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Acidic Marine Biotoxins Implicated in Diarrhetic Shellfish Poisoning

Eoin P. Carmody

Department of Chemistry, Cork Institute of Technology, Cork, Ireland.

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Acidic Marine Biotoxins
Implicated in
Diarrhetic Shellfish Poisoning

Eoin P. Carmody
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Implicated in
Diarrhetic Shellfish Poisoning

Eoin P. Carmody B.Sc.

Department of Chemistry
Cork Regional Technical College
Cork, Ireland

Supervised by Dr. Kevin J. James

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for the Degree
of
Doctor of Philosophy

May 1996
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Eoin P. Carmody

ABSTRACT

An okadaic acid (OA) etiology has been demonstrated for most outbreaks of diarrhetic shellfish poisoning (DSP) in Europe. An isomer of OA, dinophysistoxin-2 (DTX-2), has recently been found in Irish waters and, in 1991 and 1994, was the major toxin during prolonged infestations in cultivated mussels (*Mytilus edulis*) along the south-west coast of Ireland.

The OA class of DSP toxins have been analysed by HPLC using modified versions of three established fluorescent derivatisation methods. A 'dual tag' method for the confirmation of specific toxins has also been developed.

Survey work has been carried out over the past four years on several mussel culturing areas in the south-west of Ireland. Results of seasonal, geographical, as well as horizontal and vertical variations in shellfish toxicity are reported. Evidence that there is a correlation between changes in temperature and subsequent appearance of high cell counts of *Dinophysis acuta* in the surface water column exists. The monitoring of oceanographic profiles may be useful in predicting future toxic events.

Two commercially-available ELISA methods, designed to determine OA were examined for potential use in the analysis of samples containing both OA and DTX-2. Rapid DSP-screening of shellfish in Ireland and other European countries may thus be possible using ELISA.

International collaborative studies have been undertaken on new DSP toxins, DTX-2 and DTX-2B, which have been isolated in our laboratory. The cytotoxic and protein phosphatase activity of DTX-2 is presented. Mass spectral data for these toxins using LC-ISP-MS, FIA-MS and negative-ion FAB MS/MS techniques is reported. Preliminary findings using ozonolysis for the detoxification of contaminated shellfish is also presented.

The synthesis of a new fluorogenic reagent for the analysis of carboxylic acids by HPLC is described. The reagent was found to react readily with saturated fatty acids in aqueous solution, under mild reaction conditions.
ACKNOWLEDGEMENTS

I wish to express my thanks and appreciation to the following people. Firstly, to my supervisor, Dr. Kevin James, for his advice and encouragement during the course of this project. Without his foresight and knowledge none of this research would have been possible. Also, a special thank you to the other members of the team, Sean, Ian, Ambrose, Alan and Marian.

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Dr. Hideo Naoki, Suntory Institute for Bio-organic Research, Osaka, Japan.
Prof. Y. T. Yashomoto, Suntory Institute for Bio-organic Research, Osaka, Japan.
Bantry Bay Mussels Ltd.

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Finally, I'd like to thank my parents and brother and sisters, Deirdre, Niamh and Ruaidhri, for their constant life long support.
To Mam and Dad

And in Memory of Granny and Granda
For all at last return to the sea- to Oceanus, the ocean river, like the ever-flowing stream of time, the beginning and the end.

Rachel Carson (1907-1964)
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Introduction:
Toxic Marine Algae and their effect on marine aquaculture

*One cannot explore new countries in express trains*

August Kekulé (1829-1896)
Dept. warns on shellfish

THE Department of the Marine warned yesterday that waters off a stretch of the West Cork coast were toxic as a result of seasonal algal bloom. The Department issued the warning about the Roaringwater Bay area following results of water samples. Results of other samples from Oysterhaven, Co. Cork, are currently awaited.

The SHB warned the public against eating mussels, oysters, clams and similar bi-valve shellfish from the Roaringwater Bay area. Consumption of shellfish from the area may result in diarrhoeic shellfish poisoning.

The Health Board will inform the public when the danger has passed and it was safe again to consume these shellfish. The high toxicity levels — scoring two on a scale of zero to four in the West Cork water samples — are a result of the microscopic algae, Dinophysis, which is common along the southwest coast during the summer.

The Department of the Marine has operated monitoring stations along the coastline for the last 10 years as a result of this. Roaringwater Bay is the first reported case of such algal bloom to occur in Irish waters this year. It has occurred earlier than usual, as it normally occurs between June and the end of August.

Red tide scare

A MOST unusual outbreak of 'red tide' has occurred in the sea off Kenmare, Co. Kerry, resulting in a public warning from the Southern Health Board about eating shellfish caught in the area.

It was thought that the 'sain' — a natural bloom of microscopic algae in sea water — occurred only in summer or autumn temperatures but it appeared in the last week of January in Killmackillogue Harbour.

However, the Board yesterday disclosed that the most recent scientific tests had shown that the area was again free of this 'sain'. Recent storms and high tides are believed to have stirred the virus from the seabed.

Urgent plea for research

Report: EDDIE CASSIDY

A CALL was made yesterday for the Department of Marine to establish a fisheries research centre in West Cork, as a Bantry-based shellfish company officially launched a new £190,000 vessel.

SOUTHERN HEALTH BOARD

MUSSELS, OYSTERS, CLAMS AND SIMILAR BI-VALVE SHELLFISH

Further to previous notices, toxicity has returned to bi-valve shellfish in:

ROSSCARBERY BAY AREA

from which the public are advised, in the interest of public health, to cease collecting bi-valve shellfish for human consumption.

Toxicity tests are continuing and the public shell be kept informed.

Signed: MEDICAL OFFICE FOR HEALTH
Community Care Office, Skibbereen, Co. Cork.

SOUTHERN HEALTH BOARD

MUSSELS, OYSTERS, CLAMS AND SIMILAR BI-VALVE SHELLFISH

The public are advised in the interest of public health to cease the practice of collecting bi-valve shellfish for human consumption from:

ROARINGWATER BAY AREA

Toxicity tests are ongoing and the public will be kept informed.

Signed: Area Medical Officer for Health, West Cork Community Care Office, Skibbereen.
1.1 NATURAL HISTORY OF *Mytilus edulis*

Almost all of the commercial production of cultured mussels in the world consists of the common edible mussel *Mytilus edulis* and the Mediterranean *Mytilus galloprovincialis*. *Mytilus edulis* reaches sexual maturity in the first year and spawns with the rising temperature in the spring and summer. Eggs and sperm are shed separately and fertilisation takes place in open water. The free swimming larvae remain plankton for between ten to fifteen days depending on food supply, temperature and settling sites. Young mussels are very sensitive to light and require a high light intensity in the region where they settle.

Generally, spawning peaks in mid-summer with larvae being found from early spring to late autumn. However, in certain parts of Europe, spawning occurs throughout the year.

1.1.1 Generalised life cycle of the common mussel

Mussels normally occur in large banks or shoals but the reason for this is not fully understood. The life cycle of the mussel is illustrated in Fig. 1.01. The mussels attach to the substratum by means of *hyssus* which they secrete. They normally prefer to attach themselves to fibrous materials but they have been found to settle on other mussels, and shellfish, as well as rocks, stones and seaweeds. The mussel may discard its byssus and can either crawl about, or secrete a bubble of gas, and drift with the current or tide in order to find a more suitable environment. This ability to move about is an important factor in mussel cultivation. Care must be taken in transplanting mussels so as to duplicate the conditions under which they were formerly living with respect to light intensity, temperature, salinity etc. or there is a danger of their detachment and migration.

1.2 THE HISTORY OF MUSSEL CULTURE

France is the only country with a long history of mussel culture. The culture of mussels in France began with the shipwrecking of an Irish sailor named Patrick Walton at the Point of Escale, near the port of Esnodes, in the autumn of 1235. Faced with the problem of survival, Walton first fashioned a small wooden punt-like boat with which he could move about the soft tidal flats. He then designed and built a large net suspended on the flats by poles driven into the mud with which he hoped to catch land and sea birds.
Walton soon discovered that the supporting poles quickly became encrusted with mussels and that the shellfish on the poles grew much faster than those living on the bottom. Further experimentation and exploitation of this discovery by Walton eventually led to the so-called “bouchet system” of mussel culture which is still in practice today with only minor modifications.

**Fig. 1.01**  
*Generalised life cycle of the common mussel*  
*(Courtesy of BIM “Aquaculture Explained” booklet No.2)*
1.2.1 History of mussel cultivation in Ireland

1.2.1.1 Bottom culture method

The principle of bottom cultured mussels involves locating the mussel beds, fishing the beds of seed mussels and moving them, by dredger, to sheltered inshore waters. Here, the mussels are put on plots, where they are protected from winter storms and grow rapidly, due to increased nutrients. After eighteen months to three years, the mussels are ready for harvesting.

The main source of seed still remains the Irish Sea coasts of Wicklow and Wexford, where beds of mussels settle in shallow coastal waters. Some of the best seed locations are in the lee of sand banks which run parallel to the coast. Transplanting gets underway during the summer, following the location of the seed beds with the assistance of underwater cameras. In Wexford, boats take seed by sea to their growing beds while in other locations the seed is transported by road, which can be quite expensive.

Total bottom mussel production over the past few years has averaged 10,000 tonnes. The bulk of bottom mussels went for processing in the past, but in the future more will be exported in an unprocessed form. The ‘Lett group’ Ltd., in Wexford was for many years, the largest mussel processor in Europe, but has now commenced depuration needed for the sale of fresh mussels. The greatest tonnage of mussels is produced by the bottom culture method. This is practised mainly in two areas of Ireland, Wexford harbour and Castle-Maine harbour, Cromane, Co. Kerry. Bottom mussels are also grown in Waterford harbour, Youghal harbour and Lough Swilly.

1.2.1.2 Suspended culture method

All the survey and analytical work conducted in the current work was carried out on mussels from the West Cork and Kerry regions, where suspended mussel culture is practised. With the introduction of raft culture, rope mussel growing commenced in Ireland some seventeen years ago. Rope mussels sell at an average price of £500 per tonne, which is more than double the price of bottom mussels. This is due to a number of factors including the fact that the shell is cleaner, meat content is good, there is a lack of grit and the product is evenly sized.
This method of production originated in the north of Spain. The original centre of Spanish mussel culture was on the Mediterranean coast near Barcelona, where the techniques used were the same as those practised in France. In 1946, the first attempt was made to grow mussels in the Galician Bays on the Atlantic coast of north-west Spain. The Galician bays are sunken valleys 7 to 11 km long, 2 to 5 km wide and as much as 60 m deep in places. As a result, a new technique was required for mussel cultivation. The solution was based on the Japanese raft method which had long been used for oyster cultivation in the embayments of Japan. Suspended mussel culture employs a method by which long ropes are lowered into the sea, supported by rafts or long-line systems in order to catch the drifting mussel spat and to support the growing mussel.

1.2.1.2.1 Rafts Rafts vary in design depending on the resources available locally. They are usually based around a Catamaran design as shown in Fig 1.02(a). They use two floatation hulls, between which are strung cross beams bearing the mussel ropes which hang down into the water. The mussel-rafts also act as working platforms for handling the mussels.

1.2.1.2.2 Long-lines This method is now preferred to rafts as it is less expensive. Oil drums or specially designed flotation barrels are used to support a stout double headed-rope from which the mussel ropes are suspended. A long-line system is shown in Fig 1.02(b). A typical 85m long double head-rope, supporting 340 x 7m long, mussel ropes, each pegged at 50cm intervals, can yield up to 20 tonnes of mussels.
Seed mussels are collected locally from rocks or planktonic settlements and are later grown to full size on ordinary nylon ropes which have been pegged at intervals of 50cm. The pegs increase the surface area of the rope for spat collection and prevent the bunches of growing mussels from sliding down the
rope and falling off. As the mussels grow, they begin to overcrowd the rope and must be *thinned off* in order to allow those remaining to grow to a reasonable size. These *thinnings* can then be placed in a nylon mesh tube, known as a mussel-stockling, which binds the seed mussels together and yet allows them to push their way out through the mesh as they grow.

On the larger mussel farms, some of the hard manual work has been replaced by machinery such as hydraulic cranes, which are used to lift the mussel ropes aboard the harvesting vessels. The mussels are then stripped from ropes / mesh, washed, cleaned and sorted by automatic grading machines.

Most suspended culture production now comes from long-lines located in Bantry, Kenmare, Roaringwater and Clew Bay. Suspended culture also takes place in Killary, Bellacreagher, Dunmanus and at locations in Donegal.

The majority of mussels cultured in Ireland are exported for the French market. Ireland produces 2% of the world market of cultured mussels (Fig. 1.03).

---

**World Production of Cultured Mussels**

- **UK** 2%
- **Spain** 39%
- **USA** 5%
- **Denmark** 15%
- **Ireland** 2%
- **Netherlands** 22%
- **France** 11%
- **Germany** 4%

Fig. 1.03 *Pie-Chart representing the world production of cultured mussels*  
(Data courtesy of BIM)
Bantry Bay Mussels Ltd is a company involved in the farming and processing of rope cultured mussels which are grown in Bantry Bay and adjoining bays. The company is owned by a group of local Bantry people. In its first year of production it processed and sold 950 tons of mussels. To date, this has grown to in excess of 3000 tons per annum, giving employment to forty people. The company has been approved for Grant Aid under an IDA / EGAF Project. An expansion programme will allow this company to process in excess of 20 tons of mussels per day. It uses a patented production process where by mussels are processed, vacuum packed and frozen with all the natural juices retained inside the shell. The mussel juices act as a natural glaze to the meats, so that when heated the frozen mussels taste exactly the same as if cooked from fresh. This has the advantage of enabling the company to supply their customers with a quality product throughout the year.

Cork RTC has recently collaborated in the setting up and validating of a fully equipped on site chromatography laboratory for the chemical analysis of diarrhetic shellfish poisoning (DSP) toxins.

There are two periods in the year when mussels are not of proper quality for processing. In the March-April period, the mussels spawn and cannot be harvested during this time. In July-October the mussels may contain DSP toxins which makes them unsuitable for human consumption during this period. Bantry Bay Mussels have their own cold-storage facilities in Bantry for between 800 and 1000 tons of product. This means that they can have supplies of mussels, which have been harvested in peak condition to supply their customers even when they are not processing.
Approximately 97% of its total production is for the export market. Its main markets are France, Italy, Portugal, Switzerland, Cyprus, Germany, Norway, Sweden, UK and Malta, with some of the products being re-exported from France to places like Austria, Lebanon and North Africa (1).
1.3 TOXIC PHYTOPLANKTON BLOOMS

Toxic phytoplankton blooms consist of millions of single-celled plants that are carried around with the ocean currents and often invisible to the naked eye. These plant cells depend on supplies of sunlight and nutrients for their existence. Their distribution is usually restricted to the surface few tens of metres of the seas. Below these depths the environment is too dark and these tiny plants represent the start of the marine food chain. It is no coincidence that established sea fishery areas are regions where these plants thrive. It is the ecology of phytoplankton that is responsible for the diversity in productivity of different marine regions. Phytoplankton are so named as they are primarily plants (*phyto*) and because they drift around in the sea, having no independent means of movement (*planktos* = made to wander). Some phytoplankton have small flagella, but even these are powerless to swim against the ocean currents. They form part of the major grouping of plants known as the *algae*, which range in size from single-celled organisms up to the highly organised seaweeds which may achieve lengths of 30 metres.

The following table shows some of the common names, classes and genera of the more familiar phytoplankton groups (Table: 1.01).

**Table: 1.01**

<table>
<thead>
<tr>
<th>Common name</th>
<th>Class</th>
<th>Common genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue-green algae</td>
<td>Cyanobacteria</td>
<td>Oscillatoria</td>
</tr>
<tr>
<td>Red algae</td>
<td>Rhodophyceae</td>
<td>Rhodella</td>
</tr>
<tr>
<td>Cryptomonads</td>
<td>Cryptophyceae</td>
<td>Cryptomonas</td>
</tr>
<tr>
<td>Chrysomonads</td>
<td>Chrysophyceae</td>
<td>Aureococcus</td>
</tr>
<tr>
<td>Silicoflagellates</td>
<td>Bacillariophyceae</td>
<td>Dictyocha (= Distephanus)</td>
</tr>
<tr>
<td>Diatoms</td>
<td>Raphidophyceae</td>
<td>Chatoeceros, Rhizosolenia, Leptocylindrus, Nitzschia, Thalassiostra</td>
</tr>
<tr>
<td>Chloromonads</td>
<td>Xanthophyceae</td>
<td>Heterosigma</td>
</tr>
<tr>
<td>Yellow-green algae</td>
<td>Eustigmatophyce</td>
<td>(very rare)</td>
</tr>
<tr>
<td>Coccolithophorids</td>
<td>Prymnesiophyce</td>
<td>Emiliania</td>
</tr>
<tr>
<td>Prymnesiomonads</td>
<td></td>
<td>Isochrysis</td>
</tr>
<tr>
<td>Euglenoids</td>
<td>Euglenophyceae</td>
<td>Eutreptiella</td>
</tr>
<tr>
<td>Parasideomonads</td>
<td>Prasinophyceae</td>
<td>Tetraselmis, Micromonas</td>
</tr>
<tr>
<td>Green Algae</td>
<td>Chlorophyceae</td>
<td>Rare in the marine environment</td>
</tr>
<tr>
<td>Dinoflagellates</td>
<td>Pyrrophyceae</td>
<td>Ceratium, Gonyaulax</td>
</tr>
<tr>
<td></td>
<td>Dinophyceae</td>
<td>Dinophysis, Protoperidinium, Prorocentrum</td>
</tr>
</tbody>
</table>
Of the groups listed, the commonest forms encountered in coastal marine samples are diatoms (*Bacillariophyceae*) and dinoflagellates (*Dinophyceae*).

A single litre of water taken from the sea surface may contain thousands of phytoplankton cells. These organisms have an immense diversity with more than 10,000 species, ranging from the primitive blue-green algae (recently renamed the Cyanobacteria) to the more complex diatoms, dinoflagellates and green flagellates. However, the one thing that most have in common, is the production of chlorophyll, for photosynthesis.

Phytoplankton are of critical importance to the aquaculture industry. They provide the food supply for shellfish, which extract them from the water by filtering them through their gill-like apparatus. The viability of commercial bivalve production operations ultimately depends on a rich supply of phytoplankton.

1.3.1 Blooms

The term given to a proliferation of phytoplankton cells is ‘bloom’. Thus the terms ‘phytoplankton bloom’ or ‘algal bloom’ are quite common. Most blooms are natural events and quite harmless, even beneficial to the marine ecosystem. Some blooms are so intense that they discolour the water. Very occasionally, blooms can contain harmful or toxic species. On other occasions, the bloom itself can cause detrimental changes in water quality and these are generally referred to as harmful blooms. Before discussing the species responsible for harmful blooms, it is useful to appreciate the type of environment or even time of year when blooms, either harmful or beneficial can be expected. A few hundred species can from time to time occur in exceptionally high concentrations. Cell counts of several million cells per litre can result in an obvious discolouration of the sea surface. This discolouration is typically brown, red-brown or red-orange, and hence this phenomenon is commonly referred to as a ‘Red Tide’.

A few species (approximately 40 have been described to date ) have the capacity to produce potent toxins that can find their way through fish and shellfish to humans. To have this effect, cell concentrations of certain species need only be of the order of a few hundred cells per litre. Such a situation is called a ‘toxic bloom’ or ‘toxic algal bloom’. The term ‘toxic bloom’ has often been confused with the term ‘red tide’. An important distinction is that a red tide refers to a situation where phytoplankton cells have achieved
such high concentrations that the sea-water surface is physically discoloured, but this need not be the case with a toxic bloom.

1.3.1.1 A brief history of red tides

Red tides and toxic blooms can be collectively referred to as harmful algal blooms. They have been with us for a long time and it is generally considered that the earliest references to a harmful bloom appears in the Old Testament in the book of Exodus. One of the plagues resulted in river waters turning red, becoming undrinkable and the fish in the river died. An explanation for this is that bloom-forming algae became so concentrated that indiscriminate kills of both fish and invertebrates resulted from anoxic conditions. Despite being plants and producing oxygen through photosynthesis, high respiration rates or dense populations of algae can strip the water of oxygen either at night or in low light levels. Alternatively, once the bloom has finished, decay by bacteria may produce anoxic conditions.

Ancient sailors often reported sailing through blood-red coloured seas during certain seasons. It was also suggested that the Red Sea acquired its name from such a report. An early case of a toxic bloom affecting humans occurred in 1793 when Captain George Vancouver and his crew landed in British Columbia, at a site now known as Poison Cove. Fatalities occurred after the consumption of shellfish presumably contaminated with dinoflagellate toxins. George Vancouver noted that it was taboo for the local Indian tribes to eat shellfish when the sea became phosphorescent due to dinoflagellates.

In this century the instance of harmful blooms has apparently been on the increase. In 1933, cultured pearl oysters worth about £2 million were killed in Japan. In 1972, millions of pounds worth of caged fish were lost through toxic phytoplankton in the Japanese Inland Sea. Similar levels have occurred around the European coast, notably in Norway and Denmark. Toxins can be accumulated by the animals which feed on them and transmitted through the food chain. The death of 14 humpback whales in Canada in 1987 was attributed to food-chain contamination; they had been eating mackerel which contained algal toxins. The toxins can affect human health and the resulting economic implications can be very serious for producers. Toxicity can manifest itself even when the toxin-producing species are present in low numbers, as few as 100 to 200 cells per litre of water.
The earliest scientific reference to a bloom in Irish waters was an observation at the end of the 19th century by a former Inspector of Fisheries, W. S. Green, who realised that brown slime on mackerel nets was caused by an excess of planktonic algae. In 1978 and 1979 red tides of *Gyrodinium aureolum* occurred on the south-west Irish coast and lead to mass mortalities of caged fish. This resulted in a prohibition of finfish farming development in the area for ten years.\(^5\)

It is uncertain whether we are experiencing an actual increase in the occurrence of harmful blooms or if the increased frequency of reports is due to a greater degree of monitoring, resulting from an expanding exploitation of the coastal zone through aquaculture. Recent evidence from Japan has, however, suggested a relationship between frequency of red tides and pollution levels.\(^6\)

From what is currently known of harmful blooms, they can be characterised into three different groups.

1.3.1.2 **Red tides of otherwise harmless algae**

Dense blooms of algae cause water quality problems and, in particular, the depletion of oxygen levels. This can be due to the high respiration of the plant cells at night or in dim diffuse light, but is more commonly associated with bacterial respiration during the decay of the bloom. Apart from general problems resulting from this process (asphyxia), fish stock will not only be stressed but, as a result of filtering more water through the gills to gain their normal ration of oxygen, the algae may clog the gills intensifying the problem.

1.3.1.3 **Blooms of organisms non-toxic to humans but harmful to aquatic organisms**

Some algae species can seriously damage fish gills, either mechanically or through the production of haemolytic substances. Caged fish do not have the freedom to swim away from affected areas; and they appear to be extremely vulnerable to such noxious blooms.

1.3.1.4 **Blooms of organisms producing toxins poisonous to humans**

These are populations of species which produce potent toxins that find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses.
1.3.2 Oceanographic features relating to algal blooms

Phytoplankton or algal blooms arise essentially from a period of growth, where the water column is such that they can be kept in the surface light-rich layers with an adequate supply of nutrients. There are a number of natural instances where this can be expected to happen. A prolonged period of calm and bright warm weather can allow a major bloom to develop. Blooms are also associated with the development of fronts. One example is off the south-west coast of Ireland, where upwelling brings cool, nutrient-rich water to the surface, allowing growth and reproduction of the algae at a greatly increased rate. These factors may explain the occurrence in the Irish Sea during July 1994, when a brown slick elicited extensive media reports with headlines such as ‘Large bloom off east coast no threat, Department says’ (from The Irish Times July 30th 1994) and ‘Slime slick drifting northwards’ (from Cork Examiner July 24th 1994) appearing in some of the national newspapers.

1.3.2.1 Seasonal cycles

The climate in most of Europe is temperate and the year can be easily divided into the familiar distinct seasons. In temperate climates, terrestrial plants respond by adapting their growth and reproduction to these seasons. This is not only in response to available light, but also to temperature, which varies throughout the year over the approximate range -10 to 30°C. However, sea-water temperatures do not vary as much. This is particularly important in winter in mid-latitudes where water temperatures may fall to only around 4-5°C near coasts and the winter minimum may be much warmer offshore at around 9-10°C.

