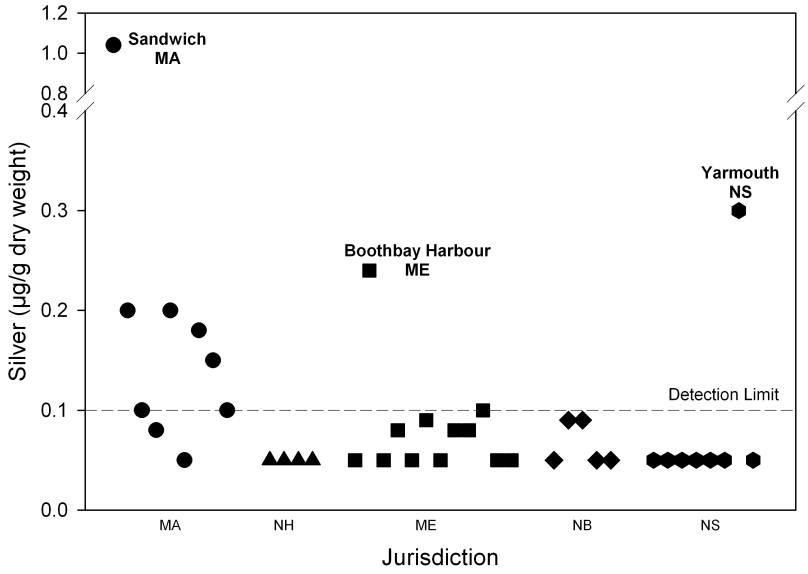

Figure 2. 9-year median silver concentrations at Gulfwatch sites, 1993-2001.

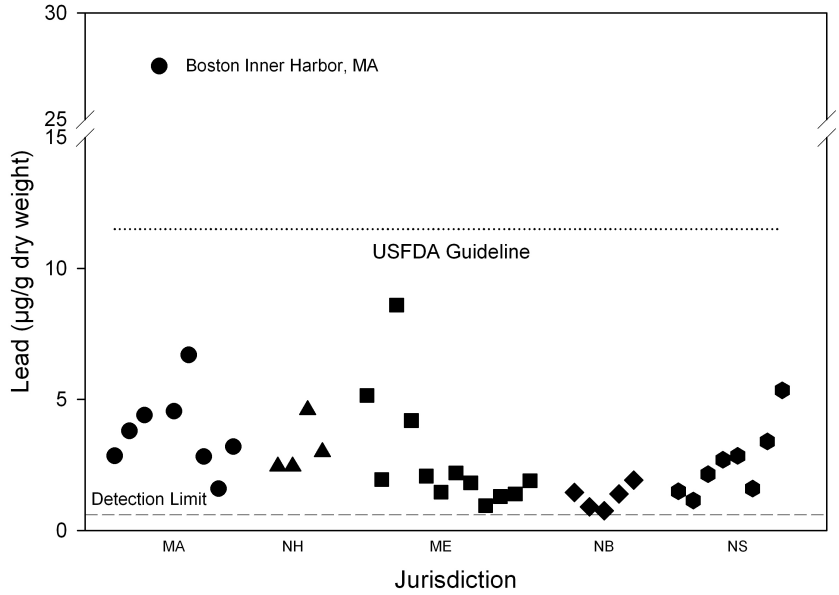

Figure 3. 9-year median lead concentrations at Gulfwatch sites, 1993-2001.

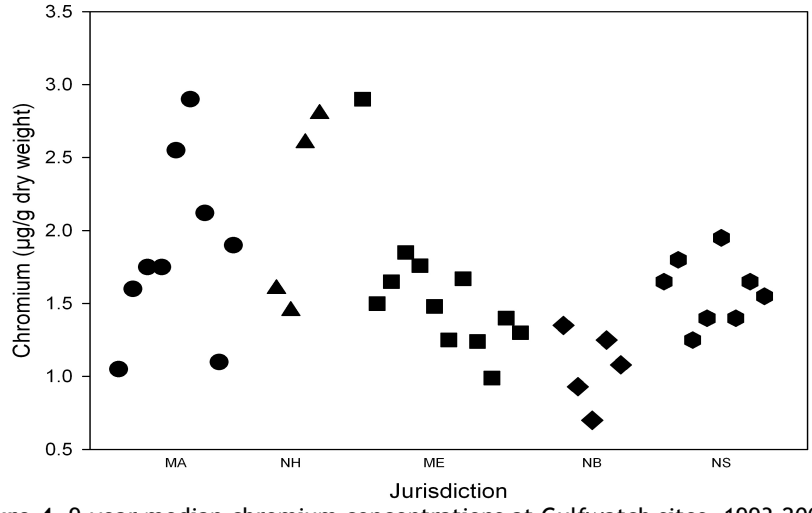

Figure 4. 9-year median chromium concentrations at Gulfwatch sites, 1993-2001.

Pesticides were more frequently detected at sites in the southern Gulf (Figure 5). Of the 16 targeted pesticides, p,p'-DDE was the most frequently detected at all sites. Only four pesticides were found at >50 % of sites, while mirex and aldrin were never detected (Figure 5; Table 2). Trend analysis indicated a significant ($P<0.01$) decline for p,p'-DDE from south to north (Figure 6). In general, PCB concentrations in US jurisdictions were elevated by one or more orders of magnitude compared to Canadian sites (Figure 7).

Table 2. Overall frequency of detection for each pesticide.

Pesticide	% Detection
p.p"-DDE	97
p,p'-DDD	62
cis-Chlordane	51
Dieldrin	51
o,p'-DDD	49
Lindane	41
trans-Nonachlor	35
p,p'-DDT	27
o,p'-DDE	22
a-Endosulfan	14
o,p'-DDT	14
Heptachlor-epoxide	11
b-Endosulfan	5
Heptachlor	3
Mirex	0
Aldrin	0

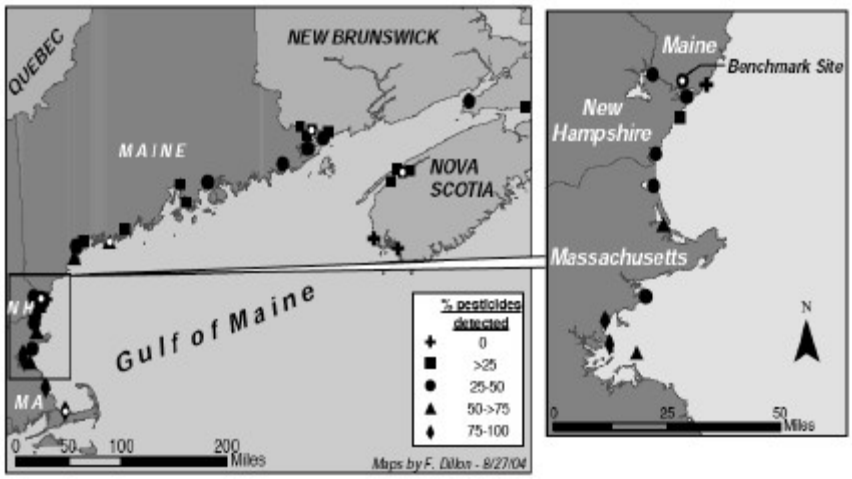

Figure 5. Percentage of the number of pesticides detected at Gulfwatch sites: 1993-2001.

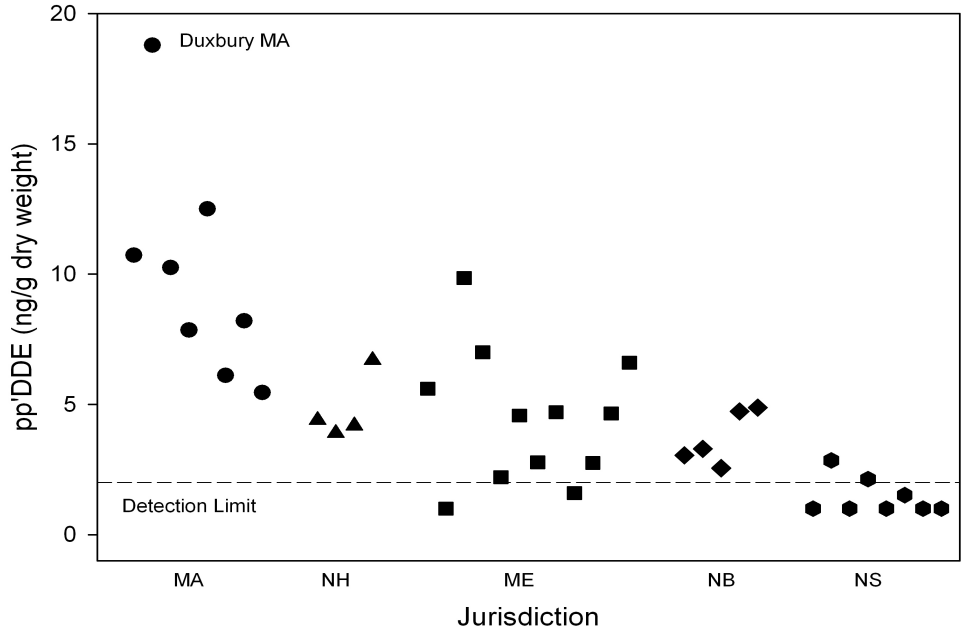

Figure 6. 9-year median pp'DDE concentrations at Gulfwatch sites, 1993-2001.

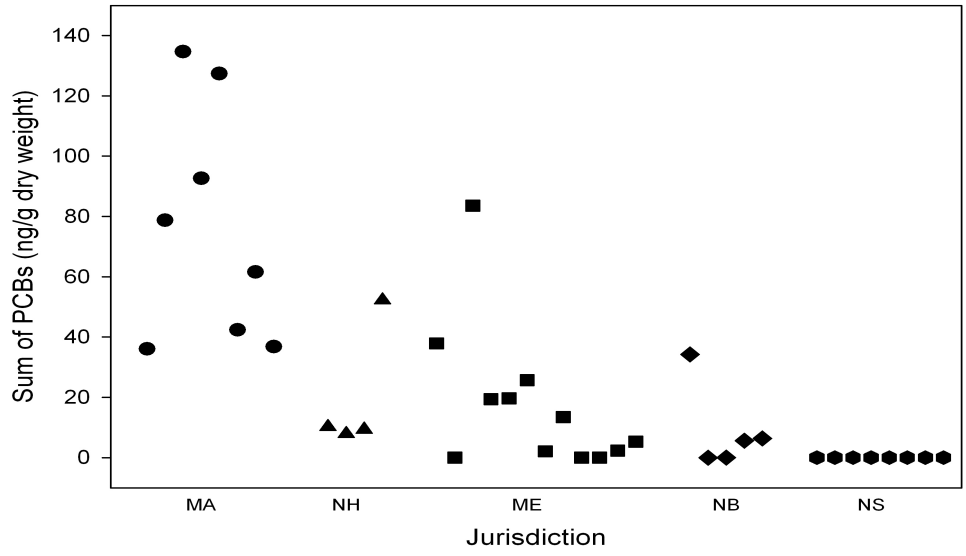

Figure 7. 9-year median PCB concentrations at Gulfwatch sites, 1993-2001.

Temporal Trends at Benchmark Sites in the Gulf of Maine

Trends were only analysed at Gulf of Maine benchmark sites for which there were 8-9 years of data. The Sandwich, MA site was the only benchmark site where silver was consistently detected over 9 years. A declining trend in silver tissue concentrations was observed (Figure 8). A significant ($P<0.05$) temporal decrease for lead was observed at three of the five benchmarks sites (Figure 9), Sandwich, MA (MASN), Hospital Island, NB (NBHI) and Digby, NS (NSDI). A significant decline in chromium concentrations was detected at Sandwich, MA ($P<0.01$) and Digby, NS ($P<0.05$) (Figure 10). There was only one benchmark site, Clark's Cove, ME, where there was a significant temporal decline ($P<0.01$) for p,p'-DDE (Figure 11). No significant temporal trends in total PCB concentrations were found at the three benchmark sites where concentrations were consistently above detection limits (Figure 12), Sandwich, MA (MASN), Clark's Cove, ME (MECC) and Kennebec River, ME (MEKN).

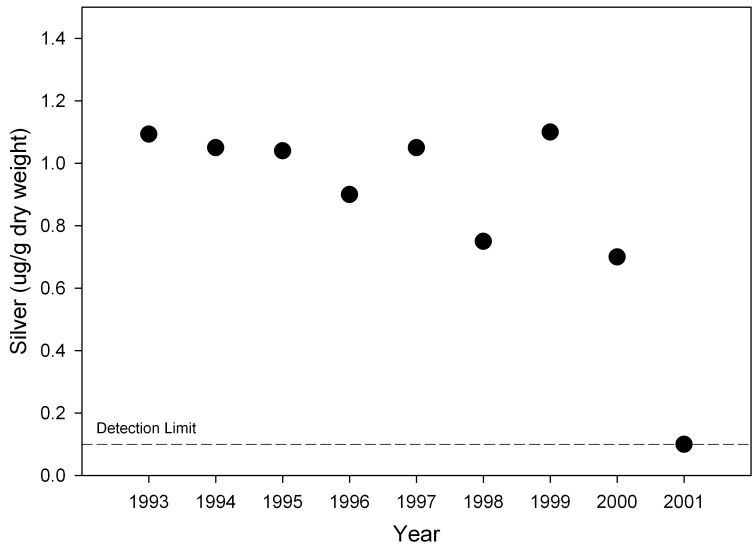

Figure 8. Annual median silver concentrations at Sandwich, MA: 1993-2001.

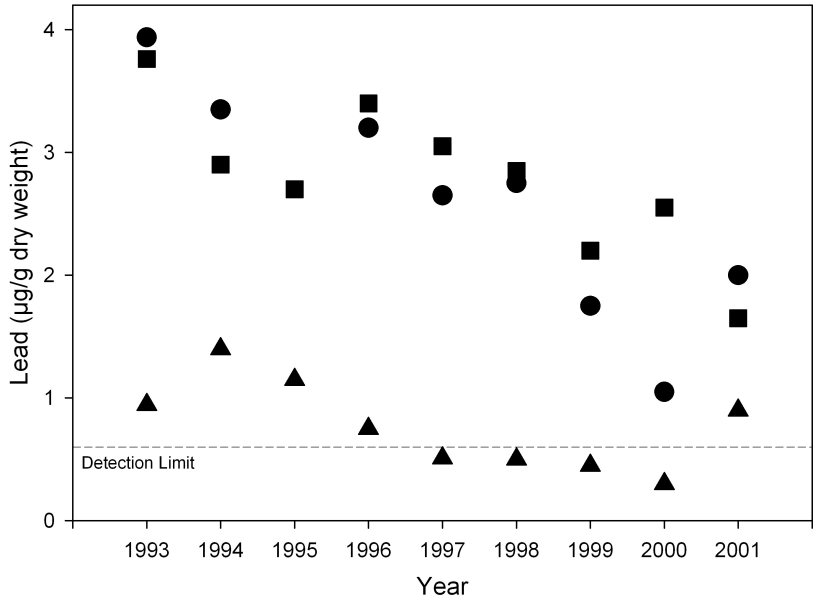

Figure 9. Annual median lead concentrations at Gulfwatch sites: 1993-2001. MASN = square; NBHI = triangle; NSDI = circle.

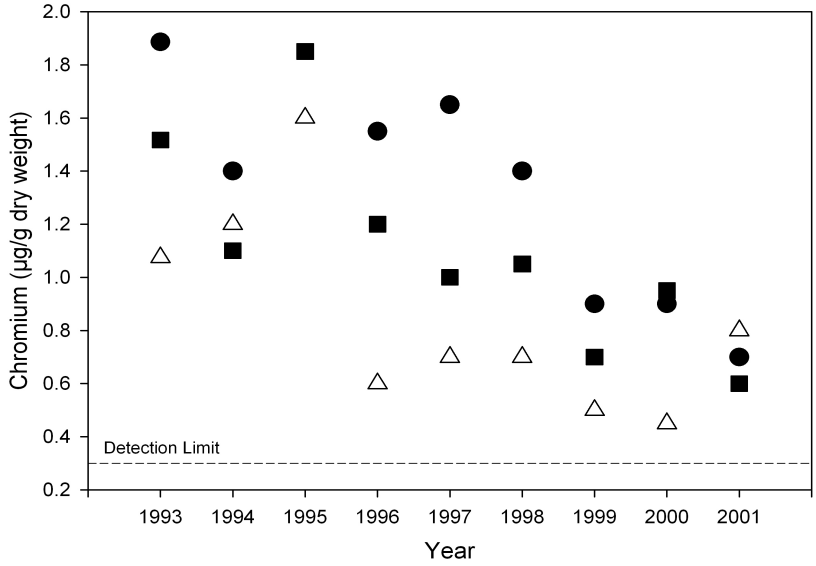

Figure 10. Annual median chromium concentrations at Gulfwatch sites: 1993-2001. MASN = square; NBHI = triangle; NSDI = circle.

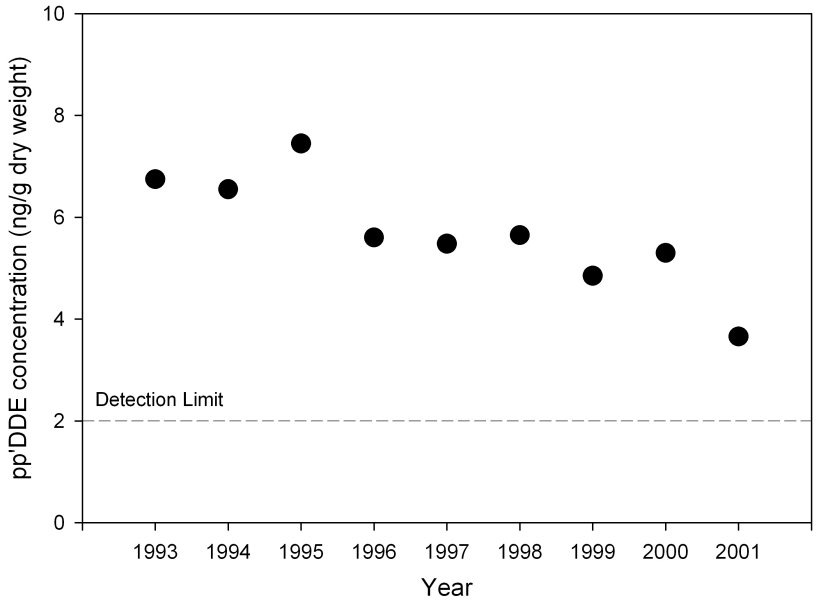

Figure 11. Annual median pp'DDE concentrations at Clark's Cove ME: 1993-2001.

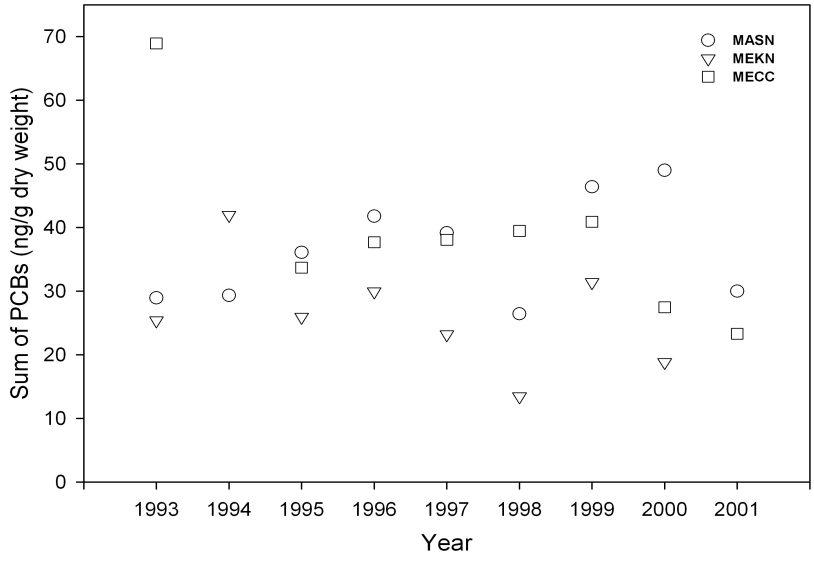

Figure 12. Annual median PCB concentrations at Gulfwatch sites: 1993-2001.

Discussion

Data for the first nine years of Gulfwatch contaminant monitoring in the Gulf of Maine were compiled and analysed. Many contaminants, including total chlorinated pesticides and total PCBs, showed a definite gradient of higher tissue concentrations in the southwestern end of the Gulf (Massachusetts, New Hampshire and southern Maine) with lower concentrations at the northeastern part (northern Maine, New Brunswick and Nova Scotia). This is consistent with earlier analyses of Gulfwatch data (Chase *et al*. 2001, Jones *et al*., 2001) and suggests greater organic chemical contamination in the more industrialised and populated southwestern Gulf. Thus, the mussel tissue levels reflect loadings in the environment, validating the Gulfwatch approach. The only significant temporal trends indicated declines in contaminant concentrations. In a previous analysis using data from 1991-1997 at the same five benchmark sites, virtually all trace metal had decreasing trends, and a mix of increasing and decreasing trends were observed for organic compounds (Chase *et al*. 2001). The longer-term database used in the present study makes analysis more robust and is more representative of long-term trends.

A few sites had markedly higher metal concentrations. Median silver concentrations in mussel tissue were low or undetected at most sites, with elevated concentrations observed only at three sites. Biota in coastal waters that receive municipal sewage effluent are known to have elevated exposures to silver (Buchholz ten Brink *et al*. 1996, Sanudo-Wlhemly and Flegal, 1992). Silver is up to 1000 times more concentrated in the coastal waters of Massachusetts than in other Gulf of Maine waters, in part because of its use in the state's photographic and jewelry industries (Krahforst and Wallace, 1996). No significant local sources of municipal waste in the Sandwich, MA area are known, so the high levels of silver may be a function of transport and deposition of sewage-derived particles from upstream sources (Bothner *et al*., 1993) that are sequestered in Cape Cod Bay and accumulated by mussels. The median concentration of lead was generally <5 µg/g DW, except at the Boston Inner Harbor and Boothbay Harbor sites. Elevated lead levels in Boston's Inner Harbor are consistent with the area's heavy industrial activities and recent data for lead in sediments (http://www.epa.gov/emap/ nca/html/regions /northeast.html). However, lead levels in mussels (Figure 3; Pala *et al*. 2003) and in soft shell clams, *Mya arenaria* (Schwartz *et al*. 1996) at sites in other areas of Boston Harbor were generally ≤5 µg/g DW and well below the USFDA guidance level. Elevated lead levels in mussels from Boothbay Harbor, ME were probably the result of local, as yet unidentified sources (Getchell, 2001).

A more comprehensive analysis of the 9-year Gulfwatch database is currently underway. Gulfwatch continues to illustrate the role of mussel tissue monitoring in coastal chemical exposure assessment and ecological risk assessment.

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HEAVY METALS, ORGANOCHLORINE PESTICIDES AND CHLOROBIPHENYLS IN BIVALVE MOLLUSCS FROM THE ATLANTIC AND MEDITERRANEAN COAST OF SOUTH SPAIN.

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Abstract
The geographical distribution and fluctuations of heavy metals (Cu, Cd, Pb and Hg), polychlorinated pesticides (DDT, Lindane and HCB) and 7 chlorobiphenyl congeners (CB28, CB52, CB101, CB118, CB138, CB153 and CB180) concentrations in several shellfish species from the Atlantic and Mediterranean littoral of the Andalusian Community (South-Spain) are presented. This study is part of the last six years (1998-2003) of the monitoring programme of shellfish harvesting zones along over 900 km of coastline in the South of Spain with different marine environments (i.e., estuaries, bays and coastal). The selected bivalve mollusc species, including *Donax trunculus*, *Chamelea gallina* and *Scrobicularia plana*, among others are representative of the shellfish production and sentinel for the monitoring programme. The results showed the existence of differences between the Atlantic and Mediterranean areas and zones affected by anthropogenic pollution sources and river effluents containing a particular metal-enriched composition.

Introduction
Heavy metals and organochlorine compounds are known to be involved in bioaccumulation processes in marine ecosystems, which are considered a serious hazard to the living organisms as well as to the human being (Furness and Rainbow, 1990; Kennish, 1996; Crompton, 1997). Their persistence in the environment and toxicity justify the inclusion in shellfish safety programmes. Cadmium, lead and mercury show a high potential risk at very low concentrations. Copper, an element considered necessary for life, may cause health problems to humans if concentrations exceed certain limits. Although use of some organochlorine compounds have been terminated or suspended a few years ago, they are not easily degraded. In addition, they are lipid-soluble and are not easily metabolised or excreted from the marine organisms. As a consequence, these compounds are stored in fatty tissues by means of bioaccumulation through the food chains. Since 1994, the Andalusian Autonomous Government has implemented a Quality Control Programme of shellfish production areas along the Atlantic and Mediterranean Coasts in the south of Spain. This shellfish watch programme is being carried out in the Laboratory of Quality Control of Fishing Resources, where several biological and chemical parameters are monitored in bivalve molluscs, according to EU and Spanish regulations, including biotoxins, microorganisms as well as heavy metals (Cd, Pb, Hg and Cu). Nevertheless, other potential chemical pollutants like PCBs, polychlorinated pesticides and radionuclide have been also incorporated.

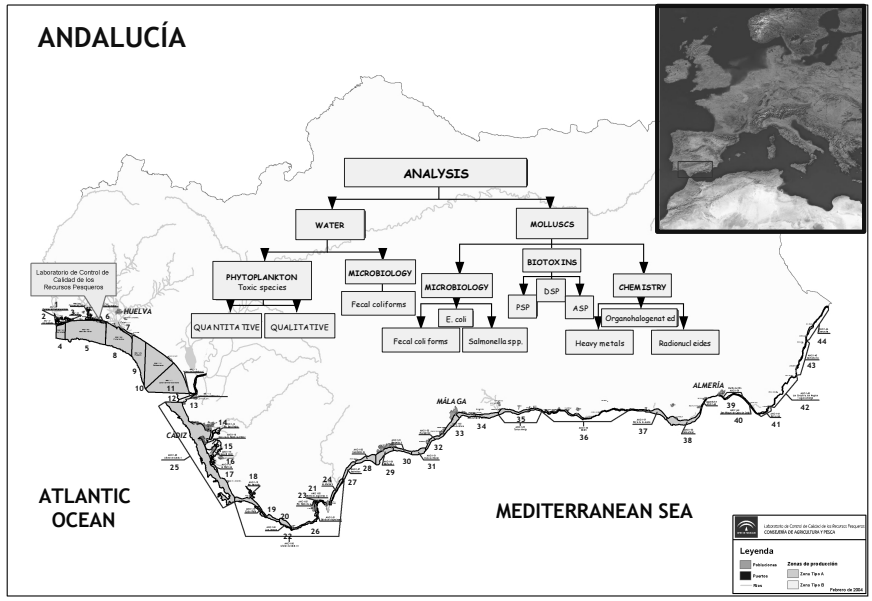

Figure. 1. Map of the Atlantic and Mediterranean Coasts in the South of Spain, showing the shellfish production

The abundance and variety of shellfish species along this coast, as well as the potential influence of densely populated areas (e.g. sewage discharges) justify the interest of monitoring pollutant contents. Other important sources are large rivers, mainly in the Atlantic Coast, like the Guadiana and Guadalquivir Rivers as well as other rivers with lower flow but with a higher content in heavy metals because of mining activities and rocks weathering upstream, like the Tinto and Odiel Rivers (van Geen *et al.*, 1991). In order to guarantee an appropriate shellfish quality, in case that pollutant values would exceed the limits in any shellfish sample, action procedures are prompted to ensure that the shellfish production area is not being further harvested until the metal levels decrease again below the regulatory limits. Thus, species of that area are collected and analysed as soon possible.

Materials and Methods

Sample processing

As shown in the map (Figure 1), the Andalusian Coast has been divided in 44 shellfish production areas. The shellfish species (Table 1) were selected as the most representative of each area. They were collected periodically since 1998 to 2003, according to a periodicity of at least twice a year for heavy metals (Cd, Pb, Hg and Cu) and yearly for pesticides (DDT, lindane and hexachlorobenzene) and PCBs (CB28, CB52, CB101, CB118, CB138, CB153 and CB180). Samples were collected by ship, dredged up, classified and sent to the laboratory for analysis. Once in the laboratory, the shellfish were processed and sufficient amount (30 g wet weight for heavy metals and 90 g for organochlorine) of edible tissue was homogenised by ultraturrax, freeze dried and stored under dry atmosphere until analysis.

Table 1. Shellfish used as sentinel species collected in the production areas.

Atlantic Coast		Mediterranean Coast	
Zone	***Specie***	**Zone**	***Specie***
1	*Cerastoderma edule* (BE)	**21**	*Callista chione* (CF)
2	*Ruditapes decussatus* (AF)	**22**	*Callista chione* (CF)
3	*Ruditapes decussatus* (AF)	**23**	*Cerastoderma edule* (BE)
4	*Donax trunculus* (CQ)	**24**	*Callista chione* (CF)
5	*Chamelea gallina* (CH)	**26**	*Paracentrotus lividus* (ER)
6	*Cerastoderma edule* (BE)	**27**	*Callista chione* (CF)
7	*Donax trunculus* (CQ)	**29**	*Callista chione* (CF)
8	*Chamelea gallina* (CH)	**28**	*Callista chione* (CF)
9	*Chamelea gallina* (CH)	**30**	*Venerupis rhomboides* (AC)
10	*Chamelea gallina* (CH)	**32**	*Callista chione* (CF)
11	*Donax trunculus* (CQ)	**33**	*Callista chione* (CF)
12	*Scrobicularia plana* (SP)	**34**	*Callista chione* (CF)
13	*Scrobicularia plana* (SP)	**35**	*Callista chione* (CF)
14	*Scrobicularia plana* (SP)	**36**	*Callista chione* (CF)
15	*Ruditapes philippinarum* (AJ)	**37**	*Chamelea gallina* (CH)
16	*Scrobicularia plana* (SP)	**38**	*Chamelea gallina* (CH)
17	*Donax trunculus* (CQ)	**39**	*Chamelea gallina* (CH)
18	*Scrobicularia plana* (SP)	**40**	*Donax trunculus* (CQ)
19	*Donax trunculus* (CQ)	**41**	*Chamelea gallina* (CH)
20	*Donax trunculus* (CQ)	**42**	*Chamelea gallina* (CH)
25	*Paracentrotus lividus* (ER)	**43**	*Chamelea gallina* (CH)
26	*Paracentrotus lividus* (ER)	**44**	*Donax trunculus* (CQ)

Analytical methods

Heavy metals were determined by atomic absorption techniques after microwave acid digestion with HNO_3 65 %. Cadmium and lead were determined by electrothermal (graphite furnace) atomic absorption spectrometry (ETAAS) with Zeeman background correction, Cu by air-acetylene flame atomic absorption spectrometry (FAAS) and Hg by cold-vapour atomic absorption spectrometry (CVAAS). Data quality assurance has been carried out by means of participation in QUASIMEME Programme, as well as with an appropriate CRM (BCR 278, mussel tissue), with satisfactory results.

Lindane (γ-hexachlorociclohexane, γ-HCH), DDTs and hexachlorobenzene (HCB) as well as PCB congeners no. 28, 52, 101, 118, 138, 153 and 180, were analysed after Soxhlet extraction (hexane-acetone, 20 % v/v) and purification with alumina eluted with n-pentane for lipids removal, according to the procedure described by González-Quijano and Fumega (1996). Subsequently, the extract was divided in two fractions for each group of compounds using a silica gel column eluted with

isooctane and isooctane-diethylether mixture and both were determined by gas chromatography with electron capture detection (GC-ECD).

Results and Discussion

Only species with a significant amount of data have been processed. In general, as shown in the box-whiskers plots, the four metals showed in general values below the limits by legal regulations for bivalve molluscs, which have been considered as a reference for this work (EC, 2001; EC, 2002; RD 1991).

Cadmium (Figure 2) showed highest concentrations in *Chamelea gallina*, especially in zones 8, 9 and 10, compared with nearby areas (i.e. zone 5) along the Atlantic coast, whilst the two sampled areas along the Mediterranean (37 and 38) also exhibited relatively high values of this metal in comparison with other species and areas. However, none exceeded the legal limit (1 mg/kg wet w.).

Lead (Figure 3) only presented remarkable concentrations above the valid limit (1.5 mg/kg wet w.) in *Scrobicularia plana* (13) close to the Guadalquivir River Estuary and *Donax trunculus* (44), and rarely in *Scrobicularia plana* (16) and *Chamelea gallina* (37).

Copper (Figure 4) has two legal limits in the Spanish regulation, 20 mg/kg w.w. in all the species, with the exception of 60 mg/kg wet w. in *Donax trunculus*. Highest values have been obtained in samples from zones 7-11, particularly in *Donax trunculus* (11). This metal has a characteristic geographical distribution due to the influence of metal inputs from rivers and mining areas. Thus, copper in *Chamelea gallina* and *Donax trunculus* along the coastal belt from the Tinto and Odiel River Estuaries to the Guadalquivir River Estuary also presented remarkable values in comparison with samples collected along the Mediterranean coast (i.e., *Chamelea gallina* in zones 37-38 and *Donax trunculus* in zone 40). As a reason for this particular metal distribution, river flows containing higher metal loads due to anthropogenic activities (mainly, mining and industrial effluents) and rocks weathering may produce metal enrichment in their Atlantic estuarine and coastal habitats.

Regarding mercury (Figure 5), this metal have never exceeded the assigned limit value (0.5 mg/kg wet w.) and it is only remarkable in single samples from zones 3, 15 and 40.

Concerning pesticides, as shown in Figure 6, the highest levels of ΣDDT (sum of 2,4'- and 4,4'-homologues of DDT, DDD and DDE) have been found in mollusc samples harvested along the Atlantic Coast, specially in those sampling stations closer to estuaries and inner bays. Regarding lindane and HCB, their levels are much lower and without extreme variations along the Andalusian Coast. Although the use of DDTs as pesticides was banned, the results showed their persistence in the environment.

On the other hand, among the investigated PCBs (Figure 7), congeners no. 153 and, in less extent, 138 have shown to be the most abundant in the targeted species. In this case, their distribution follows a similar pattern that found for the pesticides, with higher concentrations in the Atlantic Coast rather than the Mediterranean. The influence of fluvial inputs can be again pointed out. Nevertheless, the levels of these chlorobiphenyls may be considered in most cases as relatively low.

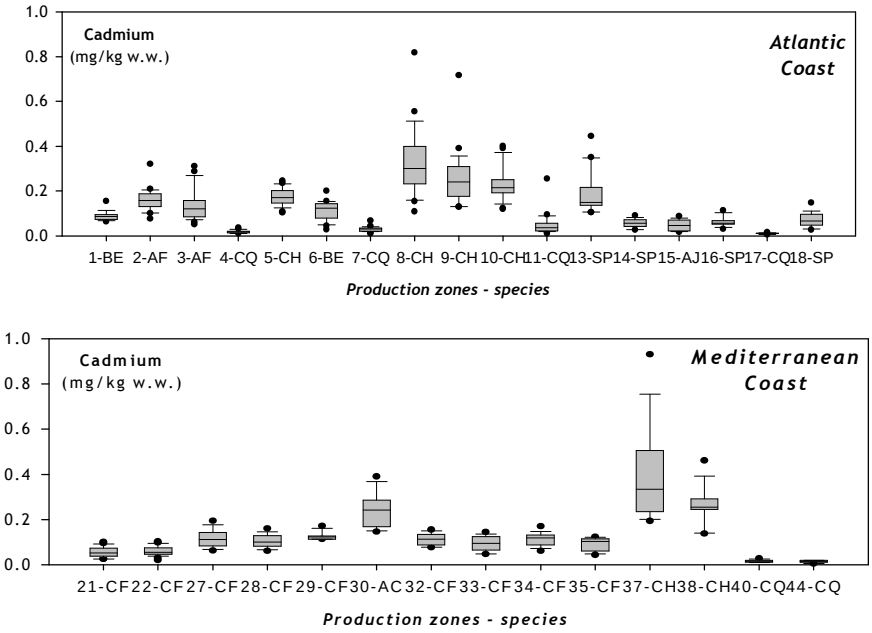

Figure 2. Box-Whisker plots of cadmium in shellfish from the Atlantic and Mediterranean Coasts (1998-2003).

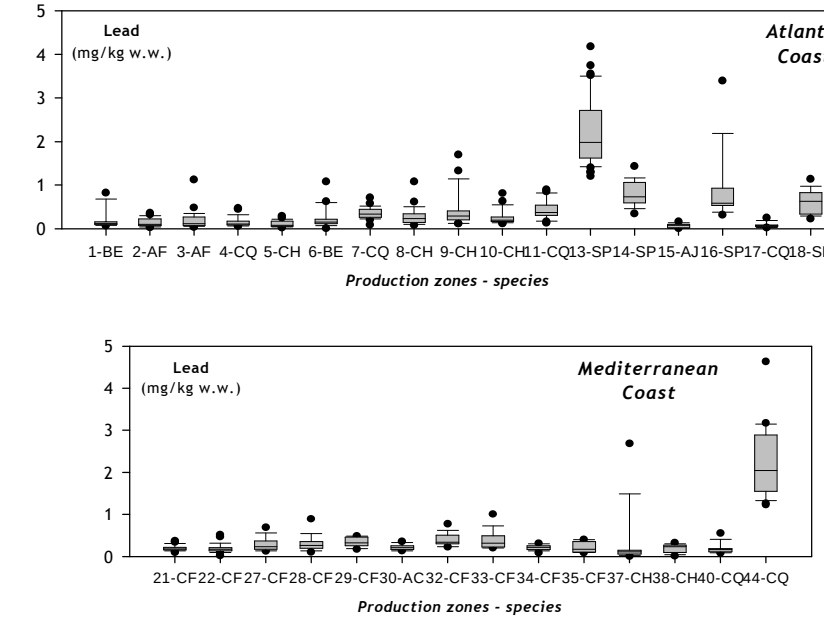

Figure 3. Box-Whisker plots of lead in shellfish from the Atlantic and Mediterranean Coasts (1998-2003).

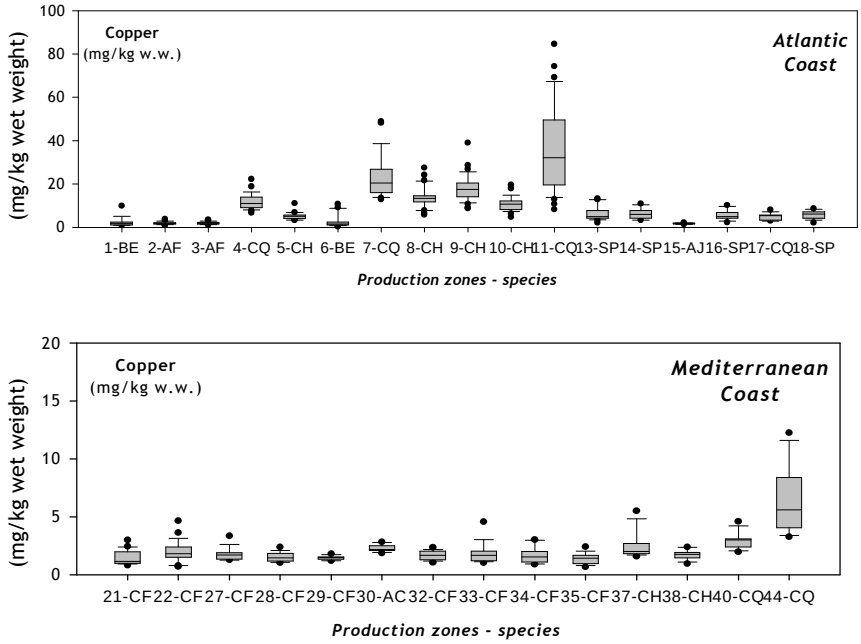

Figure 4. Box-Whisker plots of copper in shellfish from the Atlantic and Mediterranean Coasts (1998-2003).

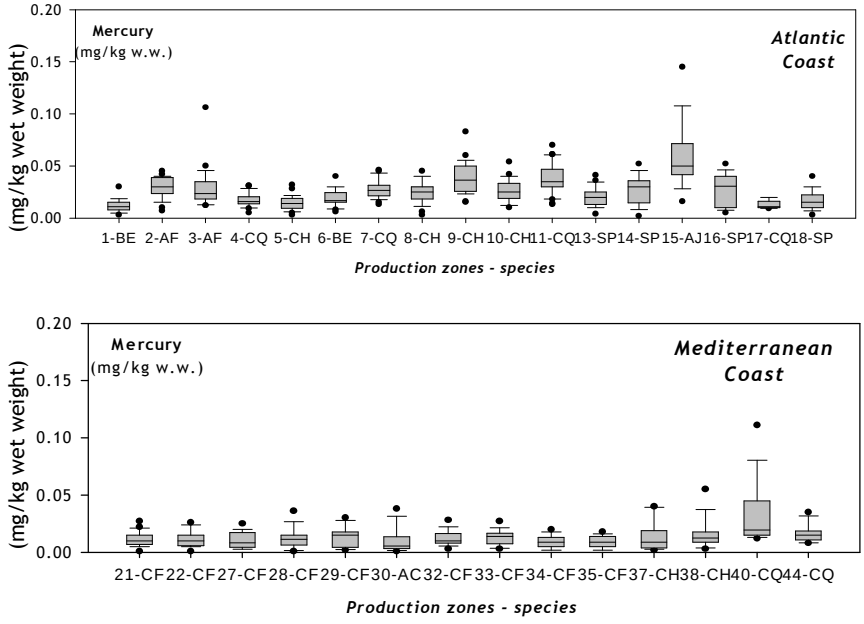

Figure. 5. Box-Whisker plots of mercury in shellfish from the Atlantic and Mediterranean Coasts (1998-2003).

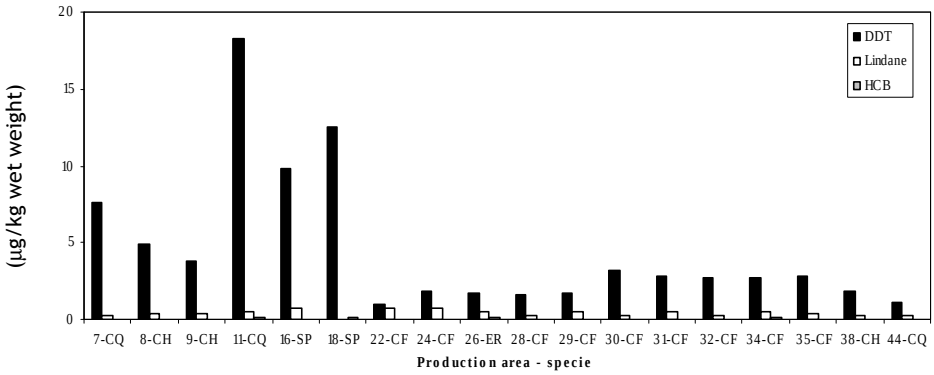

Figure 6. Distribution of ΣDDT, Lindane and HCB (µg/kg wet weight) in shellfish from the Atlantic and Mediterranean Coasts of the South of Spain (1999).

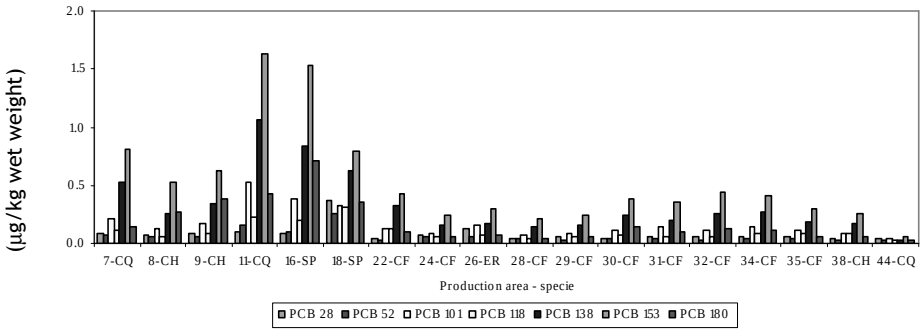

Figure 7. Distribution of PCBs (µg/kg wet weight) in shellfish from the Atlantic and Mediterranean Coasts of the South of Spain (1999).

Acknowledgements

We gratefully thank all the staff of the *Laboratorio de Control de Calidad de los Recursos Pesqueros*, and especially those who have worked in the Department of Chemistry, Rosa Palomar, Yolanda Contreras and M. Carmen Sanchez. We also thank the contribution of Carlos Jiménez and José L. Marenco from the *Consejería de Agricultura y Pesca* of the Andalusian Autonomous Government. Special thanks to Manuel Aguilar from the *E.P. Desarrollo Agrario y Pesquero* for his support and confidence in this Project.

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CHEMICAL TESTING AT THE PUBLIC ANALYST'S LABORATORY, GALWAY.

*Caroline Lardner, Andrew Flanagan, Nora Madden, Louise Mannion.

Abstract

The Western Health Board's Public Analyst's Laboratory Galway, provides a Chemical Analytical service in the testing of food, water, pharmaceuticals and miscellaneous toxicological samples, for the Western region. The main work of the laboratory is the chemical analysis of foodstuffs for parameters concerning food safety, composition, quality, nutrition and consumer information. In the laboratory, fish and fish products are tested for the presence of biogenic amines, benzo- [a]-pyrene, sulphur dioxide, dioxins and marine biotoxins. In the monitoring of marine bio-toxins (DSP, AZP and ASP toxins), the Health Board's authorised officers (Environmental Health Officers) submit largely retail- and catering-level products acting as a second check on the efficiency of control at production level by the Marine Institute / Dept. of Marine. Processed and imported products are included in the monitoring, together with raw Irish produce. The surveillance is currently at a relatively low level (~100 samples p.a.), in part reflecting the low level of contaminated produce on the market. There are three Public Analysts' Laboratories in the Republic of Ireland, located in Dublin, Cork and Galway; these are Official Food Laboratories within the Department of Health/Health Board Food Control system and they provide a service in the testing of food, waters/effluents, medicines and other miscellaneous samples to ensure compliance with national and EU legislation and standards (Figure 1). Due to the widespread adulteration of food and drink in the mid 1800s, the U.K. Public Analyst Service was formed, and the first Irish Public Analyst was appointed in Dublin in 1862.

The Public Analyst's Service

The Public Analyst's Laboratory in Galway provides a Chemical Analytical service in the testing of food (including a Service Agreement with the FSAI), water (for Health Boards, Local Authorities, the public and industry), pharmaceuticals (including a contract with the Irish Medicines Board) and provides a Toxicological service for the Western region. Air Quality is monitored for Galway City Council and the Department of Environment. The main work of the laboratory is the chemical analysis of foodstuffs (~ 3,500 samples tested p.a.) for parameters concerning food safety, composition, quality, nutrition and consumer information. These parameters include contaminants/residues e.g. fungal toxins (aflatoxins, ochratoxin A, etc.) and processing contaminants (e.g. gluten and acrylamide), additives such as sulphur dioxide and nitrate/nitrite, composition (protein, fat and salt), quality (e.g. DPTG in used frying oils) and labelling. The laboratory also deals with complaint samples which arise when consumers find contamination, infestation, spoilage or extraneous matter in foods. In the laboratory, fish and fish products are routinely tested for the presence of biogenic amines in scombroid fish (e.g. tuna and mackerel), heavy metals Pd, Cd and Hg (e.g. swordfish and tuna), benzo-[a]-pyrene in smoked fish and fish oils/supplements, sulphur dioxide in crustaceans, dioxins (CALUX assay – research project) and marine biotoxins in molluscs. In 2000 the Western Health Board, FSAI, Molluscan Shellfish Safety Committee and the Department of the Marine…/Marine Institute agreed a supportive monitoring role for the Western Health Board (w.r.t marine bio-toxins) targeting largely retail- and catering-level products. This monitoring should help ensure that shellfish from unauthorised sources are not being marketed, and it should act as a second check on the efficiency of production- level control by the Department of Marine/Marine Institute.

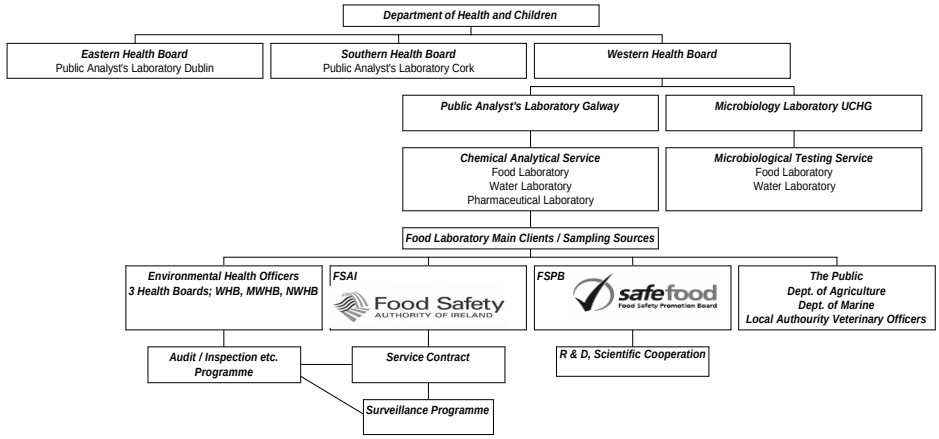

Figure 1. The Public Analyst's Service

Sampling is undertaken by Environmental Health Officers and testing is carried out by the Public Analyst's Laboratory for the presence of DSP toxins, Okadaic Acid, DTX-1 and DTX-2, and AZP toxins using LC-MS. HPLC is used to analyse for the presence of ASP toxins. Processed and imported products are included in the monitoring, together with raw Irish produce. The surveillance is currently at a relatively low level (~100 samples p.a.), in part reflecting the apparently low level of contaminated produce on the market. The testing results from 2001-2003 inclusive (1 sample excessive out of 480 tested) are generally satisfactory (Table 1); they reflect both the phytoplankton levels in the production bays and the regulatory authorities' and industry's efforts to prevent contaminated shellfish getting to the market.

Table 1. Marine Biotoxin Results 1996-2004

Year	No. of Samples	Toxins	'Unsatisfactory[‡]' samples	Analytical Method
1996-98	41	**DSP**	0	ELISA
1999	100	DSP	0	ELISA
2000	173	DSP/AZP	0	ELISA +Cell Assay
2001	211	DSP/AZP/ASP	1[†]	LC-MS + HPLC
2002	161	DSP/AZP/ASP	0*	LC-MS + HPLC
2003	108	DSP/AZP/ASP	0	LC-MS + HPLC
2004	100[#]	DSP/AZP/ASP	----	LC-MS + HPLC

[‡] Corresponds to greater than 16µg DSP/100g edible tissue, 16µg AZA/100g edible tissue or 20µg Domoic Acid/g edible tissue.
[†] One sample of blue mussels was found with excessive DSP (58µg/100g edible tissue). This sample was found to have been placed on the market by an unauthorised harvester.
[*] The presence of elevated (but not excessive) ASP toxins was detected in a number of mussels.
[#] Projected number of samples for 2004.

The ongoing co-operation afforded to us by the Marine Institute is much appreciated.

MEASUREMENT OF CHEMICAL CONTAMINANTS IN SHELLFISH FROM SCOTTISH WATERS

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Abstract
Samples of blue mussels (*Mytilus edulis*), scallops (*Pecten maximus*) and oysters (*Crassostrea gigas*) from sites around Scotland were collected during March/April and August/September 2002. The polycyclic aromatic hydrocarbon (PAH), trace metal, chlorobiphenyl (CB) and organochlorine pesticide (OCP) concentration and composition has been determined in all three species from the two sampling occasions. The total measured PAH concentration in 23 out of the 28 mussel samples analysed was <150 ng/g^{-1} wet weight. On only two occasions was a concentration >250 ng/g^{-1} wet weight recorded. One sample (Loch Roag, summer) contained PAHs at a concentration, and with a relevant PAH distribution, such that the product was at risk of being tainted. All samples of oysters contained PAHs at a concentration <150 ng/g^{-1} wet weight, regardless of season. The PAH concentration in scallop gonad was, without exception, greater than in the corresponding adductor muscle. Trace metal distribution in mussels, oysters and scallops showed that the concentrations generally do not exhibit a high variance between sites. Very few samples exhibited Cu, Zn, Cd, Hg or Pb concentrations greater than the EC food safety limits. The CB concentrations determined in mussels were generally within the Ecotoxicological Assessment Criteria (EAC) established by OSPAR for mussels. An EAC has not been established for either oysters or scallops, but CB concentrations were within the EAC established for mussels. Organochlorine pesticide concentrations were generally within ranges found in previous studies.

Introduction
The European Union (EU) Shellfish Hygiene Directive (91/492/EC) includes the requirement to assess a range of elements of end product quality in shellfish offered for sale for human consumption. Therefore there is a need to conduct a targeted programme to provide new data on the concentrations of priority contaminants in harvested shellfish and to assess the contaminant levels in cultivated and natural populations of a number of shellfish species throughout Scottish coastal waters. Samples of shellfish from a number of production areas were collected in both March/April and August/September in 2002. Twenty-eight blue mussel (*Mytilus edulis*) samples, 9 Pacific oyster (*Crassostrea gigas*) and 15 king scallop (*Pectin maximus*) samples were collected. The number of samples per species reflected the relevant commercial importance of the products. The purpose of the work was to provide baseline information to the Food Standards Agency Scotland (FSA) on concentrations of contamination in these shellfish species in Scottish coastal waters and to meet obligations under 91/492/EC.

Materials and Methods
The number of mussel, oyster and scallop sampling sites selected for this exercise per Region, reflect the distribution and scale of shellfish and scallop harvesting areas by the Scottish shellfish industries (Figure 1). The samples of mussels and oysters collected were returned to Fisheries Research Services Marine Laboratory (FRS ML) where twenty mussels from each sample were shucked and the wet tissue homogenised for PAH, trace metal and CB/OCP analyses. Five oysters from each sample were treated as above. The FSAS selected the scallop sampling locations and

chartered commercial fishing vessels to provide trawled scallop samples for this exercise. On delivery to the FRS ML, between five and ten scallops (depending on the size of the individual animals in the sample supplied) were opened and the adductor muscle and gonad tissue removed. Both muscle and gonad tissues were homogenised separately.

Analytical methods

Analyses of these samples determined the soft tissue concentrations of polycyclic aromatic hydrocarbons[1] by GC-MSD; trace metals cadmium, copper, mercury, arsenic, lead, nickel and zinc by ICP-MS, chlorobiphenyls CBs 28, 52, 101, 118, 153, 138 and 180 and organchlorine pesticides[2], eg dichlorodiphenyltrichloroethane (DDT) by GC-ECD. Full details of the analytical methods are reported in McIntosh *et al.*, 2003. Quality assurance was provided through successful participation in the QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe) Laboratory Performance Study scheme. Furthermore, all methods are accredited by the United Kingdom Accreditation Service to ISO 17025.

Results and Discussion

Polycyclic aromatic hydrocarbons

The total measured PAH concentration determined in winter sampled mussels ranged from 16.7 ng g^{-1} wet weight tissue (Loch Roag, Western Isles) to 278 ng/g^{-1} wet weight tissue (Loch Striven, Strathclyde) and demonstrated considerable variability in concentration both within and between regions (Figure 2). The PAH concentrations determined in mussels from Strathclyde (winter samples) were significantly different (p=0.002) than for those from the other regions. The total measured PAH in mussels collected in the summer ranged from 24.7 to 158 ng/g^{-1} wet weight with one (exceptional) value of 620 ng/g^{-1} wet weight (Loch Roag, Western Isles). Total measured PAH concentrations between <50 and 150 ng/g^{-1} wet weight tissue in post-spawning mussels can be considered as typical background/reference values. PAH concentrations of between 50 and 150 ng/g^{-1} wet weight can be considered as background for pre-spawning mussels (Webster *et al.*, 2003). Mussel tissue PAH concentrations in the range 150 – 250 ng/g^{-1} wet weight may indicate an acute exposure or low-level chronic exposure to PAH contaminants. PAH concentrations >250 ng/g^{-1} wet weight tissue are likely to be indicative of a more severe (eg emergency) or long term chronic exposure. When interpreting the significance of or possible source of PAH contamination, percentage composition as well as total concentration should be considered. In the case of Loch Roag, the PAH concentration increased from 16.7 ng/g^{-1} wet weight in the winter sample to 629.5 ng g^{-1} wet weight in the mussels collected during the summer. The increase in concentration between winter and summer contrasts with the more conventional behaviour as observed at Loch Leven and Loch Striven (Figure 2). Furthermore,

[1] Total measured PAH: the 2- to 6-ring parent and branched PAH compounds naphthalene, 2-methyl naphthalene, 1-methyl naphthalene, C2, C3 and C4 naphthalenes; acenaphthylene (152); acenaphthene (154); fluorene (166); phenanthrene (178), anthracene (178), C1-C3 178; dibenzothiophene, C1-C3 dibenzothiophenes; fluoranthene (202), pyrene (202), C1-C3 202; benzo[c]phenanthrene (228), benz[a]anthracene (228), chrysene/triphenylene (228), benz[b]anthracene (228), C1-C2 228; benzo[b]fluoranthene (252), benzo[k]fluoranthene (252), benzo[e]pyrene (252), benzo[a]pyrene (252), perylene (252), C1-C2 252; dibenz[a,h]anthracene (278); indenopyrene (276), benzoperylene (276), C1-C2 276.

[2] HCB, a-HCH, (-HCH, heptachlor, heptachlor epoxide, oxychlordane, a-chlordene, (-chlordene, a-chlordane, (-chlordane, trans-nonachlor, DDE, DDD and DDT

although the winter PAH percentage composition was that of a 'typical' mussel profile, the summer sample was dominated by the naphthalenes and 3-ring PAHs and there was twice as much DBT in the summer sample than in the winter mussels. The significant reduction in the proportion of 4- to 6-ring PAH was also very marked (Figure 3). The data are consistent with an acute petrogenic input, most likely a hydrocarbon spill, impacting on the area where the mussels were sampled. PAH concentrations of this magnitude, and where there is a high proportion of naphthalenes, have resulted in tainting of *Nephrops norvegicus* (Moffat *et al.*, 1998) and a suspect taint in mussels (Topping *et al.*, 1997).

Trace Metals

Trace metal concentrations in mussels generally do not exhibit high variance between sites. The concentrations were within the ranges reported previously for mussels from Scottish coastal waters (Brown and Balls, 1997). The exceptions to this were a wider range of cadmium concentrations. The trace metal distribution in oysters generally showed little difference between sites. An exception to this was the zinc concentration determined in the sample from Kyle of Tongue, 343μg/g^{-1} wet weight compared to an average of 205 μg/g^{-1} from the other five sites. The concentrations of trace metals determined in scallop muscle and gonad tissues were all, with one exception, within the given Guideline and Imperative values for shellfish (Henderson and Davies, 2001). The exception was the zinc value from one sample (of two) from scallop gonad tissue from the Moray Firth (Box M2), 103 μg/g^{-1} wet weight (Figure 4). Mercury concentrations in all shellfish analysed were, in the main, below the analytical detection limit with only 18 % of samples containing detectable concentrations. All values determined were well below the value of 0.5 μg g^{-1} EC maximum limit (Comm. Reg. 466/4001/EC, amended by Comm. Reg. 221/2002/EC). Lead values in all shellfish ranged from 0.11 - 1.35 μg/g^{-1}, the maximum concentration being determined in mussels from Loch Sunart, possibly due to historical mining activity in the area. This was the only sample with a value greater than the 1 μg/g^{-1} EC limit.

Chlorobiphenyls (CBs) and Organochlorine pesticides (OCPs)

Ecotoxicological Assessment Criteria (EAC) (OSPAR 2000) can be used to assess data and identify potential areas of environmental concern based the ICES 7 CBs (CBs 28, 52, 101, 118, 153, 138 and 180) which have also been recommended by the European Union Community Bureau of Reference due to their relatively high concentrations in technical mixtures and their wide chlorination range. The EAC for the sum of the ICES 7 CBs for blue mussels is in the range 0.75 ng/g^{-1} to 7.5 ng/g^{-1} wet weight. All samples of mussels returned values less than 10 ng g^{-1}, with the exception of the sample from Loch Striven which contained a total of sum of ICES 7 CBs of 13.9 ng/g^{-1} wet weight. The sum of the DDTs ranged from 0.25 ng/g^{-1} in mussel from the Kyle of Tongue to 4.51 ng/g^{-1} wet weight in the sample from Loch Striven. The sum of the chlordanes ranged from < detection threshold (Loch Kishorn and Loch Etive) to 1.69 ng/g^{-1} wet weight in the sample from Sandsound in Shetland. The concentrations of other (organochlorine) pesticides determined were generally low ranging from less than detection threshold to less than 1.0 ng/g^{-1} wet weight.

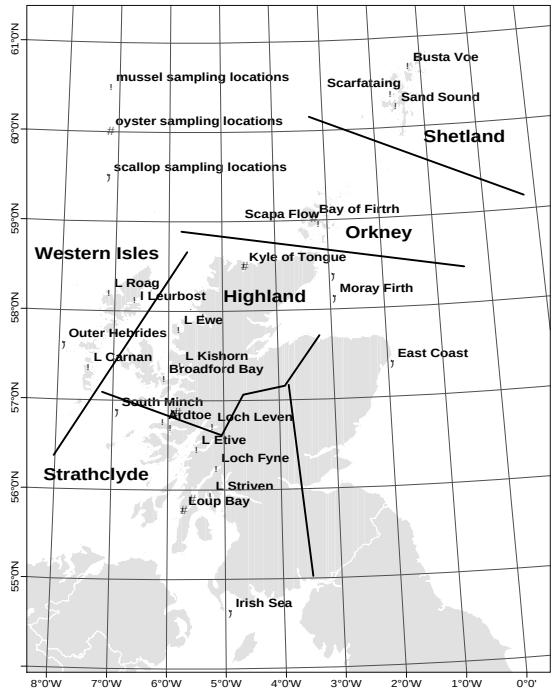


Figure 1. Sampling locations for mussels, oysters and scallops, 2002. The Regions, borders for which are shown by the solid lines, are named in **bold**. L = Loch.

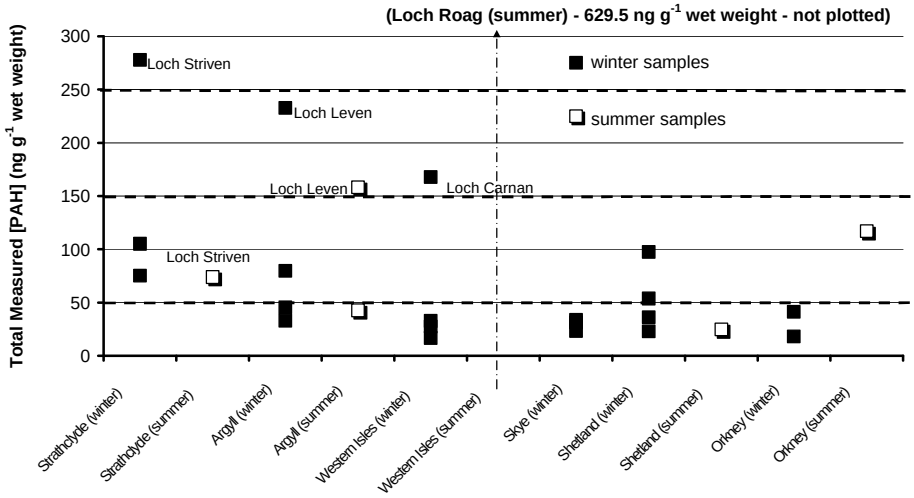
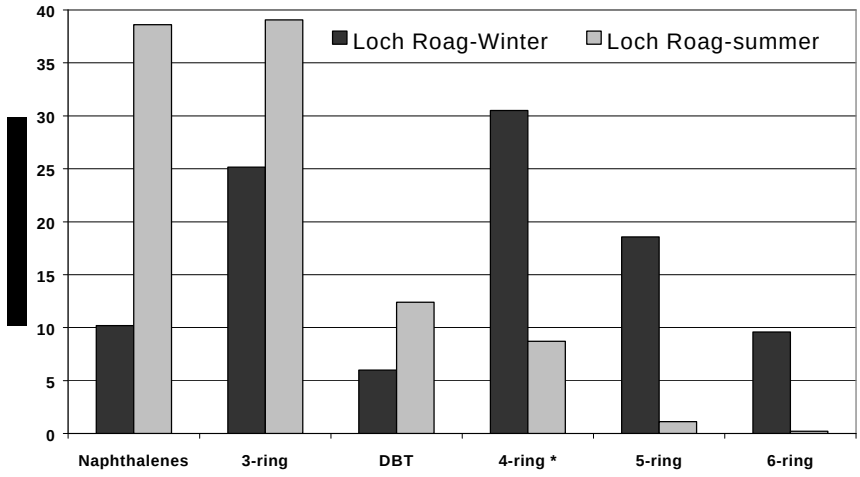


Figure 2. Total measured PAH concentration in mussels, winter and summer 2002. (Argyll includes Loch Etive, Loch Leven, Loch Ailort and Loch Sunart)



*4-ring *m*/*z* 202 and *m*/*z* 228

Figure 3. PAH percentage composition in mussels (*Mytilus edulis*) from Loch Roag (Western Isles) sampled during winter and summer 2002.

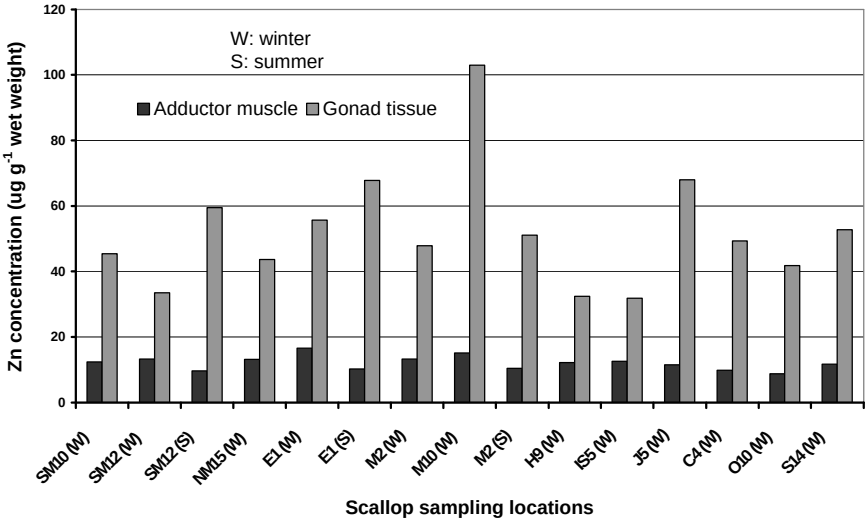


Figure 4. Zinc concentrations in scallop (*Pectin maximus*) adductor muscle and gonad tissue sampled from various offshore locations during winter and summer 2002. Samples are gathered from a specific box, as adopted in part of the Scottish shellfish biotoxin monitoring programme (Petrie, 2004). (SM-South Minch; NM-North Minch; E-East coast; M-Moray Firth; H-Hebrides; IS-Irish Sea; J-Jura; C-Clyde; O-Orkney; S-Shetland)

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PATHOLOGICAL STUDY OF AZASPIRACID POISONING IN MICE

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Abstract
Azaspiracid poisoning first occurred in 1995 after ingestion of mussels, *Mytilus edulis*, produced in Ireland. The lethal toxicity in mice by oral administration was 200~500 μg/kg (b.w.) for the major causative toxin named Azaspiracid. Pathologically, multiple organ damage (inflammation in the lung, fat accumulation in the liver, erosion of small intestine and stomach, and necrosis of lymphocytes in the thymus and spleen) were seen. A chronic exposure experiment revealed gas accumulation in the digestive organs as well as death at subacute levels, and some tumors were observed in mice exposed for up to 1 year.

Introduction
Azaspiracids (AZA) have been reported to cause symptoms including nausea, vomiting, diarrhea and stomach cramps in humans, and an acute bioassay using mice showed some neurological symptoms (McMahon and Silke, 1996, 1998). To clarify the pathological changes, and to assess the risks posed by AZA, this work studied morphological changes in the organs of mice following acute and chronic exposure to azaspiracid-1.

Materials and Methods
In the present experiments, azaspiracid–1 (AZA-1) was used for the pathological studies. AZA-1 was extracted from blue mussels, *Mytilus edulis*, collected in Ireland in 1996 and 1997 (Satake *et al.*, 1998a,b). A stock solution of AZA-1 was prepared by dissolving 1.0 mg of the toxin in 2 ml of aqueous 50 % ethanol. Two separate experiments were conducted for the study of subacute poisoning in mice. In the first experiment, 5 – 10 mice were exposed at levels from 1 – 50μg/kg (b.w); in the second experiment ca. 20 mice were dosed at levels ranging from 5 – 20 μg/kg (b.w.). A total of 39 male ICR (4 week) mice was use for acute poisoning experiment, the 25 surviving mice were used for a recovery experiment. Additionally, 126 mice were used for chronic experiments (+control n=49). The AZA was dissolved in 0.2 mL portions of saline and administered orally by gastric tube. Mouse organs were fixed with 10 % formalin and embedded in paraffin; these sections were stained with H and E and PAS and observed by light microscopy. .

Results and Discussion
1. Acute poisoning in mice
The lethal toxicity of AZA to mice by oral administration was in the range 200~500μg/kg (b.w.). Mice showed individual differences in sensitivity to the toxin. In particular it was observed that older mice died at the lowest dose levels (Ito *et al.*, 2002). Pathologically, multiple organ damage was observed including inflammation or edema in the lung, fatty change and single cell necrosis in the liver, erosion both in the stomach and small intestine, and lymphocyte necrosis in the thymus and spleen (Ito *et al.*, 2000). The time period for recovery varied between different organs ranging from 10 days in the lymphoid tissues, 15 days in the liver, 8

weeks in the lung, but the recovery in the gastro-intestinal mucosa was not complete 3 months after withdrawal (Ito *et al.*, 2002).

2. Subacute poisoning in mice

In the first chronic exposure experiment, high levels of mortality were observed at the highest dose level of 50 µg/kg twice weekly with 100 % of mice dying within 5 months. With repeated p.o. administration at 20 µg/kg twice weekly mortality was still 30 % after 5 months (Figure 1). No effects, however, were observed at the lowest level of 1 µg/kg. Based on these results the second experiment used a narrower and lower dose range from 5 – 20 µg/kg. In the second study, 9 of the 20 mice died after 15 administrations at 20 µg/kg. In these mice, a large amount of gas accumulation in the digestive organs (stomach, small and large intestines) was common (Figure 2). There are a number of possible mechanisms that could have led to the observed gas accumulation including: 1) As the observed recovery from the damage to the lamina propria was slower than 3 days, the 2 treatments per week could have exacerbated the erosion in the small intestine. This could, therefore, have resulted in complete erosion of the villi on the surface, with the result that gas could not be absorbed 2) interstitial inflammation in the lung was also observed in these mice and, thus, due to the lowered respiratory function, CO_2 could not be carried from the intestine. The results indicate that 20 µg/kg, (~10 % of the lowest acute lethal dose) becomes a lethal dose by ingestion after relatively few exposures.

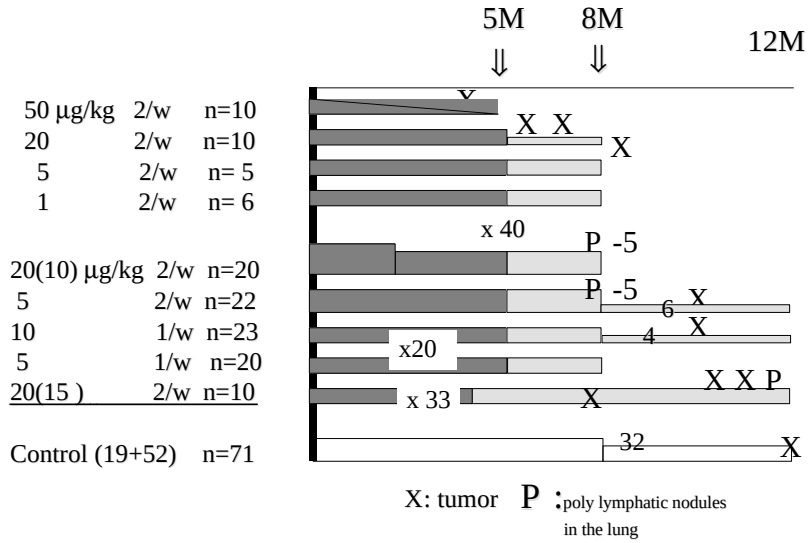

Figure 1. Outline of chronic administration of AZA and appearance of tumors and poly-lymphatic nodules in the lung

3. Tumor appearance in mice

In the first chronic experiment, lung tumors were observed in 1 of the 10 mice given the highest exposure dose of 50 µg/Kg and in 3 of the 10 mice given 20 µg/Kg. The second study used more animals in an attempt to confirm the first study. Indeed, a further 5 tumors were observed in the second study. Thus, in total , tumors were observed in 9 out of 126 treated mice (X in Figure 2). The highest dose that was not lethal at chronic exposure (boundary dose) consistently caused tumors in both experiments, i.e. 3 tumors were observed in both experiments one and two for the

mice exposed at 20 (or 15) µg/kg. Amongst the 9 tumors observed, 7 were lung tumors (Figure 3) and 2 were malignant lymphomas (Figure 4).

Only twenty mice could be observed after 12 months, while the others were either sacrificed after 8 months, or died during dosing period. More tumors may have been observed if all mice had survived up to 1 year. Because 10 mice of 27 sacrificed at 8 months had multiple lymphatic nodules in the lungs (P in Figure 2), some of them might correlate with malignant lymphoma.

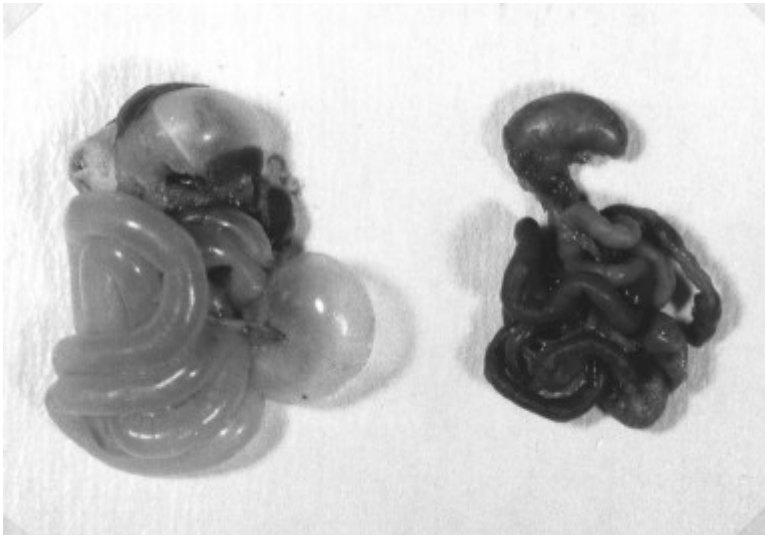

Figure 2. Gas accumulation in the digestive organs of mice following repeated doses of 20 µg/Kg (b.w.) of AZA-1. (right: control).