The amount of daylight that penetrates the sea surface is important for phytoplankton. In winter, the daylength and light penetration is reduced which results in a lower phytoplankton biomass in the sea. Nutrients are physically mixed up into the sea surface due to storms, and their concentrations are at their highest at this time of year. The classical annual cycle of phytoplankton growth for temperate climates such as Ireland starts with an increase in phytoplankton cells in spring and this is usually referred to as the Spring Bloom. This bloom is caused by the increase in daily available irradiance, due to increasing daylength and the sun rising higher in the sky. This bloom has important consequences for further production in the year, because it strips surface waters of...
nutrients, such as nitrates and phosphates. Thus, in summer when light conditions are plentiful, phytoplankton growth is severely limited by the lack of nutrients. Replenishment of nutrients to the sea surface is inhibited by the development of thermal stratification forming a *thermocline*, which acts as a diffuse barrier between warmer surface layers and cooler nutrient-rich deep water. Towards the end of this season, cooling of surface water and increased turbulence, combine to destroy the thermocline, which results in the mixing of nutrients back up to the sea surface\(^9\).

### 1.3.2.2 Mixing and upwelling

In summer, the water column stratifies into two layers; a warm surface layer which is depleted in nutrient and a cool bottom layer which is nutrient rich. Phytoplankton cannot survive in this lower layer as there is insufficient light. If, however, there was a physical process which could uplift the lower layer towards the surface, then phytoplankton could flourish or bloom. In coastal waters, there are two processes which can cause this. The first is physical *mixing* due to tides, physical obstructions such as headlands or directly from wind-mixing. The second is known as *upwelling*\(^9,10\). If enough physical turbulence is applied to a water column, then thermal stratification may be destroyed and the result is a mixed water column. A common instance of this is around headlands, where the physical turbulence, caused as the tide moves water around these obstructions, contributes to large amounts of mixing energy which drives nutrients to the surface. This can stimulate phytoplankton growth in these region and localised patches of large phytoplankton populations can be found\(^11,12\). The most common cause of *upwelling* is the presence of alongshore winds. Winds drag water along the surface of the sea initially in the same direction. This can cause water to move away from the coast. Since water seeks its own level, as surface water moves away from the coast it becomes replenished by deeper water. The net result is the movement of nutrients towards the sea surface, and consequently phytoplankton flourish on these occasions.
1.3.2.3 Flushing of bays
A narrow embayment will be flushed with sea-water from outside the bay with every tide. The incoming or floating tide brings sea-water which mixes with water inside the bay and part of the water originally inside the bay will be removed with the outgoing or ebbing tide. Thus, the water inside the bay is to a certain extent purified with every tide. The relative amounts of purification with each tidal cycle can be identified by the flushing time of the bay. This number gives information on the degree of dilution the embayment incurs each day.

The relevance of this to phytoplankton is that if the flushing time is much faster than the growth rates of phytoplankton then blooms will tend not to develop inside the bay and the phytoplankton will be representative of that of the sea outside the bay. If flushing is weak, then blooms will occur locally inside the bay which may be particularly important if the bay is receiving nutrient effluent either directly or by land run-off. As it happens, growth rates of phytoplankton also vary considerably. Diatoms are however faster growing than dinoflagellates. Diatoms can divide about once per day sometimes faster but dinoflagellates only divide every 3 days

1.3.2.4 Fronts
Fronts or boundaries between different types of water masses exist throughout the world's oceans. A type of front, quite common off the north-western European coastline, is one known as a tidal front. They arise because some regions of the sea have very strong tidal streams and are relatively shallow. Therefore, they fail to thermally stratify in summer and a thermocline does not develop. One such area covers most of the Irish Sea where tidal streams are strong and the water column is relatively shallow (<60 m deep). The Celtic Sea to the south and the Atlantic ocean to the north do stratify and, where these waters meet, pronounced tidal fronts are found. They can be recognised by the difference in sea surface temperature on either side of the front. Tidal fronts are sites of intense biological activity, caused by the presence of nutrients at the sea surface coupled with reasonable water column stability. Algal blooms of either diatoms or dinoflagellates at these fronts are common in summer. The fronts of NW France and at the southern end of the Irish Sea are particularly noted for very high concentrations of the red tide organism Gyrodinium aureolum in summer.
1.3.2.5 Other factors (Ships' ballast)

One reason for the global spread of 'red tides' may be the transportation of cysts from one area to another in ships ballast tanks. It has long been recognised that ships carry marine organisms across the world's oceans in their ballast water. Studies carried out in Tasmania have found more than 300 million toxic dinoflagellate cysts in one vessel alone. It has been argued that one PSP-producing dinoflagellate species first appeared in Tasmanian waters twenty years ago and coincided with the development of a local wood-chip industry. Empty vessels that begin a journey in a foreign harbour, pump water and sediment into their tanks for ballast but when wood chips are loaded in Tasmania the tanks are discharged. Cysts easily survive the transit cruise and colonise the new site. The Australian government has now issued strict guidelines for discharging ballast water in their ports. Unfortunately most other nations do not have such restrictions.

1.4 HARMFUL ALGAL BLOOMS AND THEIR CAUSATIVE ORGANISMS

As mentioned earlier, phytoplankton can produce potent toxins which find their way through the food chain to humans and are responsible for a variety of gastrointestinal and neurological illnesses. To date, these have been classified into the following four main syndromes:

a) Amnesic Shellfish Poisoning (ASP)

b) Neurotoxic Shellfish Poisoning (NSP)

c) Paralytic Shellfish Poisoning (PSP)

d) Diarrhetic Shellfish Poisoning (DSP)

Table: 1.02 is a summary of the Harmful Algal Blooms and their Causative Organisms.
### Table 1.02  *Major types of Harmful Algal Blooms and their Causative Organisms*

<table>
<thead>
<tr>
<th>Bloom Types</th>
<th>Species</th>
<th>Type</th>
<th>Major Problem Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red tides of otherwise harmless algae</strong></td>
<td>Noctiluca scintillans</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gonozaulax polygramma</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scrippsiella trochoidea</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trichodesmium erythraeum</td>
<td>Cyanobacterium</td>
<td></td>
</tr>
<tr>
<td><strong>Alae producing toxins poisonous to humans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSP</td>
<td>Alexandrium acatenella</td>
<td>Dinoflagellate</td>
<td>Australia, Japan, USA</td>
</tr>
<tr>
<td></td>
<td>Alexandrium catenella</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alexandrium fundyense</td>
<td>Dinoflagellate</td>
<td>Europe, Japan, USA</td>
</tr>
<tr>
<td></td>
<td>Alexandrium tamarensense</td>
<td>Dinoflagellate</td>
<td>Spain, Australia</td>
</tr>
<tr>
<td></td>
<td>Gymnodinium catenatum</td>
<td>Dinoflagellate</td>
<td>Phillipines, Brunei</td>
</tr>
<tr>
<td></td>
<td>Pyrodinium bahamense</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td>DSP</td>
<td>Dinophysis acuta</td>
<td>Dinoflagellate</td>
<td>Europe, (Ireland)</td>
</tr>
<tr>
<td></td>
<td>Dinophysis acuminata</td>
<td>Dinoflagellate</td>
<td>Chile</td>
</tr>
<tr>
<td></td>
<td>Dinophysis fortii</td>
<td>Dinoflagellate</td>
<td>Europe, (Ireland)</td>
</tr>
<tr>
<td></td>
<td>Dinophysis norvegica</td>
<td>Dinoflagellate</td>
<td>Japan</td>
</tr>
<tr>
<td></td>
<td>Dinophysis mitra</td>
<td>Dinoflagellate</td>
<td></td>
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<tr>
<td></td>
<td>Dinophysis rotundatum</td>
<td>Dinoflagellate</td>
<td></td>
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<tr>
<td></td>
<td>Prorocentrum lima</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prorocentrum minimum</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td>ASP</td>
<td>Pseudonitzschia pungens f. multiseries</td>
<td>Diatom</td>
<td>Canada</td>
</tr>
<tr>
<td></td>
<td>Pseudonitzschia pseudodelicatissima</td>
<td>Diatom</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pseudonitzschia australis</td>
<td>Diatom</td>
<td></td>
</tr>
<tr>
<td>NSP</td>
<td>Gymnodinium breve</td>
<td>Dinoflagellate</td>
<td></td>
</tr>
<tr>
<td>Ciguatera Fishfood Poisoning</td>
<td>Gamblierdiscus toxicus</td>
<td>Dinoflagellate</td>
<td>Australia, Caribbean, Pacific Islands</td>
</tr>
<tr>
<td>Cyanobacterial Toxin Poisoning</td>
<td>Anabaena flos-aquae</td>
<td>Cyanobacterium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microcystis aeruginosa</td>
<td>Cyanobacterium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nodularia spumigena</td>
<td>Cyanobacterium</td>
<td></td>
</tr>
<tr>
<td>Algae non-toxic to humans but harmful to aquatic organisms</td>
<td>Chaetoceros convolutus</td>
<td>Diatom</td>
<td>Canada</td>
</tr>
<tr>
<td></td>
<td>Chaetoceros peruvianum</td>
<td>Diatom</td>
<td>Scotland</td>
</tr>
<tr>
<td></td>
<td>Gyrodinium aureolum</td>
<td>Dinoflagellate</td>
<td>Europe, Japan, Australia, Europe</td>
</tr>
<tr>
<td></td>
<td>Chrysochromulina polyplepsis</td>
<td>Prymnesiophyte</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chrysochromulina leadbeteri</td>
<td>Prymnesiophyte</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prymnesiurn parvum</td>
<td>Prymnesiophyte</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prymnesiurn patelliferum</td>
<td>Prymnesiophyte</td>
<td></td>
</tr>
</tbody>
</table>
1.4.1 Amnesic shellfish poisoning (ASP)

Toxic algal blooms are not limited to dinoflagellates. The diatom *Pseudonitzschia pungens* was found to be responsible for the intoxication caused by the production of the neurotoxin, domoic acid (Fig. 1.04).

The first incidence of ASP occurred on the Atlantic coast of Canada, at Prince Edward Island in 1987\(^{(17)}\). The outbreak resulted in three fatalities, with adverse effects on over 150 consumers of the toxic mussels, 22 people were hospitalised with 10 of these requiring intensive care. At present, ASP is predominantly a Canadian occurrence, however *Nitzschia* species appear globally. Low levels of domoic acid have, however, been detected in Spain and New Zealand\(^{(18,19)}\).

In 1991, hundreds of pelicans and cormorants, were washed up on beaches near Santa Cruz, California, after feeding on tainted anchovies in Monterey Bay. Also, in that same year, people in Washington state were poisoned by domoic acid in razor clams\(^{(20)}\).

Scientists now believe that a bizarre attack by crazed seabirds near Santa Cruz in 1961, which may have inspired Alfred Hitchcock’s film *The Birds* (Fig 1.04 insert) was a result of domoic acid poisoning\(^{(21)}\).

Domoic acid is an analogue of kainic acid which is an excitatory dicarboxylic amino acid. These materials are competitive for glutamate receptors in the central nervous system. One subtype of the glutamate receptor is responsible for the specific binding of domoic acid and kainic acid\(^{(22)}\).
The symptoms appear up to 12 hours after the consumption of the shellfish. They include nausea, vomiting, abdominal cramps, kidney failure, disorientation and, in some cases, loss of memory and death due to respiratory failure. The principal symptom in the critically ill, surviving patients appears to be a permanent loss of the short-term memory, hence the name ascribed to this syndrome (23, 24).

The standard mouse bioassay for PSP can detect domoic acid but is not sensitive enough to detect the regulatory level set by the Canadian Government. Therefore, a reversed phase HPLC method, with UV detection at 242 nm, was developed for domoic acid analysis in shellfish. This method was adopted as an official first action by the AOAC in 1990. This was only the second official method, and the first physico-chemical method, for marine toxins approved by the AOAC (17).

1.4.2 Neurotoxic shellfish poisoning (NSP)

NSP was first reported in eastern Mexico and the south east coast of America, but more recently in New Zealand. Gymnodinium breve (Ptychodiscus brevis) is the causative organism and produces the brevetoxins (Fig. 1.05). These compounds owe their toxicity to the fact that they bind to a different site on the sodium channels than saxitoxin and cause an opposite effect to the latter.

The symptoms of NSP (which are similar to PSP) include diarrhoea, nausea, vomiting and abdominal pain, followed by muscular aches, dizziness, anxiety, sweating and peripheral tingling which occur 3-4 hours after consumption of the toxic material (25-27). Another mode of human exposure to brevetoxins is by the inhalation of sea spray containing fragments of P. brevis cells (28). They are also associated with the killing of fish, invertebrates, seabirds and possibly lead to mortalities in dolphins and manatees (29).

The brevetoxins can be detected by immunoassay, mouse bioassay, sodium channel assay and high-performance liquid chromatography.
Fig. 1.05  Structure of the two groups A) and B) of NSP toxins
1.4.3 Paralytic shellfish poisoning (PSP)

Of all seafood poisoning, PSP poses the most serious threat to public health, and the economic damage caused by accumulation of the toxins in shellfish are immense. PSP occurs in both cold and warm waters throughout the world. Up to 18 different PSP toxins, including saxitoxin, neosaxitoxin and gonyautoxins can be present in a variety of shellfish. (Fig. 1.06)

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**Fig. 1.06** *Structures of 18 PSP toxins*
A number of dinoflagellate species are known to produce the toxins: *Alexandrium* spp. (*Gonyaulax* or *Protogonyaulax*), *Gymnodinium catenatum* (30,31) and *Pyrodinium bahamense* var. *compressum*. Some strains of fresh-water blue-green algae, *Aphanizomenon flos-aquae*, have also been found to produce saxitoxin and neosaxitoxin(32,34).

One of the first recorded incidences of PSP (See extract of ship’s-log below) occurred on 15 June 1798, during the second of three exploratory voyages made by Captain George Vancouver to the coast of the Pacific north-west, aboard the *Discovery*(3).

In one of these they stopped to breakfast, where finding some mussels, a few of the people ate them roasted; as had been their usual practice when any of these fish were met with, about nine o'clock they proceeded in very rainy unpleasant weather down the south-westerly channel, and about one landed for the purpose of dining. Mr Johnstone was now informed by Mr Barre, that soon after they had quitted the cove, where they had breakfasted, several of his crew who had eaten of the mussel were seized with a numbness about their faces and extremities; their whole bodies were very shortly affected in the same manner, attended with sickness and giddiness...... One man only in the Chatham's boat was indisposed in a similar way. Mr Johnstone entertained no doubt of the cause from which this evil had arisen, and having no medical assistance within his reach, ordered warm water to be immediately got ready, in the hope, that by copiously drinking, the offending matter might have been removed. Carter attracted nearly the whole of their attention, in devising every means to afford him relief, by rubbing his temples and body, and applying warm cloths to his stomach; but all their efforts at length proved ineffectual, and being unable to swallow the warm water, the poor fellow expired about half an hour after he was landed.... There was no doubt that this was occasioned by a poison contained in the mussels he had eaten about eight o'clock in the morning; at nine he first found himself unwell, and died at half past one; To this bay I gave the name of Carter's Bay, after this poor unfortunate fellow; it is situated in latitude 52°48’, longitude 231°42’: and to distinguish the fatal spot where the mussels were eaten, I have called it Poison Cove, and the branch leading to it Mussel Canal.

The saxitoxins block sodium current through most known classes of voltage-activated sodium channels by a direct binding reaction and block the flux of sodium ions in and out of nerve and muscle cells(35,36).

Established methods for the detection of PSP toxins include mouse bioassay (37, 38), house-fly assay(39), immunoassays(40), a sodium channel binding assay(41), a tissue culture assay(42), capillary zone electrophoresis(43,44) and fluorescent high-performance liquid chromatography (HPLC) with either pre-column(45) or post-column oxidation(46) of the toxins to produce fluorescent products.

The symptoms occur within 30 minutes of eating; starting with tingling, itching or burning sensations in the face, especially on lips and tongue, which finally spread out over the whole body down to the feet. Systemic effects are headaches, dizziness,
faintness, heavy thirst, loss of balance, light-headedness, and finally blurring of vision and temporary blindness. Death occurs within 12 hours from respiratory arrest \(^{(24)}\).

To date, PSP is not a problem in the Republic of Ireland but these toxins have been found in shellfish from Belfast Lough. Our laboratory has identified saxitoxin, neosaxitoxin and gonyautoxin in mussels from this Lough using pre-column oxidation prior to HPLC analysis (James & Sherlock, unpublished).
1.5 DIARRHETIC SHELLFISH POISONING (DSP)

1.5.1 Introduction

Diarrhetic shellfish poisoning (DSP) is a syndrome named after the predominant human symptom; diarrhoea, gastroenteritis, following the ingestion of shellfish. Outbreaks of DSP are currently limited to cold and warm temperate areas of the Atlantic and Pacific oceans, although cases have been reported from the tropical Indo-Pacific region. The symptoms of human intoxication associated with DSP have been known since the 1960's. *Dinophysis* and *Prorocentrum* spp. have been suspected as the causative agent in DSP for almost as long. Over 10,000 cases have been reported throughout the world since 1976. The toxicity arises from shellfish that have been feeding on toxic phytoplankton, particularly *Dinophysis* and *Prorocentrum* spp. However Yasumoto *et al.* was the first to isolate and characterise a causative toxic compound from Japanese *Dinophysis.*

1.5.2 The history of Diarrhetic Shellfish Poisoning

1.5.2.1 The first report of DSP

The first suspected incident of DSP was thought to have occurred in the 1960s in the Eastern Scheldt district of The Netherlands, where consumers of mussels fell ill and reported symptoms of vomiting and diarrhoea. A major DSP incident also occurred in Japan in the 1970's. In 1976, 42 people suffered food poisoning after eating mussels from the Sanriku Coast. A similar incident occurred in the same season the following year (1977), but there was an increase in both the amount of poisoning and the amount of shellfish farms affected around the Tohoku District. On this occasion, clams and scallops were also found to be responsible for the poisoning. As no pathogenic micro-organisms were found in left-over foods or in the excreta of victims, it was presumed that an unknown toxin was the source of contamination.

The symptoms observed in the victims were diarrhoea (92%), nausea (80%), vomiting (79%) of cases and abdominal pain (53%) of cases. Symptoms occurred between 30 minutes and a few hours after the consumption of contaminated shellfish and lasted for a maximum of 3 days.
Shellfish samples collected from the affected areas were extracted with various solvents. Toxicity tests by means of intraperitoneally injecting mice with dilute solutions of the extract were carried out and the minimum dose of toxin required to kill a 20g mouse in 48 hours was defined as a mouse unit (MU). The majority of the toxin accumulated in the digestive glands of the shellfish. The toxin was found to be lipophilic and 12 MU of toxin was determined as the amount of toxin needed to cause mild poisoning in an adult\(^{51}\).

**1.5.2.2 The organisms responsible for DSP toxicity in Japan**

Public concern over the 160 cases of food poisoning arising from the consumption of mussels between 1976-1977 in Japan, led to the establishment of a regular phytoplankton monitoring program. Studies of Okkirai Bay revealed that the occurrence of a dinoflagellate *Dinophysys fortii*, paralleled, both in time and quantity, with the variation of mussel toxicity. Shellfish toxicity was variable, depending on the season of the year, the region the shellfish came from and the depth at which they were located in the water. This was evidence that toxicity resulted from shellfish feeding on toxic plankton. The dinoflagellate, *Dinophysys fortii*, was found in the sea water around the toxic period. Plankton samples were collected and classified by size using sieves of different mesh. All the plankton samples were tested for toxicity by mouse bioassay. Toxicity was detectable only in a sample trapped in a 40-95 mm fraction. *D. fortii* was not only concentrated exclusively in this fraction but also its abundance was proportional to the toxicity levels of the plankton samples. The plankton toxin was indistinguishable from the mussel toxin in both gel permeation chromatography and partition chromatography. It was concluded therefore that *Dinophysys fortii* was the causative organism responsible for toxicity.

The toxin responsible for toxicity was named dinophysistoxin and the syndrome diarrhetic shellfish poisoning (DSP) after its predominant symptom\(^{51}\). In 1982, dinophysistoxin-1 (DTX-1) was isolated from the hepatopancreas of mussels and its structure determined\(^{53}\).
1.6 THE ISOLATION AND STRUCTURAL DETERMINATION OF DSP TOXINS

The DSP problem was found to be more complicated than initially thought, with the discovery of the presence of several toxins in the so called DSP-Complex. These toxins are subdivided into three main groups:

i) Okadaic acid and its derivatives
ii) Pectenotoxins
iii) Yessotoxins

1.6.1 Isolation and structural determination okadaic acid and its derivatives

\[ \text{Fig. 1.07  Structure of okadaic acid and its derivatives} \]
In 1981, Tachibana et al isolated and determined the structure of a cytotoxic component of two marine sponges *Halichondria okadai* and *H. melanodocia*\(^{(55)}\). The component, a complex polyether derivative of a C\(_{38}\) fatty acid was named okadaic acid (Fig. 1.07). In 1982, okadaic acid (OA) was identified as a toxic component from mass cultured *Prorocentrum lima*, a benthic marine dinoflagellate\(^{(56)}\). Later the same year the structure of DTX-1 was determined by Murata *et al.*, to be 35S-methyl okadaic acid \(^{(53)}\). The total synthesis of OA was achieved by Isobe *et al.* in 1985 and required over one hundred synthesis steps\(^{(57-59)}\).

### 1.6.1.1 Isolation and structural determination of dinophysistoxin-2 (DTX-2)

The shellfish monitoring programme conducted by the Department of the Marine in Ireland and in our laboratories revealed a new toxin of the okadaic acid class\(^{(60)}\). The toxin was isolated and identified by Canadian researchers and named dinophysistoxin-2 (DTX-2). Analysis of a mussel extract taken from Bantry Bay, by liquid chromatography-ionspray-mass spectrometry (LC-ISP-MS), indicated that DTX-2 was an isomer of okadaic acid. The toxin was extracted from mussel hepatopancreas with methanol and acetone, and isolated using several chromatographic steps. The structure of DTX-2 was determined by nuclear magnetic resonance (NMR) spectroscopic analysis of the isolated product (Fig. 1.07)\(^{(61,62)}\).

### 1.6.1.2 Isolation and structural determination of dinophysistoxin-3 (DTX-3)

In 1985, Yasumoto *et al.* reported the isolation and structural determination DTX-3 from toxic scallops \(^{(63)}\). DTX-3 was found to be a mixture of 7-O-acyl derivatives of DTX-1 (Fig. 1.07). It is, in fact, a mixture of DTX-1 ester derivatives with different fatty acid moieties at carbon 7. DTX-3 can be converted back into the free fatty acids and DTX-1 by hydrolysis. To date it has not been found in phytoplankton samples which suggests that acylation of DTX-1 to DTX-3 takes place in the hepatopancreas of the shellfish \(^{(58)}\). There was a noticeable loss in toxicity when a dried sample of DTX-3 was exposed to air. Purification of the deteriorated product confirmed that it was in fact 7-O-palmitoyl DTX-1 and it lacked any significant toxicity. The fact that palmitic acid is a saturated fatty acid led Yasumoto *et al.* to believe that the toxicity of DTX-3 was related to the unsaturation in the acyl moiety.
The 7-O-acyl derivatives of OA were isolated from Dutch mussels in 1986 and Irish mussels were found to contain 7-O-acyl derivatives of DTX-2\(^{(64)}\). Conversion of okadaic acid, DTX-1 and DTX-2 to their acyl ester derivatives appears to be related to their concentrations in the shellfish. The production of 7-0-acyl compounds by shellfish is believed to be a detoxification process since they are less toxic than their parent compounds.

1.6.1.3 Isolation and structural determination of dinophysistoxin-4 (DTX-4)

DTX-4 is the latest toxin to be isolated which belongs to the okadaic acid class of DSP toxins (Fig. 1.07). Investigation of DSP toxins in *Prorocentrum* species revealed that the butanol-soluble fraction of a strain of *Prorocentrum lima* isolated from Nova Scotia, Canada give positive results in the mouse bioassay for DSP toxins. Purification by chromatography yielded a purified, polar, water soluble toxin which was subsequently named DTX-4 \(^{(65)}\). This toxin is a sulphonate ester of OA, and represents the first polar DSP toxin and was found to be sparingly soluble in chloroform. Consequently, quantitative analysis of DTX-4 by the established ADAM-HPLC method \(^{(66)}\) is not possible.
1.6.2 Isolation and structural determination of the pectenotoxins (PTX)

In 1983, Yasumoto et al., using a complex chromatographic procedure, isolated another class of DSP toxins known as pectenotoxins, from toxic scallops (Fig. 1.08).

![Structure of the pectenotoxins](image)

The pectenotoxins (PTX) were named after *Patinopecten yessoensis* scallops from which the toxins were extracted. From a chemical point of view, pectenotoxins are different to the okadaic class of DSP toxins in that they have a longer carbon backbone (C₄₀), a C₃₃ lactone ring and a novel dioxabicyclo moiety. Five neutral pectenotoxins were isolated, PTX-(1-5), but only the structures of PTX-1 and PTX-2 were determined initially. Subsequently, two more acidic pectenotoxins, PTX-6 and 7 were isolated from toxic scallops, and the structures of PTX-3 and PTX-6 were determined. PTX-7 was shown to be 7-epi-PTX-6 and PTX-4 to be 7-epi -PTX-1. However, there was not enough compound available to determine the full structure of PTX-5.

PTX-2 was found to be produced by *Dinophysis fortii* and it is thought that the other toxins were produced as a result of the oxidation of PTX-2 in the hepatopancreas of the shellfish.
1.6.3 Isolation and structural determination of the yessotoxins (YTX)

The third class of DSP compounds are called yessotoxins (Fig. 1.09). Yessotoxins, like pectenotoxins, were isolated from toxic *Patinopecten yessoensis* scallops collected from Mutsu Bay, Japan in 1986.

![Structure of yessotoxins](image)

The first compound isolated was called yessotoxin-1 (Fig. 1.09). The structure was determined, by means of N.M.R., to be a polyether toxin with a molecular formula of $C_{55}H_{36}O_{21}S_2Na_2$ which partly resemble the brevetoxins. It has contiguous *trans*-fused ether rings, one eight-membered, one seven-membered and nine six-membered rings and is substituted with two hydroxyl, six methyl, two exo-methylene and two sulphate ester groups\(^{(69)}\). Later, 45-hydroxy yessotoxin was isolated from scallops \(^{(68)}\).
1.7 PHYTOPLANKTON RESPONSIBLE FOR DIARRHEIC SHELLFISH POISONING

1.7.1 Identification of toxin-producing organisms

The symptoms of human intoxication associated with DSP have been known since the 1960's. *Dinophysis* and *Prorocentrum* spp. have been suspected as the causative organisms in DSP for almost as long (47, 51). Outbreaks of DSP in Japan in the 1970's led to the identification of DTX-1 as the major toxin. The causative organism was *D. fortii* (51). However the inability to culture *Dinophysis* spp. in the laboratory has made confirmation of their toxigenicity difficult. Several *Prorocentrum* spp. have been successively cultured in the laboratory allowing confirmation of their toxigenicity.

Recently, in work carried out by Lee et al., sixteen species of unialgal samples of dinoflagellate, either wild or cultured, were tested for production of diarrhetic toxins such as OA, DTX-1, and pectenotoxins. Of the cultured *Prorocentrum* spp. that were examined only *P. lima* was found to be toxic, and contained both OA and DTX-1.

Seven of the *Dinophysis* species were confirmed to produce either OA and/or DTX-1. *D. acuta* (Vigo, Spain), *D. acuminata* (Le Harve, France and Tokyo Bay, Japan) and *D. fortii* (Inland Sea, Japan) contained OA only. *D. mitra* (Mutsu Bay, Japan), *D. roundata* (Mutsu Bay, Japan), *D. norvegica* (Sogndal, Norway) and *D. tripos* (Kesen numa, Japan) contained DTX-1 only. *D. acuta* (Sogndal, Norway) and *D. norvegica* (Arendal, Norway) contained OA and DTX-1. *D. fortii* (Mutsu Bay, Japan) contained DTX-1 and PTX-2. Intraspecies variation in toxin content and profiles was noticed which leads to difficulties in correlating *Dinophysis* populations with shellfish toxicity (50).

1.8 A GLOBAL PERSPECTIVE IN RELATION TO INCIDENCES OF DSP AND THE PRESENCE OF TOXIC ALGAE

1.8.1 Europe

To date, Europe has experienced the greatest problems with DSP. The unpredictable incidence of toxicity, with its occurrence in all of the climatic regions of Europe, has resulted in severe economic consequences for shellfish producers. Shellfish production areas have been closed for periods from a few weeks to a year in Spain, Portugal,
France, The Netherlands, Sweden, Norway, Ireland and Italy, due to positive animal bioassay responses or human toxic incidents.

(a) The Netherlands

The first incidents of DSP in Europe occurred in 1961 when there were reports of mussel poisoning in the Eastern Scheldt region of The Netherlands. Similar incidents occurred in the Eastern Scheldt and Dutch Waddensea regions throughout the 1970's. In addition, there were 30 incidents of shellfish poisoning in 1981 on one of the Dutch Wadden Isles. OA was the toxin responsible and *D. acuminata* is the causative organism of DSP in The Netherlands.

(b) France

In 1983, approximately 3,000 DSP-type intoxications were recorded following the consumption of mussels. Studies carried out in South Brittany during 1983-1984, revealed that numerous DSP intoxications were recorded following blooms of *D. acuminata* along the French coast. From 1984-1990, significant portions of the French coastline suffered from regular occurrences of *D. acuminata* most of which coincided with reports of DSP contamination in shellfish. OA was the toxin responsible and *D. acuminata* is the predominant causative organism of DSP in France.

(c) Spain

From 1977-1981, incidences of DSP have been recorded along the north-western coast of Spain where both *D. acuta* and *D. acuminata* were associated with a DSP episode which caused 5000 cases of gastroenteritis. Between 1990 and 1993 monitoring of plankton hauls carried out in Ria de Pontevedra (N-W Spain), revealed that OA was the predominant DSP toxin, with small quantities of DTX-2, being detected in 57% of all samples analysed. The species responsible for DTX-2 production appears to be *D. acuta*, since during an unusual proliferation of this species in December 1992, the occurrence of *D. acuta* coincided with the period when DTX-2 was detected in the algae samples. Both *D. acuta* and *D. acuminata* are the predominant causative organisms, with *Prorocentrum lima* also thought to be a contributor to DSP toxins in Spain.
(d) Portugal

In Portuguese coastal waters, DSP toxins have been detected in bivalves since 1987 although no human intoxications have been reported. DSP toxins were detected soon after the appearance of *D. acuta*, *D. acuminata* and *D. sacculus*. In 1994, DTX-2 was identified in Portuguese shellfish using HPLC with the standard supplied by our laboratories. Both okadaic acid and DTX-2 were observed in all the samples studied, except for one period in April when okadaic acid alone was present. DTX-2 was the predominant toxin in some of the samples analysed.

(e) Scandinavia

In Scandinavia, the first case of DSP in humans probably occurred in 1968 in Norway. It was reported as an 'unidentified mussel poisoning'. During 1984 and 1985, over 400 people living in the south-west coast of Norway became ill following consumption of blue mussels. This was the first confirmed outbreak of DSP in Norway. The cause of the illness was identified as toxins from *Dinophysis* spp. The marked decrease in the number of intoxications is due to the implementation of monitoring programmes for DSP toxins which were established in Norway in 1986. In Sweden, several people who had consumed blue mussels from the west coast developed symptoms of DSP following the occurrence of a dinoflagellate bloom in October 1984. The toxin concentration was higher than 170 mouse units (MU)/kg of mussel meat. This outbreak occurred at the same time as the outbreak in Norway, showing heavy contamination of the coastline in western Sweden and south-west of Norway. There was also a report showing that Danish mussels exported to France in 1990 caused poisoning in 415 people. The mussels contained 170 μg okadaic acid per 100 g of mussel meat.

The outbreak of DSP in Norway and Sweden was due mainly to *D. acuta*. However, analysis of mussels harvested at Sogndal in the Sognefjord, Norway during 1986 showed that DTX-1 was the major toxin along with small amounts of yessotoxin. Thus, mussels from this location had the same toxin profile as Japanese scallops. In contrast, OA was the principal toxin in mussels from another part of Norway (Arendal) resembling the situation in other European countries. This is noteworthy, as *D. acuta* and *D. norvegica* are likely to be responsible for infestation of the mussels in both areas.
(f) **Italy (Northern and North-western Adriatic Sea)**

A DSP outbreak occurred in Slovenia in the autumn and winter of 1989. Of the eight *Dinophysis* species detected in the Seca and Strunjan regions, *D. acuminata* and *D. fortii* were the most dominant species\(^{86}\).

From the months of September and October 1990, and May 1991 mussels from the Gulf of Trieste were found to contain high levels of DSP toxins. Toxic levels of OA and DTX-1 were observed in mussels after *D. fortii* blooms in autumn (1990), but not after *D. acuminata* blooms in spring (1991). It was concluded that *D. fortii* was the probable source of DSP toxins in the Northern Adriatic Sea\(^{87}\).

Also, in June 1989 and June 1991, mussels along the coast of Emilia-Romagna (North-western Adriatic Sea) in Italy showed high levels of DSP toxins with a ban on shellfish harvesting being imposed until the following January. In 1989 *D. fortii* was the dominant dinoflagellate in the area in June and July, *D. sacculus* in August and September, *D. fortii* and *D. caduata* in October and November and *D. tripos* in December. OA was found to be the toxin responsible for this toxicity\(^{88,89}\).

In July 1990, mussel samples collected from Trieste, Rimini and Ancona in Italy were found to contain toxic levels of okadaic acid. Discrepancies were observed in the Rimini sample between mouse assays and HPLC results, suggesting the presence of other toxins\(^{90}\). New toxins were isolated and were found to have cytotoxic activity against P388 murine leukemia cells. They absorbed UV light of 205 nm but they did not form fluorescent esters with 9-anthryldiazomethane. However, the limited sample size did not allow further characterisation of the toxins\(^{91}\).

(g) **Germany**

DSP has occurred in Germany since 1986 when okadaic acid was detected in mussels from the Wadden Sea\(^{92}\). Between 1986 and 1989, DSP toxins were detected in bivalves from the coasts of German Bight, North and East Frisia where *D. acuminata* was the predominant causative organism.

(g) **Scotland**

In July 1992, a brown-red discolouration of a 10 km stretch of water occurred along the eastern shore of the upper part of Loch Long (a fjord-like sea loch), on the west coast of
Scotland. Mussels were collected from the area and tested for toxins. All samples tested positive for DSP toxins. At four sampling stations, the numbers of *D. acuminata* ranged from 8,000 cells/L (Portincaple), to 103,000 cells/L (Ardmay), 916,000 cells/L (Arrochar) to 942,000 cells/L (Finnart). *D. acuta* and *D. norvegica* were also present in all four stations. There were no reported cases of human intoxications as the area is not used for commercial shellfish farming.

(h) Ireland

Diarrhetic toxicity in shellfish was first recorded in Ireland in 1984. Cases of DSP have occurred each year from 1987 to date. An isomer of OA, dinophysistoxin-2 (DTX-2) was identified for the first time in mussels from Ireland (61) and both OA and DTX-2 were present during a DSP episode in 1991 (60). DTX-2 was found to be the predominant diarrhetic shellfish toxin in Ireland. In the period July-December 1991, mussels from Bantry Bay in South-West Ireland were found to have a complex toxin profile with OA, DTX-2, 7-O-acyl derivatives of DTX-2 and DTX-1 detected (94). In 1994, there was again a major infestation of mussels but the toxin profile was different from that observed in 1991. OA, although present at low levels, did not play a significant part in this DSP episode. DSP in shellfish in Ireland is associated with blooms of *Dinophysis acuta* and *D. acuminata*. During these studies, the detection of high levels of DTX-2 in mussels followed shortly after the observation of high cell counts of *D. acuta* (5,000-15,000 cells/L) in the cultivation areas (95). *D. rotundata* and *D. norvegica* were also observed in Irish waters (96).

**Summary:**

Therefore, *D. acuta* and *D. acuminata* seem to be the main toxin producers in Europe, although other species have been implicated as well.

1.8.2 Eurasia

(a) Japan

The variation in the dominating toxins in Japan and Europe seems to be attributable to the presence of different dinoflagellates. In Japanese waters, *D. fortii* is the primary source of DTX-1, while in Europe, OA is produced mainly by the dinoflagellates *D. acuta*

37
and *D. acuminata*. 160 cases of food poisoning arising from the consumption of shellfish between 1976-1977 were reported in Japan. Studies in Okkirai Bay revealed that the occurrence of a dinoflagellate *Dinophysis fortii* paralleled, both in time and quantity, with the variation of mussel toxicity\(^{(52)}\). An additional 1257 people were poisoned between 1976 and 1984, following 34 separate DSP outbreaks\(^{(97)}\). Although other *Dinophysis* species such as *D. acuminata* were found to be responsible for DSP in Japan, blooms of *D. fortii* are the dominant species which cause DSP in this region of the world\(^{(63)}\). DTX-1 and DTX-3 are the predominant toxins in Japanese shellfish.

(b) Russia

From the far eastern coastal waters of the USSR, *Dinophysis acuminata*, *D. acuta*, *D. fortii* and *D. norvegica* were identified in Vostok Bay plankton between 1969 and 1990. The population density of these species in the areas used for off-bottom mollusc culture was found to be several times higher than that at which DSP was recorded in many World Ocean regions. According to Konovalova et al., these species, together with other dinoflagellates, have caused toxic red tides during summer/early autumn\(^{(98)}\).

(c) India

A study carried out along the coast of Karnataka state, India, between 1984 and 1986, showed that diarrhetic shellfish toxins were present in several shellfish examined. The levels ranged from 0.37 to 1.5 MU/g hepatopancreas\(^{(99)}\).

Summary

Although there are no reports of DSP episodes from the USSR or India, detection of toxin-producing flagellates and toxic shellfish indicates the possibility of food poisoning in these areas.

1.8.3 American Continent

(a) Canada

The first reported identification of DSP toxins in Canadian shellfish was in 1991 when DTX-1 was identified in mussels collected in June-August 1989 from Prince Edward Island\(^{(100)}\). In August 1990, 13 people developed gastroenteritis after consuming toxic...
mussels from Mahone Bay in Nova Scotia. DTX-1 was found to be the toxin responsible for the DSP incident. *P. lima* and *D. norvegica* were shown to be the causative organisms\(^{(101)}\). This was probably the first confirmed case of DSP in North America.

*P. lima* was isolated and brought into unialgal culture from a phytoplankton net sample from the Atlantic coast of Nova Scotia. The sample was collected at an aquaculture site immediately following an incident of DSP due to the consumption of contaminated mussels. Chemical analysis of the culture extract indicated the presence of the DSP toxins, OA and DTX-1 \(^{(78)}\). During July-August, 1990, *D. norvegica* was reported to be responsible for a DSP out-break in Bedford Basin, Canada. Phytoplankton samples taken during this period were analysed by immunoassay and chromatographic analysis confirmed the presence of OA and/or DTX-1 \(^{(102)}\).

In June 1991, several reports of DSP in Nova Scotia were documented. Although DTX-1 was detected in the shellfish, the amount was well below the toxicity limit. The toxicity was mainly due to unknown toxins which were shown to inhibit protein phosphatase activity \(^{(100,103)}\). Again, in the summer-autumn period of 1992, mussels from Mahone Bay were found to contain low levels of DTX-1 \(^{(104)}\).

\(\textbf{(b) North America}\)

The monitoring algae in the area of Long Island, USA, from 1971 to 1986 showed the presence of *D. acuminata*, *D. acuta* and *D. norvegica* in most cases. *Dinophysis fortii* was observed only in the ocean and the south shore bays. Between 1983 and 1985, four DSP-type intoxications occurred in Philadelphia and Long Island, New York after consumption of clams and mussels. *Dinophysis* or *Prorocentrum* species were associated with the episodes but no chemical analysis was carried out on the shellfish \(^{(105)}\).

In 1989, high numbers of *D. acuminata* were observed in discoloured water in Nassau County, New York. Analysis for OA revealed that mussels from two stations contained more than 0.5 MU per 100 g \(^{(25)}\).
(c) Mexico

In 1990, OA was detected in shellfish and phytoplankton taken from the Gulf of Mexico. Gulf Oysters (Crassostrea virginica) collected during autumn from Cedar Point Reef, Mobile Bay, were shown to contain OA. Dinophysis caudata, was detected in the seawater after the discovery of the toxicity. However, phytoplankton samples were not taken on the same day as the shellfish samples and therefore it was not possible to establish a correlation between D. caudata populations and levels of OA in the shellfish.

(d) Chile

DSP was first reported in the Reloncavi Estuary in Chile in 1970, when 100 people suffered intoxication after consuming mussels. D. acuta was detected in the water but not confirmed as the causative organism. In 1979 and 1986, toxic mussels occurred in the same estuary and D. acuta was present in both water samples and in the shellfish tissue. In 1980 and 1984 toxic mussels were harvested in the Jacaf Fiord (240 miles South of the Reloncavi Estuary) and D. acuta was again present.

In January 1991, a massive DSP intoxication occurred in Puerto Aguirre, Chonos Archipelago, Chile. Toxicity in the shellfish lasted six months. D. acuta was thought to be the causative organism with OA, DTX-1 and unknown toxins being detected in the shellfish. A maximum toxin concentration of 4 MU/g hepatopancreas was recorded in January. Three sets of samples were collected: Eight specimens of the mussel, Aulacomya ater, collected at Pajal Island in February 1991; 30 specimens of the mussel, Mytilus chilensis, collected at Caleta Andrade in February 1991; and canned mussels, M. chilensis, prepared in January from an unknown site. All samples were analysed by mouse bioassay and HPLC. The mouse bioassay gave a positive result for the presence of DSP toxins in all three samples. The mussels from Caleta Andrade contained DTX-1 as the major toxin and OA as the minor toxin. Also, 217 cells of D. acuta and 12 D. rotundata cells were found per gastrointestinal content of the specimen. The concentration of these species in the water was 176 cells/l.

Both OA and DTX-1 were also present in canned mussels but their concentration was too low to account for the mouse lethality, which suggested the presence of additional toxins. D. acuta was found in both gastrointestinal content of the mussels and the fluid.
in the cans. No *Dinophysis* species were detected in the water around Pajal Island at the time of sampling but 18 *D. acuta* cells per specimen were detected in the gastrointestinal content of the mussels\(^{(90)}\).

(e) Uruguay

In the latter part of 1991, the entire coast of Uruguay was closed to shellfish harvesting due to the detection of both PSP and DSP. The ban was lifted in November of the same year only to be issued again in January 1992, when DSP was detected. *D. acuminata* was found to be the causative organism, with concentrations of up to 6,000 cells per litre of sea-water being recorded in La Paloma. This was the first time this species was recorded in Uruguayan waters\(^{(109)}\).

1.8.4 Diarrhetic shellfish poisoning in other Countries

In addition to the cases mentioned, it is reasonable to assume that DSP has occurred in other parts of the world. There are, for instance, earlier reports indicating that DSP has occurred in Australia, Indonesia and New Zealand\(^{(119)}\). Between the years 1984-1986 DSP toxins were found to be present in shellfish from along the coast of Karnataka state, India\(^{(99)}\). To date, in some south islands of New Zealand, low levels of DSP toxins have accompanied by blooms of *D. acuminata* and *D. sacculus*\(^{(19)}\).
1.9 TOXICITY AND HISTOPATHOLOGICAL STUDIES ON THE EFFECTS OF DSP TOXINS

The isolation and characterisation of DSP toxins resulted from pioneering research carried out by Japanese scientists and lead to the discovery of three chemically distinct classes of toxins \(^{67,111}\). These classes of shellfish toxins have been shown to exert very different types of toxic properties. Strictly speaking, only the 'okadaic acid' class, should be referred to as DSP toxins, because pectenotoxins and yessotoxins are toxic to mice upon intraperitoneal injection (i.p.), they do not induce diarrhoea. The pectenotoxins, when administered orally to mice and rats had no obvious effect on the organs of the animals. They also cause no changes in the epithelium of the small intestine when injected intraperitoneally, but markedly increased permeability of the digestive tract and liver were observed \(^{112,113}\). However, this conflicts with a report by Yasumoto et al., where he stated that pectenotoxins were reported to cause diarrhea in mice when administered orally. However, they were less potent in diarrheagenicity and less common in occurrence than the okadaic acid class of compounds \(^{68}\). The toxic effects of pectenotoxins and yessotoxins are not fully understood due to the lack of toxin standards available to researchers.

1.9.1 Toxicity and histopathological studies on Okadaic Acid and its Derivatives

Following the incidences of DSP in Japan in 1976 and 1977, Yasumoto et al. carried out toxicity tests by means of a bioassay, on extracts from contaminated mussels. Toxins were extracted from the hepatopancreas of the mussels with various solvents and tested for toxicity by means of intraperitoneally injecting mice with dilute solutions of the extract. Depending on the dose administered the onset of symptoms varied between 30 minutes and several hours, with the mice dying between 100 minutes and 47 hours after injection. When the toxin was administered orally by means of a stomach tube, the lethal dose was found to be 16 times higher than that obtained by intraperitoneal injection \(^{52}\). Other animals, including cats, chickens, mice and rabbits have also been administered with this toxin intraperitoneally (i.p.) but were found to be less sensitive to it than the mice \(^{52,114,115}\).
Murata et al. isolated and identified DTX-1 as the toxin responsible for these DSP incidents. Bioassays revealed that the minimum dose required to kill a mouse in 24 hours by i.p. was 160 µg/kg of body weight, the mice suffered constant diarrhea before death \(^{(53)}\). In Holland, white rats fed on contaminated shellfish also suffered from diarrhoea \(^{(48)}\). Hamano et al. evaluated the enteropathogenicity of DSP toxins in intestinal loops of rabbits and mice, and in suckling mice. Crude toxin extracts of DSP, which were prepared from scallops implicated in poisoning incidents in Osaka, Japan in 1978 and 1982, showed fluid accumulation in the intestinal tracts of the animals tested. The results of the suckling mouse assay carried on OA, DTX-1 and DTX-3 suggested that all three were diarrheagenic \(^{(114,115)}\).

Further studies were carried by Terao et al. where by suckling mice were administered with varying concentrations of DTX-1 (50-500 µg/kg of body weight) by intraperitoneal injection (i.p.). After various time intervals, the mice were sacrificed and histopathological studies carried out. The toxin caused marked edema (excessive accumulation of tissue fluid) in the lamina propria of villi and severe mucosal injuries in the small intestine. Three stages of ultrastructural changes in the intestinal villi were noticed and it was concluded from these, that DTX-1 may directly attack the Golgi apparatus.

The stages are:

1. Extravasation of serum into the lamina propria of villi (abnormal separation between epithelial cells and the basal membrane).
2. Degeneration of the intestinal absorptive epithelium characterised by a marked dilation of the cisternal portion of the Golgi apparatus.
3. Desquamation or shedding of the degenerated mucous epithelium from the villous surface (The mucosal epithelial cells detach themselves from the basal membrane.).

Therefore, morphological responses of the mouse small intestine to DTX-1 are distinguishable from the responses to choleragen and entrotoxins from *E. coli* or *C. perfringens* and are similar to those of the botulinum C\(_2\) toxin or the toxin produced by *C. difficile* \(^{(112)}\).
Edebo et al. found that OA induced rapid hypersecretion in ligated intestinal loops of rats, following injection of toxin suspensions into the loops. The rat's small intestines has been shown to be one of the most sensitive and reproducible organs for studies of the diarrhetic effects caused by OA and its derivatives. On the basis of body weight, humans are estimated to be at least four times more sensitive than rats to OA \(^{(116)}\).

Lange et al. has shown that microgram doses of OA also produces rapid and characteristic changes in the mucosa morphology in ligated loops of rats. 90 minutes after administration of the toxin, histological changes in the intestinal mucosa show rapid swelling which result in shedding of the enterocyes. The enterocytes at the upper part of the villi are replaced by goblet cells, which are not affected by the toxin. There is no apparent barrier damage or inflammatory reaction and the deeper part of the mucosa are unaffected. When a high dose of toxin is used, a collapse of the villi architecture occurs. When OA was administered intravenously, less extensive changes were noticed, which suggest that the toxin targets the enterocytes \(^{(117)}\).

Yasumoto et al. demonstrated that a saturated acyl derivative of DTX-1, 7-O-palmitoyl-DTX-1, was found to have only one tenth the mouse lethality of DTX-1 and a homologue having a highly unsaturated acyl retained a third of the potency in mice following intraperitoneal injection \(^{(63)}\).

Yanagi et al. also showed that acylation of the 7-OH reduced the mouse lethality of OA but not the diarrheagenicity. The lowering of mouse lethality depended on the varying degrees of unsaturation of the acyl-derivatives. Also, the 7-O-acyl derivatives of okadaic acid showed less potency in the mouse test than okadaic acid and, like the DTX-1 acyl derivatives, potency generally increased in parallel with unsaturation in the acyl substituents \(^{(111)}\).