The ratio of tumor-bearing mice was >7 % (9/126), while control mice did not show any tumors. Since ICR mice show a relatively high ratio of spontaneous tumors at 2 years old (Giknis, 2000), Azaspiracid can be either tumorigenic itself or a promoter to the early appearance of tumors. Future work should consider a larger number of mice, not only for better statistical assessment of the observed effects but also to show whether the observed lymphomas will lead to cancer after the exposure is ceased.

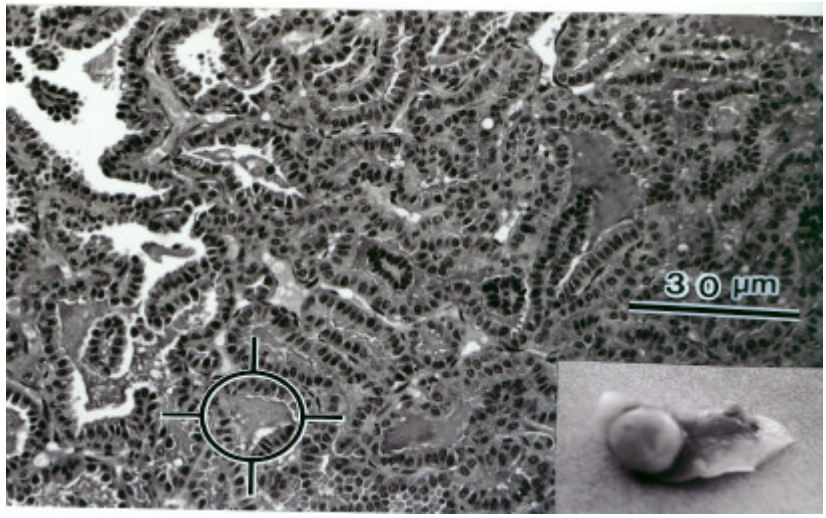

Figure 3. Lung tumor (adenocarcinoma) AZA: 20(15) µg/kg x33)

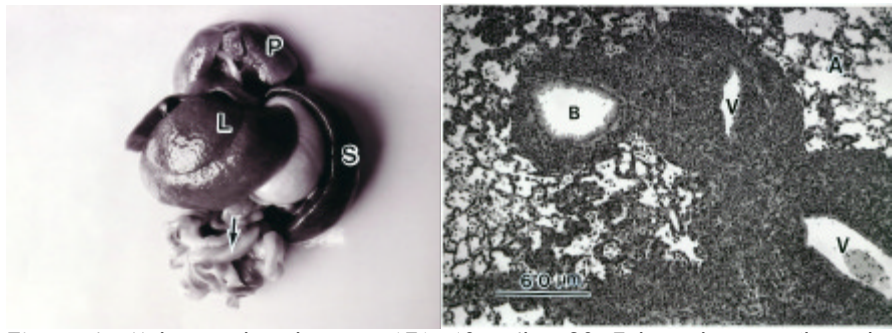

Figure 4. Malignant lymphoma. AZA: 10 µg/kg x20. Enlarged organs (lung, liver, spleen) and lymph node are filled with lymphocytes (systemically). In the lung (right), lymphocytes are increasing around blood vessels and bronchioles.

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YESSOTOXINS ALTER THE MOLECULAR STRUCTURES OF CELL-CELL ADHESION IN CULTURED CELLS

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Abstract
Treatment of cultured epithelial cells with yessotoxin (YTX) induces the accumulation of a 100 kDa fragment of the cell-cell adhesion protein E-cadherin. The phenomenon is clearly detectable after 48 hr of treatment and proceedes to complete disruption of the cellular E-cadherin pool in the following days. We found that cell treatment with YTX is accompanied by a decrease of the E-cadherin-catenin complexes extractable from epithelial cells, indicating that the molecular defect induced by YTX consists in the removal of the intracellular domain of E-cadherin, which contains the binding site for ß - and ?-catenins, as well as the pp120 protein. The disruption of the E-cadherin-catenin system we have detected is caused by very low concentrations of YTX (1-2 µg/l). Based on these observations, and the fact that E-cadherin represents a tumour suppressor, a re-orienting of investigations onto the risks posed by YTX in humans seems appropriate, towards a more thorough characterization of long term effects due to low-dose and/or repeated ingestion of YTX.

Introduction
Cell-cell contacts, involving components of the cadherin family of calcium-dependent adhesion proteins, maintain proper tissue architecture and functioning in animals. The integrity of epithelia, in particular, depends on the complex multiprotein assembly of the E-cadherin-catenin system, whose functioning and ultrastructural organization control other fundamental biological processes, such as proliferation and morphogenesis (Yap *et al.*, 1997; Christofori and Semb, 1999, Tepass *et al.*, 2000). The general organization of the cadherin-catenin system is best exemplified by the adherens junctions, where interacting E-cadherin molecules protruding from one cell interact with those of an adjacent cell, and the mechanical strength of cell-cell adhesions is given by the binding of E-cadherins to actin molecules of the cytoskeleton, which is mediated by proteins of the catenin family (Ozawa *et al.*, 1989; Christofori and Semb, 1999; Beavon, 2000). The functional roles of the E-cadhein-catenin system do not stem exclusively from its property to maintain proper cell-cell adhesion, but from its participation in signal transduction mechanisms as well (Christofori and Semb, 1999; Sharpe *et al.*, 2001; Taipale *et al.*, 2001).
Yessotoxins (YTX) represent sulfated polyether compounds produced by dinoflagellates, which can be accumulated in bivalve molluscs by filter feeding (Yasumoto and Murata, 1993). A severe acute toxicity of these compounds has been documented by *i.p.* injection (Terao *et al.*, 1990; Ogino *et al.*, 1997; Aune *et al.*, 2002) but not by oral administration (Aune *et al.*, 2002; Tubaro *et al.*, 2003; Tubaro *et al.*, 2004) in the mouse. Mouse dosing with 2-2.5 mg/kgYTX by oral intubation, in

fact, does not lead to death, although some morphological alterations have been detected in the heart by electron microscopy (Aune *et al.*, 2002; Tubaro *et al.*, 2003). These observations have stimulated the investigations aimed at the characterization of the mechanism of action of YTX and at a deeper understanding of oral toxicity of YTX with regard to long term effects. Our interest in the effects exerted by lipophilic phycotoxins on molecules involved in cell-cell adhesion, stems from the original observation that okadaic acid (OA) causes loss of cellular E-cadherin and cell detachment from culture dishes when administered to MCF-7 breast cancer cells (Malaguti and Rossini, 2002). Since YTX can be found in contaminated mussels in conjunction with OA and other algal toxins (Yasumoto and Murata, 1993; Ogino *et al.*, 1997), we have analysed the effects induced by major lipophilic toxins (OA, YTX, pectenotoxin-6) on E-cadherin. In this paper we show that the treatments of cultured cells with OA and YTX induce distinct alterations in the E-cadherin-catenin system.

Materials and Methods

Reagents and procedures used in the present study have been already described in detail elsewhere (Malaguti and Rossini, 2002; Pierotti *et al.*, 2003; Ronzitti *et al.*, 2004). If not stated otherwise, the general organization of our experiments included MCF-7 cell treatments with 1 nM YTX for 24 hr at 37° C, cell harvest and preparation of cytosoluble extracts from cell lysates, fractionation of proteins in cytosoluble extracts by electrophoresis (SDS-PAGE). The E-cadherin-catenin complexes have been immunoprecipitated by the protocol we have already described (Ronzitti *et al.*, 2004), using the HECD-1 anti-E-cadherin antibody. After fractionation, proteins were detected by immunoblotting, the membrane was developed by a chemiluminescent detection system, and results were visualised by autoradiography.

Results

Our initial experiments were devoted to a comparative analysis of the effects which different lipophilic phycotoxins could induce on E-cadherin in cultured cells (Figure 1). Treatment of MCF-7 cells with 50 nM OA for 24 hr confirmed our original observations that this toxin causes more than 70 % loss of cellular E-cadherin (Malaguti and Rossini, 2002). A thorough evaluation of the phenomenon revealed that this loss of cellular E-cadherin is accompanied by the accumulation of a 135 kDa protein, which is recognised by anti-E-cadherin antibodies, and has been proposed to represent the unprocessed precursor of E-cadherin (Shore and Nelson, 1991). Cell treatment with 1 nM YTX, in turn, causes the accumulation of a 100 kDa fragment of E-cadherin, without a concomitant loss of the intact protein within the first 48 hr of treatment (Figure 1). No detectable change in the cellular content and size of E-cadherin could be observed, instead, when MCF-7 cells were treated with micromolar concentrations of pectenotoxin-6 (Figure 1).

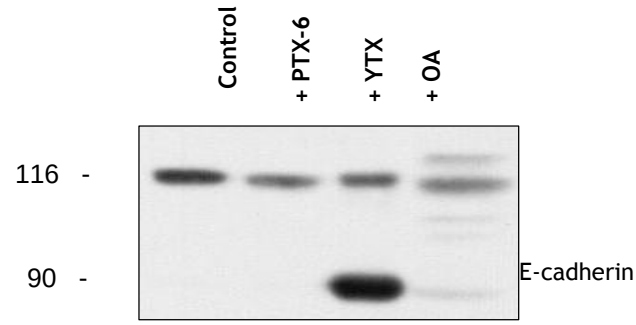

Figure. 1. Effect of MCF-7 cell treatment with okadaic acid, yessotoxin and PTX-6 on E-cadherin. Cells have been treated with 50 nM okadaic acid, 1 nM yessotoxin, 1 µM PTX-6, or vehicle, for either 24 (okadaic acid) or 48 hr (vehicle, yessotoxin, PTX-6) at 37°C, and were then used to prepare cytosoluble extracts, which were fractionated by SDS-PAGE, and were analysed by immunoblotting using the HECD-1 anti-E-cadherin antibody. Immunocomplexes were then detected by enhanced chemioluminescence and visualised by autoradiography. The electrophoretic mobilities of ß-galactosidase (116 kDa) and fructose-6-phosphate kinase (90 kDa) subunits, used as marker proteins running in a parallel lane, on the left.

Thus, the 135 kDa E-cadherin related antigen ($ECRA_{135}$), and the 100 kDa E-cadherin related antigen ($ECRA_{100}$) constitute toxin-specific molecular markers of OA and YTX actions, respectively, in cultured cells.The increase in the cellular levels of $ECRA_{100}$ is clearly detectable after 16-18 hr of MCF-7 cell treatment with YTX and proceedes to complete disruption of the cellular E-cadherin pool in the following days (Ronzitti *et al.*, 2004).

Experiments carried out with monoclonal antibodies recognizing different portions of the E-cadherin molecule revealed that the molecular defect of $ECRA_{100}$ affects the intracellular domain of the protein, which could be totally or partially lost (Ronzitti *et al.*, 2004). Since the intracellular domain of the E-cadherin protein contains the binding site for ß- and ?-catenins, as well as for pp120, we decided to probe at which extent YTX treatment could destroy the E-cadherin-catenin complexes in epithelial cells. This issue was technically approached by immunoprecipitation of material in cytosoluble extracts from control and YTX-treated cells, using an anti-E-cadherin antibody recognizing an epitope located in the extracellular domain of the protein, such as the HECD-1 monoclonal antibody (Shimoyama *et al.*, 1989). By this procedure, any protein physically associated with the intracellular domain of E-cadherin could be co-imunoprecipitated with the antigen, and would be separated from other unrelated cellular components, thereby allowing an analysis of participants to E-cadherin-catenin complexes in control and YTX-treated cells.

A preliminary study showed that MCF-7 cell treatment with YTX leads to a substantial decrease in the levels of ß- and ?-catenins associated with E-cadherin, implying that YTX causes the disruption of this type of interaction (Ronzitti *et al.*, 2004). Since it is recognised that pp120 is associated with the juxta-membrane, intracellular portion of E-cadherin (Yap *et al.*, 1998), we wished to determine whether YTX treatment of MCF-7 cells completely disrupted the E-cadherin-catenin system or was responsible for a partial alteration of the supramolecular arrangement of the multiprotein complex. We then obtained cytosoluble extracts

from control and YTX-treated cells, separated the E-cadherin-catenin complexes from other cellular proteins by immunoprecipitation with an anti-E-cadherin antibody, recovered the antigen-antibody complex, including E-cadherin-associated proteins, by the use of protein G-Sepharose, and analysed the immunoprecipitated components by immunblotting of proteins fractionated by SDS-PAGE. The results we obtained are reported in figure 2, and show that YTX caused extensive alteration of antigens in unfractionated extracts only in the case of E-cadherin, whose cellular pool was mostly represented by protein fragments, including $ECRA_{100}$. The pattern of proteins associated to E-cadherin and recovered by immunoprecipitation with an anti-E-cadherin antibody, in turn, was severely affected by cell treatment with YTX, as we detected a 40-70 % decrease in the levels of pp120 associated with E-cadherin (Figure 2). By inducing the fragmentation of E-cadherin, therefore, YTX does not only induce a loss in associated ß- and ?-catenins, but it causes the derangement of the entire set of components associated with the intracellular domain of E-cadherin in epithelial cells.

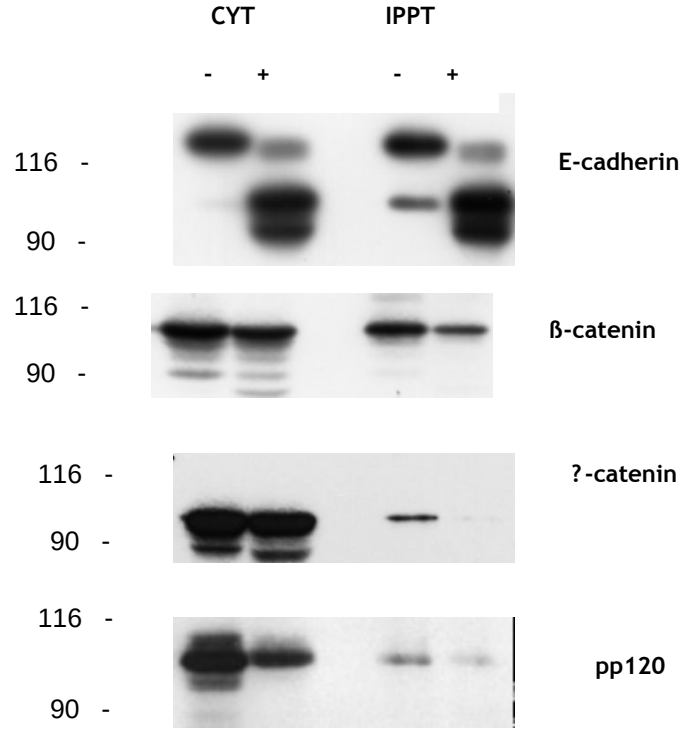

Figure 2. Effect of yessotoxin treatment of MCF-7 cells on the levels of ß- and ?-catenins, and pp120 associated with E-cadherin. MCF-7 cells were treated with (+) or without (-) 1 nM YTX for 5 days at 37°C. At the end of the incubation cells were processed to prepare cytosoluble extracts, which were subjected to immunoprecipitation using the HECD-1 anti-E-cadherin antibody. Identical aliquots of cytosoluble extracts (CYT) and of immunoprecipitated material (IPPT) were subjected to SDS-PAGE and immunoblotting, using antibodies racognizing the indicated proteins. The electrophoretic mobilities of ß-galactosidase (116 kDa) and fructose-6-phosphate kinase (90 kDa) subunits, used as marker proteins running in a parallel lane, are indicated on the left.

Discussion

The alteration of the E-cadherin-catenin system has been found in many types of cancers (breast, colon, prostate, intestine, etc.), and disruption of E-cadherin functions is believed to play a role in tumor progression, tumor spreading and metastasis formation (Birchmeier and Behrens, 1994; Christofori and Semb, 1999; Beavon, 2000). The treatment of cultured MCF-7 cells with OA and YTX was found to alter the cellular pool of the cell adhesion protein E-cadherin. The extensive loss of the cellular pool of this protein induced by a 24 hr treatment of cultured cells with OA has been found to cause cell detachment from culture dishes (Malaguti and Rossini, 2002). Although we have found that this effect accompanies a cell death response, we cannot presently exclude that OA might cause loss of E-cadherin *in vivo* and that disruption of cell-cell contacts mediated by E-cadherin might contribute to tumour promoting effects of OA (Fujiki and Suganuma, 1993). The disruption of the E-cadherin-catenin system induced by YTX has been found to result in a loss of cell adhesion only after prolonged (4-5 days) treatment of cultured cells *in vitro*, but a cell death response was not induced by this toxin in MCF-7 cells (Ronzitti *et al.*, 2004). Under our conditions, the accumulation of a fragment of E-cadherin induced by YTX was accompanied by an extensive alteration of the supramolecular arrangement of the E-cadherin-catenin complexes, comprising a decreased interaction of E-cadherin with pp120. YTX, therefore, could also induce an alteration of the signal transduction involving the Src substrate pp120 (Parsons and Parsons, 1997; Martin, 2001). The effects induced by YTX in cultured cells pose the question on whether they may have potential adverse effects in humans, as a consequence of eating shellfish contaminated by this toxin. The very low concentrations of YTX (1-2 μg/l) capable to induce the disruption of the E-cadherin-catenin system, and the possibility that this phenomenon might lead to tumor spreading and metastasis formation, could indicate a serious danger linked to ingestion of shellfish contaminated by YTX. On the other hand, the low intestinal absorption of YTX, implied by the low oral toxicity of the compound (Aune *et al.*, 2002; Tubaro *et al.*, 2003; Tubaro *et al.*, 2004), and the prolonged exposure to the toxin which seems to be required to disrupt the interaction between E-cadherin and catenins (Ronzitti *et al.*, 2004), would counterweight the concern regarding the real toxicity of YTX in humans. Based on our observations and these considerations, a re-orienting of investigations onto the risks posed by YTX in humans seems appropriate. The low acute toxicity of YTX by oral ingestion should not jeopardise further investigations onto more elusive, but potentially severe risks posed to consumers by eating shellfish contaminated by this toxin. Thus, an accurate assessment of long term effects due to low-dose and/or repeated ingestion of YTX in animals is needed.

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DEVELOPMENT OF A FUNCTIONAL *IN VITRO* BIOASSAY FOR AZASPIRACIDS (AZA) USING HUMAN COLONIC EPITHELIAL CELLS

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Abstract
Azaspiracid is a marine biotoxin found in shellfish and is responsible for gastrointestinal disturbances in humans. The aims of this study were to examine the effect that AZA-1 has on gastrointestinal cells and to develop an *in vitro* assay to assess toxicity of AZA-1. The human colon cell line - Caco-2 cells – were grown on microporous membranes until they formed an intact monolayer similar to the *in-vivo* gastrointestinal tract. The intactness of the monolayer was measured by transepithelial electrical resistance (TER) and is a useful index of the function of these cells in maintaining the transport of solutes and water. TER was measured using an electrical resistance measurement device and expressed as the change in TER with respect to time matched controls (? TER (? .cm^2). Varying concentrations (0 –100 nM) of AZA-1 were added for different time-points of 24, 48 and 72 hours. Significant reductions in TER were detected at 5, 10 and 100 nM AZA-1.This assay has proved to be sensitive for detection of AZA-1. The level of 5 nM would be analogous to a concentration of 0.0042 μg/g.

Introduction
In 1995 a novel shellfish poisoning incident was reported in the Netherlands from mussels, *mytilus edulis,* imported from Killary Harbour, Ireland. Symptoms reported included nausea, vomiting, severe diarrhoea and stomach cramps (Mc Mahon and Silke, 1996). The toxin was later identified and named as azaspiracid (AZA-1) (Satake *et al.*, 1998). To date ten additional analogues of azaspiracid have been identified (Ofuji *et al.*, 1999, 2001; James *et al.*, 2003) of which AZA-1 is the predominant analogue found in nature (James *et al.*, 2002). Initial toxicological studies reported multiple organ damage in mice when exposed to azaspiracid. The symptoms included necrosis, followed by erosion of the lamina propria of the small intestine, dilation of the stomach and fatty changes in the liver. In addition to this, necrotic lymphocytes in the thymus, spleen and Peyer's patches were also observed (Ito *et al.*, 2000). Recently chronic studies have reported the presence of lung tumours and hyperplasia of epithelial cells in the stomach of mice (Ito *et al.*, 2002).

Epithelial cells are capable of forming barriers between separate compartments. The epithelium lining the intestine acts as a barrier to the external environment. The integrity of the barrier is maintained by intercellular junctions, the most apical of which is the tight junction (Farquhar and Palade, 1963). The tight junction structure is located around the circumference of the cell where it is responsible for regulating the passage of ions and small molecules through the paracellular pathway. Altered epithelial paracellular permeability has been associated with diarrhoea in patients (Teahon *et al.*, 1992).

Materials and Methods
Azaspiracid-1 was supplied by Dr. Philipp Hess (Marine Institute, Galway, Ireland).

Cell culture
Human colon carcinoma cell line (Caco-2) was obtained from the European Collection of Cell Cultures (Wiltshire, U.K.). Cells were cultured in Dulbecco's modified Eagle's medium with GLUTAMAX-1 (Gibco, UK) containing 10 % foetal calf serum (FCS), 1 % non-essential amino acids, 50 U ml^{-} penicillin, 50 µg ml^{-} streptomycin at 37°C in a humidified atmosphere of 5 % CO_2 – 95 % air. The cells used for the experiment were at passages 43 – 55.

Transepithelial electrical resistance experiment
Caco-2 cells were cultured on polycarbonate membrane HTS Transwell™-24 inserts (0.33 cm^2, 0.4 µm pore size, Corning Costar, NY, U.S.A.). The integrity of the monolayer was monitored by measuring transepithelial electrical resistance (TER) using the REMS® robotic device (World Precision Instruments, U.K.). TER values were typically in the range of 700 $\Omega.cm^2$. The upper chamber (apical side) contained 0.25 ml medium and the lower one (basolateral side) contained 1 ml medium. Cells were exposed to AZA-1 on the apical domain. The point of addition of the drug was taken as time zero and TER across the monolayers was monitored over a range of timepoints (24 to 48 h). The TER 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. The results were expressed as the change in TER with respect to time matched controls (ΔTER ($\Omega.cm^2$)).

Alamar Blue viability assay
After incubation with AZA-1, cell media was removed from Caco-2 cells and replaced with DMEM containing 10 % Alamar Blue™ (Biosource) for 2 h at 37°C. Fluorescence was determined at 540 nm excitation wavelength and 590 nm emission wavelength using a microplate reader (Wallac 1420 Multilabel HTS counter).

Results and Discussion
As the main symptoms of AZA toxicity in humans are gastrointestinal disturbances, human intestinal cells were chosen to develop an *in vitro* assay relevant to the *in vivo* effects in humans. This also served to eliminate any species-species variations that could occur with animal based models. The human colon carcinoma cell line Caco-2 was selected for these studies due to their ability to form tight junctions and generate a transepithelial electrical resistance (TER). The effect of AZA-1 on cellular viability was initially assessed using the Alamar Blue assay. Reduction of Alamar Blue from an oxidised (non-fluorescent, blue) form to a reduced (fluorescent, red) form was used as a measure of metabolic activity. Caco-2 cells were exposed continuously to AZA-1 (0.01 – 100 nM) for periods of 24, 48 and 72 hours. This assay did not detect any loss in cellular viability but an increasing trend in viability was observed at 24 hours with concentrations of 5 nM or higher (Figure 1). This increase could be due to increased cellular proliferation although further work would be necessary in order to confirm this.

TER studies were carried out using the WPI REMS Autosampler, an automated electrical resistance measurement device. After a stable TER reading was achieved cells were exposed continuously to AZA-1 (0.1 - 100 nM) for periods of 24, 48 and 72 hours. No significant change in TER was observed at any time-point up to a concentration of 2.5 nM. A significant decrease was observed at 24, 48 and 72 hours with 5 nM AZA-1 or higher (Figure 2). This decrease in TER correlates with an

increase in paracellular permeability. The ability of AZA-1 to functionally alter Caco-2 barrier function mimics the *in vivo* situation. This disruption to the barrier function could in turn enhance antigenic exposure to underlying immune cells, further compromising barrier function (Bruewer *et al.*, 2003). An assessment of tight junction components e.g. occludin, claudin would clarify AZA-1's ability to alter paracellular permeability in this model.

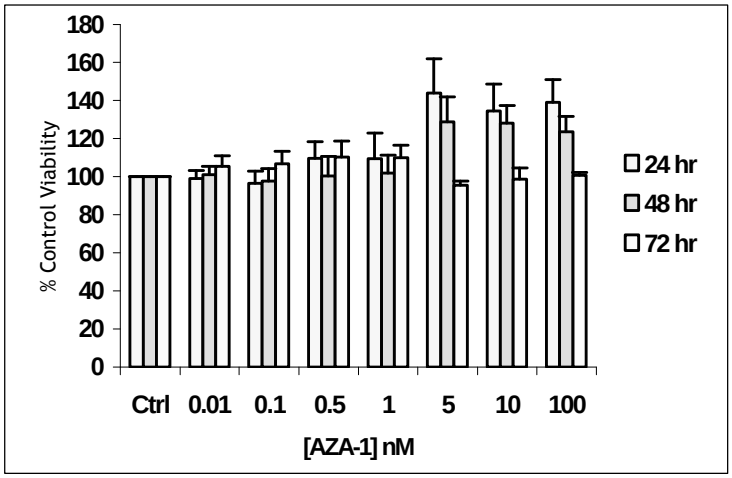

Figure 1. Relative viability of Caco-2 cells treated with AZA-1 (0.01 - 100 nM) for 24, 48 or 72 hrs.Cells were grown to confluency on 24 well plates and treated with AZA-1 (0.01 - 100 nM) for a period of 24, 48 or 72 hrs. At 24 hrs cell culture medium was removed and replaced with Alamar blue for 2 hrs. The fluorescence of each well was measured. Each value represents the mean ± S.E.M. of 3 experiments.

The current regulatory limit is 0.16 μg of azaspiracid/g edible mollusc part, which equates to approximately 190 nM. The ability of this assay to detect levels of 5 nM is potentially very useful. While the sensitivity of this model in detecting pure AZA-1 induced alterations in epithelial function are clear, it must be stressed that in a real life *in vivo* human situation, dilution factors and possible breakdown in the gastrointestinal tract would have to be taken into account in determining a NOAEL.

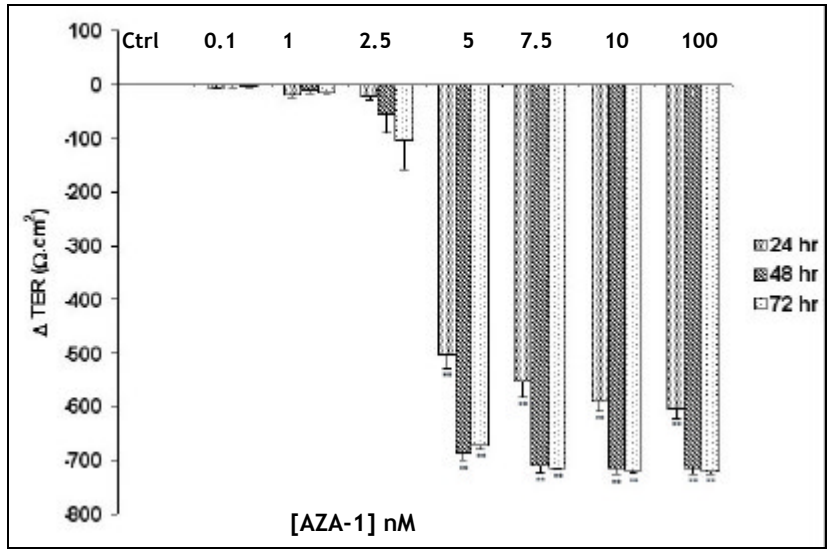

Figure 2. Effect of AZA-1 on the transepithelial electrical resistance across Caco-2 cell monolayers using the REMS autosampler. Cells were grown to confluency on HTS Costar Transwell filters and treated with increasing concentrations of AZA-1 (0.1 – 100 nM) for a period of 24, 48, 72 hrs. Each value represents the mean ± S.E.M. of 3 experiments. Indicates statistically different to control : ** $p<0.01$.

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TOXICITY OF YESSOTOXIN IN MICE AFTER REPEATED ORAL EXPOSURE

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Abstract
Studies in mice have indicated that the oral toxicity of YTX is at least one order of magnitude less than via the intraperitoneal route after single exposures to the toxin and that the heart muscle (myocardium) is the prime target organ for the toxic effects. However, more studies are needed on the oral toxicity of YTX and in particular the effects of repeated oral exposure. In the present study, YTX was given to mice twice weekly by gavage, all together 7 times at doses of 1, 2.5 and 5 mg kg^{-1} body weight. Controls received vehicle only, using the same procedure. All four groups of mice were killed 3 days after the last exposure and organs were studied by light and electron microscopy. Clinical signs or effects on weight gain were not observed in mice exposed to YTX. Pathological changes were not detected by light microscopy in myocardium, lung, liver, kidney, small intestine, spleen, thymus, brain, pancreas, testes or adrenal gland in any of the groups. Electron microscopy studies of the myocardium could not demonstrate any effects of repeated oral exposure of YTX, even at 5 mg kg^{-1} body weight per dose. Our results indicate that YTX present a small problem to human consumers of shellfish.

Introduction
Yessotoxin is produced by the algae *Protoceratium reticulatum* and accumulates in bivalve molluscs (Satake *et al.*, 1997). In blue mussels (*Mytilus edulis*) YTX is partly converted to 45-hydroxy-YTX, carboxy-YTX and other metabolites (Aasen *et al.*, submitted). Nevertheless, YTX constitutes a considerable part of the YTX group in mussels. The YTXs were removed from the Diarrhoeic Shellfish Poisoning (DSP) toxin complex and established as a separate toxin group by the EU Commission in 2002. The reason was that YTXs are not diarrhoeagenic (Daiguji *et al.*, 1998, Murata *et al.*, 1987) and have other mechanisms of action than the DSP toxins (Ogino *et al.*, 1997, de la Rosa *et al.*, 2001, Alfonso *et al.*, 2003). In addition, there are no reports of toxicity in humans caused by YTXs.

Previous studies on acute toxicity in mice have shown that YTX is far less toxic via the oral route, compared to intraperitoneal injections (Terao *et al.*, 1990, Ogino *et al.*, 1997). These data and preliminary results from oral studies using higher concentrations (Aune *et al.*, 2002) led the EU to increase the tolerance level of YTX in shellfish to 1 mg kg^{-1} shellfish meat. In a study with single oral YTX exposure of mice at 1, 2.5, 5 or 10 mg kg^{-1} body weight, no lethal effects were observed, and only slight, dose-dependent morphological changes in the myocardium were seen by transmission electron microscopy (TEM) at YTX doses of 2.5 mg kg^{-1} and above (Aune *et al.*, 2002). These changes were swelling of myocardial cells and separation of organelles close to the capillaries. In a similar study, no alterations were found in 17 organs by light microscopy in mice treated once with YTX at 1 or 2 mg kg^{-1}, or the analogues homoYTX or 45-OH-homoYTX at 1 mg kg^{-1} (Tubaro *et al.*, 2003). Using TEM, they found moderate changes (cytoplasmic protrusions) of myocardial cells in mice that were killed 24 hrs after exposure. The aim of the present study was to examine the effects of repeated oral exposure with YTX in mice. The mice were treated with YTX seven times within 21 days and weight gain and signs of disease

were monitored. Three days after the last YTX treatment, the animals were sacrificed and several tissues investigated for the presence of pathomorphological changes.

Materials and Methods

Toxin

YTX extracted from *P. reticulatum* was produced at > 95 % purity (NMR-spectrum) by Dr. Satake. The toxin was dissolved in 25 % EtOH, and delivered at 3 concentrations in 1 % Tween 60 with 1.25 % EtOH.

Animals

NMRI male mice, from Taconic MandB, Ry, Denmark with a bodyweight (b.w.) of approximately 14 g at the start of the experiment. The mice received RM1 maintenance diet from Scanbur BK, Nittedal, Norway, and mains tapwater *ad libitum*. They were not fasted. The mice were group-housed on aspen bedding (Scanbur BK) in standard Type III macrolone cages on a 12:12 light/dark cycle at 21-23°C.

Treatment

Each dose of YTX was given to three mice and five mice (controls) received the vehicle only (Tween 60 with 1.25 % EtOH). All mice received 250 µl YTX or the vehicle on day 0, 3, 7, 10, 14, 17, and 21 *per os*.

YTX doses

1.0, 2.5 or 5.0 mg kg^{-1} b.w. The cumulative dose in each group during 21 days was 7.0, 17.5 and 35.0 mg kg^{-1} b.w., respectively.

Autopsy and tissue sampling

The mice were killed on day 24, three days after the last treatment. The following organs were fixed in formaldehyde and studied by light microscopy after HE staining: myocardium, lung, liver, kidney, small intestine (duodenum and jejunum), colon, spleen, thymus, pancreas, brain, testes and adrenal gland.

The myocardium was also studied by transmission electron microscopy. Small pieces of tissue were fixed in 1 % paraformaldehyde and 2 % glutaraldehyde in 0.1 M cacodylatebuffer, pH 7.4, postfixed in 2 % osmium tetroxide in 0.1 M cacodylatebuffer for 1h, dehydrated in graded series of ethanol and embedded in Lx_{112}. Ultra thin sections were contrasted with uranyl acetate and lead citrate and examined in a Philips EM208S electron microscope.

Results

No clinical signs were observed in mice treated 7 times with YTX at doses of 1, 2.5 or 5 mg kg^{-1} b.w. There were no differences in weight gain between treated mice and controls (Figure 1). At autopsy, no gross pathological lesions were observed. No pathological effects were observed in any of the organs studied in any treatment group by light microscopy. No significant changes were observed in the myocardium by electron microscopy, including the areas surrounding the capillaries (Figure 2 A, B).

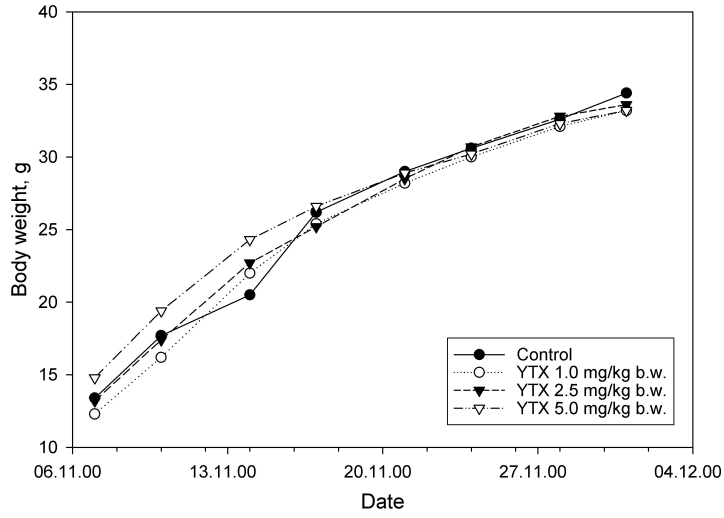

Figure 1. Weight gain, mice.

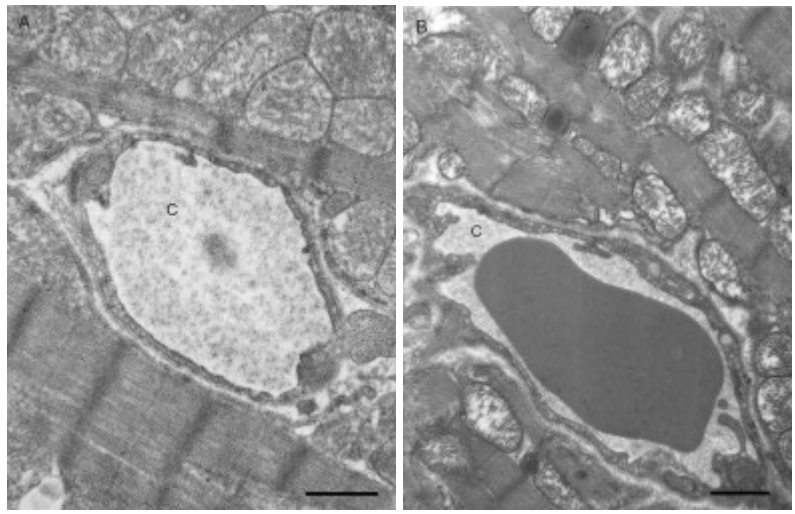

Figure 2. Transmission electron micrographs of myocardium. A) Control. B) From a mouse orally exposed to 5 mg kg^{-1} YTX 7 times during 21 days. No significant changes were observed. (C: Capillary. Bar: 1μm.)