Recently, Terao et al. studied the patho-morphological effect of administered okadaic acid, DTX-1 and DTX-3 to male ICR mice and Wistar rats. Each toxin was administered either i.p. (375 µg/kg body weight), or orally (750 µg/kg) with stomach tubes. The effects of both okadaic acid and DTX-1 on the animals organs were almost identical, except that the severity of i.p. injection was almost 7-times greater than that of oral administration. The effects of DTX-3 on the small intestine when orally administered, was similar to that of OA and DTX-1.
In contrast, the effect of i.p. injection of DTX-3 was found to be far weaker than that of OA and DTX-1. It led to dilatation of the cisternae of the Golgi apparatus; numerous vesicles occurred in the cytoplasm of the absorptive epithelium within 1 hour and then, after 4 hours these changes were completely reversed. DTX-3 also caused severe degeneration and necroses of the hepatocytes in the midzone of the liver. After 24 hours of oral administration of DTX-3, a marked accumulation of fat droplets and necrotic foci occurred in the midzonal and periportal regions of the hepatic lobule. OA and DTX-1 had no obvious effect on the liver when administered orally but they showed a weak cytotoxic effect on the hepatocytes when administered by i.p. injection.

1.9.2 Toxicity and histopathological studies on pectenotoxins (PTX)
The effects of PTX-1 on suckling mice differ considerably from those described for DTX-1. Terao et al. noted that there were no pathological findings in the small and large intestine, but the livers were markedly congested and their surfaces appeared finely granulated. Multiple vacuoles appeared around the periportal region of the hepatic lobules within 60 min of i.p. injection (1000 µg/kg body weight). Livers from mice treated with 500 or 700 µg/kg showed similar features after 2 hours. Electron microscopic studies confirmed that several portions of the microvilli of the hepatocytes became flat and the plasma membrane was invaginated into the cytoplasm. Within 30 min, the vacuoles had increased in size and most of the cellular organelles had become compressed. Within 24 hours, almost all hepatocytes containing numerous vacuoles and granules had become necrotic.

More recently Terao et al. studied the patho-morphological effect of administered PTX-1 and PTX-2 to male ICR mice and Wistar rats. Intraperitoneal injection of PTX-1 or PTX-2 resulted in marked fluid accumulation in the thorax and pericardium of the mice and rats within 4 hours. The villi of the small intestine were edematous. Histologically, capillaries in the villi were dilated and oozed flocculent materials such as blood plasma into the lamina propria. PTX-1 at the dose given i.p. did not kill the mice and rats for up to 24 hours after administration.

Non-fatty vacuoles also occurred in the hepatocytes located in the subcapsular regions of the liver. The cytoplasm of the hepatocytes was occupied by numerous vacuoles often connected with sinusoids, which led to the compression of the micro-organelles. The...
vacuoles contained erythrocytes and strands of fibrin. Occasionally, degeneration and swelling of the sinusoidal lining cells was seen and often an accumulation of blood platelets occurred in the sinusoids around the injured hepatocytes (118).

1.9.3 Toxicity and histopathological studies on the effects of yessotoxins (YTX)

The few studies that have been carried out on this toxin revealed that YTX is the most toxic of the DSP toxins (119). According to Murata et al., YTX kills mice at a dose of 100 μg/kg body weight (i.p.). They observed no fluid accumulation in the suckling mice even at the lethal dose level (69).

Terao et al. studied the toxicity of yessotoxin and desulphated yessotoxin assayed on 5 week old male mice (23-25 g) by i.p. injections. At doses above 300 μg/kg body weight, the mice displayed normal behaviour for the first hour, but then suddenly dyspnea set in and they died. Mice given desulphated yessotoxin in the same concentrations survived 48 hours. Mice receiving 500μg of YTX per kg body weight displayed severe cardiac damage. Endothelial lining cells of the capillaries in the left ventricle were swollen and degenerated. Almost all cardiac muscle cells were swollen.

In contrast, desulphated yessotoxin caused only slight deposition of fat droplets in the heart muscle. On the other hand, effects of desulphated YTX were observed in the liver and pancreas. Macroscopically, the livers were pale and swollen within 12 hrs after injection of 300 μg/kg. Fine fat droplets were found in all hepatocytes in the lobuli. Almost all mitochondria were slightly swollen and displayed reduced electron density. Pancreatic acinar cells also showed degeneration. Disarrangement of the configuration of the rough endoplasmic reticulum was prominent within 6 hours. In contrast, YTX at a dose of 300 μg/kg body weight i.p., did not cause any discernible changes in the liver, pancreas, lungs, adrenal glands, kidneys, spleen or thymus. Mice treated orally with YTX at 500 μg/kg showed no changes, while mice given desulphated YTX at the same dose developed fatty degeneration of the liver.

Yessotoxin seemed to be least toxic towards the isolated hepatocytes. Toxins induced tiny blobs on the cell surface in the concentration range 20-30 μg/ml while the spherical shape was maintained (113).
1.10 TUMOUR PROMOTING EFFECTS OF OKADAIC ACID AND ITS DERIVATIVES

The process of chemical carcinogenesis consists of two stages, initiation and promotion. Initiation is caused by a single application of a small amount of a carcinogen, which induces irreversible genetic damage to DNA. For example application of the carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), to the skin on the back of a mouse was reported to induce mutation of an oncogene\textsuperscript{120}. Agents that promote carcinogenesis from initiated cells are called tumour promoters. Treatment with DMBA, followed by repeated application of a tumour promoter, results in a high percentage of tumour-bearing mice, whereas treatment with an initiator alone or tumour promoter alone did not produce any tumours in the mouse skin. 12-O-tetradecanoylphorbol-13-acetate (TPA) for example, is the classical tumour promoter since its isolation from croton oil in the late 1970's. The mechanism of action of TPA-type tumour promoters was found to involve the binding to phorbol ester receptors and activation of protein kinase C \textit{in vitro} \textsuperscript{121,122}. Members of the OA class of DSP toxins were also shown to induce tumour formation by initiated cells. In two-stage carcinogenesis experiments on mouse skin, OA and DTX-1 showed a potency comparable with TPA. However, OA, DTX-1 and another structurally unrelated compound, calyculin A, were found to be non-TPA-type tumour promoters. Subsequently, it is thought that these compounds had their own binding protein(s) which were different from the phorbol ester receptor. It was discovered that calyculin A also binds to the OA binding protein(s). OA, and possibly DTX-1, binds to a particular fraction of mouse skin. It is of interest to note that the binding sites of OA are also present in the stomach, the small intestine and the colon, as well as in other tissues. According to Suganuma \textit{et al.}, OA and DTX-1 might also act as tumour promoters in the stomach \textsuperscript{123,124}. The possible implications of the tumour promoting capacity of the diarrheagenic toxins on human health warrants further study, particularly the implications for stimulation of growth of gastrointestinal tumours.
1.10.1 Mode of action of okadaic acid and its derivatives and their effect on protein phosphatases

The first clue to the mechanism of action of OA was the discovery that it caused long-lasting contraction of smooth muscle from human arteries\(^{(125)}\). Smooth muscle contraction is triggered by phosphorylation of a subunit of myosin, and the effect of OA was due to inhibition of myosin light chain phosphatase \(^{(126)}\). Later it was shown that OA is a potent inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), two of the four major protein phosphatases in cytosol in mammalian cells\(^{(127,128)}\).

Phosphorylation and dephosphorylation of proteins are among the most important regulatory processes in eukaryotic cells\(^{(127,128)}\). Cellular processes as diverse as metabolism, membrane transport and secretion, contractility, cell division and others are all regulated by these versatile processes. PP1 and PP2A have very broad and overlapping specificities \textit{in vitro} \(^{(127)}\). They are involved in almost all phosphatase activities in muscle and liver cells toward approximately 20 phosphoproteins with a wide array of functions.

1.10.2 Mutagenicity of OA

OA did not induce mutations in \textit{Salmonella typhimurium} TA 100 or TA 98, with or without microsomal activation enzymes added. However, it was strongly mutagenic to Chinese hamster lung cells without a microsomal activation system. In this assay, diphtheria toxin resistance (DTr) is used as a selective marker of mutagenesis. The mutant frequency was calculated to be 5500 per 10\(^6\) survivors/\(\mu\)g within the linear part of the dose-response curve (between 10 and 15 ng/ml). This value is comparable to that of 2-amino-N\(^6\)-hydroxyadenine, one of the strongest known mutagens. These results indicate that OA increased the number of DTr cells by induction of a mutation for the DTr phenotype, and not by selection of spontaneously induced DTr cells. Hokama \textit{et al.} have proposed a possible mechanism for the mutagenic action of OA. They consider that induction of the DTr mutation is not due to OA-DNA adduct formation, but more probably operates via modification of the phosphorylation state of proteins involved in DNA replication or repair\(^{(129)}\). Further research is needed to produce the necessary background data for the estimation of possible human health risk from chronic exposure to OA and other DSP toxins.
1.10.3 Immunotoxicity of OA

Effects of OA on the function of the immune system have been studied. Hokama et al. studied the effect of OA on the peripheral blood monocytes of man in vitro by means of the effects on the interleukin-1 (IL-1) synthesis. OA at concentrations of 0.1-1.0 μg/ml induced a marked suppression of IL-1 production in the monocytes. At higher concentrations, OA killed the cells. The suppressive effect of OA on IL-1 is readily reversed by specific monoclonal anti-OA. The mode of action of OA on monocytes is presently unknown.
1.11 METHODS FOR THE DETERMINATION OF DSP TOXINS IN SHELLFISH

Since the first recorded incidences of DSP in Japan in 1976 (52), public concern over the consumption of seafoods, has led to many countries setting up phytoplankton monitoring programs. The need for the setting up of ‘marine biotoxin regulations’ is of paramount importance. Environmental surveillance for toxic algal species in areas where shellfish are grown and regulatory control to ensure that toxin-contaminated shellfish do not reach the consumer are needed.

The DSP group of toxins present a significant challenge to the analytical chemist faced with the responsibility of developing methods for their analysis. The structural differences between the three sub-groups of DSP toxins (previously mentioned) further complicates the development of analytical techniques. Thus, a procedure optimised for one group of toxins is unlikely to be applicable to another group present in the sample. In view of the high toxicities of these compounds analytical methods must be capable of providing high sensitivity, while complex shellfish tissue matrices demand high selectivity.

Bioassays are the most common methods used by regulatory bodies world-wide for the determination of DSP toxins. They detect the presence of DSP toxins, but they do not give quantitative or qualitative information about the specific toxins present in the sample.

The EU Directive 91/492 states:

"The customary biological testing method must not give a positive result to the presence of diarrhetic shellfish poison (DSP) in the edible parts of the molluscs (the whole body or any part edible separately)."

However, the directive allows member states a free choice of animal bioassay to assess diarrhetic toxicity in shellfish but does not specify actual toxic limits. The consequence is a lack of harmonisation between member states and confusion with respect to interpretation of permitted toxic levels in shellfish.
1.11.1 Bioassays

1.11.1.1 Adult mouse bioassay

Following the outbreak of DSP in Japan in 1976 and 1977, it was Yasumoto et al who first used a mouse bioassay for the determination of DSP toxins. 10g of hepatopancreas from the suspected shellfish, were extracted three times with 50 ml portions of acetone for 2 minutes at room temperature. The acetone from the combined extracts was removed under reduced pressure and made up to 2 ml with 1% Tween 60 solution. 0.5-1.0 ml aliquots of this solution, or serially diluted solutions, were given intraperitoneally to female mice (15-20 g). Toxicity of shellfish was expressed in terms of mouse units per gram (MU/g) of the hepatopancreas\(^{(52)}\). The mouse bioassay proved to be quick and resulted in all lipid toxins being extracted. However, interferences include PSP toxins or free fatty acids\(^{(130-132)}\).

The Ministry of Health and Welfare in Japan adopted a modified version of this mouse bioassay as their official mouse bioassay method. This method also expresses toxicity as a mouse unit, but it is defined as the amount of toxin(s) required to kill 2 out of 3 male mice of 20g body weight in 24 hrs\(^{(85)}\). One MU (expressed as \(\mu g/Kg\) of mouse body weight) corresponds to 200\(\mu g\) okadaic acid, 160\(\mu g\) DTX-1, 250\(\mu g\) DTX-3 (7-O-palmitoyl DTX-1), 250-770\(\mu g\) PTX's and 100\(\mu g\) YTX\(^{(68)}\). The toxicity limit in Japan is 5.0 MU/100 g mussel meat\(^{(133)}\). The mouse bioassay is currently used in Belgium, Luxembourg and Spain. PSP toxins can alter the results of this method, as they are extracted together with the DSP toxins during the acetone treatment and the final extract was found to be highly toxic to mice.

Yasumoto et al. overcame the problem of PSP interferences by introducing a diethyl ether extraction of the aqueous residue after the acetone extraction. Combined ether extracts are then backwashed twice with small portions of water. The ether is then evaporated, re-suspended in 1% Tween 60 solution and injected into mice. This removed the problems of PSP interferences. However, the problem of free fatty acid interferences was not overcome. Another disadvantage of the assay is that yessotoxins were found to have a poor solubility in diethyl ether\(^{(134)}\). This test is currently used in Denmark and a similar test is used in Italy and the United Kingdom where a result is positive if 2 out of 3 mice die within 5 hrs.
A modified test is also used in France in which toxicity is expressed differently from that of the official Japanese method. Acetone is used to obtain three successive extractions from 30 g of hepatopancreas. After solvent evaporation, the residue is made up in 6 ml of 1% Tween 60 solution. 1 ml of this solution is administered to 3 male mice by i.p. injection. If the 3 mice survive for more than 5 hrs the shellfish are considered safe for human consumption. However, as a precautionary measure the mice are kept under observation for at least 24 hrs\textsuperscript{(73,74)}. This test is also used in Portugal.

*Institut Francais de Recherche pour l'Exploitation de la Mer* (IFREMER) has further modified this test so that free fatty acids are removed. After acetone evaporation, the residue is dissolved in 80% methanol and washed twice with hexane. The methanol is evaporated before injection and the residue re-suspended in 1% Tween 60. While this removes the free fatty acids, the acyl derivatives of okadaic acid, DTX-1 and DTX-2 are also removed in the hexane\textsuperscript{(132)}.

The European Communities Reference Laboratory on Marine Biotoxins, Vigo, Spain, has proposed that another modification of this assay be used as the standard European mouse bioassay. This involves replacing the diethyl ether extraction with dichloromethane in the above method. The assay is considered positive if two out of three mice die in 5 hours. This allows the detection of all DSP toxins, but one disadvantage is that free fatty acids can still interfere with the analysis leading to false positive results\textsuperscript{(132)}.

### 1.11.1.2 Suckling mouse bioassay

The toxins are extracted from the shellfish and made up in 1% Tween 60 solution as for the adult mouse test. 0.1 ml of the sample suspensions and a drop of 1% Evan's Blue solution via Teflon tubing are orally administered to 4-5 day old mice. The mice are kept at 28 °C for 4 hrs and sacrificed.
The entire intestine is removed, weighed and the fluid accumulation (FA) ratio is then calculated (FA ratio is defined as the ratio of the weight of the intestine to that of the remaining body). An FA ratio of 0.09 corresponds to 0.1 MU\textsuperscript{(114)}.

Fig. 1.10

*Shows fluid accumulation in suckling mouse caused by administration of DSP toxins*\textsuperscript{(114)}.  

This test has advantages in that it is fast, has a low threshold and is more easily related to human symptomatology than the adult mouse test. However, the test is not suited for routine use. A continuous supply of infant mice is difficult to ensure during a DSP outbreak, wounding of the infant mice is common on administration of the toxin solution, quantification of results is difficult and pectenotoxins and yessotoxins are not detected\textsuperscript{(73)}.

### 1.11.1.3 Rat intestinal ligated loop bioassay

Edebo *et al.* has quantitatively determined OA and DTX-1 from toxic mussels in ligated intestinal loops of rats. Male Sprague rats (7 week old) weighing 150-170g, are anaesthetised with ether. A longitudinal abdominal cut is performed and one ligation each adopted ca 10 cm as well as ca 25 cm aborally (distally) of Treitz's ligament. 2 ml of homogenised mussel tissue, suspended in phosphate-buffered saline, is injected into the closed loop. The skin is closed with clamps and the rats left until they are killed by cervical spine dislocation (normally 2 hrs). The clamps are removed, the loop dissected, weighed and measured. The secretion expressed in mg/cm, is calculated from the ratio of the weight of the sample loop to the length of the sample loop, minus the same ratio of the weight of a control loop to the length of the control sample loop. The toxicity limit for this test is 100 mg/cm secretion. The detection limit of the test corresponds to 0.5 μg OA.
This is a fast and sensitive bioassay and is linear in the range of 0.5-5 μg OA. However, the test is not recommended for routine surveillance and it has only been validated for okadaic acid and DTX-1 \(^{116}\).

### 1.11.1.4 Rat bioassay

Marie Kat et al. first reported a test for the determination of DSP toxins using white rats. It is currently used as the official test in The Netherlands, Germany and Ireland. The meat of ten mussels is mixed with 6 g ground normal rat feed and offered to a test animal of 100-120 g weight which has been starved for 24 hours. The refusal or partial refusal of this mixture and the consistency of the faeces produced (diarrhoea or soft faeces) point to the presence of DSP toxins \(^{48}\). From the combination of both reactions in the test animal, the severity of the toxicity can be read from Table 1.03.

<table>
<thead>
<tr>
<th>% eaten of the mussel mixture offered</th>
<th>Consistency of the faeces in test animal</th>
<th>Degree of toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100-90</td>
<td>normal</td>
<td>negative</td>
</tr>
<tr>
<td>90-50</td>
<td>normal</td>
<td>slightly toxic +</td>
</tr>
<tr>
<td>80-50</td>
<td>soft-diarrhoea</td>
<td>moderately toxic ++</td>
</tr>
<tr>
<td>&lt; 50</td>
<td>diarrhoea</td>
<td>seriously toxic +++</td>
</tr>
</tbody>
</table>

Table 1.03

*Diarrhetic mussel poisoning parameters, in relation to the % eaten of a mussel mixture offered to test animals* \(^{48}\)

This is only a semi-quantitative test and it does not detect yessotoxins or pectenotoxins. To account for the variable weight of mussels, the Irish Fisheries Research Centre, now use 10 g of mussel hepatopancreas in this test.

### 1.11.1.5 Daphnia magna bioassay

More recently, Vernoux et al. described an assay using *Daphnia magna*, a species of fresh water cladoceran invertebrate, to analyse OA concentrations in mussel extracts. A linear correlation was found between OA concentration determined by the bioassay and by liquid chromatographic analysis. The *Daphnia magna* bioassay is 10 times more
sensitive than the mouse bioassay and it was suggested that it could be used as a replacement for the latter in the screening of shellfish contaminated by DSP toxins\textsuperscript{131,135}.

1.11.2 Biochemical Assays

For ethical reasons, there is a growing concern about the continued use of mammals for bioassays. Over the past decade research has concentrated on the developing of more humane bioassays that do not involve the use of live animals.

1.11.2.1 Immunoassays

Immunoassays evolved as a medical technique to avoid the long and uncertain process of culturing pathogens. Once an animal has been exposed to a foreign substance, such as a toxin, it produces antibodies which bind to sites on the foreign molecule.

The immunoassay techniques of radio-immunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA), have been developed for the detection of OA. Levine \textit{et al.} reported the production of antibodies and the development of a RIA for OA. OA was conjugated to bovine albumin and used to immunise rabbits, which responded by producing anti-OA antibodies. Competitive binding of OA with $^3$H-OA in the test system, as measured by scintillation counting and was found to have occurred at concentrations as low as 0.2 pmoles. When the percentage inhibition was plotted against the log of the OA concentration, a linear graph resulted in the approximate range 0.2-9.0 pmoles\textsuperscript{136}.

The ELISA technique is described as follows: A microtitreplate, capable of holding 96 different samples, is coated with antibody and after any excess has been washed off, the sample is added. After binding, any excess sample is washed away and another dose of antibody is added, so that a sandwich of antibody and antigen is formed. Then another antibody, which carries on it an enzyme, is added. This binds to the preceding antibody. Finally, the enzyme substrate is added and the resulting colour change can be read. The enzyme amplifies the response. Low levels of bound antibody can produce a detectable colour change. The higher the absorbance, the more antibody is bound to the plate and the lower the concentration of DSP toxins in the sample. Several samples can be analysed in 20 minutes and the assay is sensitive to 10 ppb for okadaic acid. Thus, the
test is sensitive, specific and rapid. The ELISA procedure for OA is currently patented and marketed as a kit under the name 'DSP Check' by Sceti Ltd., Tokyo, Japan. The fact that OA is used as coating in this assay, means that these kits are expensive. Shestowsky et al. have developed an alternative immunoassay in which they use two mouse monoclonal antibodies to OA. One of these antibodies is an anti-OA monoclonal antibody (mAb) called 6/50 (idiotype (Id)) and the other is a syngenic anti-anti-OA mAb called 1/59 (anti-Id). The 1/59 anti-Id mAb is an internal image of okadaic acid and can be substituted for the OA coating on the solid phase. 1/59 anti-Id competes with free okadaic acid analyte or standard, for binding to a limited amount of anti-OA mAb in a liquid phase. The bound 6/50 is then quantified by an enzyme-conjugated anti-mouse antibody. The assay permits reliable measurement of OA in the 9-81 ppb range and is marketed as a kit by Rougier Bio-Tech Ltd., Montreal, Canada.

1.11.2.2 Protein Phosphatase Inhibition Assays
Holmes et al. reported the analysis of OA and DTX-1 in toxic shellfish by a liquid chromatography-linked protein phosphatase bioassay. The toxins were resolved by liquid chromatography, then quantified by specific inhibition of both protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) catalytic subunits, in a $^{32}$P-phosphorylase $a$ phosphatase radioassay. The concentrations of okadaic acid needed to show a 50% inhibition of PP1 and PP2A (IC$_{50}$) in the assay, were 19 nM and 0.2 nM respectively. Thus OA and DTX-1 both inhibit PP2A, 90 (±10) fold more strongly than PP1. Identification of OA and DTX-1 can be confirmed by inspection of relative PP1 : PP2A inhibition ratios.

Pectenotoxins and yessotoxins were found to be essentially inactive against PP1 and PP2A.

Simon et al. have developed a colourimetric phosphatase-inhibition bioassay for the quantitative measurement of OA and its derivatives. The assay uses semi-purified protein phosphatase PP2A-c containing extract from rabbit muscle and an artificial substrate, paranitrophenylphosphate. It has a detection limit of 0.4 pmol OA and is reported to be rapid, accurate, reproducible, specific and easily carried out.
1.11.2.3 Cytotoxicity assays

The cytotoxic effect of OA has been recognised since its isolation by Tachibana et al. \(^{(55)}\). It was noticed that when rat hepatocytes were exposed to toxic shellfish extracts, a rapid leakage of lactate dehydrogenase occurred, while non-toxic extracts had no disintegrating effect on the cell membranes \(^{(142,143)}\).

A DSP toxin cytotoxicity test has been reported, based on morphological changes of rat hepatocytes when exposed to toxins. The three classes of DSP toxins can be determined and they all produce different changes in the cell's morphology. However, the analysis is time consuming and results are confusing when toxin mixtures are involved \(^{(144)}\).

Amzil et al. have developed a novel rapid cytotoxicity bioassay for OA detection. The method evaluates the minimal active concentration (MAC) of shellfish extracts by microscopic observation of toxin-induced morphological changes on KB cells. A high correlation was found between the logarithms of the MAC of tested extracts and the OA concentrations in mussel hepatopancreas measured by liquid chromatography. Approximately half of the cells treated with an equivalent of the MAC showed epithelial features and the other half showed round features as a result of toxin modification. High doses of toxin results in all the cells becoming round. The MAC of mussel extract is measured by the incubation of 50 μl of serially diluted mussel extracts with 50 μl of a 200,000 cell/ml suspension. The minimal active quantity of OA in the test was 0.125 μg \(^{(131,145)}\).

A non-specific quantitative analysis of the toxicity of maitotoxin and OA, using the uptake of neutral red (measures cell viability) by mammalian fibroblasts in culture has been used as a toxicity measurement. More specific responses were obtained by morphological analysis. Cells exposed to OA characteristically presented a two step morphological change- they first became square with membrane microbleeding and subsequently became round.

Cells are incubated with toxic extracts for up to 24 hours at 37°C in a 5% CO\(_2\) incubator. The cells were tested for morphological changes, using a contrast transmission microscope and also for neutral red uptake, by measuring the absorbance at 540 nm. Concentrations of maitotoxin and OA in shellfish extracts were determined from dose-response curves \(^{(146)}\).
1.11.3 Instrumental / Chemical Assays

To date, chemical assays are the only assays that can determine the specific toxins involved in a DSP intoxication. Instrumental methods have the potential for fully automated, sensitive and precise quantitation of known toxins, as well as confirmation of structural identity.

High-performance liquid chromatography (HPLC) is the most valuable analytical tool for toxins because it is so well suited to the analysis of such high molecular weight and non-volatile compounds. As the DSP toxins are not naturally fluorescent, they must first be derivatised prior to analysis with a fluorescent labelling agent. Fluorescent detection is usually employed as it is many times more sensitive and selective than UV detection.

The okadaic acid class of DSP toxins are reacted with a reagent that converts the carboxylic acid moiety into a fluorescent ester. One problem, associated with this procedure, is that the resulting chromatograms are often very complex due to artefact peaks from reagent impurities and fluorescent derivatives of endogenous compounds in the sample. A number of fluorescent reagents have been used in the HPLC analysis of the okadaic acid class of DSP toxins.

A fluorimetric LC analysis of the 9-anthrylmethyl derivatives of the acidic diarrhetic toxins has become an established method and this protocol was recently subjected to critical examination. However, the derivatising reagent, 9-anthrylmethyl-diazomethane (ADAM), is somewhat unstable and problems due to artefact peaks in LC are common. Other derivatising reagents have been used for the determination of OA in shellfish, including; 1-pyrenediazomethane (PDAM), 4-bromomethyl-7-methoxy-coumarin (Br-Mmc), 9-chloromethylanthracene (CA), N-(9-acridinyl)-bromoacetamide (NABA), 1-bromoacetylpyrene (BAP) and 2,3-(anthracendicarboximido)-ethyltrifluoromethane sulphon-ate (AE-OTf). Difficulties arise with each of these methods in determining trace analytes in the presence of large reagent peaks and artefacts. The application of column switching procedures with several of these reagents has recently been employed to aid automation.

Note: The fluorimetric LC analysis of the 'OA class' of DSP toxins will be discussed in more detail in Chapter 2.
1.11.3.1 Detection of okadaic acid and its derivatives using high performance liquid chromatography coupled with mass spectrometry (LC-MS)

Mass spectroscopy (MS) is a powerful tool for the analysis of marine toxins. Besides high sensitivity and selectivity, MS can provide structural information useful for the confirmation of toxin identity, and also aids the identification of new toxins. The greatest potential of MS for the routine analysis of 'real samples' lies in its combination with liquid chromatography (LC). Although LC-MS equipment is expensive, the possibility of automation can begin to justify such an investment if large numbers of samples can be screened quickly. For research studies LC-MS will facilitate a greater understanding of the chemistry and biochemistry of marine toxins.

Initial research in mass spectral characterisation of DSP toxins was carried out using fast atom bombardment (FAB) ionisation \(^{50,85}\). Pleasance \textit{et al.} developed an LC-MS method, using an ion-spray (ISP) interface and atmospheric pressure ionisation (API), for the determination of OA and DTX-1. The LC column was connected to the ion-spray interface with a fused silica capillary. The positive ion ISP mass spectra of OA and DTX-1 were simple, with abundant peaks due to the protonated toxins \([M+H]^+\) occurring at m/z 805 and 819 respectively. Selected ion monitoring (SIM) at these m/z values was found to give the best sensitivity and selectivity for the analysis of OA and DTX-1. The method was not as sensitive as the HPLC-FLD method reported by Lee \textit{et al.}, but had the advantage that no derivatisation or complex clean-up stages were required. The detection limit was found to be 2 ng of toxin injected and linearity was observed for up to 50 ng of injected toxin. This method was used to confirm the presence of OA in \textit{Prorocentrum concavum} and in natural populations of dinoflagellates from eastern Canadian waters. This particular technique was later used to detect the new DSP toxin 'DTX-2' in Irish mussels as well as 7-O-acyl derivatives of OA, DTX-1 and DTX-2 \(^{64}\).

An improvement to the LC-MS method was reported by Pleasance \textit{et al.} using a 1mm i.d. column. DSP toxins were eluted with a linear gradient mobile phase of 40-100% aqueous acetonitrile in 20 minutes (with 0.1% trifluoroacetic acid (TFA)). A modified ISP interface was used which allowed more precise control in the positioning of the ISP needle in relation to the sampling orifice, resulting in a reduction of the background
chemical noise. These improvements resulted in a 10-fold increase in the detection limit of the method, making it more sensitive than the HPLC-FLD method developed by Lee et al. The improved detection limits permitted the analysis of whole mussel tissue\(^{(157)}\).

Quilliam et al. further improved the sensitivity of the LC-MS method by 10-fold using a methanol:water (7:3 with 0.1% TFA) mobile phase. The optimum flow-rate was determined to be 20 to 30 µl/min with 7-0-acetylokadaic acid (AcOA) being employed as an internal standard. An improved clean-up step, using aminopropylsilica SPE cartridges was used for mussel extracts. This helped protect the LC columns, reduced interferences and allowed concentration of extract to lower detection limits. It was suggested that collision induced fragmentation before the sampling orifice of the LC-MS or LC-MS/MS could be used to provide structural information about the toxins or to provide confirmatory fragment ions\(^{(147)}\).

Studies have also been carried out on the ‘OA class’ of toxins to see if derivatisation would help increase the sensitivity of the LC-MS method. Derivatisation with ADAM did not give better sensitivity than the underivatised method. This was attributed to the injection of large amounts of excess reagent and its side products and the preference of the ISP ionisation process for polar molecules\(^{(158)}\).

On the other hand, when N-(9-acridinyl)-bromoacetamide (NABA) was used to derivatise OA and DTX-1, the products formed were 5-fold more sensitive to the LC-MS method than the underivatised standards. The NABA-derivatised analytes were well resolved on reverse phase columns and showed good peak shape when protonated. It is the protonated nitrogen which also helps the sensitivity under ISP ionisation conditions, as the ion-evaporation process favours pre-formed ions in solution. Compared to OA and DTX-1, the derivatised toxins were detected with characteristic shifts in mass and retention time. Therefore, this method can be useful for additional confirmatory evidence for the identity of a particular toxin\(^{(157)}\).

The analysis of DSP toxins derivatised with Br-Mmc, using atmospheric pressure /electrospray ionisation (API/ESI) MS has been achieved by Luckas et al. However sensitivity data for the method was not reported\(^{(158)}\).
1.11.3.2 Detection of okadaic acid and its derivatives using gas chromatography

Murata et al. first applied gas chromatography (GC) for the detection of OA and DTX-1. The toxins were trimethylsilylated with TRI-SIL‘Z’ reagent either intact or after derivatisation with diazomethane into methyl esters. A glass column (3mm x 1m) packed with 2% OV-101 on 60/80 mesh Uniport HP was used for the analysis. The column temperature was maintained at 320 °C and a nitrogen flow rate at 30 ml/min \(^{(53,63)}\). The method is unlikely to find wide usage due to difficulty in the extraction and derivatisation steps.

1.11.3.3 Detection of the pectenotoxins (PTXs) by high-performance liquid chromatography

PTX-1 and PTX-4 have primary hydroxyl groups and can be converted into fluorescent esters by derivatisation with 1-anthroylnitrile in the presence of triethylamine (Fig. 1.11). Chromatographic separation was achieved on a Develosil ODS column with an acetonitrile:water (8:2) mobile phase. The excitation and emission wavelengths for the analysis are 365 and 465 nm, respectively \(^{(68,159,160)}\). PTX-2 and PTX-3 can be analysed on a Develosil ODS column with mobile phase acetonitrile:water (6.5:3.5) and UV detection at 235 nm \(^{(160)}\).

![Fig. 1.11 Reaction of 1-anthroyl nitrile with alcohols](image)

Like the okadaic acid class of DSP toxins, PTX-6 and PTX-7, possess a carboxyl group and can be detected by derivatisation with ADAM followed by fluorimetric detection. Using the Lee et al. method of analysis, they were seen to elute before OA \(^{(90,160)}\).
1.11.3.4 Detection of the yessotoxins (YTX) by high-performance liquid chromatography

Chromatographic separation of Yessotoxins and 45-hydroxy-yessotoxin has been achieved on a Develosil ODS column with a methanol : 0.1 M sodium phosphate buffer (9:1) mobile phase and UV detection at 230 nm.

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Chapter 2

HPLC Methodology for the determination of DSP toxins (‘OA Class’)

....those sciences are vain and full of errors which are not born from experiment, the mother of all certainty.....

Leonardo Da Vinci (1452-1519)
2.1 INTRODUCTION

As previously mentioned, OA has been the predominant toxin responsible for most of the outbreaks of DSP in Europe (1). However, the predominant toxin detected during Irish DSP incidences is an isomer of OA, Dinophysistoxin-2 (DTX-2) (2). To date, yessotoxins or pectenotoxins have not been detected in Irish shellfish (3) and, in view of this, the analysis of mussels in this project targeted acidic diarrhetic toxins using fluorescent HPLC methods.

2.2 THE DEVELOPMENT OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODS FOR THE DETECTION OF THE ‘OA CLASS’ OF DSP TOXINS

Chemical assays are the only techniques that can determine the specific toxins involved in a DSP intoxication. Instrumental methods have the potential for fully automated, sensitive and precise quantitation of known toxins, as well as providing confirmation of structural identity. Furthermore, methods combining chromatographic and spectroscopic techniques are valuable for the identification of new toxins.

High-performance liquid chromatography (HPLC) is a valuable analytical tool for the analysis of shellfish toxins which are non-volatile compounds. HPLC provides excellent quantitative precision and is readily automated. However, problems arise for the OA class of toxins which do not possess a chromophore for sensitive UV absorption or fluorescence detection. Either pre- or post-column derivatisation is required for the optical detection of these toxins.

DSP toxins present a challenge to the development of analytical protocols. Due to their high molecular weight and lipophilic nature, it is difficult to separate the toxins from endogenous substances in shellfish tissue. Since the DSP toxins are not naturally fluorescent, they must first be derivatised prior to analysis with a fluorescent labelling agent. Fluorescent detection is employed since it is more sensitive than UV detection. The OA class of DSP toxins can be reacted with compounds which convert the carboxylic acid moiety into fluorescent esters. One problem associated with this procedure is that the resulting chromatograms are frequently complex due to reagent impurities and other fluorescent derivatives of endogenous compounds in the sample. Like all methods based on derivatisation chemistry, effective sample clean-up procedures
are essential for reproducible quantitative analysis. To date six, different fluorogenic reagents have been successfully applied to the analysis of the OA class of DSP toxins.

2.2.1 (1) Derivatisation with 9-anthryldiazomethane and analysis by HPLC-FLD

Nimura et al. first reported the use of 9-anthryldiazomethane (ADAM) as a fluorogenic reagent (Fig. 2.01) in the HPLC analysis of picomole amounts of short chain fatty acids (C3-C18) (4). 50μl aliquots of methanolic fatty acid solutions (concentration ranging from 50-100 μg/ml ) were added to equal volumes of a 0.1 w/v% ADAM solution and allowed to stand for 60 min. HPLC analysis of 10 μl portions of this reaction mixtures was carried out. Chromatographic separation was achieved on a 250 x 4.0 mm i.d. Lichrosorb™ RP-8 (5 micron) column, with a mobile phase of acetonitrile-water (90:10) at a flow rate of 1.1 ml/min, and fluorescent detection at 412nm with excitation at 365nm.

![Fig. 2.01 Reaction of 9-anthryldiazomethane with carboxylic acids](image)

Subsequently ADAM was used for the HPLC-FLD analysis of prostaglandins (5), the fatty acid composition of human serum lipids (6) and the microanalysis of free fatty acids in the plasma of experimental animals and humans (7).

More interestingly Martinez et al. applied ADAM to the analysis of monensin and related antibiotics in beef and chicken livers (8,9). Monensin, salinomycin, narasin and lasalocid, are all ionophores, belonging to a class of compounds known as carboxylic polyethers. They all have similar chemical structures which have a close resemblance to the OA class of DSP toxins (Fig. 2.02).
Fig. 2.02  *shows the similarities in the structure of A) monensin and B) the OA class of DSP toxins*

Therefore, in 1987, Lee *et al.* developed a fluorimetric HPLC method for the analysis of the okadaic acid class of DSP toxins derivatised with ADAM. Both liquid and solid phase extraction steps were employed to remove matrix effects prior to injection. A known weight of the shellfish hepatopancreas was homogenised with 80% methanol. This extract was washed with petroleum ether and the toxins extracted with chloroform. An aliquot of the chloroform extract was dried under nitrogen, and derivatised for one hour in the dark with 0.1 w/v % ADAM in methanol. Solid phase extraction (SPE) was employed to remove the excess ADAM reagent. HPLC analysis was carried out on a Develosil® ODS column (250 x 4.2 mm) with mobile phase, acetonitrile:methanol:water (8:1:1). The fluorescent intensities of the toxin derivatives were measured at an excitation wavelength of 365 nm and an emission wavelength of 412 nm and showed good linearity for both OA and DTX-1 derivatives in the range 1-80 ng. *Zhoa et al.* has also described a method for the confirmation of specific toxins whose identity is in doubt due to overlapping contaminant peaks. The eluting peaks from the
Develosil® ODS column can be collected from the outlet of the fluorimetric detector and re-analysed on a Capceil Pack CN column with acetonitrile:water (53:43) as mobile phase.  

7-O-acyl derivatives of the OA class can be determined by the Lee HPLC method using a C18 column and eluted within 20 min using 100% methanol. However, most of the acyl derivatives are extracted by the petroleum ether in the defatting stage. 

Marr et al. found that the acyl derivatives could be converted into the free toxin and the free fatty acids by alkaline hydrolysis. An extract, purified from the digestive glands of toxic mussels was found to be contaminated with OA, DTX-1, DTX-2, and small amounts of DTX-3, (a mixture of 7-O-acyl ester derivatives of DTX-1). In addition, acyl ester derivatives of OA and DTX-2 were also detected by direct LC-MS analysis and confirmed by analysis of their hydrolysis products. This was the first report of the detection of other naturally occurring 7-O-acyl esters similar to DTX-3. Using the same hydrolysis technique, Suzuki et al. has analysed the DSP toxins and fatty acid ADAM derivatives by HPLC-FLD using a stepwise gradient mobile phase.

An improvement to the sample clean-up for the Lee method was developed by Stabell et al. Ultrasonic treatment of the samples during derivatisation was found to improve reproducibility. It is thought that the ultrasonic treatment probably results in the disruption of micelles formed by toxins and other partly hydrophobic compounds, which increased access to reactive sites on toxins by ADAM. Stabell found that the use of 0.1 g silica cartridge column and different eluent compositions in the sample clean-up gave better chromatograms.

Pereira et al. investigated the influence of solvents and the concentration of the ADAM reagent on the reliability of the Lee method. They also reported an improvement in the quality of the ADAM reagent as well as the extraction and sample clean-up procedure. Isopropanol was used in the homogenisation step and the toxins were extracted with ethyl acetate and hexane. Pereira et al. found that satisfactory and reproducible recovery yields were obtained when Tetrahydrofuran (THF) and ethyl acetate were used as solvents for the derivatisation reactions, while MeOH gave poor results. This might be attributed to poor solubility of the extract and ADAM in MeOH. This explanation was supported by the work of Stabell et al. 200 µl of 0.4 or 0.2% w/v ADAM in ethyl
acetate was recommended as the optimum conditions for use in the derivatisation step. The clean-up procedure used was similar to that of Stabell et al. The samples were analysed by HPLC using a Lichrospher-100 RP 18 column, mobile phase was acetonitrile:water (8:2), with fluorescent detection at λ<sub>ex</sub> 365 nm and λ<sub>em</sub> 415 nm<sup>15</sup>. Recently, Quilliam et al. also reported improvements to the Lee et al. method. The amount of ADAM reagent used in the Lee et al. method was found to be insufficient for complete reaction with toxins in some shellfish samples. More reliable derivatisation yields were achieved by increasing the ADAM concentration to 0.2% w/v and reacting for 2 hours at 37 °C. A LiChrospher-100 RP-18 column was found to have a high efficiency and effective separation of the toxins. A mobile phase of 80% acetonitrile in water and a column temperature of 40 °C was recommended. The sensitivity of the procedure was improved by using an λ<sub>ex</sub> of 254 nm instead of 365 nm.

Custom SPE columns were prepared from freshly activated silica purchased in bulk, as the activity of silica used in commercial SPE cartridges varies considerably between different manufacturers and between different production batches. This improved the reproducibility of the method, as well as providing considerable cost savings. The use of 10% instead of 5% methanol in chloroform, as the elution solvent in the SPE clean-up, ensured, complete recovery of the analyte and did not introduce additional interferences. The analysis of OA by in situ derivatisation with ADAM, in both pure solutions and extracts from contaminated bivalves, was studied in order to overcome the problems with the reagent stability. ADAM was prepared prior to analysis by the reaction of 9-anthraldehyde hydrazone with quinuclidine and N-chlorosuccinimide in ethyl acetate. The quantitative results were identical to those obtained using commercial ADAM (solid) reagent. However, a peak nearly overlapping with OA occasionally appeared on the chromatogram of the test solution, which was believed (in the absence of mass spectral data) to be due to an artefact formed in the derivatisation reaction<sup>16-18</sup>. 

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2.2.1.1 Internal Standards for the HPLC-FLD analysis of OA class of DSP toxins

Deoxycholic acid (Fig. 2.03) has been successfully used as an internal standard for the analysis of OA and DTX-1 \(^{14}\).

![Structure of deoxycholic acid (DCA)](image)

**Fig. 2.03**  *The structure of deoxycholic acid (DCA) used as an internal standard for the analysis of the OA class of DSP toxins*

Later, Quilliam *et al.*, reported the use of 7-O-acetylokadaic acid (AcOA) as an internal standard for the analysis of the OA class of DSP toxins. AcOA (Fig. 2.04) was synthesised by the partial acetylation of OA. The use of AcOA improved the accuracy and precision of the method, by correcting for incomplete recoveries in extraction, clean-up, and derivatisation steps, and for volumetric errors and instrumental drift.

![Structure of 7-O-acetylokadaic acid (AcOA)](image)

**Fig. 2.04**  *Structure of 7-O-acetylokadaic acid (AcOA), where \( R_1 = CH_3CO \), \( R_2 = H \) and \( R_3 = CH_3 \)*

AcOA was used instead of deoxycholic acid (DCA) as an internal standard as its relative retention time was constant for different columns and temperatures. The molecular structure of DCA (Fig. 2.03) is very different to that of the DSP toxins. DCA is much
less reactive towards ADAM than the DSP toxins, which can give rise to a positive bias in results based on DCA internal standardisation (19).

2.2.1.2 Automated HPLC-FLD method for the analysis of OA class of DSP toxins
An automated HPLC method for determining DSP toxins, using a column-switching system has been described by Luckas et al. The method has some advantage in that it avoids the necessity of complex clean-up procedures after derivatisation of shellfish extracts with ADAM. Errors, due to losses of toxin derivatives, which occur in the SPE clean-up procedures, were eliminated by this method. The chromatograms were also reported to be free of interfering peaks from matrix and ADAM reagent. However, the column-switching system is more complicated and expensive, with a highly skilled chromatographer required for its operation (20, 21).

2.2.1 (2) Derivatisation with 1-pyrenyldiazomethane and analysis by HPLC-FLD
Nimura et al. (Fig. 2.05) first described the synthesis and application of 1-pyrenyldiazomethane (PDAM) for the HPLC-FLD analysis of both short and long-chained fatty acids (22). More recently the use of PDAM as a fluorometric derivatising agent for DSP toxins has been reported. An ethyl acetate solution of PDAM (0.1% w/v) was reacted with toxic shellfish extract in methanol, at 75 °C for 30 min., followed by the clean-up procedure of Lee et al. The toxins were separated by HPLC analysis using 75% acetonitrile as mobile phase with fluorimetric detection at λ<sub>ex</sub> 340 nm and λ<sub>em</sub> 394 nm emission (23, 24).

![Reaction of 1-pyrenyldiazomethane (PDAM) with carboxylic acids](image)

Fig. 2.05 Reaction of 1-pyrenyldiazomethane (PDAM) with carboxylic acids
2.2.1 (3) Derivatisation with 1-bromoacetylpyrene and analysis by HPLC-FLD

Kamada et al. first used 1-bromoacetylpyrene (BAP) as a derivatising agent for the analysis of free and conjugated bile acids in serum. Subsequently Asukabe et al. applied BAP to the analysis of polyether antibiotics. This method differed from the former, in that Kryptofix 222 was used in place of dicyclohexyl-18-crown-6, as a catalyst for the formation of the fluorescent derivatives. Asukabe et al. studied the reactivity of salinomycin with BAP using dicyclohexyl-18-crown-6 and found that the reaction yield was very low (10%). Kryptofix 222 was found to be more effective at converting salinomycin into its 1-pyreneacyl ester (yield > 70%).

The application of BAP to the analysis of the OA class of DSP toxins was first described by Dickey et al. (Fig. 2.06). The toxin extracts were derivatised with 0.1% w/v BAP in acetonitrile (0.4 ml) and 5% diisopropylethylamine (0.1 ml), for 15 min. at 75 °C. Diisopropylethylamine, which acted as the catalyst in converting the DSP toxins into their 1-pyreneacyl esters, was found to be a more effective catalyst than Kryptofix 222. The same clean-up procedure as described by Lee et al. was used and the HPLC analysis was carried out using 75% acetonitrile as mobile phase with fluorometric detection at \( \lambda_{ex} \) 365 nm and \( \lambda_{em} \) 418 nm emission. The method was linear between 1.0 and 80.0 ng of OA injected and the lower limit of detection was 0.1 ng. BAP proved to be more stable in both the solid and solution form than ADAM or PDAM. The esterification of OA and analogues using BAP was easily controlled, and the product chromatograms contained fewer extraneous peaks than the ADAM or PDAM products.

\[
\begin{align*}
\text{COCH}_2\text{Br} & \quad \text{RCOOH} \\
\text{(C}_3\text{H}_7)\text{NC}_2\text{H}_5 & \quad \text{75°C} \\
\text{COCH}_2\text{OCOR} & \quad \text{75°C}
\end{align*}
\]

Fig. 2.06 Reaction of bromoacetylpyrene (BAP) with carboxylic acids
2.2.1 (4) Derivatisation with 9-chloromethylnanthracene and analysis by HPLC-FLD

Zonta et al. applied a HPLC method using 9-chloromethylnanthracene (CA) as a derivatising agent for the analysis of OA in mussels. The toxins were extracted from the mussels by the Lee et al. method. 1 ml of the chloroform extract was taken, the solvent evaporated and 200 µl of 0.1% w/v CA in methanol and 100 µl of 25% w/v tetramethylammonium hydroxide (TMA) added to the residue (Fig. 2.07). The mixture was reacted at 75 °C for 30 min. and the Stabell et al. clean-up procedure was applied. The toxin derivatives were separated on a LiChrosorb RP-8 column using a gradient programme with acetone and water:acetonitrile (1:1) as mobile phase solvents, and fluorometric detection ($\lambda_{ex}$ 366 nm and $\lambda_{em}$ 404 nm). However, this method was not as sensitive as the ADAM method, but was able to detect the toxins at quantities below the regulatory limits in Japan. The method was also used in the determination of free fatty acids (e.g., linolenic acid), which may interfere with the DSP bioassay. DSP toxins were observed in the lipid fraction of the extract whereas no OA was detected in the analysed samples.