Discussion

In this experiment, with oral gavage of YTX 7 times at 1.0, 2.5 or 5.0 mg kg^{-1} within 21 days, no clinical signs of disease, effects on weight gain or histopathological changes by light microscopy were observed. Using TEM, no significant differences between mice dosed with YTX and controls were observed, even in areas subjected to changes in previous studies on acute oral YTX exposure (Aune *et al.*, 2002; Tubaro *et al.*, 2003). The apparent difference in effect between acute and repeated oral exposure to YTX could be explained by the variation in the period from treatment to necropsy. In the studies on acute toxicity, the mice were killed 40-75 min or 24 h after treatment while in this study on repeated exposure

necropsies were performed 3 days after the last treatment. It might be that repair mechanisms have healed the toxic effects in the myocardial cells during the 3 days after the last exposure, which would be compatible with the finding that mice surviving more than five hours after YTX treatment, will not die from YTX (Ogino *et al.*, 1997). Such repair mechanisms could also explain why possible cumulative toxic effects were not observed in this experiment because the interval between each treatment were at least 3 days, possibly allowing repair to occur between each exposure. Whether cumulative effects do occur after YTX exposures at these dose levels and periods is an open question. In a study with daily oral treatment of mice with 2 mg kg^{-1} for 7 days and necropsies 24 h after the last treatment, similar changes as observed in the acute studies were described, indicating that cumulative effects are not important after repeated YTX exposure for one week (Tubaro *et al.*, 2004). In addition to repair mechanisms, pharmacodynamic adaptations can modulate or reduce the toxic effects during repeated exposure to toxins.

The no observable adverse effect level (NOAEL) of YTX was estimated by the EU at 1 mg kg^{-1} body weight based on results from acute oral exposure of mice. A tolerance level of 1 mg kg^{-1} shellfish meat was established, assuming an average shellfish consumption of 100g and applying an uncertainty factor of 600 instead of the arbitrary factor of 100 due to the lack of data and indications of effects on the cardiac muscle.

In the present study, YTX was given seven times to mice within three weeks, without apparent toxic effects even at YTX doses of 2.5 and 5 mg kg^{-1} body weight. These results indicate that even repeated exposure to relatively high levels of YTX presents little if any threat to consumers.

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SHELLFISH SAFETY AND QUALITY AS A SOURCE OF COMPETITIVE ADVANTAGE-THE NEW ZEALAND EXPERIENCE

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Abstract
New Zealand (NZ) is a small country with a population of only four million, distant from its markets, yet it exports Greenshell® Mussels (*Perna canaliculus)* to over sixty-four countries throughout the world. While this may be partly due to the uniqueness of this gastronomic delight, access through sixty four government food safety agencies requires that NZ has in place internationally recognised and accepted standards, particularly for microbiological and marine biotoxin management of its commercial shellfish growing areas.

State of the art standards are critical for providing the shellfish industry with competitive advantage in marketing their shellfish product in another country. The adequacy of standards, the regulatory system and the international competitive advantage that New Zealand might have, have been tested a number of times this century already with: New Zealand being the first country to approve LCMS methods for regulatory testing for marine biotoxins; the fraudulent production of boxes and shellfish in other countries and the labelling of that shellfish as NZ shellfish; the first country to approve the relay of shellfish from norovirus contaminated areas; the regulation and removal from regulatory control of pectenotoxin 2 seco acid; Missions to NZ in late 2003 by the United States Food and Drug Administration and the European Commission to conduct detailed paper and field audits of the New Zealand Shellfish Quality Assurance Programme.

It is issues like these that are the test of the competitive advantage that NZ shellfish have in the international marketplace.

Introduction
To overcome distance from all markets, the need to test and manage brevetoxins, domoic acid, okadaic acid, pectenotoxins, yessotoxins and saxitoxins and the NZ Government requirement that the shellfish industry pay for all costs incurred in complying with shellfish safety regulatory requirements, NZ Shellfish (being all those in academic, regulatory, research, laboratory, shellfish industry parts of the NZ shellfish programme) has been able to develop, over the last decade or so, a competitive advantage in safety and quality to facilitate the trade of shellfish into over 64 countries.

The base for this has been developed for over a decade through a synergistic combination of factors which includes the NZ Shellfish brand, strategic planning, partnerships, reputation, technical innovation and the shellfish product itself. The application to these factors, of the mysterious NZ quality, “kiwi ingenuity”, comprising the No. 8 wire approach to the resolution of problems, provides NZ shellfish safety and quality with the competitive advantage that it is renowned for. While personnel changes over time in the NZ Shellfish partnerships does limit the progress of programme development, this is negated somewhat in NZ through a progressive shellfish industry and a consistent desire to “do things better” while remaining within the bounds of public health protection. Along with consistent shellfish safety leadership through 3 decades and a general lack of bureauacracy, hierachic committees, political influence and procrastination, new ideas and

approaches to shellfish safety and quality are developed, progressed and decisions made in a timely manner.

NZ Shellfish Strategy
Improving export competitiveness requires a strategy at the national level to improve the shellfish business environment, export competitiveness and food safety advantage. NZ Shellfish and the NZ Food Safety Authority (NZFSA) have been addressing this since the 1980s through the NZ Seafood Standards Council (NZSSC), as evidenced in the NZSSC Strategic Plan.

The NZSSC comprises a NZFSA Director, 5 seafood industry representatives (of which 2 are shellfish industry) 1 consumer representative and two Seafood Industry Council (SEAFIC) Executives. The purpose of the NZSSC is "to be the consultative forum ---- in the preparation of food safety standards, other technical standards and protocols that are necessary for market access of seafood; and to provide leadership, analysis and advice that contributes to the development of cost-effective sustainable standards and risk mitigating strategies, that achieve performance comparable to international best practice in risk management ands consumer protection". Successful national export strategies are based on identifying the competitive advantage of a product at a national level and capitalising on it.

SEAFIC is also a formal Industry Advisory Council to Government –advising on setting standards of seafood safety, specifying competence and diligence requirements and developing negotiating strategies for access to overseas markets.

Partnerships and Core Competency
The development of long term partnerships has provided NZ Shellfish with the base for developing sustainable core competencies. While there are many points of view on what the latter means, in relation to the different parts of the NZ Shellfish Quality Assurance Programme (NZSQAP) the following 3 descriptions are most apt

1. "An area of specialised expertise that is the result of harmonising complex streams of technology and work activity", Prahalad and Hamel (1990);
2. "Aggregates of capabilities, where synergy is created that has sustainable value and broad applicability", Gallon, Stillman and Coates (1995); and
3. "A combination of complimentary skills and knowledge bases embedded in a group or team that results in the ability to execute one or more critical processes to a world class standard", Coyne, Hall and Clifford (1997).

It is this core competency approach that provides NZ shellfish with competitive advantage in safety and quality, as demonstrated in the development, approval and implementation of LC-MS testing for the various toxin groups.

Marine Biotoxins
The NZ Marine Biotoxin Technical Committee (MBTC) (comprising the author, a representative of the shellfish industry and the manager of the marine biotoxin programme for recreational harvest areas) has been meeting since 1993 to discuss and address all aspects of the regulatory management of marine biotoxin management including development and publication of the 1996 National Marine Biotoxin Management Plan, regulatory levels of respective biotoxins, test method changes, phytoplankton action levels, management plan requirements, hydrolysis of samples, rules for reviewing management plans, protocols for dealing with new toxins and metabolites of known toxins and management of the Marine Biotoxin

Science Workshops. While the author has been the Chair of the MBTC since 1993, the representatives of each of the other two groups have only changed once. This invaluable consistency in MBTC membership and institutional memory provides a strong partnership base for the deliberations of the MBTC.

To encourage attendance by observers, the MBTC meets in different parts of NZ – at the last meeting, 14 observers from industry, regulators, research and laboratories attended. The MBTC meetings provide a "no surprises" forum between the shellfish industry and regulators and decisions are by consensus with the NZFSA having the authority to make the final decision, if necessary in the interest of public health. New Zealanders do not "beat about the bush", so from time to time there is "blood on the floor" while controversial items are discussed – but decisions do get made and NZ marine biotoxin management has made it's world leading advances, such as the requirement that the Cawthron Institute Phytoplankton Monitoring Laboratory receive NZS/ISO/IEC 17025 accreditation (which it received in 1996) prior to phytoplankton monitoring being accepted as part of the regulatory marine biotoxin monitoring programme and the 2000 MBTC meeting where it was agreed that the two NZ marine biotoxin testing laboratories could proceed with the development of LC-MS as a replacement method for mouse bioassays from decisions made at these meetings.

The six monthly Marine Biotoxin Science Workshops (MBSWs), which have been conducted by the NZFSA on behalf of the MBTC since 1993, provides all researchers, students, academics, shellfish industry, laboratories and regulators involved in any way with marine biotoxins and toxigenic phytoplankton with the opportunity to meet, discuss marine biotoxin issues and present progress in their respective areas in a series of 20 minute papers. To minimise costs and facilitate attendance of the 50-70 participants, the MBSW is held at no cost to participants, centrally in Wellington, and when it can be managed - when an international marine biotoxin expert is visiting NZ. The proceedings are published informally. It is these Workshops that have provided the optimisation of relationships of persons and agencies involved in marine biotoxins, contributing to the development of the core competencies mentioned earlier.

Noroviruses

While the emphasis in the NZSQAP over the last decade has primarily been in marine biotoxins, partnerships in the field of norovirus management had been strengthening since the mid 90s.This partnership took a sudden boost in 2002 when the Institute of Environmental Science and Research Ltd offered Catherine Seamer, Advisor (Seafood), NZFSA, a PhD scholarship (1/2 time) in norovirus detection methodology. As a direct result of this new partnership, an inaugural norovirus workshop was organised by NZFSA to bring together researchers, students, regulators and shellfish industry to discuss the status of norovirus research and issues relating to norovirus management in shellfish growing areas. A significant outcome of this workshop was the decision to hold a National Shellfish Virus Workshop in early October 2003, associated with the visit to NZ of Professor Joan Rose from Michigan State University. Auckland Regional Council, the largest local government body in NZ, offered its Council Chambers gratis and over 100 attendees listened to and discussed presentations from norovirus analysts and researchers, local government planners, intertidal oyster farmers, shellfish regulators, a Medical Officer of Health and a waste disposal consultant.

The norovirus partnerships were further strengthened by the visit to NZ of Dr David Lees, CEFAS, as part of an EC Shellfish Mission Audit as this provided NZFSA with the opportunity to arrange an informal evening meeting with David on norovirus issues, attended by representatives of regulatory, laboratory, research and shellfish industry parties from the wider norovirus partnership.

SQAWK
Partnerships between regulators and their contacts are further strengthened through the three yearly NZFSA Shellfish Quality Assurance Workshops (SQAWK) which are held for 5 days to update and refresh the health protection officers responsible for growing area classification and marine biotoxin management in all aspects of their field work. Shellfish industry and microbiological and marine biotoxin laboratory analysts also participate and attendance by members of the Australian Shellfish Quality Assurance programme further enrich this partnership.

Shellfish Industry Research Partnerships
For many years the NZ Mussel Industry Council (NZMIC) has participated in joint funding research partnerships with: Universities in seed production and genetics; the Cawthron Institute in toxic microalgae, (taxonomy, toxicity and physiology), foam fractionation (*Gymnodinium catenatum* cyst removal); and AgResearch Ltd (isolation of PTX, YTX and analogues, preparation of toxin standards, toxicological studies and ELISA development). NZMIC also funds and manages an Environmental Management System for its farms and the National Spat Transfer Programme to minimise the spread of *Gymnodinium catenatum*.

In January 2004, Sealord Shellfish, the largest processor and marketer of Greenshell® Mussels in New Zealand, was awarded organic certification for some 6,000 tonnes of its 18, 000 tonnes annual production. While it will take Sealord Shellfish several years to recoup its research investment in this international ground breaking accreditation, the competitive advantage that Sealord Shellfish and the New Zealand mussel industry will receive for this new quality initiative is immeasurable.

NZ Shellfish "Brand" and Technical Advances
NZ Shellfish has developed a strong brand equity type reputation internationally for over a decade for effective, innovative and practical solutions to shellfish safety issues that are equivalent to or exceed the requirements of importing countries. For example, the development of a DSP/NSP bioassay screen test method in 1993, the NZS/ISO/IEC 17025 plus other requirements for phytoplankton monitoring prior to its introduction into marine biotoxin management in 1996, the development of the Test method Validation Guide between 1998 and 2002, the approval for LCMS for .DA/OA/PTX/YTX in 2001, the approval to relay intertidal oysters from norovirus contaminated areas in 2002, the 2003 requirement to hydrolyse all samples requiring OA analysis, and the incorporation of mandatory E.coli testing for shellfish into the draft Animal products (Specifications for Bivalve Molluscan Shellfish) Notice 2004.

Position branding of NZ Shellfish safety and quality measures can be a significant barrier to other entrants e.g. in the area of new methods for marine biotoxin detection, the validation work required by NZFSA for the 2001 approval of LC-MS was so rigorous that validation of LC-MS methods became a barrier for other laboratories wishing to use LC-MS or other new methods.

Summary
The long term synergistic relationships (at personal and agency level) between the shellfish industry, researchers, laboratories and regulators enables NZ Shellfish to have a sustainable competitive advantage in the international marketplace through kiwi ingenuity and the effective application of the developed core competences. This is an advantage that enables the NZ shellfish industry to survive against its competition over a long period of time.

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MANAGEMENT OF QUALITY SCHEMES IN IRISH AQUACULTURE

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Abstract
The volume and value of Irish aquaculture output was 44,152 tonnes with a value of €40.8 million euros in 2003. Board Iascaigh Mhara (BIM) is the Irish Seafood, Fisheries and Aquaculture development agency and has a history of promoting the sustainable development of the Irish sea fish and aquaculture industry. As part of the development of the aquaculture section, BIM over the last 3 years has established independent certified quality assurance schemes for the industry. The Irish Quality Mussel Scheme has been running since the beginning of 2003. The Irish Quality Mussel scheme is an independently certified assurance system, whereby the highest standards of quality and safety can be assured throughout the production chain. Promotion of the Irish Quality Mussel Scheme will be facilitated through the Quality Seafood Programme. This programme will act as the vehicle for communicating quality achievements in the marketplace from the wholesaler to the consumer via the use of a programme symbol. For the oyster industry, Supreme D'Irelande, a packed oyster quality assurance scheme has been developed by BIM and running since 2002. The standard was developed with the view of penetrating the EU market with a high quality product capable of competing with the best product from other countries.

Paper
BIM is the Irish semi-state organisation tasked with the sustainable development of the Irish seafood sector. BIM's aim is to promote development at sea and ashore, and to support diversification in the coastal regions so as to enhance the contribution of the seafood sector to employment, income and welfare, both regionally and nationally. BIM works with the industry, and market forces drive the industry. Whatever the market demands defines the job specifications for BIM as the national development agency. From the sea to the table, BIM assists with technical innovation, quality assurance schemes, marketing and investment. Irish aquaculture is clearly defined by goals and aims under government policy. Rope mussel production is concentrated along the Irish western and south-western seaboards. Important growing areas include Bantry Bay, Kenmare River and Roaringwater Bay along the south coast, Killary Harbour and in sheltered bays along the Donegal coast. Bottom mussel cultivation is mainly focused along the east coast in bays at Waterford and Belfast, and Lough Foyle in the north west coast. Rope mussel production and value from 1980 to 2003 is illustrated in Figure 1. Since 1980, production has steadily increased and in 2003, amounted to almost 9,000 tonnes, valued at €7.5 million. Figure 2 shows bottom mussel production covering the same period. The trend in this sector since 1980 has also seen an increase in production. In 2003, almost 30,000 tonnes were harvested, valued at almost €16 million. Combined rope and bottom mussel production for 2003 amounted to more than 39,000 tonnes, valued at almost €23.5 million.

From these current figures is could be assumed that the industry has consistently gone from strength to strength. This, however, is not the case: In 1999 and 2000, the industry experienced extended biotoxin closures and faced a very bleak outlook. The effect of these protracted closures can be seen in Figure 1 in terms of reduced volume of harvested product and subsequent loss in value. In addition, what cannot be seen from this graph is the loss of market confidence that occurred as a result of these problems. In order for the Irish rope mussel industry to survive, radical

changes had to be made to the Irish biotoxin monitoring system so as to regain confidence in the market.

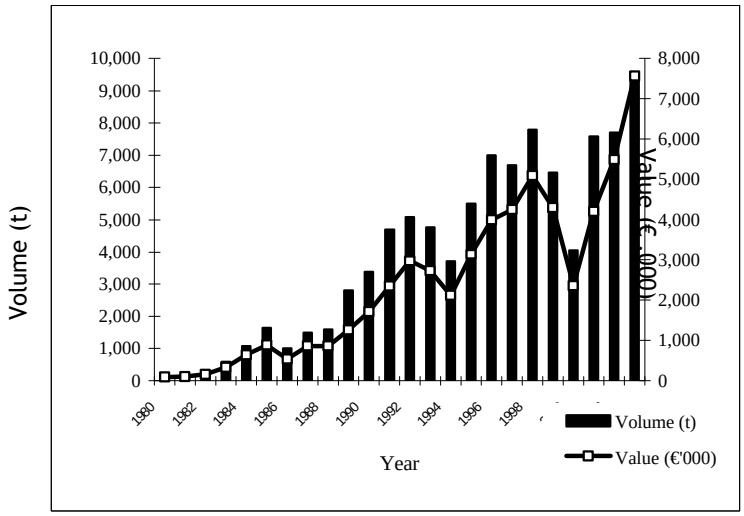

Figure 1. Irish Rope Mussel Production 1980 – 2003

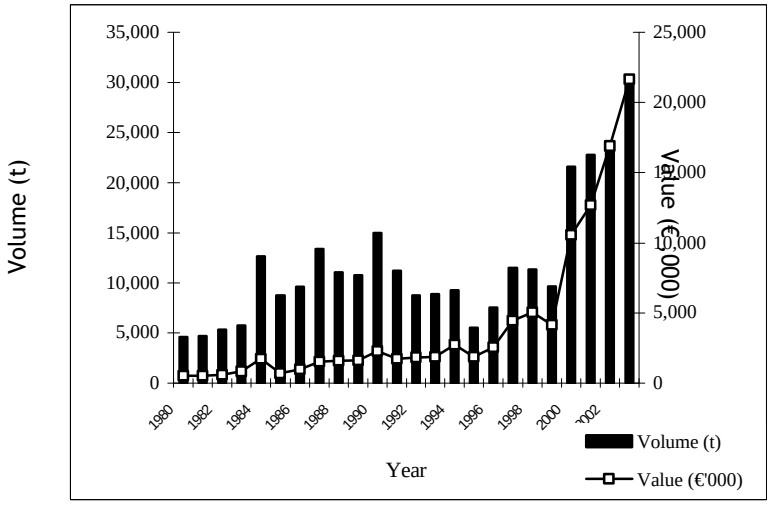

Figure 2. Irish Bottom Mussel Production 1980 -2003

As a result of strong political commitment to resolving the problem, the Molluscan Shellfish Safety Committee (MSSC) was established. This committee is an industry/state partnership with all interested parties sitting around the table discussing all issues relating to shellfish production and consumer safety.

The committee instigated a full review of the monitoring programme, and as a direct result the Marine Institute invested heavily in both personnel and equipment. This commitment by government and the agencies means that the Irish aquaculture industry now has a world-class monitoring system for both the industry and its customers. Quality assurance (QA) is part of quality management, focusing on

providing confidence whereby quality requirements are fulfilled. QA is what the consumer enjoys if quality control has been effective. QA as the base of a quality scheme is a unifying force with all members committed to meeting laid down standards. It is also a powerful marketing tool used to sell product. The market quickly identified QA as one of its requirements, and from this the Irish Quality Mussel Scheme (IQMS) was initiated. It was officially launched at the Brussels Seafood Exhibition in 2003. The IQMS is an independently certified assurance system for both bottom and rope mussels whereby the highest standards of quality and safety are assured throughout the production chain. This third party certification body – the Irish Food Quality Certification (IFQC) – is accredited to EN45011, which is a European standard for certification bodies. A number of steps had to be taken to establish the IQMS. Firstly, standards had to be written and to do this, a Technical Advisory Committee (TAC) was formed with all interested parties represented. This included the FSAI, BIM, growers, processors and IFQC. The standard was drawn up based on the existing code of practice for mussels, i.e. best practice in traceability and good management practices.

The standard was broken in to three sections, namely: harvester, shore-side and processing. The harvester section deals with the mussels in the water up to harvesting, the shore-side section deals with selling the fresh product and finally the processing section is self-explanatory. In order to become a certified member of the IQMS, certain procedures have to be gone through. Firstly the grower, harvester or processor makes an enquiry to BIM directly about certification. BIM then sends out information and an application form. The applicant completes the form and returns this with the fee to IFQC. The IFQC then sends out a copy of the standard, which also contains information regarding the documentation that will be required during the audit. At this stage, BIM provides all the technical assistance required. Some additional work might be required collecting and writing the documentation. Financial assistance may also be available through approved grant programmes. The applicant will also receive a self-assessment form, and when BIM and the applicant feel they are ready to be audited, the IFQC is contacted. When the applicant is ready, an audit is carried out by IFQC, which then goes to the client and also to the TAC. The TAC makes a decision on the basis of this report to either recommend the client for certification or to defer and ask for additional information. When the TAC is satisfied, the client can then be recommended. Once an applicant becomes a member of the scheme, regular audits occur. For processors, this is twice yearly while for boats it is annually. Now that the IQMS is up and running with certified members, the question is what can BIM do to promote the scheme? The answer is the Quality Seafood Programme (QSP). Central to this programme is that all mussels are produced to IQMS requirements and once part of the programme, members can use a scheme symbol. This symbol is adapted in every language according to a member's requirement. The QSP acts as the vehicle for communicating quality achievements in the marketplace from wholesale to consumer level. Unfortunately up until now, Irish oysters remained relatively unknown amongst many consumers because the majority of oysters grown in Ireland are sold in bulk to the French market. With this in view, BIM, decided to work on establishing an identity of excellence for Irish oysters at consumer level. To achieve this, BIM produced a quality scheme for packed Irish oysters named Supreme D'Irelande. As the name suggests, the standard is intended for the highest quality Irish oysters. Similar to the mussel quality scheme, a standard was drawn up. The standard contains a multitude of restrictions that guarantee the constant quality of Supreme D'Irelande oysters. Supreme D'Irelande requirements can be summarised in three main areas: oyster quality, traceability, controls and distinctive packaging. Oyster quality requirements

include meat index, shell shape, specific internal shell colourings and strength of shell obtained after a minimum period of hardening. Full traceability must be achieved from hardening site through to each packed box of oysters. Only distinctive and appropriate Supreme D'Irelande oyster lid and label must be used on the final packed oyster box.

In conclusion, the Irish Quality Mussel Scheme as part of the Quality Seafood Programme and Supreme D'Irelande are the marketing tools of Irish seafood to leverage value that is underpinned and differentiated by quality assurance and origin.

PROFICIENCY TESTING FOR THE QUANTITATIVE DETERMINATION OF DOMOIC ACID, THE AMNESIC SHELLFISH POISONING TOXIN

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Abstract
In 2003 the proficiency testing scheme, QUASIMEME, extended its scope to include testing for domoic acid, thereby creating the first proficiency testing for marine biotoxins at global level. This paper presents the results of the first two rounds, which were run as development exercises in 2003 and 2004. The test matrices included standard solutions, crude and cleaned extracts as well as biological tissues at levels appropriate to check the performance of laboratories around the two limits of 4.6 and 20 mg kg^{-1}. Standard solutions were dilutions of certified standards, whereas the tissue reference materials and extracts were prepared from naturally contaminated matrices. The homogeneity of tissue test materials was tested at the stage of preparation while the stability of biological tissues and extracts was tested at three different temperatures over 6-8 weeks. Tissues and extracts were sufficiently homogenous and stable to be fit for purpose. Participating laboratories were asked to quantitatively determine and report the sum of the concentrations of domoic acid and epi-domoic acid. Methods applied by the 36 participants included a variety of extraction and clean-up techniques and separation based on immuno-affinity as well as high-performance liquid chromatography with ultraviolet and mass spectrometric detectors.

Overall, the results of the two exercises suggested that the proficiency testing could be continued on a routine basis, thereby allowing official laboratories to continually check and compare internationally their performance on unknown test samples.

Introduction
Amnesic shellfish poisoning toxins are a group of water-soluble neurotoxins, the principal compound being domoic acid. The production of domoic acid has been related to the pennate diatom *Pseudo-nitzschia spp.*, which may accumulate in filter-feeding bivalves and pose a risk to human health. The toxic effects of domoic acid in humans were described following an incident in 1987 on the Canadian Coast, where 107 people became ill with gastro-intestinal and neurological symptoms. Following the AOAC validation of a HPLC-based method for the determination of domoic acid in shellfish, EU Directive 97/61/EC prescribes that methodology based on HPLC should be used to determine the content of domoic acid in shellfish. This directive, similar to other regulations worldwide, fixes the permissible limit of domoic acid to 20 mg kg^{-1}. More recently, the levels permissible in the scallops, *Pecten maximus* and *Pecten jacobaeus*, were further regulated in the EU (2002/226/EC) whereby the maximum concentration in edible parts of scallops was limited to 4.6 mg kg^{-1}, where the toxin level in the whole flesh exceeded 20 mg kg^{-1}, if a restricted harvesting regime was applied. While the production of certified standards and reference materials by the National Research Council Canada provides adequate tools for in-house validation and quality control in the determination of domoic acid, international harmonization also requires proficiency testing as external control of official testing. EU directives 93/99/EEC and Commission Decision 1999/312/EC require official laboratories to work to internationally accepted criteria such as ISO 17025 and the use of proficiency testing as far as

appropriate.This paper reports on the first international proficiency testing for domoic acid, the amnesic shellfish poisoning, in shellfish.

Materials and Methods

Preparation and dispatch of test materials

The preparation of test materials was described in detail in the QUASIMEME 2003 and 2004 reports of rounds 34 DE-9 and 36 DE-9. Briefly, 2 standards, 2 tissues, 1 crude extract and 1 SAX-cleaned extract were prepared and distributed to the participants with only an indication of the range of concentrations prior to analysis of the materials. The standards were dilutions of the certified standard DACS-1C (NRC Canada), and were ampouled under nitrogen prior to the exercises. The shellfish tissues were prepared from scallop gonads or from whole scallop tissue (both *Pecten maximus*). The crude extract was prepared using an extraction solvent of 50 % MeOH. The SAX-cleaned extract was prepared using a crude extract in 50 % MeOH and cleaning it up over a set of strong anion exchange SPE cartridges. Homogeneity of the materials was determined for the tissue reference materials by the supplier (Marine Institute, Ireland), and all materials showed adequate homogeneity (< 5 % CV). Stability of DA in the test materials was evaluated by the supplier (Marine Institute, Ireland) both for the extracts and the tissue materials; stability of standards was demonstrated over long-term use in the 2 exercises. Figure 1 shows that the stability of a whole scallop tissue is only ensured at temperatures < -18 °C (freezer), while at fridge and room temperature degradation starts within a few days. Therefore, all the materials were shipped on ice.

Statistical Analysis

In these exercises, the assigned values for each material were derived from the maxima of population measurement functions (PMF) obtained from the results of all participants using Cofino Statistics (Cofino *et al* (2000) and Cofino *et al* (2004)) which were compared in detail to other robust statistics (Wells *et al*, 2004). Within and between laboratory CVs were also determined using Cofino statistics. The total allowable error (acceptable deviation from the assigned value) was derived as the sum of a constant and a proportional error, 0.1 µg g^{-1} and 12.5 %, respectively. Each participant was assigned a z-score for the result obtained for each test material. Z-scores are:

z = (result – assigned value)/ total allowable error

A z-score is considered very good if $0 < z < 1$, acceptable if $1 < z < 2$, examination of the procedure is recommended strongly if $2 < z < 3$ and the performance of the method is considered faulty if $z > 3$.

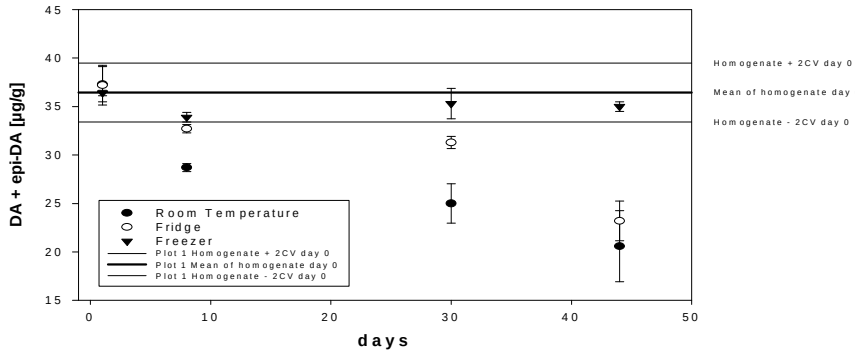

Figure 1. Stability of the whole scallop homogenate at three temperatures (freezer, fridge and room temperature).

Results

The materials were adequately homogenous and stable, as demonstrated through the use over two rounds (Table 1). The overall performance was satisfactory with 21 of a total 36 and 20 of a total of 34 laboratories achieving 100 % acceptable results ($0 < z < 2$) in round 34 and 36, respectively. Also, 28 (27) labs achieved 75 % or greater (80 % or greater) acceptable results in round 34 (round 36). The assigned values of the 2 standard solutions and the whole scallop homogenate did not vary notably between the 2 exercises, suggesting that the performance of the laboratories was very stable over the 2 exercises.

Table 1. Performance characteristics for DA in QUASIMEME proficiency testing

Assigned value mg kg^{-1}	Round *	Matrix	No of labs submitting results	No of labs with $0 < z < 2$	*Within-lab CV*$^{+}$ [%] RSD_r	*Between-lab CV*$^{+}$ [%] RSD_R
0.92	34	Standard	36	28 (33)$^{\$}$	5.1	10.5
0.94	36	solution	33	29	4.6	10.2
4.71	34	Standard	36	30 (34)$^{\$}$	1.5	3.8
4.70	36	solution	33	28	1.0	2.5
2.75	34	SAX-cleaned scallop extract	36	26 (31)$^{\$}$	2.3	5.9
7.87	36	Crude scallop extract	33	26	2.9	6.5
3.96	36	Scallop gonads	34	27	6.7	14.7
33.3	34	Scallops	36	30 (30)$^{\$}$	4.9	11.7
33.4	36	whole flesh	34	26	5.0	11.7

*34 = Data from report on round 34 DE-9, exercise 580 (QUASIMEME, 2003)
*36 = Data from report on round 36 DE-9, exercise 596 (2003/4) (QUASIMEME, 2004)
$ = Values in parentheses are after correction of reporting errors (QUASIMEME, 2003)
+ = Within and between lab CVs were derived from labs with acceptable z-scores

It was also noted that the between laboratory CVs tended to increase with decreasing concentrations. Evaluation of the data showed that the main sources of error were in reporting (5-6 labs) and calibration (5-8 labs) but minor differences were also noted in the performance of extraction procedures (3 labs) and detection procedures (1 lab).

Conclusion

The overall performance of the two rounds of this development exercise for the determination of domoic acid in biological tissue was very satisfactory. The materials provided for the study were suitable for the tests both in terms of the stability and homogeneity as well as the concentrations of the determinands in the respective matrices. The specific errors of individual laboratories have been identified and it is possible for these to be rectified. In some cases, there was a clear improvement in performance/reporting of a laboratory from round 34 to round 36. In view of the satisfactory level of performance of these participating laboratories there is little to be gained in continuing with the development exercise for the ASP toxins. This was also reflected during discussion at the QUASIMEME workshop in Galway, Ireland, June 19th 2004, where possible ways forward were discussed.

The QUASIMEME organisers look to offer a routine exercise for this determination in the near future, with 2 exercises per year. The results are also encouraging to extend the proficiency testing of shellfish toxins into other areas of instrument-based detection of shellfish toxins. The participants of the QUASIMEME workshop in 2004 suggested that the next group to be targeted be Okadaic Acid and analogues.

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CONSUMER PROTECTION FROM VIRAL CONTAMINATION OF SHELLFISH AND: DATA AND RECOMMENDATIONS FROM THE EU "VIRUS SAFE SEAFOOD" PROJECT

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Abstract
Consumers are increasingly turning to seafood, for a fresh and dietetic product whose safety must be guaranteed. Above all, it must be "virus-free". The European Commission funded a project called 'virus safe seafood' which had several objectives:
- to determine human enteric viruses contamination in sewage, streams and shellfish. After validation, methods were applied to detect main pathogenic viruses such as norovirus, enterovirus, rotavirus, astrovius and hepatitis A virus, on naturally contaminated samples collected in northern and southern Europe. The results obtained clearly showed that environmental contamination is linked to circulation of virus in the local population and thus varies from one country to another. Another important finding was the lack of correlation between *E. coli*, F+RNA phages and the presence of human enteric viruses in shellfish collected in France or in the Netherlands.
- To develop a new tool to study virus behaviour in the environment. The innovative aspect of this project was to use recombinant Virus Like Particles (VLPs) to evaluate how effective procedures for the inactivation / removal of viruses in marine waters and shellfish tissues are. Different types of VLPs (fluorescent or not) and different detection methods were evaluated depending on the parameter to be assessed.
- to study shellfish depuration: a depuration pilot was built in a professional farm and used fro experimentation either with shellfish contaminated either artificially (with F+ RNA phages or VLPs) or naturally. This pilot could tested various parameters such as temperature and feeding under conditions similar to those used by shellfish farming professional. The results obtained with VLP-contaminated shellfish compared with those obtained with naturally contaminated shellfish, demonstrated that shellfish depuration was inadequate.
All the data obtained during this European project showed that to protect consumers, efforts must be made to limit the viral contamination of growing areas (especially sewage input) and to take the viral input into consideration during outbreak in the population (months of risk).

Introduction

Outbreaks of human gastroenteritis are a major source of viruses and bacteria in the environment (Payment and Hunter 2001). Even when sewage is treated, significant microbial fluxes are apparent in coastal areas affected by rivers or direct input (Loisy *et al* 2005a, Le Cann *et al*. 2003, Schijven *et al*. 2003, Griffin *et al*. 2003, Schvoerer *et al*. 2001, Jiang *et al*. 2001). In addition, estuarine systems, where shellfish are grown, may be under the influence of faecal contamination through run-off from adjacent land used for agricultural (Pommepuy *et al*. 2005, Van den Berg *et al*. 2004a, van Heerden *et al*. 2003). Indeed, a strong association between microbial concentration in water and rainfall has already been demonstrated (Miossec *et al*. 1998). Thus, seafood grown in coastal waters is exposed to these different sources of contaminants (Van den Berg *et al*. 2004a, Lodder *et al*. 1999).

When faecal contamination is detected, depuration systems are used to eliminate potential pathogens from shellfish. Nevertheless, outbreaks have been reported even from shellfish which meet microbial standards (Bosch *et al*. 2001, Le Guyader *et al*. 2003). In fact, few depuration treatments in professional tanks have been evaluated for viral depuration of naturally-contaminated shellfish (Lees 2000). And, very few studies (Henshilwood *et al*. 2003, Doré *et al*. 2003b, Schwab *et al*. 1998) have been carried out on norovirus inactivation and/or removal by depuration, although noroviruses are the main causative agents in shellfish outbreaks (Butt *et al*. 2004, Kopmans and Duitzer 2004, Le Guyader *et al*. 2003 and 1996). Until recently, lack of information on viral depuration of naturally-contaminated shellfish in professional tanks was mainly due to the absence of reliable techniques to detect norovirus, that cannot be cultivated (Duizer *et al*. 2004) - and to the difficulties encountered when working with naturally-contaminated shellfish.

To overcome the problem, virus like particles (VLPs) were evaluated as a surrogate to mimic viral behaviour. These were used in studies on the transit of viruses during sand filtration (Redman *et al*. 2001) or their behaviour in seawater (Caballero *et al*. 2004, Loisy *et al*. 2004). Their specificity (absence of pathogenicity, stability and structural resemblance to virus) suggests that they could be an efficient tracer to study the behaviour of viruses in harvesting areas and during shellfish depuration.

Taking the recent advances in molecular technologies into account (Pommepuy and Le Guyader 1998), the Virus Safe Seafood project was established, and submitted to the EU in 1999. The main objectives were to provide useful information on viral shellfish contamination, validate professional depuration processes and propose actions to prevent contaminated shellfish from being marketed.

Materials and methods

Sampling

In The Netherlands, *Crassostrea gigas*, oysters and water were sampled on a monthly basis between October 2000 and December 2001. In Sweden, semi-treated sewage waters were collected every week at three sewage water treatment plants in the Stockholm area and seawater water samples were collected every month over one year. In Spain, sewage samples were collected from the urban area of Barcelona from November 2000 to December 2002. In France, shellfish samples were collected monthly during twenty months at two sites for oysters –(*Crassostrea gigas*) - and two other sites for clams (*Ruditapes philippinarum*).