![Reaction of 9-chloromethylnanthracene (CA) with carboxylic acids](image)

Fig. 2.07  Reaction of 9-chloromethylnanthracene (CA) with carboxylic acids

2.2.1 (5) Derivatisation with 4-bromomethyl-7-methoxycoumarin and analysis by HPLC-FLD

More recently, Hummert et al. studied 4-bromomethyl-7-methoxycoumarin (Br-Mmc) for its suitability in the pre-column derivatisation of OA and its derivatives (Fig. 2.08). An alternative to the Lee et al. clean-up method was proposed. The toxins were extracted with dichloromethane after homogenisation of shellfish hepatopancreas in 80% methanol. The dichloromethane was removed and the residue purified on a silica gel cartridge. Samples were washed with ethyl acetate and ethyl acetate/isopropanol (1:1) and the analytes eluted with isopropanol/methanoic acid (1:1). The solvent was removed under nitrogen and the residue was made up in 170 µl acetone. A 5 µl portion of 0.1%
18-crown-6 ether in acetone, 25 μl of 0.15% Br-Mmc in acetone and 1-2 mg potassium carbonate were added. The reaction mixture was kept at 25°C for 2 hours. The derivatised toxins were analysed by HPLC, using a mobile phase composition of acetonitrile:water (7:3), with detection at λ_{ex} 325 and λ_{em} 390 nm. The clean-up method was reported to have the following advantages over the ADAM method. Firstly, the additional removal of the major lipid content of the raw extract, by extraction with the n-hexane can be omitted. Secondly, the purification step on silica is carried out before derivatisation to avoid the partial decomposition of the formed derivatives. Therefore, the additional clean-up step after derivatisation is not necessary. The Br-Mmc and its derivatives are more stable than ADAM and its reaction products. \(^{(21,29)}\)

![Reaction of 4-Bromomethyl-7-methoxycoumarin (Br-Mmc) with carboxylic acids](image)

2.2.1 (6) Derivatisation with 2,3-(anthracenedicarboximido)ethyl trifluoromethane sulphonate and analysis by HPLC-FLD

Ohrui et al. recently reported another highly sensitive HPLC method for the determination of acid DSP toxins. The extraction procedure was a slightly modified version of the Pereira et al. method \(^{(15)}\). Toxic samples were derivatised with 2,3-(anthracenedicarboximido)ethyl trifluoromethanesulphonate (AE-OTf) in acetonitrile (100 μl of 2.25 mM), in the presence of tetraethylammonium carbonate (TEAC) in acetonitrile (75 μl of 1.5 mM) (Fig. 2.09). The mixture was allowed react for 15 min. at room temperature. The solvent was removed under nitrogen and the residue was cleaned up on a silica cartridge. The residue was rinsed with 200 μl dichloromethane and 4 ml dichloromethane : acetone (97.5:2.5), following the elution of the analytes with 2 ml dichloromethane : acetone : methanol (95:5:10). The derivatives were first separated on a phenyl-silica column and then the target fraction was introduced into a separate
ODS column by a valve switching device. The toxins were separated by HPLC using 80% methanol as mobile phase with fluorometric detection ($\lambda_{ex}$ 248 nm and $\lambda_{em}$ 462 nm). The analysis of 7-O-acyl derivatives of the toxins was also reported with only slight modifications of the mobile phase compositions being required for their separation (30).

![Reaction of 2,3-(anthracenedicarboximido) ethyltrifluoromethane-sulphonate AE-OTf with carboxylic acids](image)

**Fig. 2.09** Reaction of 2,3-(anthracenedicarboximido) ethyltrifluoromethane-sulphonate AE-OTf with carboxylic acids
2.3 MODIFICATION TO HPLC PROTOCOLS

Extensive experience in using several of the afore mentioned analytical methods for the analysis of acidic DSP toxins has lead to modifications being made to a) ADAM method (Lee et al.), b) ADAM in situ method (Yoshida et al.), and c) BAP derivatisation methods (Dickey et al.). The aim was to optimise these methods, for the analysis of DSP toxins pertaining to the west coast of Ireland (31-33).

2.3.1 a) Modifications to the ADAM method (Lee et al.)

The Lee et al. method is used extensively world-wide, although, due to instability of the ADAM reagent, several laboratories have not successfully applied this method to the analysis of shellfish. A modified Lee et al. method has been adopted as the method of choice, with the best sensitivity, for the analysis of DSP toxins in our laboratories.

2.3.1.1 Modifications to the extraction procedure

In the original procedure developed by Lee et al., the toxin content of 1g of the hepatopancreas of shellfish was extracted with 4 ml of 80% methanol (169). Survey work carried out by our laboratory have revealed seasonal, geographic and individual variation of DSP toxin content in cultivated mussels (Chapter 3), which led to difficulties in obtaining a representative sample using such a small amount of material. To help overcome this problem, mussel growers were instructed to sample the top, middle and bottom of an individual mussel line, and to combine these to give one composite sample, which in turn was sent to our laboratory for analysis. The sample size of 1g of hepatopancreas taken for extraction (Lee et al. method) was increased to 6g and extracted with of 80 % methanol (12 ml).

It is important to note that the chloroform extracts, obtained by the Lee et al. extraction procedure, do not contain the 7-O-acyl derivatives of acidic DSP toxins, as they are removed by the light petroleum wash (M. Fernandez, personal communication). Therefore to determine the concentration of these toxins the extraction procedure was modified as follows. The DSP toxins in the shellfish hepatopancreas were extracted with acetone, which was then removed under vacuum and the aqueous residue extracted with diethyl ether (34).
2.3.1.2 Modifications to the derivatisation procedure

**ADAM concentration:** It has been suggested by several authors that the derivatisation method described by Lee *et al.* is insufficient for complete reaction with toxins in some shellfish samples \(^{(19)}\). For this reason, all samples and standards were derivatised with 0.2%w/v, instead of a 0.1% w/v, ADAM in methanol solution for 2 hours at room temperature. The samples are placed in an ultrasonic bath for 2 min. after the addition of ADAM and at half hour intervals during derivatisation. The ultrasonic treatment of the samples during derivatisation with ADAM was found to improve reproducibility. This may be as a result of increased access by ADAM to reactive sites on toxins due to disruption of micelles formed by toxins and other partly hydrophobic compounds \(^{(14)}\).

To increase the solubility of ADAM in methanol, the solid is first dissolved in a few drops of acetone and then diluted to the required volume with methanol. Filtering of the ADAM solution prior to use also resulted in cleaner chromatograms.

**7-O-acyl derivatives:** The analysis of 7-O-acyl derivatives of DSP toxins by this method involves the hydrolysis of an aliquot of the light petroleum extract to convert them into the free DSP toxin forms. This solution is then neutralised with acid and the DSP toxins extracted and determined by the modified Lee *et al.* method.

2.3.1.3 Modifications to the clean-up procedure

**Solvent considerations:** Chlorinated solvents such as chloroform are susceptible to degradation due to the formation of free-radicals. Light, heat and oxygen can initiate formation of free-radicals that generate decomposition products such as phosgene, hydrogen chloride and oligomers \(^{(35)}\). For this reason, commercial chloroform is usually stabilised with varying percentages of ethanol. This can lead to poor toxin recoveries using the Lee *et al.* clean-up method, since chloroform is used to wash the silica resin and chloroform:methanol (95:5) is used to elute the toxin derivatives. Consequently, if the ethanol content of the chloroform is too high, or fluctuates in concentration from one batch to another, then varying amounts of toxin derivatives may elute in the washing stage.

Normally, good recoveries of derivatised toxins \((94 \pm 5 \%)\) were obtained when the SPE tubes were washed with chloroform stabilised with 1% ethanol, (Lab-Scan, Dublin).
However, on some occasions OA peaks were not observed in the chromatograms of both OA standards and toxic samples analysed by the Lee et al. method. Trouble-shooting of the method confirmed that the problem was related to the chloroform used (Batch No. 0351/5) in the silica washing steps. When an older batch of chloroform (Batch No. 2588/4) was used, the OA peaks appeared as normal. Subsequent gas chromatographic analysis (Fig. 2.10) of the different batches revealed that Batch No. 0351/5 was in fact stabilised with between 1.3-1.5% ethanol, while Batch No. 2588/4 had an ethanol content less than 1%.

On examination of the manufacturers specifications it was noted that the specification for chloroform (Batch No. 0351/5) was 'about 1% ethanol'. The difference in results obtained with such a small variation in the manufactures specifications for ethanol content may explain the difficulties experienced by some laboratories with this method. Quilliam et al. recently reported that 1.15 ± 0.05% was the optimum ethanol content for chloroform used in the Lee et al. clean-up procedure with a custom silica SPE (19). Ethanol content less than this leads to the inadequate clean-up of samples while higher percentages of ethanol gives poor toxin recoveries. It has been suggested that the ethanol, and chloroform decomposition products be removed from the chloroform using an activated alumina column and then to subsequently adjust the ethanol content to the optimum percentage for efficient silica clean-up (19).

These problems have been overcome in our laboratory by using chloroform stabilised with 50ppm amylene, which was spiked with ethanol to produce a concentration of 1.15% v/v. The ethanol content was routinely monitored, by GC analysis, to ensure the reproducibility of toxin recoveries.

Figure 2.11 shows a toxic mussel sample derivatised with solid ADAM and chromatographed on a C-18 column.
Fig 2.10

Gas chromatogram obtained for the analysis of commercial 'HPLC grade' chloroform spiked with isopropanol as internal standard.

Retention times are: ethanol 1.272 min, chloroform 1.849 min. and isopropanol 3.703 min. (See Appendix No. 2 for experimental conditions)
HPLC Detection: A recent report has suggested that an $\lambda_{\text{ex}}$ of 254 nm rather than 365 nm increases the sensitivity of the Lee et al. method (19). However, as this also results in a loss of selectivity, it is preferable to use the 365 nm excitation wavelength.

Fig. 2.11

Shows a Chromatogram of a toxic mussel sample from site #1 15/9/91, containing OA (16.46 min) and DTX-2 (18.95 min) derivatised using the solid ADAM method with a mobile phase of acetonitrile:water (80:20).
(Data file S1D15M9)
2.3.2 b) Modifications to the ADAM (in-situ) method (Yoshida et al.)

The in-situ ADAM method was carried out as described by Yoshida et al. The ADAM solution is filtered before use and 200 µl is then used to derivatise both the chloroform extracts of shellfish and the toxin standards. Modifications to the Lee et al. procedure as described were also incorporated into this procedure.

2.3.2.1 Preparation Of ADAM Solution (in-situ ) method

A solution of 9-anthryldiazomethane, which can be used directly as a derivatising solution for the analysis of the acidic DSP toxins, was prepared following a method of Pleasance et al. (17). This, in turn, was a modification of a method originally developed by Yoshida et al. (16). 9-anthraldehyde hydrazone was prepared by following the procedure of Nakaya et al. (36) involving the reaction of 9-anthraldehyde with hydrazine hydrate (Fig. 2.12). 9-Anthraldehyde hydrazone was oxidised with an organic oxidant, N-chlorosuccinimide, in the presence of a basic catalyst, quinuclidine (Fig. 2.13).

2.3.2.2 Preparation of ADAM solution from 9-anthraldehyde hydrazone

A solution of 8.8 g of 9-anthraldehyde in 150 ml of ethanol was stirred with 8.5 g of hydrazine hydrate(80%) at room temperature for 3 hours. After 10 min. a noticeable colour change was observed in the reaction mixture with the initial yellow colour changing to a dark yellow product. The solid product was filtered and dried under vacuum. The product was recrystallised from ethanol, yielding red/yellow crystals which showed a sharp melting point at 124-126 °C.

![Synthesis of 9-anthraldehyde hydrazone](image)

Fig. 2.12 Synthesis of 9-anthraldehyde hydrazone
2.3.2.3 Synthesis of 9-anthraldehyde hydrazone

An ethyl acetate solution of 9-anthraldehyde hydrazone (500 µl, 35 mM) was mixed with quinuclidine (500 µl, 70 mM) and N-chlorosuccinimide (500 µl, 7 mM). All the reagents were dissolved in ethyl acetate. The mixture was allowed to stand at room temperature for 1 hr. before use.

![Synthesis of 9-anthryldiazomethane (ADAM)](image)

**Fig. 2.13** Synthesis of 9-anthryldiazomethane (ADAM)

**Fig. 2.14** Chromatogram of a toxic mussel sample from site #10 (23/9/91) containing OA (10.87 min) and DTX-2 (11.90 min) derivatised with the ADAM in-situ method using a mobile phase of acetonitrile:methanol:water (80:10:10). (Data file 5-8-93.301)
2.3.3 c) Modifications to the BAP method (Dickey et al.)

2.3.3.1 Modifications to the Extraction and Clean-Up procedure
Since both the Dickey et al. (27) and the Lee et al. method use the same extraction and clean-up procedures, any modifications made to these stages of the Lee et al. method were also incorporated into the Dickey method.

2.3.3.2 Modifications to the Derivatisation procedure
A number of factors were examined in the course of optimisation studies on this derivatisation, including temperature, time and base concentration. Thus, using reaction temperatures for derivatisation ranging from 40°-90° C, it was found that although peak areas increased with temperature, interfering artefact peaks from reagent decomposition were observed above 75° C. At this temperature, maximum peak areas were observed after 15 min. and remained unchanged up to 30 min. The time used in the protocol was therefore set at 20 min. Improved derivatisation, as a result of increasing base concentration was also observed and 5% diisopropylethylamine was found to be optimum. At base concentrations higher than 5% interfering artefact peaks appeared in the chromatograms (Fig. 2.15).

2.3.3.3 Modifications to the HPLC procedure
Similar HPLC conditions to those of Lee et al. were used so that retention time data for toxins analysed under the same conditions, but with different derivatising agents, can be compared. With the BAP method, excitation and λ<sub>em</sub> of 365 nm and λ<sub>ex</sub> of 418 nm are always used.
Fig. 2.15

Shows a chromatogram of a 10ng (on-column) OA standard (11.32min.) derivatised using the BAP method with a base concentration of 10%. Note the artefact peaks at 16.24, 17.08 and 17.9min. (Data file 10-8-95.1510)

Fig. 2.16

Chromatogram of a toxic mussel sample from site #9 8/6/93 containing OA (11.9 min.) and the internal standard DCA (19.10 min.) derivatised using the BAP method with an optimum base concentration of 5%.

(Data file 10-2-94.401)
2.3.4 Modified analytical methods

The modified versions of methods a) Lee, b) Dickey and c) Yoshida are detailed below.

2.3.4.1 Modified extraction procedure

2.3.4.1.1 Extraction procedure for the acidic DSP toxins

Preparation of shellfish samples

The digestive glands (hepatopancreas) were cut from mussels (*Mytilus edulis*) and stored at -20°C prior to extraction. A portion of homogenised shellfish hepatopancreas equivalent to 6g, was extracted with methanol:water (4:1, 12ml) and, after centrifugation at 3,000 rpm (10 min.), an aliquot (2.5 ml) of the supernatant was washed with light petroleum (40-60°C), 2 x 2.5 ml, by vortex mixing for 1 min. The upper layer was discarded. Water (1ml) and chloroform (4ml) were added to the remaining solution and the mixture was vortex mixed for a further 2 minutes. Note: If the layers did not separate, the mixture was centrifuged in glass tubes for 1 min. The lower chloroform layer was removed and retained. Additional chloroform (4ml) was added to the remaining solution and the solution was vortex mixed for a further 2 min. The lower layer was removed and combined with the first chloroform extract. This combined 'chloroform extract' was made up to 10ml and stored in a freezer (-20°C) until required for analysis. (Note: If the sample was not to be analysed within a 1 week period of time the chloroform extract was blown down under nitrogen and sealed in a glass vial.)

2.3.4.1.2 Extraction procedure for the 7-0-acyl derivatives of the acidic DSP toxins

The shellfish hepatopancreas (6g) was homogenised with acetone (12 ml). The homogenate was centrifuged at 3,000 rpm (10 min) and 2.5 ml of the supernatant was transferred to a test tube. The supernatant was evaporated to dryness and the residue extracted with diethyl ether (2 x 2.5 ml) by vortex mixing for 1 min. The combined ether layers were evaporated to dryness under nitrogen.
2.3.5 Modified derivatisation procedures

2.3.5.1 Derivatisation with 9-anthryldiazomethane (ADAM)
ADAM solution (0.2% w/v) was prepared by dissolving ADAM (6 mg) in acetone (50 µl), making up to 3 ml with methanol and filtered (0.45 µm membrane) for immediate use. Standard toxins and sample extracts were evaporated under nitrogen and treated with ADAM solution (200 µl), ultrasonicated for 5 min and allowed to stand for 1 hr. All solutions containing ADAM were stored in amber vials to protect them from light. After 1 hr. in the dark the solutions were evaporated to dryness under nitrogen and redissolved in chloroform:hexane (1:1).

2.3.5.2 ADAM in-situ derivatisation procedure
Freshly prepared ADAM solution was filtered (0.45µm membrane) and 200 µl used to derivatise the residue from an aliquot of the chloroform extract (0.5 ml) which had been evaporated to dryness using nitrogen. This mixture was ultrasonicated for 2 minutes, and at 30 min intervals during derivatisation. After 1 hr in the dark, the solution was evaporated to dryness under nitrogen and redissolved in chloroform:hexane (1:1).

2.3.5.3 Modified BAP derivatisation procedure
Shellfish extract or OA, DTX-1 and DTX-2 standards (0.050-0.250 µg) in methanol (0.1 ml), deoxycholic acid (0.10 µg) (internal standard) in acetonitrile (0.1 ml), BAP and diisopropylethylamine (0.04 µl, 5% in acetonitrile) were mixed, ultrasonicated for 5 min and heated at 75°C for 30 min, while protected from light. Solvent was removed under nitrogen and the residue was reconstituted in chloroform:hexane (50:50, 1 ml). The mixture was ultrasonicated for 10 min. and heated in the dark at 75°C for 30 min. The solution was evaporated to dryness under nitrogen and redissolved in chloroform:hexane (1:1).

2.3.6 Modified clean-up procedures
A silica solid phase extraction (SPE) cartridge (Supelclean LC-Si, 3 ml, Supelco) was conditioned with chloroform:hexane (50:50 v/v, 3 ml) and the sample was applied to the conditioned cartridge. After washing with chloroform:hexane (50:50 v/v, 5 ml) followed by chloroform (5 ml), the toxin esters were eluted with chloroform:methanol (95:5 v/v, 5
ml). After evaporation to dryness under nitrogen (Turbo Vap LV, 40 °C), the residue was reconstituted in methanol (200 μl).

2.3.7 Modified HPLC procedures

HPLC analysis, using 10-20μl of sample or standards, was conducted at 30°C using isocratic solvent mixtures, acetonitrile:methanol:water (80:10:10 or 80:5:15). An Ultremex C-18 column (5 μm, 4.6 x 250 mm) with a mobile phase flowrate of 1.0 ml/min or a midbore Ultremex C-18 column (5 μm, 3.2 x 250 mm) with flowrate 0.5 ml/min were used. Injection volumes of 10-20 μl were used. The toxin derivatives were monitored using a fluorimetric detector, with λ<sub>ex</sub> 365 nm and λ<sub>em</sub> 412 nm (anthyl-methyl derivatives) or λ<sub>em</sub> 418 nm (pyrene-acyl derivatives).

All results were based on peak areas related to a standard calibration using OA (Sigma Chemical Co.) which was subjected to the same derivatisation and SPE procedures (5 - 20 ng OA on-column). The detection limits for OA determination with ADAM and BAP were 0.1 ng and 0.4 ng respectively (3:1, signal:noise ratio).

2.3.8 Analysis of 7-0-acyl derivatives and acidic DSP toxins

The residue from the ether extract was dissolved in 90% methanol/0.5 N NaOH (2 ml). After 1 hr at room temperature, the solution was neutralised with 0.1 N acetic acid, added to 10 ml water and extracted with ether (3 x 6 ml). The combined ether extracts were diluted to 20 ml, and an aliquot (1 ml) was removed and the solvent evaporated. The residue was derivatised and subjected to the clean-up and HPLC analysis, as previously described above.

Note: Calculations for converting 'ng of toxin on-column' to both 'μg of toxin per g of hepatopancreas' and 'μg per 100g of mussel meat' are given in Appendix No. 3
2.3.9 Comparison of the analytical methods (a-c) for DSP analysis

Calibrations

Certified standard lyophilised mussel material containing DSP toxins (MUS-2, NRC, Canada) were used to check the validity of both analytical methods. Calibrations using both derivatisation methods were linear for solutions containing 0.05 - 0.25 µg OA/ml (Fig. 2.17) which represents 5 - 25 ng on-column. (ADAM method \( r = 0.998 \) and BAP method \( r = 0.996 \)). A similar result (Fig. 2.18) was obtained for DTX-2 standards and this indicated that these methods can be successfully applied to the analysis of DSP toxins in Irish shellfish. For routine chemical analysis, the concentrations of DTX-1 and DTX-2 are expressed as OA equivalents.

![Okadaic acid calibrations using ADAM and BAP](image)

Fig. 2.17  Okadaic acid calibrations using ADAM and BAP
**Comparison of BAP and ADAM derivatisation methods**

As can be seen from Fig. 2.17 and Fig. 2.18, the sensitivity of measurements using BAP (detection limit, 0.4 ng) is approximately 25% of that using ADAM. The fluorescent response using ADAM was approximately four times greater than with BAP for DTX-2 (Fig. 2.18) and other toxins. One major problem with the ADAM method is the reliance on the purity of the purchased solid ADAM reagent. In our experience, large variations in the quality of commercial batches of the ADAM reagent can result in artefact peaks in the HPLC chromatograms. Good quality ADAM will give chromatographic baselines which are comparable to those obtained using BAP. It is recommended that this reagent be sub-divided into small aliquots and stored at -70°C, preferably in complete darkness in a dessicator, and should only be made up in solvent immediately before use. Daily preparation of fresh ADAM *in-situ* solution resulted in chromatograms of intermediate quality (Fig. 2.15). The quality of the prepared ADAM solution is dependant on the quality of the starting materials. (Both quinuclidine and N-chlorosuccinimide are extremely hygroscopic) While these materials are more stable than ADAM, their quality also deteriorates noticeably with time. Therefore, this method was only used when difficulties arose with the availability of pure ADAM.
Summary

The ADAM in-situ method is useful when high sensitivity is required or when the quality of purchased ADAM is poor. Both the BAP and ADAM methods proved useful for routine analysis. However, most analyses of DSP toxins were carried out using ADAM derivatisation because of its higher sensitivity. The cleaner baselines obtained using the BAP method rendered this procedure the method of choice for purity checks (e.g. purity of isolated DTX-2 and DTX-2B).

Extraction, Derivatisation and Clean-up steps for the Routine Analysis of DSP toxins

Fig. 2.19  Diagram shows a summary of sample preparation for the routine analysis of the 'OA class' of DSP toxins

Instrumentation:

The HPLC system consisted of a pump (LC-10AD), column oven (CTO-10A) and an RF-551 fluorescence detector (Shimadzu) with an autosampler (ISS-100, Perkin Elmer). The analytical HPLC columns used were an Ultremex C-18, 5μm, 4.6 x 250 mm and 3.2 x 250 mm, (Phenomenex, UK), with precolumns Ultremex C-18, 5μm, 4.6 x 30 mm and
3.2 x 250 mm respectively. LC injector (Rheodyne model #7125) and in-line filter (0.5 μm x 3 mm, Rheodyne) was also used.

Solvent evaporation under nitrogen was carried out using a Turbo Vap LV evaporator, (Zymark). Sample preparation required the following equipment: homogeniser (Ultra-Turrax T25), centrifuges (Beckman model J2-21 and Easyspin, Sorvall Instruments), vortex mixer (Maxi-Mix II, Thermolyne type 37600) and a sonic bath (Sonicor SC-42).

**Software**

Chromatographic data handling was performed using an Axxi-Chrom 717 chromatography data station (Axxiom Chromatography). Data was transferred to Microsoft Excel® for further graphical manipulation.

**Diarrhetic shellfish toxin standards.**

Okadaic acid (OA) (95%, Sigma, Gillingham, UK) and dinophysistoxin-1 (DTX-1) (Calbiochem-Novabiochem, Nottingham, UK) were purchased. A certified standard lyophilised mussel material containing diarrhetic toxins (MUS-2, National Research Council, Halifax, Canada) was reconstituted in methanol immediately prior to use to gave a mixture with 2.5 μg total toxins/ml (2.29 μg OA and 0.21 μg DTX-1/ml). Standard DTX-2 (95%) was isolated from contaminated mussels in our laboratories (31).

**Chemicals**

9-anthryldiazomethane (ADAM) was purchased from Serva Feinbiochemica (Heidelberg, Germany). 9-Anthraldehyde hydrazone was synthesised from 9-anthraldehyde and hydrazine hydrate (80%, Aldrich). ADAM in-situ was prepared from quinuclidine and N-chlorosuccinimide (Aldrich). 1-bromoacetylpyrene (BAP) was purchased (Aldrich) or synthesised from pyrene following the method of Spijker et al (37). BAP was purified before use by chromatography on silica (silica gel 60, 70-230 mesh) with elution using hexane/dichloromethane /cyclohexane to give pale yellow needles, m.p. 129-130°C. BAP base catalyst diisopropylethylamine (Aldrich ), and internal standard deoxycholic acid (Sigma).

Water, methanol, acetonitrile, hexane, light petroleum (40-60°C), chloroform (stabilised with 1% ethanol) and chloroform (stabilised with amylene, 50 ppm) were HPLC grade (Labscan, Dublin, Ireland). All solvents were filtered through 0.45 μm filters (Gelman Ltd., Dublin).
2.4 THE DEVELOPMENT OF A ‘DUAL-TAG METHOD’ FOR THE CONFIRMATION OF SPECIFIC OA TYPE TOXINS PRESENT IN TOXIC MUSSEL SAMPLES

2.4.1 Introduction

As is the case with most natural products, chromatographic methods of analysis have proved invaluable for the study of marine toxins. In addition to the need to separate toxins from the other components in complex sample matrices before detection, it is often necessary to achieve the resolution of individual members of families of toxins.

2.4.1.1 HPLC separation of ADAM-BAP toxin derivatives

Initial work carried out on an Ultremex C-18, 5µm, 3.2 x 250 mm (Phenomenex) column using varying mobile phase compositions, failed to resolve the ADAM and BAP derivatives of the specific toxins.

Since ADAM and BAP have the basic structure of anthracene and pyrene respectively, a column capable of resolving both anthracene and pyrene from each other was thought to be the most likely candidate for the resolving of specific toxin derivatives.

An extensive literature search as well as technical advice from of ‘Phenomenex UK’ Ltd. (Mr. Alan Nesbit), suggested the use of an ‘Envirosep™-PP’ column. (Fig. 2.20).

![Fig. 2.20](image)

Chromatogram of a 16 component PAH mixture with 3 surrogates which were resolved on an ‘Envirosep™-PP’ column with detection at λex:254nm and λem 390nm. Note that anthracene (peak7) elutes 3.5 min. before pyrene (peak9).

(Data courtesy of Phenomenex UK’ Ltd.)
The column is constructed of a polymeric bonded silica stationary phase which separates the analytes using both reverse-phase partition and size-exclusion principles. (exact details were not available from ‘Phenomenex UK’ Ltd.).

The main application of this column is in the analysis of polycyclic aromatic hydrocarbons (PAH) in environmental samples. PAHs are compounds produced naturally from oils, tars, coal and petroleum products. Compounds such as naphthalene, phenanthrene, anthracene and pyrene are among the PAH’s capable of being resolved by this column.

A custom built column was supplied for the present work by ‘Phenomenex UK’ and it had the following dimensions:

\[\text{Envirosep}\,^\text{TM}-\text{PP}\]

- Analytical column (00F-3029-R0): Length: 150 x 3.2mm (midbore)
- Guard column (03a-3029-R0): Length: 30 x 3.2mm

2.4.2 ‘Dual-tag’ method

The test makes use of both the ADAM and BAP derivatisation methods. The test involves derivatising two portions (subsamples) of the mussel ‘chloroform extract’ separately with both the ADAM and BAP reagents. The two subsamples undergo the same derivatisation and SPE protocols outlined previously. The subsamples are then combined and injected as a single sample into the HPLC.

The method is designed for qualitative analysis, and is based on the comparison of the relative retention times for both the ADAM and BAP derivatives of a specific toxin as they appear on the chromatogram. As previously discussed, the BAP method is approximately four times less sensitive than the ADAM method and four times the amount of chloroform extract is needed to give the same peak area as the ADAM derivatives \(^{(33)}\).

As the BAP excitation and emission wavelengths have been shown to give cleaner baselines the combined ADAM-BAP sample was monitored at \(\lambda_{\text{ex}}\) 365nm and \(\lambda_{\text{em}}\) 418nm.
The separation of both OA and DTX-2 was optimised under isocratic conditions using Hipac-B® HPLC optimisation software. Data acquired from two trial runs using 75% and 65% methanol (organic modifier) were used to obtain optimum mobile phase conditions. Tables 2.01 and 2.02 summarise the data yielded from trials 1 and 2 respectively.

Table: 2.01  Trial Data 1 [75%]

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<th>Peak As</th>
<th>Peak Assignment</th>
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<td>16.950</td>
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Table: 2.02  Trial Data 2 [65%]

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<td>32.580</td>
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Using a 'Global Rs min.' model, a mobile phase composition of 58% organic modifier was found to give optimum separation (Fig. 2.21 and 2.22) of the various toxin derivatives and two reagent impurities. (Table: 2.03.)
Table: 2.03

<table>
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Using File: ADBAP3.HBM. Friday, 24-November-1995

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Fig. 2.21

Computer simulated chromatogram of (a) BAP impurity, (b) ADAM-OA, (c) ADAM-DTX-2, (d) BAP-DTX-2 and (e) ADAM impurity, with all peaks eluting under 36 min. Flow rate 0.5ml/min.

(Data file ADBAP3.HBM)
The 'response map' generated from the data obtained from Trial No. 1 and Trial No. 2. The plot shows the variation of selected optimisation (Rs) and the retention time of the last peak (tR) versus mobile phase composition (% solvent B).

(Data file ADBAP3.HBM)
2.4.3 Application of the ‘Dual-tag’ method

2.4.3.1 Analysis of DSP standards and mussel samples

Using a mobile phase composition of 58% methanol: 42% water the method was applied to a standard mixture consisting of 5ng OA-ADAM, 25ng OA-BAP, 10ng DTX-2-ADAM and 50ng DTX-2-BAP on-column (Fig. 2.23).

<table>
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<th>Solute</th>
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<td>BAP-OA</td>
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<tr>
<td>ADAM-DTX2</td>
<td>30.29</td>
</tr>
<tr>
<td>BAP-DTX2</td>
<td>31.97</td>
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</tbody>
</table>

Fig. 2.23

Chromatogram of ADAM and BAP derivatives of a standard mixture containing both OA and DTX-2
(Data file adhpmus58)

The ADAM-BAP confirmatory test was then applied to a ‘real sample’ of toxic mussels containing both OA and DTX-2 (with DTX-2 being the predominant toxin present in the sample). The sample extraction, derivatisation and clean-up procedures were as described previously. The chromatogram showed clearly the ADAM and BAP derivative peaks for both OA and DTX-2, with detection at \( \lambda_{ex} \) 365nm and \( \lambda_{em} \) 418nm, and mobile...
phase composition of 58% methanol, 42% water and a flow rate of 0.5ml/min (Fig. 2.24).

**Retention times**

<table>
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</table>

**REAL-MUSSEL SAMPLE**

Chromatogram of ‘toxic mussel’ sample derivatised with both ADAM and BAP and resolved on an Envirosep™-PP column with a flow rate of 0.5ml/min, mobile phase 58% methanol and detection using RF-551 with gain x16. (Data file bpadmus58)
2.4.3.2 HPLC confirmation of OA type toxins

Interestingly, DTX-2 has been wrongly identified in the phytoplankton *P. lima*, by authors who relied on LC-MS (even when using authentic DTX-2). In this instance, it appears that the LC is more important than MS. The main problem is that impurities present have virtually identical spectroscopic signals to those of known DSP toxins. These impurities are structurally similar (usually isomers) and as a result present identical mass spectrum profiles.

More recently, in the summer of 1995 our laboratory was involved in the setting up and validation of a fully equipped on-site chromatographic laboratory (Bantry Bay Mussels Ltd) for the routine chemical analysis of DSP toxins in cultivated mussels (*Mytilus edulis*). The toxin profiles have on some occasions been quite complex, leading to difficulties in the identification of specific toxins. To overcome this problem and make use of the existing resources available on-site the ‘dual-tag’ test was developed.

**Discussion:**

The ‘dual-tag’ method is applicable when the confirmation of both OA and DTX-2 is required. Further improvement of the separation of both toxins could be achieved using a gradient rather than an isocratic mobile phase. Increasing the column length would also increase the number of theoretical plates. This in turn would give better resolution and allow the method to be applied to other DSP toxins such as DTX-1 and DTX-2B which have been detected in mussels in the West Coast of Ireland.

*Chromatography instrumentation available to the Processing Laboratory at Bantry*

The HPLC system consisted of a pump (LC-10AD), column oven (CTO-10A) and an RF-551 fluorescence detector (Shimadzu). The analytical HPLC columns used are an Ultremex C-18, 5μm, 4.6 x 250 mm and 3.2 x 250 mm, (Phenomenex), with precolumns Ultremex C-18, 5μm, 4.6 x 30 mm and 3.2 x 250 mm respectively. LC injector (Rheodyne model #7125), and an in-line filter 0.5 μm x 3 mm (Rheodyne) were also used. Chromatographic data handling was performed using an Unipac® chromatography data station (Shimadzu).
2.5 BIBLIOGRAPHY


Chapter 3

Detailed Monitoring Programme

Science is built up of facts, as a house is built of stones; but an accumulation of facts is no more a science than a heap of stones is a house.

Hopi Poincaré (1854-1912)
3.1 INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is a syndrome named after its predominating human symptom, diarrhoea and gastroenteritis, following the ingestion of shellfish. The toxicity arises from shellfish that have been feeding on toxic phytoplankton, particularly *Dinophysis* and *Prorocentrum* spp. (1). Toxins accumulate in the digestive glands of bivalve molluscs. The polyether carboxylic acid, dinophysistoxin-1 (DTX-1), was identified in *Dinophysis fortii* (2) and DSP in Japan is due mainly to the presence of this compound. Although DSP has been reported throughout the world, it has occurred with alarming frequency in Europe in recent years and Kumagai *et al.*, have shown that the structurally related compound, OA, was responsible for most of these outbreaks of DSP (3). The unpredictable incidence of toxicity, with its occurrence in all of the climatic regions of Europe, has resulted in severe economic consequences for shellfish producers. Shellfish production areas have been closed for periods from a few weeks to a year in Spain, Portugal, France, The Netherlands, Sweden, Norway, Ireland and Italy due to positive animal bioassay responses or human toxic incidents (4). Usually, only low levels of DTX-1 have been found in European shellfish but an exception was in Sogndal, Norway (5) where it was the predominant toxin. An isomer of okadaic acid, dinophysistoxin-2 (DTX-2) was identified in mussels (*Mytilus edulis*) from Ireland (6) and both OA and DTX-2 were present during a DSP episode in 1991 (7). The presence of multiple 7-O-acyl derivatives of OA, DTX-1 and DTX-2 (Fig. 1.07, $R_1 = $ acyl) in shellfish has also been demonstrated using liquid chromatography-mass spectrometry (8). These compounds are esters with fatty acid constituents that range from tetradecanoic (C14:0) to docosahexaenoic (C22:6ω3). These compounds have not been detected in dinoflagellates and it has been proposed that they are produced in the digestive glands of shellfish (9).

The OA group of compounds are potent inhibitors of protein phosphatases and are used as cellular reagents for biochemical research (10). Advantage of this property was employed recently to detect new toxins using a combination of chromatography and protein phosphatase assays (11). The discovery of the potent tumour promoting properties of OA (12) will also have implications for the permitted toxin levels in shellfish and the efficacy of methods for toxin detection currently used by regulatory authorities. Fluorometric HPLC analysis (13) of the 9-anthrylmethyl derivatives of the acidic
diarrhetic toxins has become an established analytical method and was used in this study to determine the toxin profiles in shellfish.

A research programme was initiated in 1991 to examine the toxin profiles in mussels (*Mytilus edulis*) in South-West Ireland where the largest rope-cultured mussel production takes place. Prolonged bans on harvesting have been enforced during the summer months for several years in this area, as a result of DSP and prolonged toxic episodes in 1991.

![urgent plea for research](image)

**Report: EDDIE CASSIDY**

A CALL was made yesterday for the Department of Marine to establish a fisheries research centre in West Cork, as a Bantry-based shellfish company officially launched a new £190,000 vessel.

*Fig. 3.01*

*News-clip from the 'Cork Examiner' dated 9*th* of February 1991, calling for research into the study of toxin profiles in shellfish.*

A comprehensive programme of sampling of shellfish (N=290) was undertaken in 1991 with over 240 mussel samples collected in a six month period. Initial HPLC analysis for DSP toxins, was carried out by Mr. Keith Thomas and Ms. Mary Lane. Some of these samples were re-analysed and detailed toxin profiles were recorded for a number of other collected shellfish samples. This study also involved the examination of a number of shellfish cultivation areas (See Fig: 3.02) in 1993 (N=58) and 1994 (N=164). An optimised analytical protocol for the determination of diarrhetic shellfish toxins was developed. There are difficulties in obtaining representative samples of shellfish for diarrhetic toxin analysis due to wide variations in toxin levels between individual cultivated mussels. It has been shown that adjacent mussels can differ considerably in toxin levels. To obtain a representative sample, mussels were collected from the top, middle and bottom of an individual mussel line (approximately 5m length), and combined these to give one composite sample (> 50 mussels).

Raw data on the cell counts of *Dinophysis* species and temperature profiles for the various sites were obtained from Ms. Mary Shannon and Mr Denis O'Driscoll, Bantry Bay Mussels Ltd.
Fig. 3.02: Map of Bantry Bay, showing the main sampling areas (A and B) as well as the other locations sampled during monitoring programme (1991-1995).
3.2 EXPERIMENTAL RESULTS

3.2.1 DSP TOXIN PROFILE IN 1991

Eleven sites were selected in two regions of Bantry Bay, Co. Cork, where major mussel cultivation is undertaken (Bantry Harbour: sites #1-8 where site #5 and #6 taken as one, Glengarriff Harbour: sites #10-13) (Fig. 3.02). The period of intoxication of mussels commenced early in July 1991 and persisted until the middle of November 1991, during which time a ban on harvesting was imposed on the shellfish farms. There were some residual levels of toxins after this period, but the total (OA + DTX-2) content was much less than 0.8 µg/g hepatopancreas, the regulatory limit in Ireland (Table 3.01) (Figs. 3.04 and 3.05).

The major diarrhetic toxin found in this study was DTX-2 which was present throughout the period of intoxication but with its isomer, OA, present at significant levels early in this period. During the course of this study, standard DTX-1 was made available to us (a gift from Professor T. Yasumoto). However, this toxin was only detected at low levels (<0.1 µg/g) in a small number of the samples examined during mid-August and October 1991.

Fig. 3.03 Chromatograms of mussel extracts, showing the 9-anthrylmethyl esters of OA, DTX-2 and DTX-1. (a) Mussel sample from site #1 12/8/91 (Data file S1D12M8) and (b) Mussel sample from site #10 28/8/91 (Data file 10D28M10).
Average DSP toxin levels (1991) (μg/g hepatopancreas)

<table>
<thead>
<tr>
<th>July-Dec. 1991</th>
<th>Week No.</th>
<th>OA</th>
<th>DTX-2</th>
<th>OA</th>
<th>DTX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>8th July</td>
<td>#28</td>
<td>0.13</td>
<td>0.14</td>
<td>0.07</td>
<td>0</td>
</tr>
<tr>
<td>14th July</td>
<td>#29</td>
<td>0.27</td>
<td>0.54</td>
<td>0.41</td>
<td>2.2</td>
</tr>
<tr>
<td>22nd July</td>
<td>#30</td>
<td>0.73</td>
<td>0.4</td>
<td>0.7</td>
<td>0.46</td>
</tr>
<tr>
<td>29th July</td>
<td>#31</td>
<td>0.26</td>
<td>0.13</td>
<td>0.77</td>
<td>0.55</td>
</tr>
<tr>
<td>6th August</td>
<td>#32</td>
<td>0.22</td>
<td>0.25</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>12th August</td>
<td>#33</td>
<td>0.25</td>
<td>1.68</td>
<td>0.09</td>
<td>0.27</td>
</tr>
<tr>
<td>19th August</td>
<td>#34</td>
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<td>1.52</td>
<td>1.05</td>
<td>1.03</td>
</tr>
<tr>
<td>26th August</td>
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<td>0.15</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
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<td>0</td>
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<td>0.03</td>
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</tr>
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<td>1.63</td>
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</tr>
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<td>13th October</td>
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<td>0.1</td>
<td>1.45</td>
</tr>
<tr>
<td>20th October</td>
<td>#41</td>
<td>0.05</td>
<td>1.07</td>
<td>0.06</td>
<td>0.3</td>
</tr>
<tr>
<td>3rd November</td>
<td>#42</td>
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<td>0.15</td>
<td>0.06</td>
<td>0.11</td>
</tr>
<tr>
<td>10th November</td>
<td>#43</td>
<td>0.25</td>
<td>2.3</td>
<td>0.26</td>
<td>2.2</td>
</tr>
<tr>
<td>17th November</td>
<td>#44</td>
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<td>0.48</td>
<td>0.04</td>
<td>0.23</td>
</tr>
<tr>
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<td>#45</td>
<td>0</td>
<td>0.4</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>14th December</td>
<td>#46</td>
<td>0.04</td>
<td>0.1</td>
<td>0</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 3.01  
*Average levels of OA and DTX-2 in mussels during the 1991 toxic period*

7-O-acyl derivatives of DTX-2 were also observed in chromatograms where the level of DTX-2 was relatively high, leading to additional unidentified peaks. Hydrolysis of extracts, using methanolic sodium hydroxide, prior to derivatisation resulted in a simplified chromatogram as the acyl groups are removed by this procedure.
Fig. 3.04: Average toxin content of mussels from Bantry (sites # 1-8) during the 1991 toxic period

Fig. 3.05: Average toxin content of mussels from Glengarriff (sites # 11-13) during the 1991 toxic period
3.2.1.1 Other acidic components present in Irish mussels during 1991

While DTX-2, OA, DTX-1 and acyl derivatives were detected in Irish mussels in the course of this study, other unidentified components were also present. It is likely that these compounds contained a carboxylic acid group, as they were detected by derivatisation with ADAM and BAP. However, their toxicity in shellfish, if any, remains unknown, as insufficient quantities of mussels were available for toxin isolation.

Previous work by Yasumoto et al. has established that PTX-6 and PTX-7 appear as peaks in the chromatograms of contaminated mussels, analysed by the ADAM method (Lee et al.), and that they elute in the region between ADAM-OA and the ADAM reagent peaks. For example, the chromatogram of a sample taken in week #36 from site #10 (Fig. 3.06) showed an acidic contaminant which eluted at 12.6 minutes just before OA (13.3 min.) and DTX-2 (14.8 min.). This peak appeared at a similar position (relative to OA) to that of PTX-7 in a chromatogram published by Yasumoto. However, no standard PTX-7 was available to confirm this.

![Chromatogram of a mussel extract, sampled from site #10 23/9/91, showing the 9-anthrylmethyl esters of OA, DTX-2 and an unidentified component tR=12.6 min.](Data file S10D23M9)
3.2.1.2 Horizontal and vertical variation in toxicity

Samples of mussels, collected from sites that were in close geographic proximity, often had large variations in total toxin levels (Fig. 3.07).

In addition, there was a variation in toxin levels in mussels with the depth at which they were cultivated. Fig. 3.08 shows the data from a single length of cultivated mussels where the DTX-2 levels were determined at each 0.5 m in depth. However, no consistent pattern of variation with depth was observed and in other instances, toxin levels increased with depth.

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**Fig. 3.07**  *OA and DTX-2 content of mussels from 10 sites 12/8/91*

Vertical variation in the toxin content of mussels.

*Note: Black line at 0.8 represents the toxic limit*
Large geographic variability in toxin levels were observed, even between adjacent cultivation areas (Table 3.02). This was particularly apparent at the beginning, and at the end, of toxic episodes. Thus, week #33 at the start of a toxic episode in 1991, showed one very high level of toxins (6.3 µg (OA + DTX-2)/g HP), four samples in excess of the regulatory limit, 0.8 µg/g, and the remaining samples below this limit. Week #43, at the end of the toxic episode produced five samples at, or above, 0.8 µg/g and the remaining at 0.2 µg (OA + DTX-2)/g HP, or less. This contrasted with a more even distribution of toxicity during toxic episodes, as shown for weeks #34 and #38. Differences in toxicity that occur at the beginning of major intoxications, presumably reflect normal toxigenic dinoflagellate population variations. Natural detoxification processes apparently operate, which account for the rapid decline in toxin levels at the end of these toxic episodes but this can also lead to local variability.

<table>
<thead>
<tr>
<th>Site</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>#33</td>
<td>6.3</td>
<td>0.9</td>
<td>0.5</td>
<td>*NA</td>
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<td>2.1</td>
<td>1.5</td>
<td>0.4</td>
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<td>&lt;0.2</td>
</tr>
<tr>
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<td>2.1</td>
<td>*NA</td>
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<td>4.8</td>
<td>2.7</td>
<td>1.9</td>
<td>1.3</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
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<td>2.6</td>
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<td>1.1</td>
<td>3.1</td>
<td>*NA</td>
<td>3.4</td>
<td>3.0</td>
<td>2.7</td>
<td>2.3</td>
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<tr>
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<td>0.8</td>
<td>&lt;0.2</td>
<td>0.8</td>
<td>3.7</td>
<td>1.1</td>
<td>&lt;0.2</td>
<td>1.3</td>
<td>0.2</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

Table 3.02:

Geographic and seasonal variation in the diarrhetic toxin content of mussels from eleven sites during the 1991 toxic period. Values refer to the total µg (OA+DTX-2)/g mussel hepatopancreas in Bantry Bay (area A, 7 sites and area B, 4 sites, see Fig 3.02). *NA = sample not available
3.2.2 DSP TOXIN PROFILE IN 1993

Detailed sampling was not undertaken in 1993. The majority of work undertaken in 1992 involved the setting-up of analytical protocols and reanalyses of samples collected from various sites during the 1991 toxic period.

During 1993, low levels of diarrhetic toxins were detected, with levels only exceeding the regularity limit in mussels sampled for week #35 (Fig. 3.09). DTX-2 was again the predominant toxin in late summer.

![Graph showing OA and DTX-2 levels in 1993 mussels](image)

Fig. 3.09

*Average levels of OA and DTX-2 in mussels, taken from sites #4, #8, and #10 during the 1993 toxic period.*

*(Line at 0.8 μg/g of HP represents regularity limit)*

Large geographic variations in both toxin levels and the ratio of OA and DTX-2 in mussels were again observed, even between adjacent cultivation areas (Fig. 3.10). Unlike the 1991 toxin profile, DTX-2 was present in samples taken at an early stage in the monitoring program, with OA only appearing when high DTX-2 levels were present (Fig. 3.09).
Fig. 3.10

Geographic variation in the diarrhetic toxin content of mussels sampled from seven different sites on (a) 22/8/93 and (b) 29/8/93

Fig. 3.11

Chromatograms of a mussel extract, sampled from (a) site #10 19/9/93 (Data file 22-9-93.701) and (b) site 4# (Data file 13-8-93.501) showing the 9-anthrylmethyl esters of OA and DTX-2
3.2.3 DSP TOXIN PROFILE IN 1994

In 1994, there was another major infestation of mussels, but the toxin profile was different from that observed in 1991. Although OA was present at low levels during the toxic period, it did not play a significant part in this DSP episode, with DTX-2 being the predominant toxin detected. OA was first detected in week #26 (< 0.13 µg/g HP) with DTX-2 not being detected until week #30-31 (0.43 µg/g HP). Fig. 3.12 shows two typical chromatograms from mussels sampled from site #4 during weeks #29 and #39, respectively.

![Chromatograms of 9-anthrylmethyl esters of mussel extracts](image)

Fig. 3.12 Chromatograms of 9-anthrylmethyl esters of mussel extracts, sampled from: (a) site #4 20/7/94 (Data file 21-7-94.301) (b) site #4 26/9/94, showing high levels of DTX-2 and trace levels of OA. (Data file 28-9-94.801)

Fig.’s 3.13 and 3.14 show the data obtained from weekly sampling of shellfish at site #4, in 1991 and 1994, respectively. The comparison of these toxin profiles demonstrates the increasingly predominant role of DTX-2 in DSP toxicity in the region. In 1994, OA levels were usually less than 0.7 µg/g HP, whereas maximum DTX-2 levels of 6.7 µg/g HP were recorded.
Neighbouring regions examined in 1994 also had high levels of DTX-2 during the late summer period; Kenmare Bay (6.4 µg/g HP), Dunmanus Bay (6.6 µg/g HP) (Fig. 3.15). Trace levels of DTX-1 were also detected in samples taken from Dunmanus Bay during week #31 (Fig. 3.16)
Fig. 3.15  *Weekly toxin content of mussels sampled from Dunmanus Bay during the 1994 toxic period (Line at 0.8 μg/g HP represents regularity limit)*

Fig. 3.16  *Chromatograms of prenacyl derivatives of both standard mixture and mussel extracts, containing OA, DTX-2 and DTX-1*

(a) *Standard mix of three toxins (Data file 3-8-94.401)*

(b) *Mussel sample from Dunmanus Bay 18/94 (Data file 3-8-94.101)*
3.2.3.1 Other acidic components present in Irish mussels in 1994

Chromatograms of mussel extracts from Ardgroom in Kenmare Bay, sampled on the 30/8/94, showed large unidentified peaks eluting before and after the DTX-2 peak (Fig. 3.17). The fact that these unidentified peaks did not occur in either standards and blanks prepared/analysed on the same day as the samples, would imply that the peaks were not impurities introduced by the analysis, but present in the mussels themselves. These peaks subsequently appeared in chromatograms of samples from Bantry (Fig. 3.18) and Dunmanus.