Concentration of enteric viruses in sewage and shellfish

At the start of the EU project Virus-Safe Seafood, methods for virus concentration from water samples and for RNA extraction were evaluated. The method using a lysis buffer to disrupt cells and particles, followed by binding of RNA to silica beads was found to be the best method for water samples when compared to the commercial Qiagen and Boehringer kits (Villena *et al*. 2003). For oysters, the protocols were based on dissected tissue. Two methods were developed: concentration of viral particles by ultracentrifugation or by PEG precipitation prior to RNA extraction. The commercial RNeasy Plant minikit Qiagen (Courtabeauf, France) was used to extract nucleic acid. Using this kit was found to best eliminate the various inhibitor compounds present in shellfish extracts (Virus Safe Seafood, 2003; Lodder-Verschoor *et al*, 2004).

Detection of viruses, E. coli and F-RNA specific phages.

Viruses were detected by RT-PCR and hybridization using the same primer sets for water and shellfish samples. The presence of the pathogenic viruses - Hepatitis A Virus (HAV), rotavirus, astrovirus, norovirus and enterovirus -, was analyzed using methods standardised during the project (Bosch *et al*. 2001, Villena *et al*. 2003, Le Guyader *et al*. 2000, Vennema *et al*. 2002). For *E. coli* and phage analyses, tissues and liquor were homogenised in a warring blender with 1 volume of a 10 % (wt/vol) NaCl solution. *E. coli* contamination level was determined by conductance measurement (NF-VO8-106) according Dupont *et al*, (2004 and 1996). Phages assays were determined with standardised methods (ISO 10705-1, 1995).

Virus-like particles (VLPs)

Construction and production of VLPs were assessed as described in previous papers (Berois *et al*. 2003, Charpilienne *et al*. 2001). Different VLPs were constructed: rotavirus VLP2/6, fluorescent GFP-VLP2/6 and DsRed-VLP2/6 (Dundr *et al*. 2002). They were used 1) in reconstitution experiments by adding VLPs or pseudo-viruses to shellfish and 2) to test reproducibility and sensitivity of detection. Different methods were developed based either on flow cytometry or ELISA (Caballero *et al*. 2004, Loisy *et al*. 2004). For shellfish analysis, a sensitivity threshold of 10^4 VLP particles was achieved (Loisy *et al*. 2005b). Experiments were performed with VLPs and other viral strains, i.e. bovine (BRV) and human (HRV) rotaviruses.

Depuration experiments:

Depuration tank. Experiments were carried out in a pilot unit (Prat ar Coum, Ets Madec): four independent tanks (3.2m x 2.2m x 0.80m), with aeration, heather/cooler equipment, seawater recycling (5-20 m^3/h) allowing simultaneous testing of the different depuration conditions (Pommepuy *et al*. 2003). Naturally-contaminated shellfish were collected from B harvesting areas (classification by EU regulation). Oysters were then immersed in the tanks at different temperatures

(from 8 to 25°C). Every two days, the oysters were sampled and analysed for F-specific phages. Each sample was composed of at least 6 oysters and duplicate analyses were performed. Artificially-contaminated shellfish with rotavirus, VLPs and phages (MS2): A batch of 10 kg of oysters was immersed in seawater at room temperature and contaminated for 24 hours with bovine stool which was positive for rotavirus and MS2 culture. The seawater was treated by filtration and UV before recycling. The water temperature in the tank was maintained at 25 °C throughout the experiments (11 days). Rotaviruses and F-RNA phage analyses were performed periodically (day 0, 2, 4, 7, 9 and 11). VLPs were also used to study the behaviour of virus in seawater and during the depuration process (same experimental conditions as above). Water control experiments: Phage behaviour in seawater was tested in the tanks (without shellfish) at different temperatures to compare it with their behaviour during shellfish decontamination at the same temperatures (from 12°C to 25°C). Behaviour of VLP rotavirus 2/6, bovine and human rotavirus strains was studied at 5 and 25°C in marine water. Elisa, and western blot methods were used to quantify viral particles. Rotavirus infectivity was measured by determining the $TCID_{50}$ in MA-104 cell monolayers.

Statistical analysis
The variable *E coli* and F-specific phages were transformed by the log (x+1) function for statistical analysis. The regression model and logistic regression were applied. The significance of the regression lines was assessed by a Student *t* test applied to the regression slope. The loss of culturability was calculated by applying the following law of logarithmic decrease: $N = N_0 \times 10^{-t/T90}$,

where N is the concentration at *t* time in hours, N_0 the initial concentration, T90 is the time required for a decimal reduction of the initial microbial population. The same law[3] of logarithmic decrease was used to calculate the depuration time, DT90 is the time required for a decimal reduction of the initial microbial population in shellfish. T90 and TD90 were expressed in days.

Results and Discussion
Detection of viruses in the participant countries
Sewage waters from three locations (Spain, Sweden and The Netherlands) and shellfish from harvesting areas (France and The Netherlands) were analysed.

In Sweden, 52 % of raw sewage samples were found positive for norovirus. A peak in contamination occurred in February - April 2001. The genotypes detected in sewage water were similar to those found in patient stools such as the Rotterdam, Lordsdale, Southampton and Queens Arms genotypes. Enteroviruses were found in about 60 % of the sewage samples. In contrast to norovirus, no seasonal variation could be noted for enterovirus, nor could any direct association between the presence of enterovirus in sewage water and the number of patients with clinical symptoms, such as meningitis, be found.

In Spain, rotavirus was by far the enteric virus most commonly found in sewage samples, which correlated with the epidemiological data of children's gastroenteritis in the same area. A clear seasonality was observed for astroviruses, which correlates with the pattern reported in clinical studies. Hepatitis A virus was rarely isolated in these samples and no clear seasonality was observed for the occurrence of rotavirus in raw sewage.

[3] where the DT90 replaces T90

As in the Swedish samples, enteroviruses were the most frequently detected viruses in The Netherlands. They were found in sewage samples (Van den Berg *et al*. 2004b) and in 22 % of oyster samples (Lodder-Veershoor *et al*, 2004). These viruses and noroviruses were also detected through-out the year in the effluent waters from the two sewage treatment plants near the harvesting areas (Van den Berg *et al*. 2004a). Rotaviruses were detected in half of the raw and treated sewage samples, but astroviruses in only some raw sewage samples and not at all in the effluent waters. Noroviruses and rotaviruses were detected in oysters but HAV and astrovirus were not found. Enteroviruses and rotaviruses, but no astroviruses and noroviruses, were detected in the shellfish harvesting waters.

In France, the most frequently detected viruses in shellfish were enteroviruses (32 % of samples), whereas fewer samples were positive for noroviruses (8 %). These results are consistent with those obtained in the Netherlands. No rotavirus was found and HAV was present in only one sample (1/77). The presence of viruses in shellfish was detected by RT-PCR and hybridization, but their presence was confirmed by sequence analysis.

No relation was found between the presence of human enteric viruses and *E. coli* classification (Table 1a) and the concentration of F-specific phages in oysters (Table 1b). These results are consistent with those found in Spain, Greece, Sweden and The Netherlands (Myrmel *et al*. 2004, Hernroth *et al*. 2002, Formiga-Cruz *et al*. 2002 and 2003, Lodder-Vershoor *et al*, 2005). In the latter country, F-specific phages were detected at high levels during the months of September to April. Of the 60 oyster samples with low levels of F-specific phages (<50 Plaque Forming Units (PFU)/50g shellfish flesh), 14 samples contained enterovirus, whereas these viruses were not detected in samples with high levels of F-specific phages.

Table 1a. Percentage of norovirus (NoV) positive samples as compared with *E coli* classifications obtained in French samples.

% of total samples (*E.coli** Classification)	% of NoV positive samples
72 % (< 230 *E.coli*)	3.9 % (2/51)
17 % (230 < x < 4 600 *E.coli*)	8.3 % (1/12)
10 % (> 4 600 *E.coli*)	12.5 % (1/8)

**E.coli* are expressed in colony forming units (CFU)/100g of total flesh
NoV: positive samples detected by RT-PCR and confirmed by hybridization.

Table 1b. Percentage of norovirus (NoV) positive samples as compared with F-RNA specific phage concentration obtained in French samples.

% samples (F-RNA phage concentration)	% of NoV positive samples
58 % (<150 F- RNA phage)	7.9 % (3/38)
42 % (>150 F- RNA phage)	3.6 % (1/28)

F-RNA phages are expressed in PFU/100g of total flesh.
NoV: positive samples detected by RT-PCR and confirmed by hybridization.

In contrast with these results, cumulative data from Great Britain have indicated a good relationship between F-RNA phages and the occurrence of norovirus in shellfish (Lees 2000, Doré *et al* 2003). The shellfish concentration reflects viruses circulating in the population and the impact of human wastes on oyster quality (Kohn *et al*, 1995). The different epidemiological status of the population from one country to another may explain the significance of correlations between phages and viruses

when they are present in contaminated shellfish, as suggested by Hernroth *et al*. (2002) and Ueki *et al*. (2005). Another discrepancy between our results and the results from the other countries is the absence of shellfish contamination by HAV during the study (Lodder-Veershoor *et al*, 2004, Di Pinto *et al*. 2003, Formiga-Cruz *et al*. 2002, Sanchez *et al*. 2002, Croci *et al*. 2000), demonstrating the importance of investigating the epidemiology of produce countries to adapt shellfish control. Following hepatitis outbreak in South Brittany (France), cockles were found to be contaminated for few months, increasing the risk of new cases (Apaire-Marchais *et al*. 1995). Implementing epidemiological surveys in the populations near harvesting growing areas could be of utmost interest to improve shellfish surveys and designate the virus to be target.

Shellfish contamination shows seasonal variations which differ from one country to another. In the Netherlands, viruses were detected in commercial and non-commercial oysters from October to June. In France, enteroviruses were present throughout the year, due to an outbreak in the population the year of the study (Chambon *et al*. 2001). In general, shellfish contamination was very low, and this could be explained by the dilution mechanism in coastal areas which would thus limit the viral contamination (Pommepuy *et al*. 2005). The methods developed are suitable to detect a few RNA copies as was demonstrated in further studies (Le Guyader *et al*. 2003, Loisy *et al*. 2004).

Viral behaviour in the environment

Human pathogenic viruses enter the marine water environment primarily through the discharge of treated and untreated sewage into surface waters. Depending on whether or not these viruses can survive long enough and in high enough concentrations, they could cause disease in individuals who are in contact with polluted recreational water or who consume contaminated seafood. Different investigations were carried out in order to answer these questions. Natural seawater was seeded with rotavirus strain, VLPs and MS2, a F-specific strain. The stability of VLPs in seawater and shellfish was evaluated and compared to the stability of native viruses (Table 2).

Table 2. Comparative survival of MS2 phage and rotavirus strains, and VLPs in seawater (T90 expressed in days)

Strain	<15°C	>20 C
MS2	2.3 - 5.5	0.5 - 1.5
Bovine rotavirus	nd	2 - 10
VPL 2/6 - GFP-VLP	nd	2 - 7

VLPs showed a low decay rate in marine water, from 2 to 7 days depending on temperature. It was interesting to note that same results were obtained with VLPs or GFP-VLPs despite a small difference in water temperature (20°C-25°C respectively) and different methods of detection (ELISA, cell culture) (Loisy *et al*. 2004; Caballero *et al*. 2004). Furthermore, additional MS2 experiments were carried out in natural seawater and large tanks ($6m^3$) to mimic natural viral behaviour. At low temperatures (12-15°C) the T90 was several days, whereas it was 0.5 to 1.5 days at 25°C (Pommepuy *et al*. 2003).

The stability of VLPs in seawater was comparable to the decay rate of infectious human rotavirus and similar to previously published results (Wait and Sobsey 2001, Bosch 1998, Chung and Sobsey 1993, Callahan *et al* 1995, Girones *et al*. 1989).

Thus, VLPs were found to be a very interesting model for survival of human enteric viruses (Loisy *et al*. 2004).

Shellfish viral depuration
Oyster were seeded with VLPs or rotavirus and then placed at 25°C in depuration tanks. Results showed a very low decrease in VLP concentrations: after 7 days of depuration, a decrease of only one log was observed (Loisy *et al*. 2005b). Rotaviruses could still be detected at day 9 and 11 by RT-PCR for the first and second experiments, respectively, with only a one log_{10}-unit decrease.

These results were compared with those obtained with naturally-contaminated shellfish. Eighteen experiments were carried out using oysters from harvesting areas. Five experiments were performed for 2.5 days (Tables 3a). The results show that 2.5 days are not sufficient to have virus safe seafood, whereas the faecal indicators are below the limit of detection (experiment J). During other experiments, the shellfish depuration was carried out over longer times (7 to 10 days). The results obtained after 7 days of depuration are shown on the table 3b where the experimental conditions (temperature of sea water, initial contamination) are indicated.

Table 3a. Effect of depuration on naturally-contaminated oysters, after 2.5 days (Prat ar Coum)

Experimental conditions		Initial contamination			Final contamination		
N° Exp.	Water Temp.	*E. coli**	F-RNA**	Enteric Virus	*E. coli**	F-RNA**	Enteric Viruses
I	15°C	$4.2\ 10^2$	$4.6\ 10^2$	Presence	<dl	$1.4\ 10^2$	Presence
J	25°C	$4.2\ 10^2$	$7.6\ 10^2$	Presence	<dl	dl	Presence
M3	25°C	<dl	$3.4\ 10^2$	Absence	<dl	dl	Absence
V1[A]	25°C	nd	< dl	Presence	<dl	<dl	Absence
V2[A]	25°C	nd	<dl	Presence	<dl	<dl	Absence

nd: not done; dl: detection limit (30 *E. coli*/100g; 100 F-RNA/100g); * Expressed in CFU/100g; ** Expressed in PFU/100g; [A] oysters were fed with phytoplankton

Table 3b. Effect of depuration on naturally-contaminated oysters, after 7 days (Prat ar Coum)

Experimental conditions		Initial contamination			Final contamination		
N° Exp.	Water Temp.	*E. coli**	F-RNA**	Enteric viruses	*E. coli**	F-RNA**	Enteric viruses
J	12-13 °C	4.1 10^1	4.1 10^2	Presence	< dl	1.1 10^2	Presence
K	12-13 °C	< dl	3.3 10^2	Presence	< dl	1.2 10^2	Presence
R1[B]	15 °C	6.6 10^1	1.5 10^4	Presence	< dl	dl	Absence
R2[B]	20 °C	6.6 10^1	1.5 10^4	Presence	< dl	dl	Absence
H	20 °C	< dl	3.9 10^2	Presence	< dl	< dl	Presence
R3[B]	25 °C	6.6 10^1	1.5 10^4	Presence	< dl	dl	Presence
L2[B]	25 °C	4.2 10^2	7.7 10^4	Presence	< dl	1.7 10^3	Absence
O1	25 °C	< dl	< dl	Absence	< dl	< dl	Absence
S[B]	25 °C	1.4 10^2	8.1 10^3	Presence	< dl	5.9 10^2	Absence
O2	25 °C	<dl	1.2 10^2	Presence	< dl	< dl	Absence
K	25 °C	4.5 10^1	4.1 10^2	Presence	< dl	< dl	Presence
M2[B]	25 °C	2.9 10^3	2.6 10^3	Presence	< dl	2.0 10^2	Presence
O3	25 °C	< dl	dl	Presence	< dl	< dl	Presence

nd: not done; dl: detection limit (30 *E. coli*/100g; 100 F-RNA/100g); * Expressed in CFU/100g; **Expressed in PFU/100g; [A] oysters were fed with phytoplankton; [B] shellfish were immersed close to a sewage outfall for 1 or 3 weeks before depuration.

In one experiment (O1), shellfish were virus free, *E. coli* and phages contamination very low (under detection limit). For other experiments (R1, R2, L2, S, and O2), at least seven days were needed to have negative viral results. In five experiments (H, R3, K, M2 and O3) viruses were still present in shellfish after 7 days, even when depurated at 25°C. For most of the experiments at 20°C and 25°C, the detection limit was reached within 2 days for *E. coli* and in less than 5 days for F-RNA phages (but L2, S, M2). Phage depuration time (DT90) varied from 7 to 20 days at low water temperatures (<13°C) and ranged from 0.77 to 6.2 days at high water temperature (>20 °C).

In naturally-contaminated shellfish, we observed the presence of different viruses as well as a viral concentration which varied from one sample to another. In experiment R3, positive results were observed at T0 for rotavirus and enterovirus, at T1 for norovirus, at T1, T3 and T10 for astrovirus (Figure 1). Furthermore, the effect of the temperature on F-RNA phages was clearly demonstrated, the concentration decreased faster at 25°C than at 15°C.

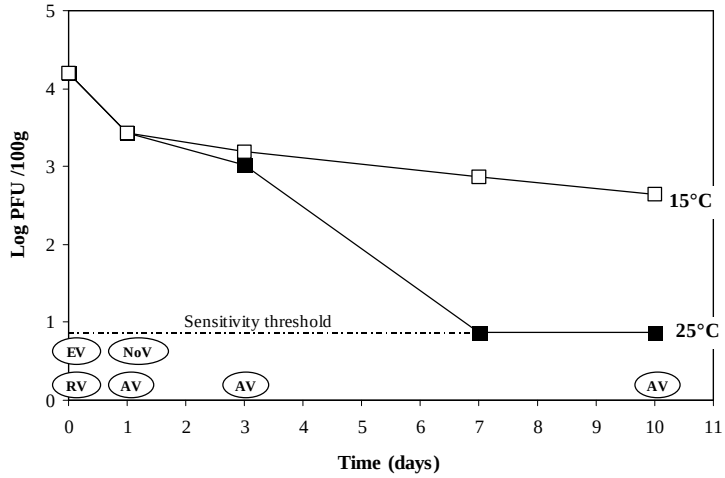

Figure 1. Depuration of naturally-contaminated oysters for 10 days in the depuration tank –Prat ar Coum (F-RNA kinetics at 25° and 15°C). Viral results were obtained in shellfish depurated at 25°C at T0 – R3 experiment - (EV: enterovirus, RV: rotavirus), T1 (NoV: norovirus, AV: astrovirus), T3 (AV) and T10 (AV).

With the aim of increasing the efficiency of viral depuration, we applied specific diets (algae feeding, addition of suspended matter) or nutrient deprivation (dry storage before depuration, depuration with filtered seawater) to oysters stored at natural seawater temperatures (10°C-17°C). No major effect was observed for the treatments when shellfish were stored at low water temperatures (12-15°C). At 18°C, the metabolism was reduced and not re-activated by the different parameters. We also increased the seawater temperature to 25°C to deplete the intestine content in order to eliminate viruses (Heral, personal com.). Unfortunately, results indicated that viruses could be still be detected even after 7 days at 25°.

Viral shellfish depuration: interest and limits.
The pilot plant at Prat ar Coum, allowed depuration efficiency to be tested under conditions close to those applied by professional users in the field. In our experiments, temperature increase (up to 20°C) was the only one parameter to drastically increase the phage depuration. These results are consistent with those of other studies mainly based on phage depuration assessment (Doré *et al*. 2003 and 1995, Lees 2000, Sobsey and Jackus 1991). The rapid elimination of phages at a high temperature was probably due to phage inactivation, as was suggested by experiments in seawater in which the phage decay rates (T90) were significantly correlated with temperature. However, shellfish seems to protect viruses, as previously suggested by Metcalf *et al* (1995). In comparison, the T90s of phages in water were found to be 3 times lower than those found in shellfish during depuration at the same temperature (Pommepuy *et al*. 2003). Moreover, if we compare the decay rates observed with different strains in water (MS2, VLPs, rotavirus strains), the decay rate also depends on the strains. Differences in depuration rates have been noted in oysters contaminated by poliovirus type 1 or hepatitis A virus (Sobsey *et al*. 1987). These data raise the possibility that a non-

infectious VLP of the same virus may be a better surrogate for depuration studies than are unrelated viruses (Loisy *et al*. 2005b).

Naturally-contaminated oysters tested in our experiments could be depurated efficiently in 4-5 days for F-RNA specific phages. However, at 20°C and more, viruses still persist in oysters even after at least 7 days of depuration. Furthermore, concerning depuration applications, considerations must be taken regarding this process's applicability to other shellfish (mussels, cockles, etc.) and its undesirable effects (*Vibrio* re-growth, shellfish mortality, etc.) which have been observed under some particular environmental conditions. Thus, this technique partially solves the problem, but cannot be a real guarantee of virus-safe seafood.

Conclusion

The overall objective of the Virus Safe Seafood EU project was to provide useful tools to evaluate human viral contamination in shellfish and innovative technology for their quality control and depuration. To this end, the input and the resistance of major human enteric viruses were investigated by assessing harvesting areas quality and the shellfish depuration process was evaluated. Various tools were evaluated: routine methodologies, using Virus Like Particles as a model to study viral behaviour in water and shellfish and a pilot unit for real scale testing of factors which could improve viral depuration in near-environmental conditions.

The results obtained for sewage waters close to harvesting areas demonstrated the presence of different enteric viruses. Pathogenic viruses in sewage and contaminated shellfish are dependent on the circulation of viruses in the population, i.e. on epidemics. The detection methods are now rapid, specific and efficient enough to undertake a survey of the sources, which may contribute to the deterioration of water quality in shellfish growing areas.

Using innovative tools such as VLPs contribute to improving knowledge about viral behaviour in water and shellfish. The major interests of VLP are the absence of pathogenicity and a structure identical to native viruses, which allow shellfish contamination to be simulated without introducing pathogens into the environment. Comparison of these results with those observed with naturally-contaminated shellfish during depuration clearly demonstrated the difficulties of eliminating viruses during the process. This also suggests that further investigations should be conducted in harvesting areas to preserve water quality from microbial contamination, rather than focusing on shellfish depuration alone. Thus, to protect consumers from viral risk, which although low but exists, efforts must be made to limit viral contamination of growing areas (sewage input and harvesting water quality). The implementation of a "warning system" adapted to coastal viral contamination could be appropriate to limit this risk (Le Saux *et al*.in press). This system would take into account the epidemiological data from the human population and events which could degrade the water quality. Identification of potential problems before they arise would help to preserve water quality. By providing action plans, it could effectively limit the risk associated with the sale of contaminated shellfish.

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MICROBIAL GROWTH IN MODIFIED ATMOSPHERE PACKAGED BLUE MUSSELS (*MYTILUS EDULIS)*

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Abstract
Microbial growth was investigated in live blue mussels (*Mytilus edulis*) packaged in modified atmosphere (MA) and stored at -1 and 4 ^{o}C for 19 days. The mortality of mussels was 0.8 % after 19 days of storage with lowest mortality at -1 and in CO_2 atmosphere. The increase in bacterial numbers in the mussel flesh was low, 1- 2 log units, measured as Aerobic Plate Counts, H_2S-producing bacteria, and psychrotrophic bacteria. There was significant lower bacterial growth ($p<0.05$) in an atmosphere with CO_2:O_2 compared air and N_2:O_2 and at -1 ^{o}C. An initial level of < 3 mg Tri-Methylamine Oxid-N (TMAO-N)/ 100g was found in the mussels, detectable levels of trimethylamine-N (TMA) were observed after 11 days of storage and TMA-N increased to 1 – 3 mg/100g in 19 days. Total Volatile Nitrogen (TVN) increased from 3.4 to 18 mg/100g in 19 days and may be an important chemical indicator to be used in defining the shelf life. Low temperature (-1 ^{o}C) did not significantly increase the shelf life, but the superchilled temperature will control pathogenic growth. MA packaging of live blue mussels is a promising packaging technology for retail distribution, with a shelf life of 11-12 days based on sensory scores.

Introduction
Modified atmosphere packaging (MAP) has been used to enhance shelf life of different seafood products and has been reviewed by several authors in the last decade (Brody 1989, Ogrydziak and Brown 1982, Parkin and Brown 1982, Pedrosa-Menabrito and Regenstein 1990, Skura 1991, Stammen *et al*. 1990, Reddy *et al*. 1992, Sivertsvik *et al*. *2002*). Crustacean shellfish keep up to 30 % longer at 0°C in a modified atmosphere than in other types of packaging, and the onset of blackspot in shell-on products is delayed (Cann 1988). A MA of 80 % CO_2 and 20 % air was found to be the preferred atmosphere for storage of freshwater crawfish (*Procambaris clarkii*) tail meat compared to 100 % CO_2 or air (Gerdes *et al*. 1989). CO_2-enriched atmosphere was found to increase the shelf-life of whole cooked shrimp by 200 %, compared with shrimp stored on ice and exposed to air (Sivertsvik *et al*. 1997). CO_2-atmospheres have also been found beneficial for storage of fish-cakes (Yokoseki *et al*. 1956), and also for chilled storage of raw squid and white octopus (Morales *et al*. 1997).

MA storage of live mussels differs from other processed MA packaged seafood in that they have a functioning immune system and that they have an active respiration during storage. The immune system protects the mussels from microbial attack and degradation. The active metabolism, aerobic and anaerobic, may on the other side produce metabolites adding unpleasant taste and flavour to the mussels in a closed packaging system. The replacement of air in the package with elevated concentration of oxygen may, however, support an extended survival time. One method with the potential to extend the period of prime quality in fish is “superchilling” or “partial freezing” (Haard 1992). In this technique, the temperature in the fish is reduced to 1 ^{o}C to 2 ^{o}C below the initial freezing point

and some ice is formed inside the product (Haard 1992, Sikorski and Sun 1994). Storage at superchilled conditions may enhance phospholipids hydrolysis and protein denaturation (Ashie *et al*. 1996), but superchilling will inhibit most autolytic and microbial reactions, and thereby in many instances increase shelf life (Chang *et al*. 1998, Huss 1995). An important advantage is that superchilled temperatures effectively will inhibit growth of pathogenic microorganisms at temperatures at 0 °C or below. The aim of this work was to examine MA packaging of blue mussels and examine effects of processing, packaging and storage temperature conditions on microbial growth, chemical spoilage products and survival rate.

Materials and Methods

Raw material and sample preparation

Farmed blue mussels (*Mytilus edulis*) were obtained from a local mussel farmer (FruMar/Mytilus AS) near Stavanger, and packaged at the packaging facility Norshell in Rogaland, Norway. Mussels were 40-70 mm long and farmed during a 3 year period in fjords with a mean salt concentration of 30 ‰, varying over a year from 20‰ to 32‰. The mussels were harvested and clots of mussels were placed in large tubs (500 L) in 3 days for conditioning in circulating seawater to imitate normal tidal waters, and to reduce stress. One sample group was exposed to changing periods with dry storage and under water storage to simulate tidal water changes. The mussels were harvested and clots of mussels were placed in large tubs (500 L) for 3 days for conditioning in circulating seawater to reduce stress. All samples were stabilised at 4.0°C before packed in plastic trays. The same day as packaging the mussels were transported on crushed ice to storage rooms with temperatures of +4 °C and ÷1 °C respectively, variables according to Table 1.

Table 1. The following packaging and storage conditions were used

No.	Gas mixture	Storage temp. (°C)	Tidal conditioning
1	O_2:N_2 = 40 %:60 %	4	Without
2	O_2:N_2 = 40 %:60 %	4	With
3	Air (sealed package)	4	Without
4	O_2:N_2 = 40 %:60 %	-1	Without
5	CO_2:O_2 = 50 %:50 %	4	Without

Temperature measurements

Sample's core temperatures were measured every 5 min during storage using electronic temperature loggers (Ebro Electronic, Ingolstadt, Germany) at both temperatures and in both packaging methods to ensure stable storage temperatures.

Packaging and packaging materials

Portions of 850 to 1050 g mussels were packaged in blue Dynopack trays No 551, PE-HD with PA/PE top film (Polimoon, Kristiansand, Norway). The gas mixture were added in gas flushing cycles from vacuum (-1 bar) to overpressure (0.1 bar) in a Dynopack packaging machine (Polimoon ibid.) before heat-sealing.

Gas mixtures and gas measurements

The gas composition (O_2, CO_2 and N_2, %) in the packages was measured in triplicate using an oxygen and carbon dioxide analyser (M.A.P. Test 4000, Hitech Instruments, Luton, UK). A 30 ml aliquot of the gas was collected through a syringe from the head space after intrusion of the top foil and analysed. Before intrusion of the syringe, a foam rubber septum (Nordic Supply, Skodje, Norway) was added to the top foil to avoid introduction of false atmosphere into the gas analyser. The

analyser was calibrated against a certified gas mixture (O_2:CO_2:N_2 1.1:44.1:54.8) and air before each sampling

Microbiological analysis
Samples of 25 g mussel tissue were taken at random and homogenised in 225 ml of 0.9 % NaCl (w/v) and 0.1 % peptone (w/v) for 120 sec in a Stomacher 400 Laboratory Blender (Seward Medical, London, U.K.). Total viable counts/ aerobic plate counts (APC) were measured after an aliquot a suitable dilution had been added to melted and temperated (44 °C) iron agar (Agar Lyngby, IA, Oxoid CM 867, Basingstoke, Hampshire, U.K.) supplemented with L-cysteine, and incubated at 20±1 °C for 3 days. Black colonies were counted as H_2S-producing bacteria, APC were counted as the total of black and white colonies. The content of psychrotrophic bacteria was determined by a spread plate count method with plate count agar (PCA, Merck, Darnstadt, Germany) with 1 % NaCl, and incubated at 8 °C for 7-10 days. Average results of duplicate measurements are presented as log colony-forming units (cfu) per gram mussel.
Chemical analysis
Tri-methylamine oxide (TMAO), tri-methylamine (TMA) and total volatile basic nitrogen (TVN) were determined in homogenised mussel meat (25 g) in duplicate using a modified Conway microdiffusion method (Conway and Byrne 1933) and expressed as mg TMAO/TMA/TVN- N/100 g product.

Determination of live mussels
Mussels were determined as live when the two half shells closed after a light knocking on the shell surface or after a gentle touch on the closing muscle with a knife. Only live mussels were subjected to microbial and chemical analysis. Number of live mussels was determined within 3 packages from each variant at each sampling.

Shellfish tissue pH
The pH of the mussel tissue was determined in triplicate using a pH meter (Beckman 72, Dan Mezansky, Oslo, Norway) on 25 g of homogenate of mussel muscle with 25 ml of 0.1 M KCl in distilled water.

Formation of exudate and exudate pH
The exudate formation was measured gravimetrically and reported as percentage of initial net weight of mussels in the package. pH in exudate was measured directly in the exudate using same instrument as above.

Sensorial off-odour determination
Samples from the storage experiment were analysed by a sensory panel of 3 panelists. The packages were allowed to acclimitise to room temperature and an off-odour formation was scored in a 10 point scale where 10 was no off-odour/seafresh odour, 1 a putrid/rotten odour and very strong and unpleasant off-odours. A score of 5-5.5 was the lower acceptance level.

Statistical analysis
Univariate analysis of variance were performed with Minitab 13.3 (Minitab, Coventry, UK) using Tukey's HSD test at level $p<0.05$ (95 %), to obtain confidence intervals for all pair wise differences between level means of temperature and packaging type on each sampling time (days of storage).

Results and discussion

Mortality

After 19 days of storage 41 of 5095 mussels (0.8 %) died during the storage period (Table 2). About 170-200 mussels were tested for mortality at each sampling day from each packaging variant. Dead mussels were found in packaging variant 1, 2 and 3 and mortality increased with storage time.

The best survival rate was observed for O_2-N_2 at -1 °C and with CO_2. Low mortality may be due to a low respiration rate in the mussels at low temperatures (-1 °C) or higher microbial inhibition (CO_2-atmosphere).

Table 2. Mortality of mussels in packages with different atmospheres and temperatures

		Packaging variants				
Days		1 O_2:N_2	2 O_2:N_2-T	3 Air	4 O_2:N_2-1	5 CO_2:O_2
4	Live	198	179	171	180	194
	No.dead (%)	0	0	0	0	0
7	Live	203	179	174	188	190
	No.dead (%)	0	0	0	0	0
11	Live	190	183	121	199	188
	No.dead (%)	0	1 (0.5)	2 (1.6)	0	0
14	Live	198	193	172	179	196
	No.dead (%)	2 (1.0)	1 (0.5)	0	0	0
19	Live	65	192	44	188	200
	No.dead (%)	1 (1.5)	6 (3.0)	18 (29.0)	0	0

Microbiological analysis

The microbiological analysis showed that there were only minor differences in bacterial growth caused by different atmospheres and temperatures (Table 3). One day after packaging the number of psychrotrophic bacteria was log 4.9 cfu/g. This was 1.7 log-units higher than the APC numbers and 2.1 log-units higher than H_2S producing bacteria. The psychrotrophic numbers were higher than the others during the whole storage period. A general trend for APC, H_2S-producing bacteria and psychrotrophic bacteria was a slow growth during the storage period. Comparison of the effects of gas mixtures on microbial growth showed that for APC there were no differences between packages with O_2:N_2, O_2:N_2-T, air, or even O_2:N_2 at -1 °C, Table 3. However, there were significant ($p<0.05$) lower growth in CO_2 atmosphere after 4 and 7 days of storage, compared to O_2:N_2 atmosphere at 4 °C. In the last part of the storage period (day 11 and 19) no differences were observed independent of temperature or package atmosphere. The mean of all samples showed that CO_2:O_2 and O_2:N_2 – 1 °C were significant lower than O_2:N_2 and air at 4°C.

The psychrotrophic counts also were significantly lower for O_2:N_2 at – 1°C and for CO_2:O_2 at 4 °C, and except for variant 1 the same result was found for H_2S-producing bacteria (Table 2). A general trend for all microbiological results was that there were some differences between the variants in the beginning of the storage time, after 4 and 11 days, but not after 14 and 19 days.

Packaging of mussels constitutes special challenges for microbial spoilage and safety compared to other seafood products. Mussels are packaged live without removing

the parts of the organisms that contain high levels of bacteria e.g. gills, intestine, surfaces as for fish products. Due to the filter feeding system, the micro flora of molluscan shellfish therefore directly reflects the environments from where they are harvested. It comprises the natural commensal microorganisms and the microorganisms accumulated from the water during feeding.

Photobacterium phosphoreum have previously been found to be the main spoilage organism of MA packaged salmon (Emborg *et al*. 2002) and on cod at 0 ºC (Dalgaard *et al*. 1993, Dalgaard 1995). *P. phosphoreum* tolerates high CO_2 concentrations and can grow on PCA with salt and are enumerated unspecified as psychrotrophic bacteria. It produces 10-100 fold more TMA per cell than *S. putrefaciens* due to the large size of the former (5 µm) (Dalgaard 1995, Dalgaard *et al*. 1996). As a consequence a lower level of *P. phosphoreum* cell per gram is necessary for the spoilage of fresh fish. The spoilage of chilled, CO_2 packaged fish is seen at a level of 10^7 *P. phosphoreum* per gram (Dalgaard *et al*. 1993). This level was not reached for any of the packages in 19 days of storage.

Chemical analysis
Measurements of TVN –N (Total volatile base nitrogen), TMAO-N (trimethylamine oxide nitrogen), TMA-N (trimethylamine nitrogen, and DMA-N (dimethylamine nitrogen) have been used to determine fish quality. Öelenschläger (1992) concludes that for ice-stored cod the best indicator for spoilage is TMA, produced from the precursor TMAO, and/or TVN-N.
Low levels of TMAO were found in the mussels, never exceeding 3 mg TMAO/100g, and the majority of the samples had levels below 1.5 mg/100g (Table 3). TMA was not detectable during the first 11 days of storage, and then the concentration increased after 14 and 19 days to levels of 1.0 – 2.8 mg/100g. There were no significant differences ($p < 0.05$) in the levels of TMAO and TMA in packages with O_2-N_2 gas mixtures, CO_2 levels or air, and also independent of tidal water conditioning and storage temperature.

Table 3. Individual differences between gas atmospheres and storage temperatures

	Storage time (days)						
	1	4	7	11	14	19	Mean all
APC (log cfu/g)							samples
1-O_2:N_2	3,2	3,8 ab	4,6 a	4,7	4,2	4,8	4,4 ab
2- O_2:N_2-T	3,2	4,3 a	4,6 a	5,4	4,8	4,9	4,8 a
3-Air	3,2	4,2 a	4,0 ab	5,0	4,3	4,8	4,4 ab
4- O_2:N_2-1	3,2	3,9 ab	4,1 ab	4,5	4,5	4,5	4,3 b
5-CO_2:O_2	3,2	3,1 b	3,5 b	4,5	4,1	4,6	3,9 c
Psychrothrophic (log cfu/g)							
1-O_2:N_2	4,9	5,6 a	5,5 a	5,6	5,3	5,9	5,6 a
2- O_2:N_2-T	4,9	5,6 a	5,9 a	5,7	5,7	5,3	5,6 a
3-Air	4,9	5,6 a	5,3 a	5,2	5,0	5,7	5,4 a
4- O_2:N_2-1	4,9	4,4 b	4,7 ab	5,0	5,1	5,4	4,9 b
5-CO_2:O_2	4,9	4,4 b	4,4 b	5,0	4,5	5,4	4,7 b
H2S-producing (log cfu/g)							
1-O_2:N_2	2,8	3,2	3,4 ab	3,9	3,3	4,0 ab	3,6 ab
2- O_2:N_2-T	2,8	3,5	4,0 a	4,3	3,6	3,9 ab	3,9 a
3-Air	2,8	3,5	3,1 ab	4,5	3,9	4,4 a	3,9 a

4- O_2:N_2-1	2,8	3,3	2,9 ab	3,9	3,1	3,4 b	3,3 b
5-CO_2:O_2	2,8	2,7	2,5 b	3,3	3,0	3,9 ab	3,0 b
pH muscle							
1-O_2:N_2	6,5	7,0	6,5	6,5	6,5 a	6,5 a	6,6 a
2- O_2:N_2-T	6,5	7,0	6,6	6,7	6,4 ab	6,2 bc	6,6 a
3-Air	6,5	6,9	6,5	6,5	6,0 c	6,1 c	6,4 b
4- O_2:N_2-1	6,5	6,8	6,5	6,5	6,2 bc	6,4 ab	6,5 ab
5-CO_2:O_2	6,5	6,9	6,7	6,7	6,0 c	6,4 ab	6,6 a
TMA (mg –N/100g)							
1-O_2:N_2	0,0	0,0	0,0	0,0	1,0	1,6	0,5
2- O_2:N_2-T	0,0	0,0	0,0	0,0	1,2	2,0	0,6
3-Air	0,0	0,0	0,0	0,0	2,2	2,6	1,0
4- O_2:N_2-1	0,0	0,0	0,0	0,0	2,0	2,8	1,0
5-CO_2:O_2	0,0	0,0	0,0	0,0	1,6	1,0	0,5
TVN (mg –N/100g)							
1-O_2:N_2	3,5	5,6	8,0	7,2	7,0 a	7,8 a	7,1
2- O_2:N_2-T	3,5	5,2	6,6	6,6	6,8 a	10,0 ab	7,0
3-Air	3,5	5,4	7,2	7,2	15,8 b	13,0 b	9,7
4- O_2:N_2-1	3,5	5,6	6,2	6,2	15,8 b	8,8 a	8,5
5-CO_2:O_2	3,5	5,4	6,0	7,0	17,6 b	12,8 b	9,8
TMAO (mg –N/100g)							
1-O_2:N_2	1,3	0,0	2,8 a	0,4	0,2	1,4	1,0
2- O_2:N_2-T	1,3	1,6	1,4 ab	0,6	0,4	0,6	0,9
3-Air	1,3	0,6	2,8 a	0,0	0,2	0,2	0,8
4- O_2:N_2-1	1,3	0,8	0,2 b	0,2	0,6	1,0	0,6
5-CO_2:O_2	1,3	0,6	0,4 b	0,2	0,0	0,0	0,2

(Table 3 Continued)

Development of total volatile bases increased from 3.5 mg/100g after 1 day of storage to levels of 8- 12.8 mg/100g, with some high spot measurements after 14 days. There were no significant differences ($p < 0.05$) in the levels of TVN in packages with N_2-O_2 gas mixtures, CO_2 levels or air during the first 11 days of storage, but some differences was observed at the end of the storage period. The low levels of TMAO and TMA render these analyses useless in detecting end of shelf, but TVN may be a very promising chemical marker.

pH increased for 4 days then slightly decreased during the rest of the storage period. Mussel pH in live mussels ranged from 7.0 to 6.0 and the pH decreased during the period from 7 to 19 days of storage. There were no significant differences ($p < 0.05$) in the levels of pH in packages with O_2-N_2 gas mixtures, CO_2 levels or air during the first 11 days, but differences were observed after 14 days of storage.

Gas measurements

CO_2 levels in the headspace of chilled MA packages were 1.6-7.6 % the day after packaging. Solubility of CO_2 increases with decreasing temperature (Carroll *et al.* 1991). The level of CO_2 increased in the headspace during storage to levels between 8 and 22 % at day 19. The level of oxygen was from 17.4 (air) to 33.6 % after one day of storage. The level of oxygen decreased during storage, and there were low levels

(<5 %) O_2 left in all packages except in the package with air (0.0 %) after 19 days of storage.

The oxygen levels after 1 day was 33 % for packages with added oxygen and 17.4 % for packages with air. Oxygen is used by the mussels' respiration and no oxygen was left in packages with air after 11 days. Oxygen level decreased in the other packages to 5.6 – 6.7 % in O_2:N_2 packages.

Exudate formation and pH in exudate
The results from formation of exudate in the packages and from measurements of the pH in the exudate are shown in Table 4. The exudate formation was high in all variants and stabilised around 20 % from day 7 of storage. The exudates in the conditioned tidal shell (variant 2) were not lower than the other non-trimmed shells. Reduction of the exudate formation must be a goal for further trials, in order to reduce the amount of over-weight the producers need in the package and in order to meet the declared net-weight towards the end of storage. The pH in the exudate did not differ between the variants. At day 19 a drop in pH was observed for all variants except no. 4 stored at superchilled conditions.

Table 4. Individual differences between exudate and exudate pH, and raw odour in blue mussels stored under modified gas atmospheres and storage temperatures.

	Storage time (days)				
	4	7	11	14	19
Exudate (%)					
1-O_2:N_2	13.4	18.2	17.8	15.8	18
2- O_2:N_2-T	17.7	21.2	20.0	19.5	22.3
3-Air	19.2	19.3	22.6	16.3	29.7
4- O_2:N_2-1	18.6	19.2	18.6	23.4	17.2
5-CO_2:O_2	16.2	19.2	14.6	19.1	17.4
pH in exudate					
1-O_2:N_2		6.7	6.7	6.8	6.3
2- O_2:N_2-T		6.6	68	6.7	6.5
3-Air		6.8	6.9	6.8	6.0
4- O_2:N_2-1		6.7	6.8	6.7	6.7
5-CO_2:O_2		6.5	6.6	6.8	6.4
raw odour					
1-O_2:N_2	9.0	7.0	7.0	5.3	4.0
2- O_2:N_2-T	7.7	6.7	7.0	5.7	4.7
3-Air	8.0	7.0	6.7	4.3	2.0
4- O_2:N_2-1	10.0	8.3	7.0	6.0	6.0
5-CO_2:O_2	7.7	8.0	7.0	5.7	4.3

Raw odour
Raw odour scores are shown in Table 4. High quality (>7.5) was maintained in all variants at day 4 of storage, but only in O_2:N_2 atmosphere at superchilled conditions. CO_2:O_2 at chilled conditions held high quality after 7 days of storage. All

variants had acceptable odours after 11 days of storage, the lowest being in the air packaged mussels. Off-odour in all packages was strong from day 11 of storage directly after package opening; however 15 minutes later most of these strong off-odours were lost. At day 14 of storage the odour-quality was only marginal in MA-packages and the air samples were spoiled, and only the superchilled samples (variant 4) were not totally spoiled after 19 days of storage.

Conclusions

In the measurements carried out in this experiments the superchilled mussels packaged in 60 % O_2 and 40 % N_2, and the chilled mussels packages in 50 % CO_2 and 50 % O_2 attained the lowest microbial numbers, lowest TMA/TVN formations and highest odour scores. Using MA for live mussels and keeping the storage temperature low a long shelf-life is obtainable. Further studies should include the combination of superchilled storage together with e.g 50-50 CO_2-O_2 mixtures. Methods to reduce off-odour upon package opening and to reduce the formation of exudate should also be sought in future studies.

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SHELLFISH MONITORING PROGRAMME IN VIETNAM AND MONITORING RESULTS FOR THE YEAR 2002.

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Introduction

Vietnam has a 3260 km coastline and many estuaries with diversified bottom conditions favourable for the breeding and growing of many species of bivalve molluscs. In order to ensure compliance with the Directive 91/492/EEC, Vietnam Minister of Fisheries has issued the Decision on promulgating Regulation on Monitoring Hygiene and Safety Conditions for Harvesting Areas of Bivalve Molluscs in July 1997. The mandatory monitoring programme for shellfish safety was established and implemented in Vietnam since July 1997. After three years of implementation, in January 2000, Vietnam has approved exporting bivalve molluscs to EU market by the Decision 2000/333/EC.

Object, scope of the Program

Object of the Programme: Baby Clam *(Meretrix lyrata)*, Clam *(Meretrix meretrix)*, Blood clam *(Tegillarca granosa),* and Sulf clam in Kien Giang province *(Paphia sp.)*.
Scope of the Programme: 19 areas of bivalve molluscs belong to 5 provinces/cities: Ho Chi Minh City, Tien Giang, Ben Tre, Tra Vinh, Kien Giang (The details are presented in Table 1).

Table 1

No	Name of harvesting area	Province/ City	Object	Size (ha)	Production measure Metric ton	Number of sampling points
1	Can Gio	Ho Chi Minh city	Baby clam *(Meretrix lyrata)*	2,550	31,227	2
			Blood clam *(Tegillarca granosa)*	40	485	
2	Tan Thanh	Tien Giang	Baby clam *(Meretrix lyrata)*	1,800	22,000	3
3	Binh Dai	Ben Tre	Baby clam *(Meretrix lyrata)*	1,818	14,544	3
			Blood clam *(Tegillarca granosa)*	322	4,186	
4	Ba Tri		Baby clam *(Meretrix lyrata)*	1,105	22,100	5
			Blood clam *(Tegillarca granosa)*	100	1,700	
5	Thanh Phu	Ben Tre	Baby clam *(Meretrix lyrata)*	450	3,500	2
			Blood clam *(Tegillarca granosa)*	75	650	

6	Ha Tien	Kien Giang	Baby clam *(Meretrix lyrata)*	42	774	1
7	Kien Luong		Baby clam *(Meretrix lyrata)*	200	1,100	1
8	An Minh		Blood clam *(Tegillarca granosa)*	75	200	2
9	Ba Lua		Sulf clam *(Paphia sp.)*	-	11,095	2
10	Cau Ngang	Tra Vinh	Baby clam *(Meretrix lyrata)*	460	500	1
11	Hiep Thanh		Baby clam *(Meretrix lyrata)*	330	2,300	1
12	Duyen Hai		Baby clam *(Meretrix lyrata)*	400	2,600	2

(Table 1 Continued)

Contents and procedures of the program

Periodical sampling

Monitored parameters, sampling frequency and standard/recommended limit of monitored parameters are described in Table 2.

Table 2

Parameter	Sampling frequency	Reference methods of analysis	Standard limits/ Recommended limits
Microbiological criteria:			
Feacal coliform	Twice a month	MPN (five-tube, three dilution)	300/100 g molluscs flesh + intervalvular liquid
Salmonella	Twice a month	Qualitative	Negative in 25g molluscs flesh + intervalvular liquid
Harmful algae	Twice a month	Taxonomy following IOC documents	Not exceeding acceptable limit
Marine biotoxins criteria:			
DSP	Twice a month	Mouse bioassay, or HPLC or LC/MS	Negative; or: - Total Okadaic acid + Dinophysis toxins + Pecteno toxins: 160 μg/kg molluscs flesh + intervalvular liquid - Yessotoxins: 1mg/kg molluscs flesh + intervalvular liquid - Azaspiracids:160 μg/kg molluscs flesh + intervalvular liquid
PSP	Twice a month	Mouse bioassay, or HPLC or LC/MS	Negative or 80 μg saxitoxin/100g clam body + intervalvular liquid
ASP	Twice a month	HPLC or LC/MS	20 μg demonic/g clam body + intervalvular liquid

Sampling for intensive inspection of harmful algae and biotoxins:
According to result from the Shellfish Monitoring Programme in Vietnam from 7/1997 and documents of the Intergovernmental Oceanographic Commission (IOC), the provisional warning limits of toxic algae species is laid down in Table 3.

Table 3.

No	Algae species	Biotoxin(s)	Warning limit (cells/l)
		Dinoflagellates	
1	*Dinophysis caudata*	DSP	500
2	*Dinophysis acuminata*	DSP	500
		Diatoms	
3	*Pseudonizschia spp*	ASP	100,000

If other harmful algae species are detected, the tentative warning limits will be limit of country which has the lowest limit in the IOC statistics. When the density of toxic algae exceeds the warning limit, or biotoxin(s) analysis by mouse bioassay/HPLC show positive results/ or exceed the acceptable limits, the intensive inspection for harmful algae and biotoxins is applied as describle in Table 4.

Table 4.

	The intensive inspection applied
Density of toxic algae exceeding warning limit, but biotoxin(s) mouse bioassay show negative results or biotoxin(s) analysed by HPLC do not exceed(s) the acceptable limits.	- Open harvesting area(s) - Increase sampling frequency (from 1 to 3 days) and the number of samples to follow the variation of algae and toxins (if any). Intensive inspection time depends on the kinetic change of algae and toxin. - Test final products processed on previous and present periods for biotoxin(s). If biotoxin(s) exceed acceptable limit(s), the final products will be blocked and destroyed.
Not dependant on the number of harmful algae, when biotoxin(s) exceed(s) acceptable limits.	- Close harvesting area(s) - Increase sampling frequency and number of samples - Test final products processed on the previous time for biotoxin(s). If their results of biotoxin(s) analysis exceed acceptable limit, the final products will be blocked and destroyed.

Monitoring data processing and notifying harvest and post-harvest treatment regime
Based on the sanitary requirements in Table 3, NAFIQAVED has responsibilities for collecting data, evaluating results of periodical samplings and/or intensive samplings, regulating and informing harvest regimes and post-harvest handling regimes for each harvest area.

The harvesting status and post-harvest treatment include the following main contents: name of harvest area, harvesting status (open/close), period of the harvesting status and post-harvest treatment. The harvesting status and post-harvest treatment notification to be sent by fax to the local Fishery Resource Protection Divisions (FRPD) and bivalve molluscs processing factories before harvesting time.

Procedures for re-opening harvesting area
Samples are taken intensively from suspended harvest areas (Table 4) and analysed according to the methods described in Table 2. Reopening of suspended harvest areas are allowed only when results of the samples taken from those areas in 2 consecutive samples show that concentrations of biotoxins meet the requirements mentioned in Table 2.

Harvesting inspection
Inspections are carried out daily during the harvesting period to:
- Assure that no raw bivalve molluscs are harvested from forbidden harvesting areas.
- Prevent mixing of bivalve molluscs harvested from different harvesting areas.
- Identify and issue certificates of origin for each lot of raw material

Inspection of conditioning and processing establishments
Inspection of health conditions at bivalve molluscs conditioning and processing establishments for:
- Only raw material in control areas where accepted by EC has processed and exported to EU
- To quarantine health conditions for conditioning establishment, satisfying EU conditions.

Results of the Shellfish Monitoring Programme for 2002:
Toxic algae
Pseudonitztchia spp. and D. *caudata*, algae species that can produce ASP and DSP respectively, had been found regularly in water samples taken from 12 harvesting areas. In contrast to the monitoring results in 2001, *D. caudata* was not found in 2002 but *Alexandrium* spp. was found once in a water sample from Can Gio area in February to May and with the lowest densities recorded in the period of July to October. The highest density of *Pseudonitztchia sp.* was 12,600 cells/litre (still below the alert limit) but the highest density of *D.caudata* reached 1,400 and 1,240 cells/litre (over alert limit) in April and May of 2002 respectively.

Marine Biotoxin
At the same time when *D.caudata* appeared in Ba Tri and Binh Dai, Beb Tre province in May, 2002, DSP was found in clam samples collected from the same areas. DSP was also detected in clam samples from Hiep Thanh (Tra Vinh province) in April 2002 when density of *D.caudata* was very high. The results show that there was not a clear link between biotoxin accumulation in bivalve mollusc flesh and toxic algae density in water. Also DSP concentration in clam flesh was not high and it disappeared in samples from the first series of samples, so the closing period was rather short.

Conclusion
The mandatory monitoring programme for shellfish safety was established and implemented in Vietnam since July 1997 and has complied fully with regulations of Vietnam as well as requirements of EU. Suspension of harvest activities was carried out at the harvest areas in Ben Tre and Tra Vinh provinces as soon as DSP found in clam flesh samples. There were no food poisoning cases due to the consumption of bivalve molluscs, containing marine biotoxins, in Vietnam. In addition, no bivalve mollusc consignment violating food safety and hygiene regulations were exported since 1997. Supervision of the harvest activities and origin certification of each batch has also been carried out in all of the harvesting areas to prevent any mix-up of raw materials harvested from different areas. This is also to ensure that all of consignments for export to EU were processed from raw materials harvested from approved areas.

RISK ASSESSMENT OF DSP TOXINS IN BROWN CRABS (*Cancer pagurus*)

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Abstract
During the summer of 2002 (July-September), more than 200 people reported ill with typical DSP symptoms upon consumption of Brown crabs (*Cancer pagurus*). The crabs causing DSP were caught at several locations along the south coast of Norway, mainly in shallow waters. Analysis of the crabs by LC-MS showed peak levels of DSP toxins at about 1,000-1,500 µg okadaic acid equivalents kg^{-1} brown meat (mainly consisting of hepatopancreas). The toxin consisted almost exclusively of fatty acid esters of okadaic acid (Dinophysistoxin-3), yielding okadaic acid upon hydrolysis. DSP toxins were not detected in white crab meat.
The incident was associated with an unusually early season of high toxic levels of DSP toxins in blue mussels (*Mytilus edulis*) in the same area, indicating that the crabs had acquired the DSP toxins from eating blue mussels.
Before the 2003 crab season, a risk assessment was undertaken on behalf of The Norwegian Food Control Authority, in order to establish a temporary tolerance level for DSP toxins in crabs. Based on crude estimates a level of Dinophysistoxin-3 at 400µg kg^{-1} brown meat (as okadaic acid-equivalents) was chosen.
The symptoms appeared slightly delayed and were shorter in duration, compared with intoxications from blue mussels, where okadaic acid/Dinophysistoxin-1 constitutes a considerable part of total DSP toxins. One possible explanation for this might be slow or incomplete hydrolysis of Dinophysistoxin-3 to okadaic acid/Dinophysistoxin-1 in the human body.

Introduction
Episodes with diarrhoeic shellfish poisoning (DSP) associated with consumption of mussels have been recorded for many years in Norway (Aune and Yndestad, 1993). During the summer of 2002, more that 200 people experienced DSP symptoms after consumption of brown crabs (Castberg *et al.*, 2004). The crabs causing intoxications were caught in shallow waters. The incident was associated with an unusually early season of high levels of DSP toxins in blue mussels in southern Norway, indicating that toxic blue mussels were the source of DSP toxins in crabs.
The Norwegian Food Control Authority issued warnings against consumption of self-caught crabs, and established a preliminary surveillance system of DSP toxins in crabs. The surveillance was based on sampling every third week at seven locations along the Skagerrak coast including the locations where the first intoxications were recorded (see Map, Figure 1).

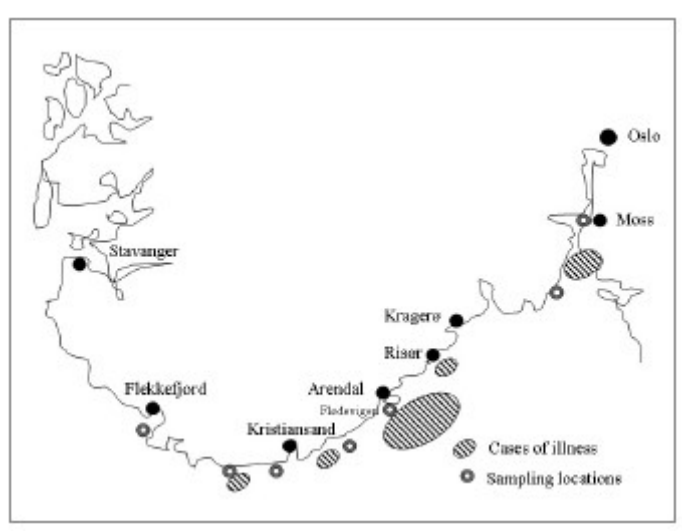

Figure 1. Map from southern Norway showing locations where crabs caught in shallow waters gave DSP and location of sampling stations for the surveillance.

Hazard identification
The DSP toxins in shellfish consist of okadaic acid (OA), dinophysistoxins (DTX) 1-2, and several 7-O-acyl fatty acid esters of OA and the DTXs (for simplicity, DTX-3). In blue mussels in Norway, DTX-3 contributes 30-70 % of total DSP toxins (unpublished results). Epidemiological studies from several countries show that DSP symptoms in man result in diarrhoea (92 %), nausea (80 %), vomiting (79 %), and abdominal pain (53 %) (Yasumoto *et al.*, 1978). The crabs inducing DSP contained only DTX-3, and the toxin was only found in the "brown meat", mainly consisting of the digestive organ, HP. There is one previous report from Portugal (Vale and Sampayo, 2002), where one person was intoxicated with DSP symptoms upon consuming green crabs (*Carcinus maenas*). Also in this case, the DSP toxins were present mainly as esters of okadaic acid.

Hazard characterisation
OA and DTX-1 are potent inhibitors of protein phosphatase (PP) 1 and 2A, especially the latter (Cohen 1989, Cohen and Cohen, 1989, Haysted *et al.*, 1898). The protein phosphatases play important roles in regulatory processes in cells. Symptoms of DSP fit well with effects of PP inhibition on membranes, cytoskeleton etc., resulting in among others leakage of fluid into the lumen. According to previous data, the Lowest Observable Adverse Effect Level, LOAEL (DSP symptoms) for DTX-1 and OA in adults are close to 38 and 48 µg, respectively (Fernandez and Cembella, 1995). At a recent event in Norway, during the grand opening of a new mussel farm, more than half of the about seventy people present developed DSP symptoms after consuming blue mussels (the organisers were warned about the risk). The level of OA/DTX-1 inducing DSP on this occasion was 55-65 µg OA-equivalents for adults (Aune 2001). The fatty acid esters of OA and DTX-1 do not inhibit PP1 or PP2A (Mountfort, D.M. *et al.*, 2001). Data on human toxicity of DTX-3 toxins are scarce. According to a previous report (Yanagi *et al.*, 1989), homologues of DTX-3 are slightly less active than OA concerning fluid accumulation in mouse intestinal loops, while their i.p toxicity in mice is markedly reduced. The biological activity of the DTX-3 toxins increases with the degree of unsaturation of the acyl side chain. DTX-3 analogues

are reported to be relatively unstable (Yasumoto *et al.*, 1985), and they are expected to be easily hydrolysed in the body, among others by lipases, giving OA/DTX-1 and -2. Since their diarrhoeagenicity is expected to be close to that of the latter, the risk of developing DSP is underestimated when using the mouse bioassay for shellfish containing significant portions of DTX-3.

Exposure assessment

Based on the first intoxications, seven locations along the south coast of Norway were selected for sampling of crabs from mid July (Figure 1). Three to five crabs were collected every third week at 15-25m depth at each location by professional fishermen (surface samples on a few occasions). Presence of OA, DTX-1 and -2 was analysed in claw and brown meat (HP) by LC-MS before and after hydrolysis (the difference being DTX-3). At some locations, DSP toxins in blue mussels were recorded simultaneously. The crabs inducing the first intoxications (in June) contained 1,000–1,500 µg OA-equivalents kg^{-1} brown meat. The toxins consisted of > 90 % of DTX-3, the rest being OA. The white crab meat from claws and the main shell contained only trace levels of DSP toxins. The levels of DTX-3 in brown meat of crabs caught at 15-25m depth varied between 20 and 550 (mostly < 300) µg kg^{-1} as OA-equivalents. The toxin levels dropped from week 33 to week 36, and the warning was lifted. Later, the toxin levels in crabs raised again, and a new wave of intoxications was reported. It was crabs caught in shallow waters, containing > 1,000 µg kg^{-1} OA-eq., that caused the DSP symptoms. The levels of DSP toxins in crabs followed the changing levels in blue mussels, but delayed for one-two weeks, indicating that crabs acquired the toxins from eating blue mussels (Figure 2).

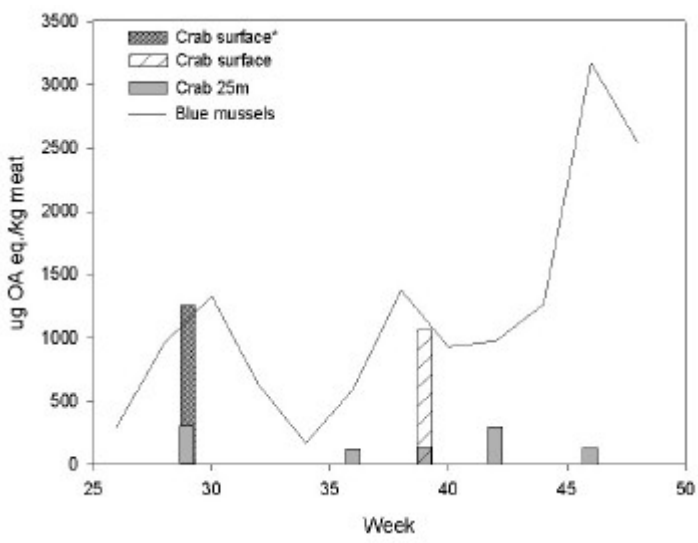

Figure 2. DSP toxins in crab brown meat and blue mussels from the Flødevigen area (*average DSP toxin level in crabs from the whole region during first intoxication episode).

The brown meat in crabs consists mainly of HP (about 80 %). A crab weighing 500g on average contains about 35g brown meat after cooking (Figure 3). An average crab meal rarely exceed 2-3 crabs of 500g fresh weight each, yielding a maximum of 70-100g brown meat. It should be mentioned that data in Figure 3 are from crabs from western Norway, while the intoxications in 2002 took place on the south-east

coast. In the latter area, the brown meat may make up a larger portion of edible parts because of less white meat in the main shell in summer.

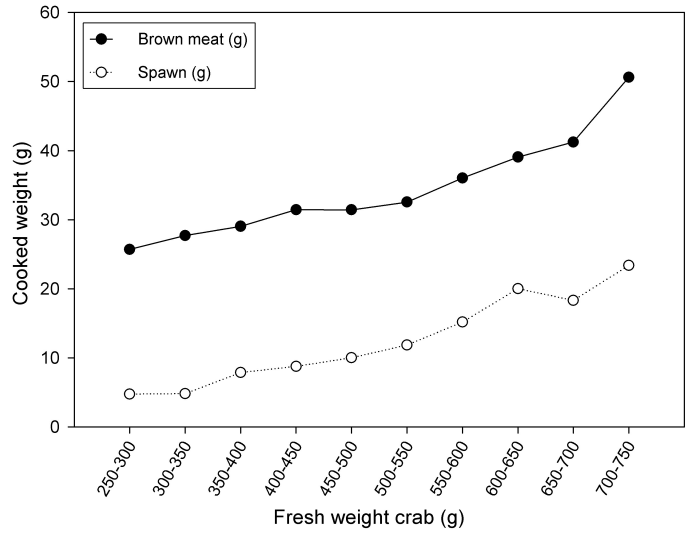

Figure 3. Correlation between brown meat (cooked weight) and fresh weight of crabs. (Data from west coast of Norway July-August 1993 by Astrid Woll).

Risk characterisation
The EU Directive (2002/225/EC) regulates the level of DSP toxins in bivalve molluscs, echinoderms, tunicates and marine gastropods, but not in crustaceans. Our results indicate a different toxin profile in brown crabs, compared with blue mussels. In brown crabs, DSP toxins are rapidly converted to corresponding DTX-3 esters, with fatty acids C14:0, C16:1, C16:0 and C18:1 (Torgersen *et al.*, 2005).
The symptoms appeared later (several hours) and were shorter in duration (about 2 days) among those intoxicated after consuming crabs, compared with persons suffering from DSP caused by consumption of blue mussels.

Leftovers from crab meals causing DSP indicated DTX-3 levels at 1,050-1,500 µg OA-equivalents Kg^{-1} brown meat. Assuming consumption of 2-3 crabs of 500g fresh weight, intoxications were associated with intake of 75-150 µg DTX-3 as OA-equivalents. This indicates slightly lower acute toxicity of DTX-3 compared with OA/DTX-1. If the relative proportion of brown meat was larger, due to low levels of white meat in the main shell at the time of consumption, the levels of DTX-3 inducing DSP were even higher.

In order to handle the acute situation, a preliminary tolerance level of DTX-3 in brown crabs was established by the Norwegian Food Control Authority. A level of DTX-3 at 400 µg kg^{-1} brown meat (as OA-equivalents) was chosen, based on: Average estimated consumption of maximum 2-3 crabs weighing 500g, giving 28-42 µg DTX-3 as OA-equivalents, respectively. An intake at this level comprise a safety factor of at least from 1.8 to 5.4 towards DSP.

Considering the reversibility of the symptoms and their lack of severity, this seems a reasonable initial way of handling the problem.

Acknowledgements
Assistance in collection of crabs by the Directorate of Fisheries is very much appreciated. Financial support was given by the Norwegian Food Control Authority.

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SANITARY SURVEY AND MICROBIOLOGICAL RISK ASSESSMENT APPROACHES IN NEW ZEALAND, MARLBOROUGH SOUNDS

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Abstract
The classification of commercial shellfish harvesting areas in NZ, particularly those areas where shellfish is destined for the European market, employs a combination of the USFDA model of Sanitary Survey predictive approach and the flesh based monitoring programme used by the European Union States. The paper expands on methods and a process used to assess the potential total impact on growing waters and discusses the resulting monitoring programme's findings in relation to the initial theoretical impact. Other issues discussed are the attempts at modelling mass transport of contaminants to and around the growing areas. Examples of rainfall issues and salinity based monitoring are discussed drawing from experience in the Marlborough Sounds and Nelson's Tasman and Golden Bay Growing Areas. Similarities and differences between growing areas are explored giving reasons for both. The resulting dataset, comprising both water and flesh bacteriological results, is used to determine safe shellfish harvesting criteria for each growing area. The resulting programme delivers a high degree of confidence to exporting companies and certifying agencies alike in NZ.

Paper
I intend to talk about New Zealand's approaches to the issues of Sanitary Surveys and Risk Assessment in New Zealand with particular reference to the way these issues are addressed in a practical sense in the Marlborough Sounds, New Zealand's premier mussel growing area.

First some background. New Zealand has since the programme was founded, drawn on the experience of Health Protection Officers (HPO's) in the delivery of the crucial Sanitary Survey work in shellfish growing areas. The United States Food and Drug Administration (USFDA) mandated the involvement of public health personnel in the 1980 Memorandum of Understanding between the United States and New Zealand Governments which allowed the market access of greenshell mussels from New Zealand into the United States. Today, all the crucial regulatory decision-makers in New Zealand's programme are in, or have worked in, a Public Health background. New Zealand's primary shellfish growing areas are spread across the country from Northland where oysters and some scallops are harvested, through the Coromandel where mussels are harvested, down to the Marlborough Sounds the premier mussel harvesting area, and finally through the South Island to Dunedin and Southland where cockles and dredge oysters are taken. New Zealand has 85 classified Growing Areas. I will confine this address to the areas that I have personal experience in, namely the Nelson Marlborough area, and the processes by which these areas are classified. This general area comprises 34 commercially harvested growing areas and includes the harvesting of rope culture mussels and oysters, feral scallops, dredge oysters and mussels, and feral cockles. I will discuss these processes generally using specific examples from the Marlborough Sounds and Tasman and Golden Bays. All areas in New Zealand that export bivalve shellfish undergo the same basic classification processes.
The Marlborough Sounds is a large drowned river valley system with a very convoluted coastline making for a complex interaction of water and land use. There are 18 classified growing areas within the broad area. The areas are

characteristically pristine coastlines with low numbers of human inhabitants and mainly pastoral and forestry farmland. The farms are within 50 metres of the coast in most cases. There is approximately 2000 hectares in farms, producing 50,000-70,000 tonnes green-weight of greenshell mussels per annum. Product is exported to 50-60 countries, with almost all harvested product destined for export, including many European Union countries. Maintenance of a high quality programme that satisfies all countries trading requirements is therefore paramount. The Marlborough Sounds Sanitation Programme is based on the USFDA requirements which has two important concepts as its cornerstone. They are firstly, predictability of pollution sources, and secondly, the ability to properly manage their impact. The USFDA system uses primarily water quality to identify and quantify pollution source risk. Many years ago the Marlborough Sounds Programme introduced the routine use of flesh quality testing in addition to water testing.

The classification process relies initially on the Sanitary Survey, and Shoreline Survey carried out by the HPO. A distinct advantage in using HPO's in New Zealand is their powers of entry under Statute to enter any private property to assess whether Nuisance conditions exist. The critical part of this initial process is to identify actual and potential pollution sources that may affect the growing area water and shellfish quality. From this information a monitoring programme is established to quantify and qualify theses sources of pollution. The sampling programme is rigorous and involves at least 30 sample days under varying climatic and environmental conditions. Such a programme will take at least 12 months to complete. The sanitary survey provides a theoretical total impact of pollution sources on the receiving waters of the growing area. The assessment takes into account the potential impact of animal and human contaminants as well as other non-microbial pollutants. The resulting dataset from the 12 months sampling effort provides the "*ground truthing*" of the initial estimates.
The actual results show that considerably less contamination, although still significant, shows up in sampling effort when compared with the theoretical impact. This is almost certainly due to the conservative nature and assumptions made in the course of the Sanitary Survey estimates. Subsequent sorting of the data to reflect when the area is open for harvesting shows that the flesh and water data is in compliance with both the USFDA and European Union Standards.

Predictability
A variety of methods are used to trigger closures in growing areas. The use of a particular type depends on what is considered the most representative of the impact of the most significant pollution source. The most prevalent method is rainfall, measured by a tipping raingauge, which logs rainfall every fifteen minutes, twenty-four hours of every day. There are some confounding issues when using rainfall, which can affect the relationship of rainfall to contamination. These include things like; differing rainfall intensities, which impact on subsequent runoff; and patchiness of rain over a large area, to name but two. Riverflow levels are also employed on occasions to trigger closures in growing areas. The confounding issue with riverflow is the ability of predicting, in some instances, when a river plume will impact on a growing area. In addition to this is the effect of other influences like tide or wind will influence the impact of the river plume by moving it elsewhere.

For this reason, other surrogate methods of predicting pollution event effects on growing areas are being investigated with some success. Salinity based monitoring on the marine farms is being used in two growing areas currently and being trialled in two others. Salinity does not offer the panacea for all situations however, and

there are inherent difficulties in getting relationships between bacteria and salinity under some situations. Again for this reason, other methods that offer potentially better relationships are being explored, the latest being turbidity. There is no evidence to present on that issue at this time, although work is planned to proceed.

Exclusion Zones
At times there are pollution sources that have much greater significance than runoff due to rainfall, and are high concentration pollution sources containing human effluent. These are the likes of sewage outfalls or marinas. New Zealand's approach to these issues is to thoroughly assess the discharge, assuming the worst case scenario, and to use dilution calculations to determine a safe exclusion zone around that area. This means that the area inside that zone is prohibited for the taking of shellfish for sale. In New Zealand it is the duty of the polluter to monitor any discharge at the source. HPO's, by virtue of their role in the oversight of Local Authority work in Environmental Health activities, has access to that monitoring data. For the purposes of the shellfish monitoring, the boundary of the exclusion zone is monitoring to give a picture over time of whether the exclusion zone distance is appropriate

Summary
In summary, the Sanitary Survey is by far the most important aspect of New Zealand's sanitation programme. The identification of pollution sources in the catchments is a key factor in decision making on safe harvesting of shellfish for sale and export. The ability to predict contamination with some certainty is essential, and is a cornerstone of the programme. Considerable effort goes into achieving this for growing areas. The special attention given to high risk discharges like sewage outfalls is essential for the safety of the harvested product.

The addition of flesh monitoring on a regular basis after growing area reopening provides a very good ongoing indication of the status of our growing areas and the safety of any shellfish harvested. Furthermore, it has been this alteration in part, that has enabled the Industry to access markets other than the United States endorsed markets, and in particular the European Union markets.

TOWARDS A WIDER APPLICATION OF RISK MANAGEMENT TO BIVALVE MOLLUSCAN SHELLFISHERIES

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Abstract
Statutory public health controls on bivalve molluscs in a number of countries date back to the early twentieth century and were largely introduced because of concerns relating to large outbreaks of typhoid. Current controls are still largely based on the same approaches and have not been significantly altered to reflect either a changed perception of the principal causes of shellfish-associated outbreaks or advances in scientific understanding of the contamination and decontamination of shellfish. In many areas, widespread commercial utilisation of shellfisheries continues to be limited by the achievable water quality and it may only be possible to overcome this by alternative approaches to public health controls. The present approaches to risk-based management of shellfisheries are reviewed and the shortcomings discussed. An international approach to addressing the problems is proposed.

Introduction
Food safety concerns regarding the consumption of sewage-contaminated bivalve molluscs, particularly with regard to their role in outbreaks of typhoid, were expressed as far back as the 1890's (Buchan 1910). Large outbreaks of typhoid associated with the consumption of contaminated bivalve mussels during the early part of the 20th century led to the establishment of national controls in a number of European countries and the United States. While legislation has evolved since then, it is largely based on the same approaches with regard to faecally-derived contamination. This paper outlines proposals for a fundamental international review of the shellfish hygiene legislation, taking into account recent advances in scientific understanding of the contamination of bivalve molluscs by viruses and good practice from the major systems. The review and proposals will concentrate on microbiological aspects, particularly those relating to faecal contamination, but many of the underlying principles which will be discussed are also applicable to other microbiological contaminants, biotoxins and even chemical contaminants.

Management of recreational waters
A similar situation has existed with the monitoring and management of recreational waters, including bathing waters. This was subject to review at a WHO expert consultation in Annapolis, USA in 1998 (WHO 1999). This identified major drawbacks with previous management approaches as follows: (i) they were based on microbiological monitoring alone; (ii) inter-laboratory and international comparability of microbiological analytical data were poor; (iii) monitoring programmes often only trigger sewage treatment and disposal improvements which occur some years later without other management actions; (iv) any controls which are applied are retrospective and exposure will already have occurred; (v) health risks are primarily associated with faecal contamination from human sources and present monitoring approaches do not distinguish between human and non-human

sources; and finally, (vi) present classifications are generally compliant/non-compliant whereas risk may, in practice, vary in severity, variety and frequency.

In considering the relationship between monitoring programmes, health effects and management practices, those involved in the recreational water area have the advantage that a number of comparatively large studies have been completed which have quantified the interaction between exposure and health effects (Kay *et al*. 1994; Prüss 1998). There have not been any similar studies in relation to bivalve mollusc-derived illness and the data available from national and international infection surveillance bodies and the scientific literature are inadequate to allow the investigation of such relationships. On the other hand, the illnesses associated with bivalve molluscs have been attributed to defined pathogens or toxins (Scoging 1991; Rippey 1994) whereas the aetiological agents responsible for causing illnesses associated with recreational water use remain at the 'speculative' level based on inference from sympomatology and onset dates (Fleisher *et al*. 1996, 1998)

The consultation at Annapolis suggested a change in the paradigm from retrospective monitoring to real time risk management of recreational waters and defined the following stages in this process.

a) Establishing a primary classification based on a sanitary inspection and initial microbiological quality assessment.
b) Reclassification to a better class if management actions are deployed to reduce human exposure (at times/places of increased risk) with a proviso that the occurrence of time/places of increased risk can be reliably predicted.
c) Definition and application of management actions.
d) Ongoing monitoring to determine whether status changes with time.

A significant requirement is the need to review whether active management plans, to allow a better classification, actually results in the required reduction of human exposure. Further to the publication of the Annapolis protocol, there have been a number of studies of the potential for real time beach management and the water quality prediction needed to underpin such approaches. The US Environmental Protection Agency has produced a review of the modelling tools that were currently, and potentially, available for use in predicting contamination events for both bathing and shellfish waters (US EPA 1999). Crowther *et al.*, (2001) used environmental variables to predict faecal indicator concentrations at recreational beaches along the Fylde coast in the UK. Morrison *et al*. (2003) investigated the use of Receiver Operating Characteristic Curve Analysis to determine the impact of preceding rainfall on *Enterococcus* concentrations in bathing water and recommended the procedure as a means of determining the appropriateness of indicator variables as predictors of bathing water quality. Kim and Grant (2004) also used statistical techniques to investigate the association between indicator variables and water quality and showed that the use of environmental variables as predictors was more successful than using the previous day's microbiological results. The Scottish Environment Protection Agency (SEPA) has undertaken a prospective project at six beaches to determine whether appropriate management action could be taken using rainfall as a predictor of poor water quality (Scottish Executive 2003). Electronic signs were erected at the beaches to display whether good or poor water quality was predicted. The results of the samples were compared to the mandatory standards in the EU Bathing Water Directive and the resultant assessment of good or poor water quality was compared to the prediction that had been displayed on the signs. The results of these comparisons were: Signage

good/water quality good, 93 %; Signage good/water quality poor, 2 %; Signage poor/water quality poor, 1 %; Signage poor/water quality good, 4 %.

Ashbolt and Bruno (2003) undertook a detailed study to investigate the application of the WHO risk assessment and management approach to recreational waters in the Sydney area in Australia. In addition to rainfall, other environmental factors such as tide, wind direction and sunlight, were deemed to be significant in predicting counts of enterococci. Some examples local information needed to supplement generic models for assessment and management were identified.

The Annapolis protocol was incorporated into the first WHO Guidelines for Safe Recreational Water Environments in 2003 (WHO 2003: Chapter 4). This WHO approach to 'real time' management of recreational waters has been incorporated into proposed amendments to the EU Bathing Water Directive 76/160/EEC (Anon 1976). This proposal incorporates the WHO recommendation of microbiological monitoring and 'beach profiling', together with a system of allowed sample discounting if a management system is in place to facilitate appropriate 'advisory' notices to the public. The guiding principle behind this system is that the public should have 'informed choice' and this is the appropriate management action to maintain public health particularly where intermittent, but predictable, pollution from non-human sources is known to affect a recreational water after heavy rainfall (WHO 2003; Anon 2004).

Current approaches to public health management of bivalve mollusc harvesting areas.

Across the range of classification, monitoring and management systems that are operated internationally for bivalve mollusc harvesting areas, there are common elements with the approach taken in the Annapolis protocol for recreational waters. For example, they may include:

a) Basal classification status determined by assessment and monitoring
b) Temporary controls for
— biotoxin incidents
— sewage pollution events
— other microbiological contamination events
• unexplained faecal indicator increases
• *V. parahaemolyticus* events
c) Seasonal classifications
d) Rainfall- or river flow-associated controls

The US programme (US FDA 2000) and those of other countries based on it, include formal requirements for sanitary surveys while this has not been the case within the EU (although this will change with new legislation to be effective from 2006). The following crucial aspects of ongoing monitoring tend to vary between different programmes: the matrix to be monitored (shellfish/water/both); the spatial coverage of monitoring points; the frequency of monitoring; analytical methodology; and whether reactive monitoring is undertaken for contamination events. There are also marked differences in the methods of data analysis used to assess statutory compliance leading to inconsistency in the manner in which classifications are determined. Elements of different programmes, such as the US use of water monitoring and the European Union (EU) use of bivalve flesh monitoring have been compared solely on the basis of their performance with respect to indicator organisms (European Commission 1996). There is a need for such

comparisons to be extended to include an assessment of the level of health protection that they offer. Given that, to date, there have been no controlled epidemiological studies for bivalves, it is not possible to do so directly. However, as improved methods for detecting viral pathogens in bivalves are developed, more data is becoming available on the presence of these in different harvesting areas, together with parallel data on the concentration of indicator organisms. This enables the performance of assessment and monitoring programmes to be judged more objectively: for example, Figure 1 shows the predicted proportion of bivalve samples that will be positive for Norovirus as the percentage compliance with a limit of 230 *E. coli* per 100g (the EU class A limit) is varied. The analysis was based on data reported by Formiga-Cruz *et al*. (2003) and *E. coli* data from the monitoring programme in England and Wales (Lee and Younger 2003). Such analyses can be extended to other aspects of monitoring and management programmes but require the acquisition of good base data - ideally this would be quantitative data for both bacterial and viral pathogens, together with a means of assessing viability of the latter.

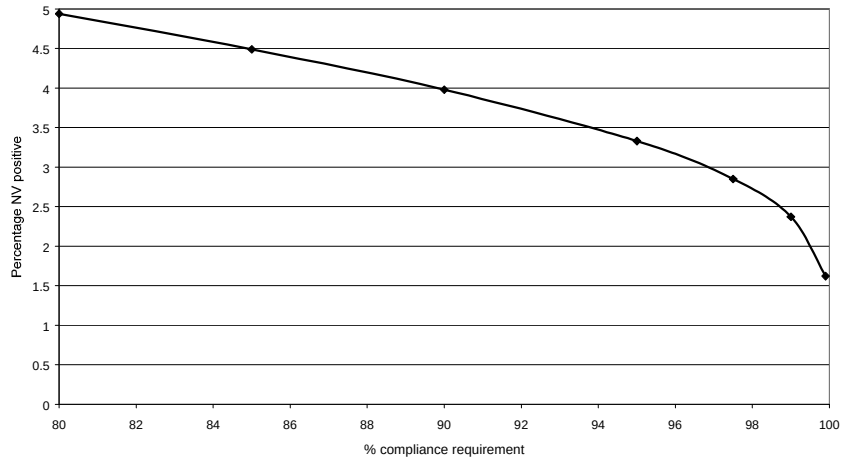

Figure 1. Proportion of samples positive for confirmed Norovirus versus % compliance requirement with 230 *E. coli* per 100g

Unpredictability of sewage impact

The use of sanitary surveys and basal monitoring results implies that, to a certain extent, the contamination arising from identified inputs is predictable and that no significant unidentified sources are present. With respect to continuous sewage discharges, the quality of the effluent may vary on a seasonal and diurnal basis and may be worse during periods of rainfall (Kay *et al*. 1999). The plumes from sewage outfalls may not behave as predicted from hydrodynamic modelling or dye tracing studies under all conditions and this may markedly affect the way that the outfall impacts on the quality of the bivalve molluscs. Added to these effects, gross contamination from normally well treated systems may arise in the event of reduced treatment during plant maintenance (planned or unplanned), or breakdown of sewage treatment plants or the sewerage system. These effects, combined with contamination arising from normal rainfall-dependent discharges from storm tanks and combined sewer overflows, may invalidate predictions of the microbiological quality of harvesting areas made on the basis of sanitary surveys and/or basal monitoring and dictate the need for well-constructed ongoing monitoring programmes (Lee *et al*. 2003).

Seasonal classifications

A number of countries classify a proportion of production areas on the basis of a perceived or demonstrable difference in contamination between different parts of the year. The differences are invariably based on the bacterial indicators used in the classification system. Such an approach may allow the commercial utilisation of areas during the periods of lower contamination when a classification based on year-round results, and the required post-harvest treatment (such as long-term relaying), may make an area of marginal commercial interest. True seasonal differences, justified by statistical analysis of data, often reflect a complex interaction between seawater temperature, rainfall effects, human population/activity changes (such as tourism) and the shellfish biology. Some of these effects may vary from year-to-year and thus forward prediction of seasonal classifications can be subject to considerable error. The matter is complicated by the relatively low frequency of sampling in most monitoring programmes and thus analysis of seasonal effects is often based on minimal data sets without any formal determination of the significance of the effects.

The present application of seasonal classifications would, in many instances, not necessarily meet the requirement identified in the Annapolis protocol for recreational waters and WHO (2003), that the periods of different risk can be reliably predicted, or that the performance of management actions should be reviewed in order to ensure that they are appropriate. For shellfisheries, there is also the need to relate differences in contamination shown by bacterial indicators to differences in risk of illness, or at least to differences in the extent of contamination by shellfish-associated pathogens. It would also seem necessary to apply an *in situ* relay time between the period of worse classification and the harvesting period, so that seasonal classifications give the same public health protection as that given by direct application of post-harvest treatment requirements to areas with year-round classifications.

Rainfall-associated controls
In a number of countries, short-term control measures, e.g. closures, may be instituted on the basis of the rainfall over a specified period exceeding a certain level (c.f. the SEPA signage project), river flow exceeding a specified rate, or the salinity remaining below a specified level. The particular trigger will depend on the environmental monitoring systems in the locality and the particular association that has been demonstrated between an environmental variable and classification compliance. The closure status in such situations is usually well-defined but the re-opening of the shellfishery is usually based on clearance times of faecal indicator bacteria, which may be hours to days and not the pathogens of interest, which may be several weeks depending on the seawater temperature (Lees 2000).

Role of pathogen monitoring
Monitoring programmes based on conventional indicator organisms may not adequately reflect health risks, especially those associated with more resistant micro-organisms such as viruses. This has led to interest in direct monitoring of pathogens, possibly in conjunction with the traditional indicators.

There are at least 6 scenarios where monitoring of pathogens in harvesting areas could be seen to have a potential role:

- Investigation of bivalve mollusc-associated outbreaks of illness
- In support of the development of risk assessments
- Investigation of the impact and persistence of contamination events (e.g. in the event of sewage treatment work or sewerage system malfunction or breakdown)
- Validation of current monitoring programmes based on faecal indicator organisms (to support decisions on programme content and data interpretation)
- Secondary monitoring to supplement programmes based on faecal indicator organisms
- Primary monitoring to replace the use of faecal indicator organisms

However, there are a number of aspects that need to be defined before proposing to use such monitoring in a statutory context. An advantage of using indicators for monitoring is that they yield a general measure of the risk of contamination from faecal sources. Individual pathogens may not be present when such a general risk of contamination exists but other pathogens may be present. Presently, it is not be practical to monitor for all possible pathogens although further developments in test methodology may make this possible. Even given such developments, there will still be the problem that new or emerging pathogens may not be detected. This may also apply to new variants of highly variable viruses such as Norovirus. However, shellfish-associated bacterial infections still occur to some extent and these would not necessarily be predicted by viral monitoring. *Salmonella* Typhi and *Vibrio cholerae* infections may result from shellfish consumption in endemic areas and the protective ability of current programmes needs to be reviewed with respect to these pathogens.

There is also the need for additional research data on which to base potential monitoring strategies. This includes the performance characteristics of the analytical tests, spatial and temporal variability in occurrence and/or concentrations of the pathogens, and the effect of (potentially species-specific)

uptake and removal. Many of these aspects have now been determined for the conventional indicator organisms such as *E. coli* (Lee and Morgan 2003; Younger, *et al*. 2003) and the introduction of monitoring for pathogens without establishing such performance characteristics could result in them being discredited.

Proposed approach

It is therefore proposed that an international review should be undertaken of the present harvesting area assessment, monitoring and management systems, in conjunction with the scientific evidence supporting such programmes and relevant risk assessments on shellfish-associated pathogens. The aim should be to:

- Assimilate good practices from existing systems
  - but to avoid an 'average' approach accommodating all but achieving little or *ad hoc* selection from different systems without considering the overall consequences
- Undertake targeted research to fill identified gaps in knowledge
- Consider synergies with initiatives in other areas
  - e.g. recreational waters (common approaches may enable sharing of resources)
- Develop assessment and monitoring systems for harvesting areas that will underpin a management system based on a presumption of public health protection and sound science

It is important that any system of active management is based on addressing the actual health risk and not manipulating compliance based on indicator organisms. It is also important that any system incorporates a formal review mechanism, as specified in the Annapolis protocol for recreational waters, to ensure that the management system is achieving the required results.

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REVIEW OF MANAGEMENT CELL

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The statutory functions of the Food Safety Authority of Ireland (FSAI) are:

- Coordinating 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- Department of Communications Marine and 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 action 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.

In 2003, 43 Management Cell Decisions were issued. There was 1 decision each where precautionary advice was issued, and the sampling frequency for oysters was changed. A total of 35 decisions dealt with situations were an assigned or provisional status had been issued for a production area (see Table 1).

Table 1. Management Cell Decisions, 2003

Original Status	Decision	Frequency
Open	Closed	2
Open	Closed Pending	0
Closed	Open	6
Closed	Closed Pending	3
Closed Pending	Open	16
Closed Pending	Closed	0
No Change in Status		8

The remaining 6 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. In the first 6 months of 2004, 17 Management Cell Decisions were issued. Two decisions were taken to report 37 and 32 sites in advance of chemical results being available using the bioassay only. 14 MCDs dealt with an assigned or "provisional" status (see Table 2).

Table 2. Management Cell Decisions, Jan to June 2004

Original Status	Decision	Frequency
Open	Closed	0
Open	Closed Pending	0
Closed	Open	10
Closed	Closed Pending	2
Closed Pending	Open	2
Closed Pending	Closed	0
No Change in Status		0

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. Further improvements to the working of the Management Cell 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 producers who are not members of any of the representative organisations.

IRISH SHELLFISH BIOTOXIN MONITORING PROGRAMME

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Introduction

Since its initial development in the early 1970s the Irish aquaculture industry has grown to be an important contributor to the national economy. There has been a steady increase, in both output and value, as well as in job creation. The total production of farmed shellfish has increased from approximately 5,000 tonnes in 1980 to 44,678 tonnes in 2003 (Figure 1), with a first sale value of €41.8m and directly employing some 1100 people (Parsons *et al*, 2004). Mussels (*Mytilus edulis*), native oysters (*Ostrea edulis*), Pacific oysters (*Crassostrea gigas*), Clams (*Tapes semidecussata*) and scallops (*Pecten maximus*) are the main species produced. With a growing recognition and awareness internationally of the potential human health effects of the consumption of shellfish containing algal toxins, a monitoring programme was established in Ireland in the early 1980s and has continued since then. In this paper the evolution and development of the programme is described and discussed.

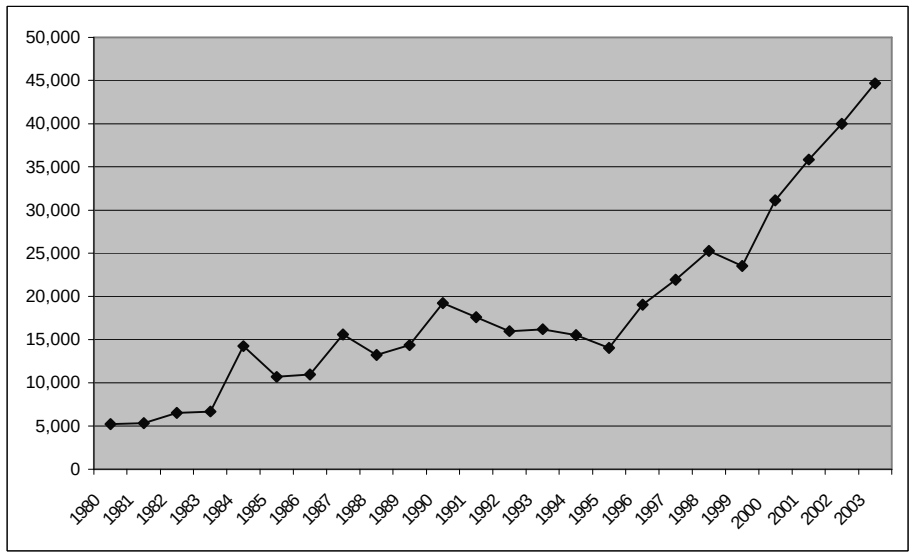

Figure 1. Shellfish production(tonnes) in Ireland 1980 – 2003

Lipophilic toxins

In the period up to the early 1980s, shellfish toxicity leading to diarrhetic shellfish poisoning (DSP) occurred so rarely in Ireland that it failed to provoke any action on the part of the regulatory authorities. However, following the first publication in the scientific literature showing the association between the occurrence of *Dinophysis spp*.and the accumulation of DSP toxins in mussels in The Netherlands (Kat, 1983) and the first records of *Dinophysis* in Ireland made at the Sherkin Marine Station on the southwest coast, the Irish Biotoxin and Phytoplankton Monitoring programme was established. The initial testing programme consisted of observations of phytoplankton samples on field trips made by Department of Fisheries staff to the southwest and the use of Rat bioassay for testing the shellfish. This testing

continued through the late '80s in the southwest, mainly in the summer months. With the expansion of the industry in the early part of the 1990s along the south and west coasts, the volume of sampling and analysis also increased. In 1991 European legislation (Council Directive 91/492/EEC) was introduced to govern the placing on the market of live bivalve molluscs and to control the toxicity therein. This Directive was subsequently amended by Commission Decision 2002/225/EC to take account of improved analytical methodologies and increased knowledge on toxicology. Initially in Ireland Okadaic Acid was shown to be the main DSP toxin present but in 1991 Dinophysistoxin-2 (DTX2) was identified for the first time in mussels from Bantry Bay on the southwest coast (Hu *et al*, 1992, Nixon and Taffe, 1993). Subsequently the toxins were monitored by bioassay and quantified by HPLC methods. In 1994 positive bioassays, due to the presence of DTX2 at high levels (McMahon *et .al,* 1996), persisted through the winter months (Figure 2) resulting in a protracted ban on the harvesting and sale of shellfish from these areas with significant financial losses for the industry. Following these protracted closures a Ministerial Task Force was established to review the monitoring programme. Recommendations of the review included year round testing of samples and the replacement of the rat bioassay with the Yasumoto 1978 mouse bioassay which was being used in most EU Member States at that time. In 1996 The Marine Institute also took over the role of coordinating the testing from the Department. By the late 90's up to 4000 bioassays were being carried out *per annum*, and a phytoplankton monitoring programme was also in place around the coast

In November 1995 at least 8 people became ill in The Netherlands following the consumption of mussels harvested from Killary Harbour, on the west coast of Ireland and subsequently a previously unknown toxin, Azaspiracid, was isolated and identified (Satake *et al*, 1998) This event marked a watershed in the monitoring programme and the reliance on bioassay testing alone was questioned. European Union legislation specified that a bioassay is to be used as the reference method, but alternative methods that could provide an equivalent level of human health protection, could be incorporated into the monitoring programme. The Irish programme changed to a 24 hour Mouse Bioassay including a clean-up step, in line with the method most widely used in Europe, and liquid chromatography mass spectrometry (LC-MS) was introduced in 2001 to allow for the identification and quantification of toxins present and chemical confirmation of the bioassay results, initially to reduce and eventually to move towards replacing animal based testing.

Table 1 shows the percentage positive bioassays observed for all species since 1994. The earlier years have high percentage positive results, but this is due to the emphasis of testing the southwest mussels alone and only in the summer months. The overall picture here however does show that there are some years when there is very little toxicity present, as low as 1.5 % for all species, but other years can be much higher, and especially in the summer months. No obvious pattern is present for these variations such as correlation to high rainfall years, warmer summers etc. A similar pattern is evident, albeit at a lower level with PSP toxicity. These figures are based on all shellfish species tested, and as rope-grown mussels are more prone to toxification than the other commonly farmed species such as oysters and clams, there may therefore be a much higher incidence of toxicity in these shellfish.

Table 1. Percentage of Positive Bioassays 1994 -2002

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

The question remains, why are these inter annual differences present, and in the absence of geographical, or inter-annual patterns the primary suspect is variability in the presence and dominance of toxic phytoplankton. The most important of these is probably the subtle switch from diatom-dominated phytoplankton in the early summer to a dinoflagellate-dominated community. The time of this switch and the ratio is different each year. Observing this however demands a high frequency phytoplankton-monitoring programme with many more sampling points and some offshore sampling. The impact on production is dramatic, and the variability from year to year is shown for the Bantry area in Figure 2. One of the difficulties with shellfish toxins is the unpredictability of the time of onset, the intensity and the duration of the event. It ranges from years that required no closures to a continuous closure of 10 months between 1994 and 95. This makes it very difficult for producers to guarantee delivery to their customers especially at shoulder periods of toxicity. Obviously, an industry that is operating under such a variable window of opportunity requires much assistance in forecasting the onset and duration of these toxic events.

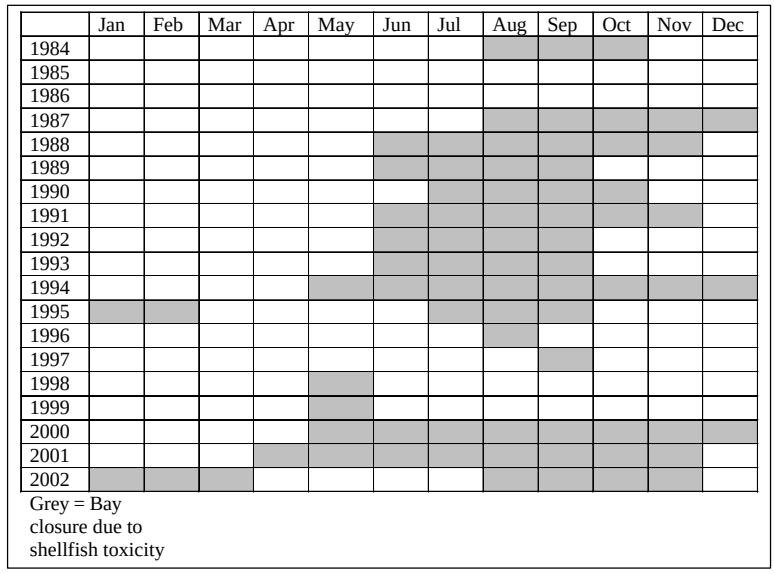

Figure 2. Variability in annual closures in Bantry Bay, SW Ireland, 1984 -2002

Current DSP Monitoring Programme

Following the operational changes outlined above, regionally based laboratories were contracted to carry out routine bioassay testing with defined quality control procedures and a laboratory accreditation plan was initiated. Approximately 2200 samples per annum are now analysed by bioassay in the regional laboratories, with chemical (LC_MS) backup being carried out in the Marine Institute. Rapid turnaround of the results of this testing programme was facilitated by the incorporation of modern communications technology to speed up the transfer of data and information between laboratories, regulatory authorities and the industry (Figure 3). This was implemented by the development of an online database, which can be accessed by the regional laboratories through the Internet. The database facilitates the decision making process as it can present all of the necessary information both current and historical in a suitable report format to help decide the status of a given shellfish production area. These reports are then issued to the various interested parties via web, fax, SMS and email. The objective is to report the result of the analysis of all samples submitted within 3 days of the samples being received in the laboratory. The development of the database system has greatly facilitated this objective, with over 95 % of test results meeting the 3-day turnaround deadline.

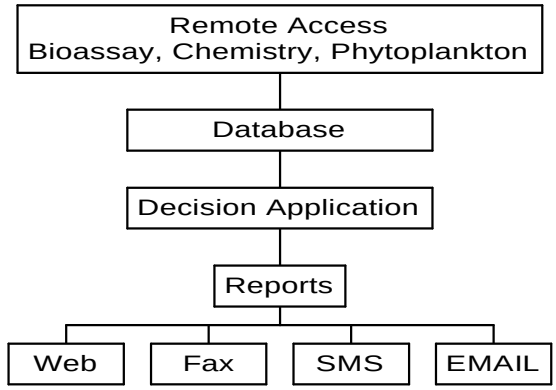

Figure 3. Biotoxin Information System

Amnesic Shellfish Toxins:

The content of domoic acid (DA) in shellfish intended for human consumption has also been regulated in the EU by the Council Directive 91/492/EEC, as amended by Council Directive 97/61/EC and Commission Decision 2002/226/EC. The maximum concentration permissible for DA in marketed shellfish or shellfish products is set to 20 mg/kg of the whole flesh or any edible part separately. The Marine Institute is responsible for the routine surveillance for amnesic shellfish poisons (DA and isomers) in live bivalve molluscs in Ireland. This monitoring entails testing of ca. 700-800 tissue samples per annum, mostly arising from the control of scallops (*Pecten maximus*) landed as fisheries products, although other shellfish species are also tested at reduced frequency. The highest concentrations of DA in shellfish from Irish waters are typically found in scallops, particular in the hepatopancreas, leading to situations where scallops must be processed to achieve toxin-free edible parts such as adductor muscles and gonads. Due to the food safety aspects and the requirement for freshness, quality of results and a fast sample turnaround are important factors in the official control of DA.

The ASP testing protocol used in Ireland, based on the EU Directive, is a three-tiered status for the individual fisheries.

Open	DA in each compartment of the scallop i.e.Adductor Muscle, Gonad, Hepatopancreas and Total Tissue is less than 20mg/Kg
Closed	DA in the adductor Muscle and/or gonad is greater than 20 mg/Kg
Limited Sale	Where DA in the Hepatopancreas is greater than 20 mg/Kg but DA in both Adductor Muscle and Gonad is less than 20 mg/Kg sale to approved processing establishments may be allowed for shucking Adductor Muscle and Gonad must be re-tested before placing on market

This scheme allows some fishing to continue in the presence of DA in the non-edible parts of the scallop. In practice, as the levels of DA in these parts of the scallop has typically been above the regulatory limit since monitoring began in 1999, the bulk of Irish fished scallops are shucked and the edible parts only are submitted for testing. These tests on representative samples from the processed batch ensure that only shucked meats below the safety threshold of 20ug/g are placed on the market.

Paralytic Shellfish Poisoning

The third group of toxins occasionally present in Irish shellfish is the Paralytic Shellfish Poison group (PSP). These are monitored in response to the presence of *Alexandrium* in seawater, using the standard AOAC PSP bioassay. In addition, the use of Immunoassay kits are being explored as valid screening alternatives to this test. The threshold of 80ug STX eq./100g flesh is used as the closure level and this has only been observed regularly as a summer phenomenon in the area of Cork Harbour on the South coast. In this area, mussels exhibit levels requiring action for a short period most years, and occasionally flat oysters are also affected. The presence of PSP in shellfish outside of Cork Harbour has been observed rarely.

Risk Based Management

In the area of food safety, the public often supports strong policies, hoping to reduce or eliminate risks to human health. But recent evidence indicates that some of these policies are not directed at the most significant sources of risk. The effect of such policies may be to misallocate resources that could improve public health if those resources were directed toward larger risks. During the early 1990s the allocation of resources to shellfish safety was totally inadequate. With the detection of Azaspiracid in the mid 90's and the extended closures in 2000-2001, pressure was placed by the industry on government to re-allocate resources and promote more informed decision-making about shellfish safety through greater use of tools such as risk assessment and decision analysis. Under the guidance of a committee of Regulators and Industry representatives (The Molluscan Shellfish Safety Committee or MSSC) a re-vamped programme was implemented in 2001 to provide sensible consumer safety but also to protect the industry from unnecessary closures. A management concept taking a holistic view of risk was initiated in 2003. Shellfish production closure and opening would now be based on all information available from bioassay, chemistry, phytoplankton, recent history and results from adjacent areas. A Management Cell, made up of representatives from the Food Safety Authority, Department of Communications, Marine and Natural Resources, Irish Shellfish Association and the Marine Institute, was established to discuss compiled information, and in the case of unusual or un-seasonal results take a measured decision. A range of improvements to the process came with the adoption of risk based management techniques. While the EU directives ultimately governed the

amount of flexibility that could be introduced into the system, a more pragmatic and appropriate level of control was introduced with the combined interest of consumer food safety and industry interests taken into account.

Conclusion

The establishment of a suitable monitoring programme is dependant on the implementation of many diverse elements. The Irish National Biotoxin Monitoring Programme is an effective implementation of phytoplankton, bioassay and biotoxin chemistry monitoring programmes. Each of these has their own strengths and weaknesses but in combination they provide a very strong programme to protect human health. Phytoplankton monitoring may provide an early warning of potential biotoxin contamination, often with results available before the shellfish tests. Identification of toxic species alone can trigger an action plan to delay harvest, or in some cases to close an area and thereby protect human health. However the patchiness of phytoplankton in the water makes it very difficult to obtain representative samples. Bioassays can provide a good indication of overall toxin load in shellfish, and some indication as to the safety of the shellfish when the toxicology of the toxin is unknown. In certain cases the bioassay can be calibrated to give a semi quantitative approximation of toxin equivalents in the shellfish and the test does not require sophisticated equipment. There is, however, some evidence that bioassays may be oversensitive to certain toxins and the reliance on bioassays alone for regulatory decision-making can be questioned. Chemical analytical techniques offer extremely sensitive methods for the quantification of the presence of biotoxins in shellfish. In many cases these methods are the only means of determining the identification of the particular toxin(s) present. In addition, due to their sensitivity these methods can detect toxin levels well below the threshold of closure, thus sometimes offering forecast information on the onset of the problem. The success of any biotoxin monitoring can be judged in terms of how consumer safety has been ensured. Since the restructuring of the programme in Ireland in 2000/2001 there have been no reports of human illnesses or product recalls associated with biotoxins in Irish Molluscan shellfish on national or international markets. The programme will continue to improve and adopt state of the art methodologies and management concepts into the future.

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USING 'REAL-TIME' STREAM FLOW AND TELEMETRY TO SAFEGUARD SHELLFISH QUALITY

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Abstract
Real-time stream flow data, plotted by telemetry, is being used as a tool to predict the likelihood of faecal contamination of blue mussels and Western Rock Oysters cultured in Oyster Harbour, near Albany, Western Australia. This shellfish growing area is subject to contamination during periods of heavy rainfall and runoff when livestock effluent and other contaminants from the catchments may be transported into the harbour via the King and Kalgan Rivers. The WA Shellfish Quality Assurance Program (WASQAP), a joint initiative between industry, the WA Department of Fisheries (DOF) and the WA Department of Health (DOHWA) has previously managed this growing area by implementing harvest closures based on rainfall triggers and a drop in salinity associated with freshwater influx from the incoming rivers. However the measurement of salinity may be inconvenient and inaccurate due to calibration of equipment, stratification of the water body and the effect of winds and tides on the path of freshwater through the harbour. Statistical analyses indicated that flow rate data from a DoE stream gauging station on the Kalgan River had a high correlation with coliform levels in Oyster Harbour. Using mobile phone telemetry, the DoE established a system to present data from the Kalgan River on their website each morning using a custom plot showing the most recent 21 days of flow rate data with a threshold line equal to 3 times the lowest flow rate in the preceding 21 day period. Data analyses indicated that when the flow rate is above the threshold line the harvest area may be at risk of contamination and harvesting should not occur. Results of a sixteenth month trial indicate the stream flow data compared against the threshold calculation provides a relatively accurate indication of contaminated water and/or shellfish.

Introduction
Ocean Foods International (OFI) produces mussels and oysters from lease areas located in Oyster Harbour, near Albany. Blue mussels are produced by long-line culture in a lease area located near the western side of the harbour whereas intertidal Western Rock Oyster grow-out areas are located on the eastern side (see Figure 1). The growing area is managed under the WASQAP and has been given a conditionally approved classification as defined under the Australian Shellfish Quality Assurance Programme (ASQAP) Operations Manual. This classification means that shellfish may be harvested directly from this growing area, except under identified environmental conditions when the harvest area will be closed for harvesting pending the results of bacteriological analyses of water (for sites see Figure 1) and shellfish samples. The ASQAP Operations manual has threshold levels for bacteriological contamination for both water and shellfish samples, and shellfish may not be harvested or sold when bacteriological results exceed these limits.

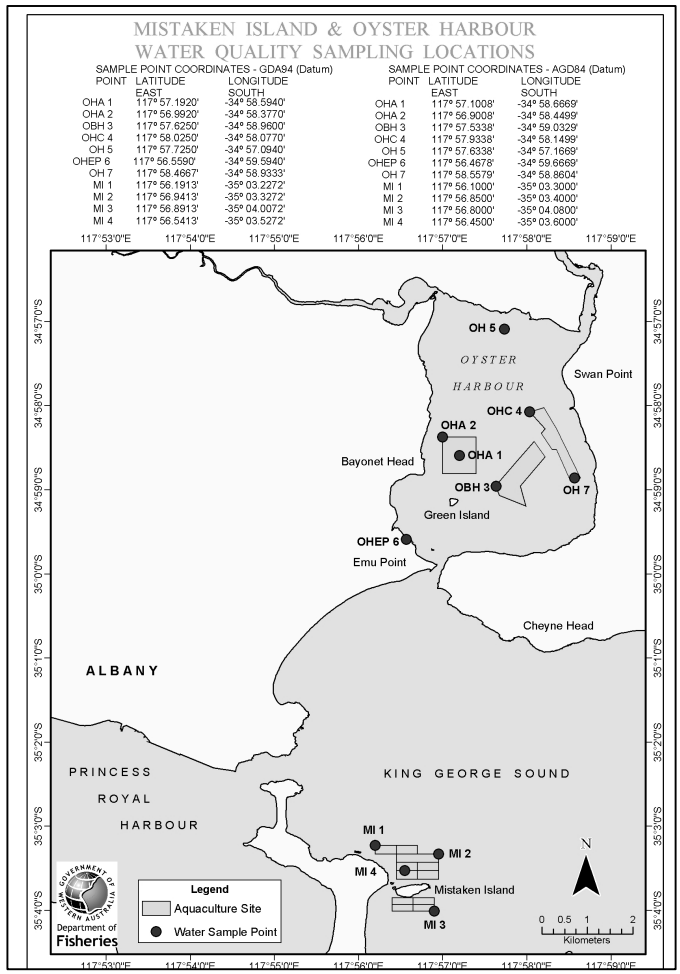

Figure 1. Lease areas and sampling sites for the Oyster harbour growing area.

In the period 1997 till 2003, the defined environmental conditions for closure of the Oyster harbour growing area related to rainfall triggers (20mm in two days or 40mm in one week) and a 25 % drop in salinity at sample point OH5 (see Figure 1). This management strategy was based on the assumption that the principal source of faecal contamination in the Oyster Harbour growing area was associated with increased flow from the King and Kalgan Rivers which drain large tracts of agricultural area and enter the Harbour at its Northern end.

Methods and Results

Analysis of Oyster Harbour water and shellfish data from 1997 to 2003 indicated that the management practices based on rainfall and salinity were not always accurate in predicting unsatisfactory water and shellfish bacteriological quality from the Oyster Harbour growing area. For example, sometimes levels of total coliforms and *E. coli*, above the threshold limits as outlined in the ASQAP Operations Manual occurred in the absence of rainfall and salinity triggers. As well, there were a number of cases

since 1997 when salinity and rainfall were outside the trigger thresholds, water samples had under threshold levels of *E. coli* and coliforms, and yet the corresponding mussel meat samples were over the threshold concentrations as defined by ASQAP. These unpredictable results resulted in extended closures and some recall of the Oyster Harbour product. It was therefore decided to look at alternative parameters to predict possible shellfish contamination.

The WA DoE routinely monitor both the King and Kalgan River some distance upstream from the river mouths. A large number of water quality parameters are measured at various times. Of these, the measurement of water temperature, total nitrogen, total phosphorus, salinity, turbidity, total suspended solids and dissolved oxygen were chosen for further study as these parameters were measured often enough to give a useful sample size. Stream flow rate is also continuously monitored, and rainfall both at Albany and Mt Barker, a town located in the catchment of the Kalgan River, some 40km from Albany, were also chosen as alternative parameters to predict contamination.

Relevant data from Oct 1997 till December 2002 was obtained from the DoE and the Bureau of Metereology. This data was compared with bacteriological data collected on the same day from water sampling point OH5, located within Oyster Harbour and yet adjacent to the entry point of the King and Kalgan Rivers, and traditionally the most contaminated of the sampling sites. Following simple calculation of the correlation coefficient this investigation showed that DoE flow rate data from the Kalgan River gauging station had a high correlation with the bacteriological results The high correlation noticed with the stream flow data was considerably better than that achieved using other water quality parameters, rainfall and/or salinity data (see Table i). More elaborate analyses indicated that a comparison of the current flow rate with a number representing three times the average flow rate data from the previous 21 days, could be used to predict the likelihood of unsatisfactory levels of faecal contamination in the Oyster Harbour water. Flow rates above this number were an indication that the harvest area was at risk. Basing the threshold level on the previous 21 days, was the most effective means to takes into account antecedent factors in the catchment area, and negates seasonal variation as well as the effects of low and high rainfall years.

Whilst the results indicated that flow rate data from the Kalgan River gauging station could be used to manage harvesting strategies in Oyster Harbour, in normal practice data from the gauging stations is stored on an 'in situ' datalogger, and downloaded every 6 months. To manage the shellfish growing area, it would be necessary for OFI personnel and staff from DOF and DOHWA to be able to electronically access the water flow results on a daily basis and using these results and a predictive model, make an informed decision on whether harvesting should be completed. Hence, to make the flow rate data available remotely and as needed, the DoE Hydrologic Technology Centre, with funding support from DOF, supplied a CDMA mobile telephone modem which was connected to the instruments at the site. An officer from the DoE Resource Information Branch in Perth established a system where the data from the site is downloaded every morning and then made available on a custom plot on the DoE River Flood Warning web page. In addition the plots are automatically emailed to WASQAP stakeholders on a daily basis. An example of a plot, incorporating flow rate and threshold line based on the predictive model is shown in Figure 2. When the flow rate is above the threshold line then harvesting is suspended pending results of bacteriological water analysis.

Table 1. Statistical Correlation (test) between water quality parameters from the King and Kalgan River gauging stations or rainfall data, and total coliform results from sampling point OH5 from 1996 to 2002. Numbers in brackets are numbers of matching data points.

	Total Coliforms at OH 5	
	No of paired samples	Correlation coefficient
Kalgan River		
Water Temp (°C)	16	-0.11
pH	16	0.13
N (tot) {TN, TN}(mg.L^{-1})	38	0.13
P (tot) {TP, pTP} (mg.L^{-1})	38	0.14
O-DO (in situ) (mg.L^{-1})	13	0.29
Turbidity (NTU)	18	0.00
TSS (mg.L^{-1})	38	0.00
Cond. Comp 25°C (in situ) (mS/cm)	7	-0.17
King River		
Water Temp. (°C)	18	-0.15
pH	18	0.23
N (tot) {TN, TN}(mg.L^{-1})	18	0.66
P (tot) {TP, pTP} (mg.L^{-1})	18	0.71
O-DO (in situ) (mg.L^{-1})	16	0.42
Turbidity (NTU)	18	0.30
TSS (mg.L^{-1})	0	Not done
Cond. Comp 25°C (in situ) (mS/cm)	7	-0.29
Rainfall Data		
Albany Rain (mm) for Previous Week	38	0.38
Albany Rain (mm) for previous 2 days	38	0.18
Mt Barker rain (mm) fro previous week		
Flow Rate Data		
Kalgan River Gauging Station	66	0.91
King River Gauging Station	66	0.24

The predictive model has been trialled in the period April 2003 till September 2004. Results of this trail are summarised in Table 2. Water and flesh sample results were analysed against the ASQAP limits for shellfish growing water (<14 *E.coli* cfu.100 ml^{-1} or 70 total coliform cfu.100 ml^{-1}) and shellfish flesh (<2.3 E. coli.g^{-1} or <100,000 aerobic plate count. ml^{-1}). The results demonstrate that 96 % of water samples taken when the flow rate was below the threshold limit fell within the ASQAP defined levels. On the single occasion when thresholds were exceeded exceptionally high tides prevented the normal flushing of the harbour. 100 % of flesh samples taken when the flow rate was below the threshold were within ASQAP limits. When the flow rate was above threshold levels, 54 % of water samples and 36 % of flesh samples showed unsatisfactory levels of faecal contamination.

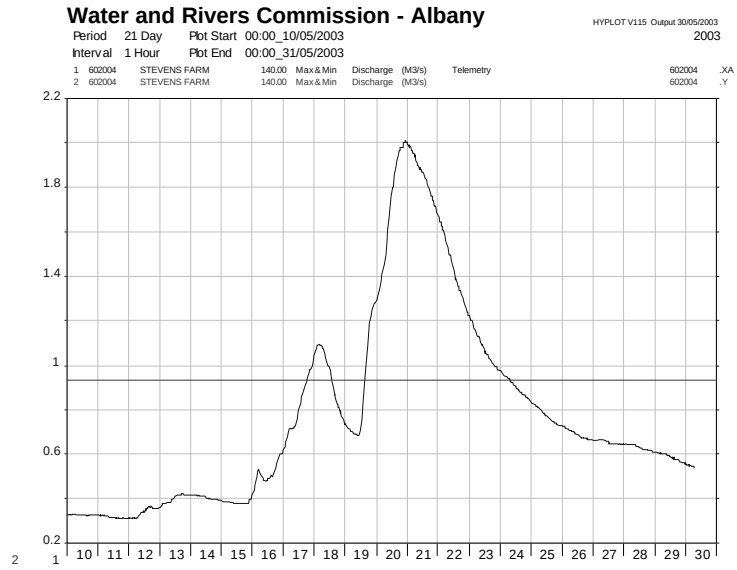

Figure 2. Example of telemetric plot showing stream flow rate and threshold levels.

Table 2. Results from Water and Flesh Samples Taken when flow rate was above and below predicted threshold levels.

Samples	Under/over flow rate threshold	No of Samples taken	% 0f samples with *E. coli*.(100 ml^{-1}) within ASQAP limits
Water samples	Under threshold	20	100 %
(E. coli must be <14 cfu.100 ml^{-1})	Over threshold	8	25 %
Flesh samples	Under threshold	20	100 %
(E. coli must be <2.3cfu.g^{-1})	Over threshold	8	50 %

Discussion and Conclusions

The Oyster Harbour rainfall and salinity predictive model had not always proved effective in safeguarding the bacteriological quality of harvested shellfish. Due to this uncertainty, the harvest area was occasionally subjected to long closures and product recalls were also necessary. Such results may have been due to a lack of correlation between salinity and the risk of contamination. In addition, measurement of salinity, apart form being inconvenient to the shellfish farmer, is subject to inaccuracies associated with efficiencies in calibration of the probe and variation in the salinity at a given point depending on the depth of measurement, particularly in a stratified water body. Also, the path of freshwater inflow through the estuary is influenced by winds and tides, and may not always cross over the sampling point.

The management of the harvesting area using real-time stream flow data and telemetry is efficient, convenient and transparent, with decision making data available to all stakeholders. Moreover, since the new model has been in operation, the closure periods have decreased and no unsatisfactory flesh results during open harvest periods, hence necessitating recalls, have occurred. The model also gives the shellfish farmer the opportunity to tailor his harvesting protocols based on the changes to the flow rate before threshold levels are reached.

However the model is still in early stages and preliminary results do indicate that the model will require adjustment to include factors such as tidal information. Such adjusting of the model, and particularly calculation of the threshold level, is likely after the results from the second winter period. At this period it is also hoped that further data analysis will allow accurate prediction of when harvesting can recommence following a fall in the flow rate. Under the current scheme, the harvest area will only be re-opened on satisfactory analytical results. Such continued modification of the model will be necessary to ensure industry profitability and public health is protected.

THE EVOLUTION OF SHELLFISH MONITORING ACTIVITIES IN A CANADIAN REGULATORY LABORATORY; AN OVERVIEW.

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Abstract
The history and evolution of the current monitoring activities for phycotoxins in bivalve shellfish by the Canadian Food Inspection Agency's (CFIA) Dartmouth Laboratory is described. The review begins with the introduction over 50 years ago of a PSP Monitoring Program, when the shellfish industry was limited to wild harvest. With the recent development of the shellfish culture industry, monitoring activities have increased as have the variety of toxins that have been identified in Canada and worldwide. Current interests are addressed including new methods, the availability of standards and reference materials. The laboratory's methods, many of which were developed in house, will be briefly described. Details of various toxic episodes and new toxin discoveries will be highlighted, as well as changes and challenges that were and will be faced by the laboratory. A brief description of the current monitoring system is presented including how outbreaks are handled from the laboratory standpoint and from the regulatory point of view. Some of the industry concerns will also be discussed.

Introduction
The Canadian Food Inspection Agency (CFIA) was created by the amalgamation of the food inspection activities of four government departments. The departments involved were the Department of Fisheries and Oceans (DFO), Health Canada (HC), Agriculture and Agri-Food Canada (AAFC), and Industry Canada (IC). The Dartmouth Laboratory of the CFIA has been identified as the centre of expertise for shellfish toxins, veterinary drug residues in aquaculture products, nutritional labelling, species identification of fish products, and elemental analysis. The laboratory is responsible for doing most of the analyses required in these fields as required by the agency. The exceptions to this are the analysis of domoic acid (DA) and paralytic shellfish poisoning (PSP). In these cases the testing activities are shared among 4 laboratories in order to deal with the workload and meet the service standards.

Paralytic Shellfish Poisoning
While the identity of the toxins was a mystery the presence of this class of toxins in Canadian waters has been known for hundreds of years. The first nation's peoples of Canada were aware that if they consumed certain shellfish from a particular area at a specific time of the year they would get ill and possibly die (Pralash, Medcof, and Tennant,1971).

Interest by government departments started in 1936 with an outbreak in Nova Scotia whereby two people died after eating red and blue mussels. However, it was the commercial harvesting and processing of shellfish to meet war time needs in 1943 precipitated the implementation of harvest controls to ensure safe products were produced. The first monitoring program was put in place to control the harvest of shellfish from the Bay of Fundy area of New Brunswick, Canada. The implementation of this monitoring scheme was met with resistance by the industry. It was felt that this was not necessary and it required further illness outbreaks in

1945 to emphasise the need for a complete monitoring program in this area (Pralash, Medcof, and Tennant,1971). Monitoring programs were expanded to include the St. Lawrence region in 1949 as a result of an outbreak in this area in the previous year with three people succumbing to the toxins effects.

Currently there is a nation wide comprehensive monitoring programme in place. Key sites are identified, and sampled periodically (weekly, biweekly or monthly) based on the toxin history of the site. The samples are subjected to toxins analysis utilizing the AOAC official method for PSP. The long established guideline of 80 ug/100 g is used to determine if areas are open or closed for harvest. Annually in Atlantic Canada there are in excess of 5000 analyses for the PSP toxins performed by two laboratories. Nationally, in excess of 10000 analyses on samples from nearly 500 sites are carried out on an annual basis.

This programme has been in place for nearly 60 years and there have not been any deaths related to PSP outbreaks in nearly 50 years. There is however periodic illnesses related to the consumption of PSP contaminated shellfish as recently as this year. These are usually due to the harvesting of shellfish from 'closed areas' and not as a result of the failure of the monitoring programme.

Domoic Acid

In 1987 a rash of illnesses occurred in the province of Quebec in people who had consumed cultured blue mussels grown in Prince Edward Island. The symptoms of the complainants varied from nausea, vomiting and diarrhea to muscle weakness, disorientation and memory loss which usually occurred 30 minutes to 6 hours after consumption. These symptoms were different from those experienced with the PSP toxins, particularly the lack of tingling and numbness. It was felt the agent responsible something other than PSP (Gilgan, Burns and Landry, 1990). This theory was furthered by the fact when that when samples were tested for PSP they showed a response that was atypical to that expected if the agent were PSP toxins. Research scientists, regulatory scientists as well as analysts worked around the clock in order to determine the causative compound which we know now as DA. Figure 1 shows the currently known analogues of DA (Quilliam, 2004).

Routine monitoring began in 1988 using the remaining extract from the PSP analysis. If no PSP analyses performed then a simple Water/Methanol extract was utilised. Currently in Eastern Canada there are in excess of 5000 DA analyses performed and nearly 10000 performed nationally. Since that time the Dartmouth laboratory has been involved directly in two episodes.

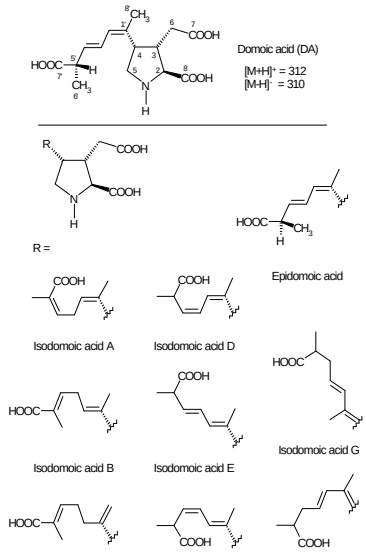

Figure 1. Domoic Acid analogues

In 1995 private laboratories testing shellfish products as part of various protocols were required to have results verified by government laboratories. In the process of auditing samples from this laboratory it was found that there was a DA outbreak occurring in scallops (*Placopectin magellenicus*) harvested from offshore banks. The outbreak was quite wide spread, with high levels of DA present in the digestive glands of the scallops. During the course of the outbreak the highest level of DA found in scallop digestive gland was 4700 ug/g. Table 1 shows the distribution of DA in the various parts of the animal.

Shortly after the discovery of the bloom offshore, results from samples taken from inshore harvest areas showed elevated levels of DA in the edible tissue. These test results resulted in a widespread closure of the areas for shellfish harvesting.

Table 1. The distribution of DA in the various tissues of a single scallop (Placopectin magellanicus)

Tissue sampled	DA concentration ug/g
Digestive Gland	3600
Gonad/Roe	43
Guts	26
Adductor Muscle	1
Digestive tract	140

The second and latest DA incident occurred in the spring of 2002. Processors were interested in harvesting product from culture sites through the ice. In order to begin processing monitoring first had to be in place. As a result of testing samples with high levels of DA were found. This was considered extremely unusual as the water temperatures were extremely cold and the common DA producing organisms in this area do not proliferate until the water temperature rise significantly. The outbreak extended from the Gaspe Peninsula to the Northumberland shore of Cape Breton Island in Nova Scotia. This event mainly affected blue mussels (*Mytilus edulis*). An interesting observation during the outbreak was that mussels and oysters (*Crassostrea virginica)* could be tested from the same vicinity but have drastically different toxin levels. At one site it was observed that mussels showed levels near 200 ug/g but the oysters in the same vicinity did not have measurable levels of the toxin (less than 0.5 ug/g). As part of the investigation of this episode the Canadian Department of Fisheries and Oceans studied the phytoplankton that was present at the contaminated areas. The causative organism of this episode was identified as *Pseudo-nitchia seriata* (Bates, 2003).

Diarrhetic Shellfish Poisons

Diarrhetic Shellfish Poisons first came to light in Canada in 1990 as a result of consumer illnesses. Thirteen people became ill after the consumption of blue mussels (Mytilus edulis). The harvest from the suspected area was stopped. Samples from that area were tested by the National Research Council, Institute of Marine Biosciences in Halifax, Nova Scotia, and were found to contain dinophysis toxin 1 (DTX-1) at a level of 0.6 ug/g. As a result our regulatory laboratory began work to develop a bioassay method for the detection of this class of compounds. Worldwide the phytoplankton responsible for these toxins is most often the *Dinophysis* spp. In Canada, the causative organism appears to be *Prorocentrum lima*. The following year another consumer illness event occurred in which 6 people were reported ill. Our laboratory's recently developed mouse bioassay method as well as an immunoassay test kit was used to determine that the DSP group of toxins was responsible. The results of these analyses were again confirmed by the NRC-IMB using LC-MS. While the two methods of analyses that were employed at this time showed correct results it pointed to the need for an actual analytical method. Work on a method was initiated based on the work of Lee *et al* (1987).

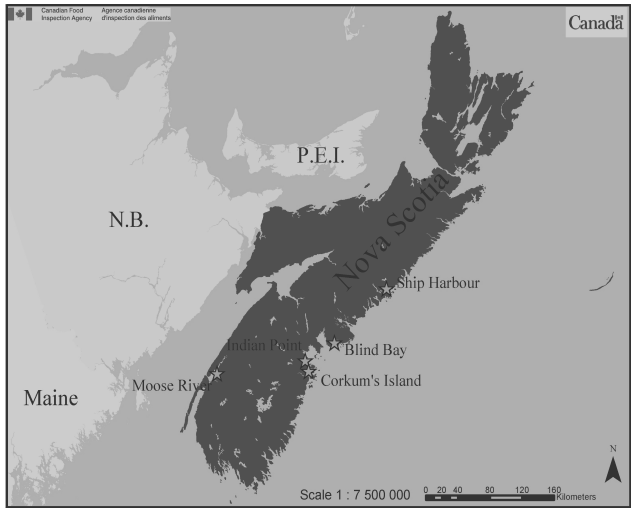

Figure 2. DSP testing sites

In 1992, as is the case now, the greatest limitation to the validation and implementation of an analytical method for shellfish toxins was the lack of adequate 'standards'. Dr. Tony deFritas of the NCR-IMB was able to supply us with a *Prorocentrum lima* phytoplankton extract that contained both OA and DTX-1. The material contained significantly high levels and could be used as a semi-quantitative stock standard which enabled our laboratory to conduct validation studies. At this time the bioassay method was still used as the routine detection method for the investigation of consumer complaints, and the NRC were still aiding us in the confirmation of positive results. The kit we were using unfortunately was designed specifically for OA, and there was a limited cross reactivity to DTX-1 the predominant toxin found in our area. The assay was also plagued with a number of false positive results. For a number of reasons this approach was abandoned.

The LC-FLD method was put in place as our routine testing procedure to monitor sites with a previous history of DSP toxin outbreaks in 1993. The period 1993-2001 settled in to a period of routine testing. These sites are shown in figure 2. Any samples resulting from consumer illness reports were also investigated for the presence of the toxins. The analytical procedure was published as a technical report (van de Riet, Burns, and Gilgan. 1995) and reviewed and accepted by Health Canada as the 'official' method for the determination of OA and DTX-1 in Canada. During this period our laboratory was involved in a two of DSP events that resulted in some expanded monitoring. In 1993, a number of people became ill after consuming mussels from the Alexander Bay area of Newfoundland. The samples tested showed levels of 4 ug DTX-1/g of digestive gland. Subsequent testing showed that the outbreak was widespread and resulted in the closure of the Bonivista Bay area shown on the map in figure 3. Another incident in 1998 in the Magdalene Islands region of Quebec resulted in the laboratory testing product associated with an illness complaint. In this case while there were only low levels of DTX-1 were present and DSP it did emphasise the need to monitor the area more closely. A routine monitoring program was established for this region.

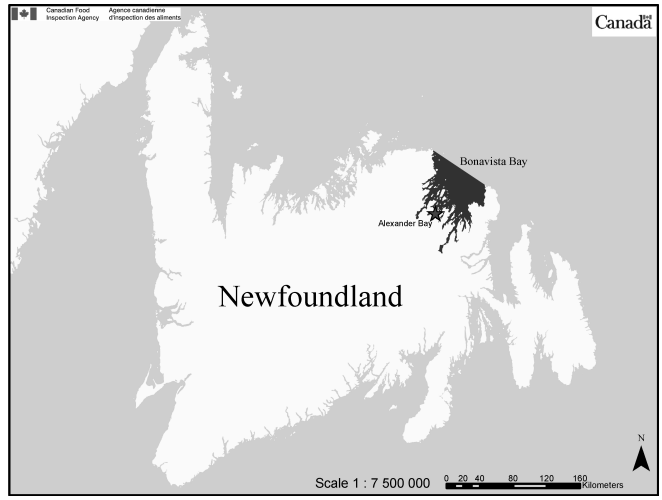

Figure 3. Bonivista Bay, NL, Canada

The future of shellfish toxin monitoring lies in the utilization of LC-Mass Spectrometry technology. In the spring of 2001, the Dartmouth laboratory secured sufficient funding for an LC-MS. After a period of familiarization, the LC-MS (Quilliam, Hess and dell Aversano, 2001) was used to look for the toxins we were currently monitoring with the LC-FLD procedure (OA and DTX-1). The results from the two instruments were compared (figure 4). The results from the two machines agree quite closely. The correlation coefficient in this case was 0.89, and the slope of the line 0.95.

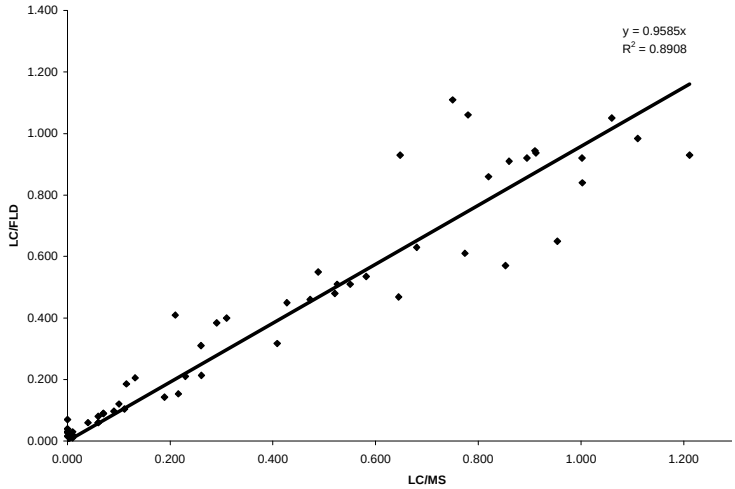

Figure 4. Correlation between LC-FLD and LC-MS analysis for DSP

In 2002 the LC-MS method by Quilliam, Hess and dell Aversano (2001) was accepted as the method of choice for routine testing. Initially monitoring was for OA and DTX-1, but after receiving a small amount of a PTX qualitative standard form the NRC-IMB it was found that we had levels of these compounds in samples from Ship Harbour, NS. These results were confirmed by M. Quilliam of the NRC-IMB.

One of the major problems with monitoring of the lipophilic shellfish toxins is the availability of standards, and standardization of methodology. At the International Molluscan Shellfish Safety Conference in Santiago, Spain in 2002, the Cawthron Institute, Nelson, New Zealand contacted laboratories that were interested in participating in an interlaboratory study on an LC-MS method for the analysis of the lipophilic shellfish toxins (Holland, and McNabb, 2003). This study provided quantitative standards, as well as sample extracts containing the entire suite of toxin of interest. Our participation in this study brought us up to date with the other laboratories that were doing similar toxin monitoring activities.

As a result of acquiring the standards and samples from the Cawthron Study, and through the generosity of M. Quilliam our laboratory has been able to expand our range of toxin monitoring to include the entire suite of DSP, PTX, and yessotoixns. As a result of this expanded capability we recently discovered the presence of Gymnodimine in Manilla Clams from Buckley Bay in British Columbia. Also, until recently YTX's have not been reported in Canadian shellfish, even though the causative organism is present. However, in the spring of this year while testing product from Newfoundland we found low levels of YTX (0.2 ug/g of digestive gland) in this material.

Tthe last conference the NRC-IMB has been at work to fill an obvious need in the shellfish toxin field that of certified analytical standards. Since the last conference they have produced numerous materials including reference materials for Pectenotoxins (PTX-2, PTX2-sa, PTX-11) Yessotoxins (YTX), cyclic imine toxins (GYM,SPX) as well as azaspriacids (AZA1, AZA123).

Conclusion

After 14 years of work in the DSP field our laboratory has made some significant contributions in the field of Shellfish toxins. These achievements include new toxin discoveries, new methodology implemented, and the addition of new standards from the NRC-IMB. We still face numerous challenges, uniformity of methodology, tolerances and guidelines and the implementation of a nationwide monitoring system.

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INDUSTRY INVOLVEMENT DRIVES INNOVATION

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Abstract
This paper will focus on industry involvement in developing and managing effective quality assurance management systems for the New Zealand industry's growing waters. Test method efficacy, regulatory enforcement and government "ownership" are the traditional foundations of shellfish safety programs. In New Zealand, where the industry is faced by unique challenges such as its distance from market, and 100 % cost recovery by Government a program widely regarded as one of the most innovative and robust in the world has emerged. New Zealand's program is characterised by industry driven pursuit of scientific advancement such as the development and adoption of chemical based test methods, a very high level of self management and an uncommon level of collaboration between regulatory authorities and industry. The net result is a program with premium efficacy and an exceptional level of voluntary compliance. From a standing start in 1993 when the first biotoxin event was experienced, a comprehensive program of monitoring and risk analysis based on phytoplankton counts, rainfall and salinity now underpins flesh testing. The industry has spearheaded the development and adoption of chemical based test methods to ensure a more robust and accurate program. Industry further imposes a series of voluntary protocols designed to minimise product quality risk. Collaboration is facilitated via The Technical Committee made up of Food Safety Authority and industry representatives, the Industry Biotoxin Committee which co-ordinates initiatives nationally and a series of individual Delivery Centres of which the Marlborough Shellfish Quality Program manages the waters of 80 % of New Zealand's growing waters.

Paper
Two of the greatest challenges facing aquaculture in the 21st century are access to good growing waters and water of the necessary quality. Ironically, an industry that is identified globally as the rising star of quality food production finds itself fighting for water quality and all too often at the bottom of a long list of competitors for water space. The solutions to both lie in science, in innovation and adaptation, in education and in modifying public opinion.

This paper discuss a variety of water quality management issues that are of relevance to all molluscan shellfish producers and illustrate for you some examples of our experience in New Zealand. Many of the challenges that we all face are outside our own direct control. We are all impacted by the activities of other resource users. This paper will explore some examples of where identifying common interests can lead to innovative win-win solutions. While we legitimately focus the majority of our efforts on science based innovations, this paper will also outline how industry is able to be a catalyst for a whole range of innovations

To understand shellfish food safety programmes in New Zealand it is essential to understand structures, the environment and relationships. There are a number of distinguishing features of the New Zealand industry:

- In 1993 when New Zealand experienced its first major biotoxin event there was no programme. Although it certainly didn't seem like a benefit at the time, this forced science, industry and regulators to collaborate to develop a programme – an invaluable legacy that endures. This collaborative approach is often not understood outside of New Zealand – and sometimes viewed with considerable suspicion. Collaboration between the parties does not imply compromising of standards or capture of the regulatory authorities by industry. Indeed the environment of mutual respect encourages extremely robust but constructive debate.
- The programme operates on a 100 % cost recovery basis. That is, industry meets the cost of all testing, regulatory services etc. As a result, industry has a deep sense of ownership and commitment to the programme and a very strong incentive to pursue innovation.
- Industry views its quality programmes as a key to its market competitiveness and hence there is a very high appreciation of the relationship between compliance and business success.
- The program is characterised by industry driven pursuit of scientific advancement such as the development, validation, and adoption of chemical based test methods, and a very high level of self management.
- The industry's approach to quality is very broad and includes such factors as public education.

The net result is a program with premium efficacy and an exceptional level of voluntary compliance.

Water Quality management Structure PP (see legend, appendix 1)
The structure is not simple, but its most important attribute is that it delivers a very high level of communication and information exchange between industry, regulators and science providers.

- The NZ Food Safety Authority, MAF Verification (MAF VA), and the District Health Boards are the regulatory authorities. *The NZFSA sets the policy and regulatory environment, maintains the relationships at government to government level, and holds ultimate responsibility for the efficacy and integrity of the biotoxin programmes. The programmes are audited by MAF Verification who maintain a North Island and South Island shellfish specialist.*
- 23 "Delivery Centres" *provide local management and apply levies to their members to pay for the programme.*
- The Industry Biotoxin Committee (IBC) o*perates nationally, representing the generic interests of the delivery centres, negotiating collective contracts and managing generic research.*
- The technical committee *comprises representatives of the NZFSA and industry and deals with matters such as amendments to the National Marine Biotoxin Management Plan.*
- Seafood Standards Council *is an official committee of the New Zealand Seafood Industry Council and is primarily concerned with the assurance of food safety for seafood produced in New Zealand.*

Most critically the delivery centres and Industry Biotoxin Committee takes an active leadership role in the development of superior protocols and test methodologies.

Biotoxins
From a standing start in 1993 when the first biotoxin event was experienced, a comprehensive program of monitoring and risk analysis based on phytoplankton counts, now underpins flesh testing. The industry has spearheaded the development and adoption of chemical based test methods to ensure a more robust and accurate program. Industry further imposes a series of voluntary protocols designed to minimise product quality risk and to manage commercial risk. It is important to understand that New Zealand's export markets are a long way away so product is often enroute to market before traditional mouse test results are available. You will appreciate that any product recall is therefore extremely expensive. The programme is based strongly around not just achieving regulatory compliance but also improving management practices. It is the latter as much as anything that has driven innovation.

Phytoplankton testing was introduced in 1994 to provide an early warning to any potential presence of biotoxin in the product. Results from phytoplankton testing were available in one day where as the mouse bioassays took up to a week meaning the potential for large product recalls was significant.

Biotoxin testing was predominately via mouse bioassay. However a high occurrence of false positive DSPs threatened the integrity of the programme and imposed significant additional costs – of special significance in a 100 % industry funded programme.

Industry strenuously pushed for the development and adoption of an alternative to the mouse bioassay. Liquid Chromatography Mass Spectrometry (LCMS) technology was well known internationally as a research tool but not as a routine testing option. Cawthron Institute researchers subsequently developed a methodology that detects the whole group ie DSP toxins
Although AZP has not been detected in NZ waters this new method can detect AZP. Negative reporting in any quality assurance programme is as important as positive reporting. Once validated and approved MSQP was the first to sign a contract to use the LCMS DSP method, and since adoption no false positives have occurred. Sensitivity is such that DSP toxins are quantified even at extremely low levels. A very significant benefit of this chemical approach is the timeliness of receiving results. The DSP results are available the day after arrival in the laboratory compared to 4 or 5 days using the mouse bioassay and have the further advantage of being readily scaleable during large-scale events. The ability to detect toxicity at very low levels is proving a useful predictive tool. An event at the end of May this year illustrates the value to industry. The LCMS results for one of our harvest areas identified increasing YTX toxicity. A week prior to the levels reaching regulatory limits industry implemented a self imposed closure. The result of this was no recall and absolute assurance of the safety of our product. Possibly the greatest benefit of all is the routine use of standards and quality controls.

Where will the future take us? The possibilities are endless – not just in the biotoxin detection field but for all parameters implicated in water quality management. As an industry it is our responsibility to clearly articulate our needs to the scientific community – and to not limit our thinking but to be very ambitious.

Bactos
While algal blooms are a natural occurrence, bacteria of concern to food safety are a result of various forms of pollution. Food safety management relies upon after the fact detection. Experience in manufacturing quality assurance clearly indicates that this approach – what we would have called "quality control" is the least reliable form of quality assurance. The far more reliable – and cost effective approach is "total quality management" where resources are directed at preventing the production of defective product. There is a very important lesson here for us. Protecting the quality of growing waters is the ultimate quality assurance procedure.

As is common in many countries, the growing waters in NZ are at the intersection – some might say collision point - between competing resource users. In our case, aquaculture, forestry, commercial fishing, agriculture, tourism and recreational users and municipalities. Aquaculture, probably more than any other primary production industry is impacted by the activities of a broad range of other users, some direct resource competitors, others indirect.

Man made hazards within our control
Man made threats are frequently insidious. The threats come in many forms- municipal sewage, septic tanks, sewage discharges from boats, effluent and fertiliser run off from farms and other land, and industrial discharges. And the sources of contamination can be hundreds of miles from the growing waters. To ensure the long term viability of our industries – and public food safety, it is suggested that we need to be more assertive in advocating water quality. We spend too much time on the back foot defending the benign nature of molluscan culture and not enough time on the front foot demanding, coercing and advocating better water quality.

This is not something that we can achieve alone. Our communities and fellow industries have to be part of the solution, and ultimately it is the ethics and behaviour of individual members of our communities that will determine our water quality. Here are some of our initiatives aimed at eliminating contamination of growing waters.

- Queen Charlotte College Aquaculture Academy – initially this project was set up to provide an opportunity for students to be aware of the variety of career opportunities available within the aquaculture industry, as well as to put something back into the community in which we farm. The benefits have been beyond our wildest expectations. The Academy is funded by five marine farmers who dedicate the profits from one mussel line each to the Academy. These funds have been used to construct a purpose built vessel to provide easy access to the water for the students. We have negotiated water space with the local regional council and the students undertake research as part of their curriculum, and for industry.

  The benefits are many. Apart from the obvious of providing real life connection between their studies and industry, the students also emerge with dive and skippers qualifications and also some industry specific qualifications.

  - A new generation of informed decision makers will emerge

  - The students obtain a valuable insight into the complexity and fragility of the marine environment
  - One senior Government Minister, who was previously ambivalent towards industry now describes aquaculture as one of New Zealand's great hopes for the future – specific as a result of his contact with the Academy

- Marlborough Sounds Advisory Group is an Advisory Committee of our Regional Council made up of Marlborough Sounds (where 80 % of NZ's aquaculture is located). Initially industry was viewed with great suspicion and at the time of joining, discussions were dominated by how the Committee should prepare submissions to object to marine farm resource consent applications. As a result an invitation was extended to join the Environment Subcommittee, one of whose goals is the elimination of all septic tank discharges into the waters. In an initiative to improve water quality the Council and Committee produced and distributed brochures to all Sounds residents advising on the proper maintenance of septic tanks. These initiatives are of immense value to industry.
- Dairy farmers fencing water ways. In a joint effort, regional councils and Fonterra (Formally the NZ Dairy Board and the largest dairy exporter in the world) have initiated programmes to assist farmers to avoid contaminating waterways. Each is driven by a different motivation, but again the efforts are of immense benefit to our industry. We therefore endorse these activities at every opportunity in the form of targeted magazine articles.
- Waterway margin restoration projects have sprung up around the country. The margins are natural filters between land and water – effectively reducing agricultural chemical and bacterial runoff into the waterways while simultaneously improving biohabitat and diversity. Again we take every opportunity to commend and encourage these initiatives.
- NZ Mussel Industry Council (NZMIC) Environmental Code of Practice (ECOP). Naturally industry must lead by example and the NZMIC ECOP is a comprehensive programme to measure, manage and mitigate environmental impact at all stages for production and processing. As an industry organisation we apply the COP to the water quality programme. For example, our vessel contracts stipulate that the contractor must comply with the relevant sections of the code.
- Beach maintenance – more walking the talk. Few things have more immediate negative impact than farming debris washing up on beaches. Industry employs two tactics to mitigate this
  - Adopt- a-beach where industry members take responsibility for maintenance of selected beaches. This has been very successful with other organisations from outside of industry, including the Department of Conservation, joining the programme.
  - Environmental mentor where industry has employed a well respected retired skipper to undertake unannounced audits on vessels, checking compliance with the Code of Practice and to advise on mitigation measures.
- Vessel inspections and education.
  - Industry was the catalyst for one Regional Council in the Far North adopting stringent measures to mitigate marine pollution. All vessels (including recreational) that enter the harbour are boarded and required to sign an undertaking not to discharge while in the harbour

and to have the toilet sealed. If they will not sign the document, they are required to leave harbour.

- In the Marlborough Sounds, before the Summer Season, in a joint initiative with the District Health Board, Regional Council, and Industry, an educational campaign will be mounted, targeting recreational users, explaining their responsibilities in relation to the marine pollution regulations. The local Port Company has undertaken to distribute the brochures to all berth holders, and the local newspaper is collaborating with the publication of informative articles.

Summary

Since the Romans developed public health infrastructure civilisations have been discharging effluent to the ocean.

We are attempting to change behaviours that are millennia old. We must constantly pursue opportunities and innovations both in the lab, in our management practices and how our communities impact on the growing waters. The innovations that will ensure our survival almost certainly already exist in other applications. Research shows that cross over between industries almost always happens by chance. But to quote Louis Pasteur

"Chance favours only the prepared mind"

We do however need – industry, science, regulators and resource managers - to be much more systematic and collaborative in that pursuit. Ultimately it will be our ability to respond to the changes taking place around us that will ensure our survival, for as Charles Darwin wrote in "The Origin of Species"

"It is not the strongest of the species that survive, nor the most intelligent, but the ones most responsive to change"

Appendix 1
Legend – Water Quality management Structure PP

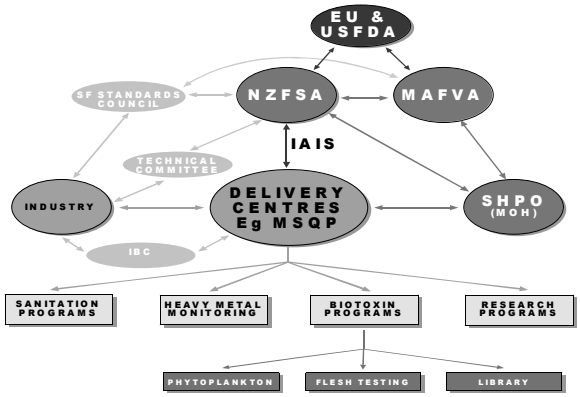

Diagram title	Official title	Description
EU and USFDA	European Union and United States Food and Drug Association	
SF Standards Council	Seafoods Standards Council	Official committee of the New Zealand Seafood Industry Council and is primarily concerned with the assurance of food safety for seafood produced in NZ.
NZFSA	New Zealand Food Safety Authority	New Zealand regulatory authority
MAFVA	Ministry of Agriculture and Food Verification Authority	Government verification authority
IAIS	Industry Agreed Implementation Standards	New Zealand shellfish standards
SHPO (MOH)	Senior Health Protection Officer (Ministry of Health)	Regulatory authority
IBC	Industry Biotoxin Committee	Represents the generic interests of the delivery centres, negotiating collective contracts and managing generic research