Large quantities of these samples were stored in the freezer at -20°C for isolation and subsequent toxicity studies. However, these compounds were found to be unstable and when re-analysed in January 1995 their concentration relative to DTX-2 had dramatically decreased.

Fig. 3.17  Chromatogram of a mussel extract, sampled at Ardgroom, Kenmare Bay 30/8/94, showing the prenacetyl derivatives of OA, DTX-2 and three unidentified components at tR.. = 10.3, 13.1 and 15.5 min. respectively. (Data file 1-9-94.101)
Fig. 3.18  Chromatograms of mussel extracts, showing the prenacyl derivatives of samples taken from site #4 on:

(a)  9/94, of OA, DTX-2 and three unidentified components at $t_R = 13.3$, 15.7 and 16.9 min. (Data file 6-9-94.651)

(b)  5/94 of OA, DTX-2 and two unidentified component at $t_R = 13.4$ and 15.7 min. (Data file 6-9-94.451)
In 1839 the genus *Dinophysis* was described by Ehrenberg *et al.* with *D. acuta* as the only, and therefore type, species (17). Since then, more than 200 species of *Dinophysis* have been described (18). Species belonging to *Dinophysis* have a cingulum and a sulcus giving the cell a dorso-ventral orientation. The cingulum is located close to the apical end of the cell, and, in many species, the epicone is not readily visible. Lists (sails) line both furrows with the list on the left side of the sulcus being especially well developed and it is supported by three ribs. Most species are strongly compressed, and the cells are therefore usually seen in lateral view. It appears that species of *Dinophysis* typically possess 18 plates with the following general plate pattern: four epithecal plates, four hypothecal plates, four cingular plates, four sulcal plates which surround the flagellar pore, and two platelets surrounding an apical pore (19-21). Many of the 200 species of *Dinophysis* cannot be clearly distinguished from the type descriptions and this genus is in need of revision. The lack of success in culturing any species of *Dinophysis* is a major obstacle to a thorough taxonomic revision. It is probable that some of the recorded morphological data refer to changes in the life-cycle of an individual species.

The most important diagnostic features in *Dinophysis* are the size and the shape of the cells, the morphology of the left sulcal list, the presence or absence of chloroplasts, and presumably plate pattern and the cal ornamentation. The following species as detailed in Table 3.03, and Fig. 3.19 have been shown to be toxin producers (1).

<table>
<thead>
<tr>
<th><em>Dinophysis</em> Species</th>
<th>Known producer of</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. acuminata</em></td>
<td>OA</td>
</tr>
<tr>
<td><em>D. acuta</em> *</td>
<td>OA and DTX-1</td>
</tr>
<tr>
<td><em>D. fortii</em></td>
<td>OA, DTX-1 and PTX-2</td>
</tr>
<tr>
<td><em>D. norvegica</em></td>
<td>OA and DTX-1</td>
</tr>
<tr>
<td><em>D. rotundata</em></td>
<td>DTX-1</td>
</tr>
<tr>
<td><em>D. tripos</em></td>
<td>DTX-1</td>
</tr>
</tbody>
</table>

**Table 3.03**

*Illustrates the known DSP toxins produced by a number of specific *Dinophysis* species.*

*Note: *D. acuta* is thought to be the producer of DTX-2 isolated from toxic Irish mussels.*
Species of the genus *Dinophysis* have a characteristic shape of greatly reduced upper cell half (epithea), mainly laterally flattened and with a well-developed sulcal list protruding like a ‘sail’. The girdle often protrudes out at the top end like a collar or ‘ruff’.

DSP in shellfish in Ireland is associated with blooms of *Dinophysis acuta* and *D. acuminata*. Monitoring programmes in recent years have revealed that high levels of DTX-2 in mussels followed shortly after the observation of high cell counts of *D. acuta* in the cultivation areas.

Fig. 3.19

*Formaldehyde-preserved cells of (a) Dinophysis acuminata, (b) D. acuta, (c) D. fortii, (d) D. norvegica, (e) D. rotundata and (f) D. tripos. Scale bar: 20μm.*

*(taken from 22)*
3.3.1 *Dinophysis acuminata*

*Identification:* *D. acuminata* is best identified by its regular oval outline and size, being 38-58 μm long by 30-38 μm wide. The cells sometimes have small protrusions (knobs) on the hypotheca (Fig. 3.20).

**Fig 3.20:** *Photograph of D. acuminata cell from water sample taken on 1st September 1994 from site # 4.*

*Ecology and distribution:* The most extensive blooms of *D. acuminata* seem to occur during the summer and autumn months (23). *D. acuminata* blooms are particularly severe along the western coasts of Europe. Annual blooms are now reported from The Netherlands with densities in excess of 40,000 cells per litre (24). Blooms have been reported from Australia (25), Denmark (26), France (27), Ireland (24), Japan (28), Norway (29,30), Spain (24), Sweden (31), and the USA (32,33).
3.3.2 Dinophysis acuta

Identification: D. acuta is larger than D. acuminata. The cells are 54-94\(\mu\)m long and 43-60\(\mu\)m wide and is among the largest species of Dinophysis. It is widest below the middle and with a pointed bottom. The main identifying feature is that this point is situated towards the sail behind the mid-line (Fig. 3.21).

Fig. 3.21: Photograph of D. acuta cell from water sample taken on 1st September 1994 from site # 8

Ecology and distribution: Like D. acuminata, most extensive blooms of D. acuta seem to occur during the summer and autumn months\(^{23}\). D. acuta has been associated with DSP outbreaks in Chile\(^{34}\), Ireland\(^{35,36}\), Portugal\(^{37,38}\), Scandinavia\(^{29,31,39,40}\), and the USA\(^{32}\).
3.3.3 Correlation between Cell counts, Temperature profiles (in water) and subsequent toxin levels in Mussels

One of the striking features of many unusual and harmful blooms is their sudden appearance and erratic fluctuations. The extent to which such blooms are actually due to deterministic mechanisms, in contrast to random forcing, is still an open issue. The use of statistical techniques that determine the extent of predictable behaviour in complex systems such as algal blooms, have to a certain extent, helped in the prediction of toxic events \(^{(41-43)}\).

Phytoplankton blooms arise essentially from a period of growth where the water column is such that they can be kept in the surface light-rich layers with an adequate supply of nutrients. As described in chapter 1, there are a number of natural instances where one can expect this to happen. A prolonged period of calm and bright warm weather can allow a major bloom to develop \(^{(44)}\).

In the summer, the water column stratifies into two layers with the formation of a \textit{thermocline} which acts as a diffuse barrier between warmer surface layers and cooler nutrient-rich deep waters. This \textit{thermocline} inhibits the replenishment of nutrients to the sea surface. Phytoplankton cannot survive in the lower layer as there is insufficient light.

Recent findings at the Marine Research Institute, University college Galway, show that large populations of phytoplankton tend to occupy the waters just above this \textit{thermocline} \(^{(44)}\). Interestingly, in the coastal waters around Ireland, the physical processes of mixing and \textit{upwelling}\(^{(45,46)}\) can uplift the lower layer towards the surface resulting in a sudden phytoplankton bloom and subsequent toxic event occurring in cultivated mussels.

The most common cause of \textit{upwelling} is the presence of alongshore winds. Winds drag water along the surface of the sea initially in the same direction as the wind. Due to the rotation of the earth, however, the water actually moves towards the right in the northern hemisphere (or left in the southern hemisphere). This can cause water to move away from the coast. Water seeks its own level, and as surface water moves away from the coast, it is replenished by deeper water. The net effect is the movement of nutrients towards the sea surface, and consequently, phytoplankton flourish on these occasions \(^{(47,48)}\) (Fig. 3.22).
Surface water moves away from the Coast

Surface water replaced by deeper water

Fig. 3.22  Alongshore winds cause upwelling of cool nutrient-rich deep water to the surface along the coast

(i) Wind starts to drag surface water in its own direction with the water moving to the right away from the coast.

(ii) Cross section A-B: The surface water moving away is filled by deep water bringing nutrients towards the surface.

The systematic monitoring of water temperatures at various depths (0-10m) can be a useful tool in the prediction of toxic blooms, with a sudden drop in surface water temperatures indicating the upwelling of deep nutrient/phytoplankton rich water. Evidence of this was found in a number of sites in Bantry Bay prior to the 1994 toxic episodes.

3.3.3.1 North Chapel (Site # 4)

Temperature profiles taken at 1m intervals from the surface down to 10m, from late July to the end of September 1994, are summarised in Fig. 3.23. This graph shows that between August 7th (week #31) and August 17th (week #33) there was a dramatic change in the temperature of the surface water column, with the greatest
decline (18°C to 14.3°C) at the surface 10m on August 13\textsuperscript{th}. This indicated that upwelling of cooler water had occurred, which was followed by the presence of large cell counts of Dinophysis \textit{acuta} on the 17\textsuperscript{th} and 29\textsuperscript{th} of August (Fig. 3.23). Subsequently large levels of DTX-2 were recorded in mussel samples taken from this site.

The \textit{D. acuta} population increased rapidly after week #32, with a maximum average count of 11,100 cells/l being recorded in week #33 (Fig. 3.23)

\textbf{Fig. 3.23}

\textit{A radar graph summarising the temperature profile for the first 10m of the water column and the subsequent appearance of large numbers of \textit{D. acuta} cells on 17/8/94 and 29/8/94.}
Fig. 3.24

Weekly variation of D.acuminata and D. acuta cell counts at site #4 in 1994

(Insert represents a magnified section showing the cell counts of D.acuminata)

The highest mussel toxicity was recorded in week #37, which was three to four weeks after the maximum levels of dinoflagellates were detected in the water, and one week after the disappearance of the dinoflagellates. Therefore, it is apparent that a period of a few weeks is necessary for the mussels to concentrate the toxins in their hepatopancreas. Mussels from site #4 detoxified themselves very rapidly after week #37 and their toxin content dropped from 6.7 to 1.2 µg/g HP by week #39 (Fig. 3.14). The level of toxicity remained between 1.2 and 2.3 µg/g HP for a further 5 weeks before dropping slowly. The reason for the levelling off of the rate of depuration is not known. However, similar trends have been observed which linked depuration rates to the slowing down of the metabolic rate of mussels in winter, due to low water temperature and the low levels of phytoplankton in the water\textsuperscript{(14,29,49)}.

3.3.3.2 West Garnish (Site #11)

Temperature profiles from July until the end of September 1994 period are summarised in Fig. 3.25. The radar graph shows that between August 7\textsuperscript{th} (week #31) and August 17\textsuperscript{th} (week #33) there was also a large change in the temperature of the surface water column, decreasing from 18°C to 14.2°C on August 13\textsuperscript{th}. This again indicated that upwelling of cooler water had occurred, which was followed by the presence of large
cell counts of *D. acuata* on August 17th (Fig. 3.25). Subsequently, high levels of DTX-2 were recorded in mussel samples taken from the site with a maximum DTX-2 levels of 6.7 μg/g HP being recorded on week #37.

The *D. acuta* population increased rapidly after week #32, with a maximum average count of 16,800 cells/l being recorded in week #33 (Fig. 3.26).

**West Garnish 1994 (Site No. #11)**

_A radar graph summarising the temperature profile for the first 10m of the water column and the subsequent appearance of large numbers of D. acuta cells on the 17/8/94._
Circumstantial evidence for the association of DTX-2 with *D. acuta* was also obtained from these studies, as the occurrence of DTX-2 in Irish mussels, followed soon after high *D. acuta* cell counts were found in the cultivation areas.

In 1994, the first appearance of *Dinophysis* at site #4 and site #11 occurred in week #22, when low cell counts of *A. acuminata* were detected. This species remained the predominant dinoflagellate until week #30. The appearance of *D. acuminata* in the water was probably responsible for the presence of trace levels of OA in mussels from both sites in week #25. Low cell counts of *D. acuminata* (< 200 cells/l) were detected for most of the 1994 toxic period. However, due to the fact that both *D. acuminata* (1.50, 51) and *D. acuta* (1.51) have been shown to be producers of OA, it is not possible to say which species contributed to the OA content in mussels throughout the toxic period.

Significant cell counts of *D. acuta* did not appear at site #4 until week #30, when this species became the predominant dinoflagellate. The resulting toxin profile of the mussels in week #31, showed that both DTX-2 and OA were present (Fig. 3.14). The *D. acuta* population increased rapidly after week #31, until a maximum average count of 11,100 cells/l for site #4 and 16,800 cells/l for site #11 were recorded in week #33 (Fig. 3.26). All *Dinophysis* species had totally disappeared by weeks #36 and #37.
3.4 DISCUSSION

There are difficulties in obtaining representative samples of shellfish for diarrhetic toxin analysis due to wide variations in toxin levels in individual cultivated mussels. It has been shown that adjacent mussels can differ considerably in toxin levels\textsuperscript{(14)}. We have also found that there can be large geographic variability in toxin levels, even between adjacent cultivation areas (Table 3.02). This was particularly apparent at the beginning and at the end, of toxic episodes. Thus, week #33 at the start of a toxic episode in 1991, showed one very high level of toxins (6.3 μg (OA + DTX-2)/g HP), four samples in excess of the regulatory limit of 0.8 μg/g, and the remaining samples below this limit. Week #43 at the end of the toxic episode, produced five samples at, or above, 0.8 μg/g and the remaining at 0.2 μg (OA + DTX-2)/g HP, or less. This contrasted with a more even distribution of toxicity during toxic episodes, as shown for weeks #34 and #38. Differences in toxicity that occur at the beginning of major intoxications presumably reflect normal toxigenic dinoflagellate population variations and this may explain the difference in toxin profiles observed in 1994 (Fig. 3.14). In 1994, OA levels were usually less than 0.7 μg/g HP, whereas maximum DTX-2 levels of 6.7 μg/g HP were recorded.

Neighbouring regions examined in 1994 also had high levels of DTX-2 during the late summer period; Kenmare Bay (6.4 μg/g HP), Dunmanus Bay (6.6 μg/g HP) (Fig. 3.15). Trace levels of DTX-1 were also detected in samples taken from Dunmanus Bay during week #31 (Fig. 3.16). Natural detoxification processes apparently operate which account for the rapid decline in toxin levels at the end of these toxic episodes but this can also lead to local variability.

Rope cultivation of mussels adds an additional complicating factor in that vertical variation in toxin levels was also observed. Thus, examination of one length of cultivated mussels showed DTX-2 levels four times higher above 2.5 m than below that level (Fig. 3.08). As a result of both geographic variability and the variation in toxin levels with depth of cultivation, a good sampling protocol is critical for accurate toxicity assessments. Therefore, it is advisable to use a much larger sample of mussel hepatopancreas than the 1g, which was specified in the original ADAM-HPLC method of Lee \textit{et al.}\textsuperscript{(13)}. Another consequence of sample variability is the need for rapid screening methods for DSP testing. This would permit a greater number, and a higher
frequency, of sampling. An ELISA method (DSP-Check, Sceti, Japan), that can detect both OA and DTX-2, has recently been evaluated (52) (Chapter 4) and was found to be a suitable screening method for these toxins.

The predominant role of DTX-2 in DSP in Ireland is unique but there have been recent reports of this toxin in other parts of Europe. DTX-2 has been detected in dinoflagellates in Spain (53) where its presence coincided with high cell counts of Dinophysis acuta. Circumstantial evidence of the association of DTX-2 with D. acuta was also presented in this chapter, with high levels of DTX-2 being observed in mussels following the appearance of high cell counts of this dinoflagellate in the cultivation areas. However, in addition to DTX-2, substantial levels of OA were found in these shellfish and the latter was always the predominant toxin.

An understanding of oceanographic features is also important in the understanding of toxic episodes. There is evidence that a correlation exists between changes of water temperature and the subsequent appearance of high cell counts of D. acuta in the surface water column. The study and monitoring of local oceanographic profiles may be useful in the predicting future toxic event.
3.5 MATERIALS AND METHODS

Reagents

Okadaic acid (OA) (Sigma Chemical Co., UK), dinophysistoxin-1 (DTX-1) (Calbiochem Novabiochem, UK) and 9-anthryldiazomethane (ADAM) (Serva Feinbiochemica Gmbh., Heidleberg, Germany) were purchased and all reagents were stored at -20°C. Standard dinophysistoxin-2 (DTX-2) was prepared from methanol extracts of naturally contaminated mussel hepatopancreas using silica chromatography, gel permeation, octadecylsilane (C-18) solid phase extraction and C-18 preparative HPLC. This standard DTX-2 was confirmed to be 95% pure by the HPLC analysis of both its ADAM and bromoacetylpyrene derivatives\(^{52,54}\).

A certified standard lyophilised mussel material containing diarrhetic toxins (MUS-2, National Research Council, Canada) was reconstituted in methanol immediately prior to its use and this gave a mixture with 2.5 µg total toxins/ml (2.29 µg OA and 0.21 µg DTX-1/ml).

Extraction and derivatisation procedures

Mussel samples (40-50 g total meat) were collected and only the hepatopancreas was used for extraction, following a modified procedure of Lee et al., (1987). A portion of homogenised shellfish hepatopancreas equivalent to 6 g was extracted with methanol/water (4:1) (12 ml) and, after centrifugating at 3,000 rpm (10 min), an aliquot (2.5 ml) of the supernatant was washed with petroleum spirit (40-60°C), 2 x 2.5 ml, by vortex mixing for 1 min. The upper layer was discarded each time and water (1 ml) and chloroform (4 ml) were added to the combined residual solution and vortex mixed for 2 min. After centrifugation (5 min), the lower chloroform layer was transferred, using a pipette, to a volumetric flask (10 ml). The chloroform extraction was repeated and the extracts were combined (excess water was carefully removed) and made up to 10 ml with chloroform. An aliquot of the chloroform extract (0.5 ml) was evaporated under nitrogen and derivatised using either ADAM or BAP (200 µl, 0.2%). Reaction conditions for ADAM : 1 hr. at room temp. and protected from light. Solid ADAM (6 mg) was dissolved in acetone (50 µl) and made up to 3 ml with methanol and filtered (0.45 µm membrane) before use. Alternatively, ADAM was generated \textit{in situ} from anthracene carboxaldehyde hydrazide by reaction with quinuclidine and N-
chlorosuccinimide. Reaction conditions for BAP: 15 min at 75°C and base catalysed using diisopropylethylamine. The resultant mixture was subjected to clean-up on a silica SPE cartridge (Supelclean™ LC-Si, 3 ml, Supelco). The cartridge was conditioned with chloroform:hexane (1:1, 3 ml) and the sample applied to the cartridge. After washing with chloroform:hexane (1:1, 5 ml), followed by chloroform (5 ml), the toxin esters were eluted with chloroform/methanol (95:5, 5 ml). After evaporation to dryness under nitrogen (40°C, Turbo Vap LV Evaporator), the residue was reconstituted in methanol (200 µl) for analysis by liquid chromatography.

Liquid chromatographic analysis

HPLC analysis was conducted at 30°C using isocratic solvent mixtures, acetonitrile:methanol:water (80:10:10 or 80:5:15). An Ultremex C-18 column (5 µm, 3.2 x 250 mm, Phenomenex), flow-rate 0.5 ml/min or a Supelcosil LC-18-DB column (5 µm, 4.6 x 250 mm, Supelco), flow-rate 1.0 ml/min were used. 10-20µl of sample was injected with detection using a Shimadzu RF-551 fluorimetric detector (ADAM: λ<sub>ex</sub> 365nm, λ<sub>em</sub> 412nm and BAP: λ<sub>ex</sub> 365nm, λ<sub>em</sub> 418nm). Data collection and analysis was achieved using an Axxiom 717 chromatographic system. All results are based on peak areas related to a daily calibration using four OA standards which were subjected to the same derivatisation and SPE procedures (5-20ng OA, on-column) and concentrations of DTX-2 are therefore expressed as OA equivalents. A certified standard lyophilised mussel material containing DSP toxins (MUS-2, NRC, Canada) was also used as a periodic check on the validity of the analytical method.

3.6 BIBLIOGRAPHY


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Chapter 4

Evaluation of ELISA methods for the determination of DTX-2

There are no sciences, only science and its applications.

Louis Pasteur (1822-1895)
4.1 INTRODUCTION

Rapid screening for toxins in shellfish products is an important aim for the use in regulatory control of product quality. The following work involves the validation of commercial enzyme linked immunosorbent assay (ELISA) kits for potential application in Ireland and other European countries for the determination of algal toxins. Commercially available ELISA kits have been developed in Japan (Sceti. Ltd.) and Canada (Rougier Bio-Tech). The potential for application in Ireland will depend on the evaluation of these methods. The ELISA methods have been developed for OA determination and the proposed study will explore the effectiveness of these methods for the determination of this toxin and its isomer DTX-2. Validation will depend on the results of a full comparative study between these methods and the established HPLC procedures.

4.1.1 Immunological methods

The identification and assay of trace contaminants in biological material is most commonly done by chemical tests, especially spectroscopic methods. If very small quantities of material must be assayed, traditional chemical methods may fail and one approach has been to use radio-chemical tracers and other similar methods (radio-immunoassay). Unfortunately, in the assay of complex systems such as shellfish meat, it is not always possible to add enough radioactive material to label detectable quantities of a particular biochemical, either because of dilution of the label or because of danger to the organism. Immunological procedures provide the solution to these difficulties because they make it possible to assay trace amounts of organic contaminants in complex mixtures.

In response to the injection of a foreign substance into a higher animal, an Antibody (Ab) is produced that can react with the target substance. Antibodies are serum proteins called immunoglobulins. Immunoglobulins are large glycoproteins with molecular weights ranging from 150,000 to 900,000. Any substance that can elicit antibody production is called an antigen (Ag). An antibody produced by exposure to an antigen has the important property of reacting specifically with the antigen that stimulated its production and not with most other antigens. Similarly, the antigen fails to react with any antibody other than that which it elicited. In addition to the original antigen, there
are other substances that react with a specific antibody, though often with a somewhat lower efficiency. This weaker reaction is called a cross-reaction. One kind of cross-reaction is that which takes place when antigen-A reacts partially with anti-B and antigen-B partially with anti-A. Asymmetric cross-reactivity also occurs; that is, antigen-A reacts with anti-B, but antigen-B does not react with anti-A. Cross-reaction occurs when there is a chemical similarity between analytes but they are not identical (e.g. stereoisomers). During recent years, the limitation of serum as a source of antibodies has been overcome with the development of techniques to manipulate and culture mammalian cells that synthesise antibodies in vitro. Normally each cell and its progeny produce a single antibody species termed monoclonal antibody. After the antibody has been prepared, the antibody-antigen reaction is carried out merely by mixing the two substances in a buffer and incubating until the reaction is complete. The reaction is remarkable in that it usually proceeds in complex mixtures containing other proteins, small molecules, polysaccharides, which have no effect on the rate or extent of reaction.

4.1.2 Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) has become one of the most widely used serological tests for antibody or antigen detection. This test involves the linking of various "label" enzymes to either antigens or antibodies. Two basic methods are used: the double antibody sandwich method and the indirect immunosorbent assay.

4.1.2.1 The double antibody sandwich assay is used for the detection of antigens

In this assay, specific antibody is placed in the wells of a microtiter plate. The antibody is absorbed onto the walls of the wells and sensitises the plate. A test antigen is then added to each well. If the antigen reacts with the antibody in the well, the antigen is retained when the well is washed to remove unbound antigen. An antibody-enzyme conjugate specific for the antigen is then added to each well. The final complex is formed of an outer antibody-enzyme, middle antigen and inner antibody; that is a layered (Ab-Ag-Ab) sandwich. A substrate that the enzyme will convert to a coloured product is then added and any resulting product is quantitatively measured by optical density scanning of the plate. If the antigen has reacted with the absorbed antibodies in the first
step, the ELISA test is positive. If the antigen is not recognised by the absorbed antibody, the ELISA test is negative because the unattached antigen has been washed away and no antibody-enzyme is bound.

4.1.2.2 The indirect immunosorbent assay for the detection of antibodies

In this assay, antigen in appropriate sensitising buffer is incubated in the wells of a microtiter plate and is absorbed onto the walls of the wells. Test anti-serum is added and, if specific antibody is present, it binds to the antigen. Unbound antibody is washed away. An anti-antibody that has been covalently coupled to an enzyme, such as horseradish peroxidase, is next added. The antibody-enzyme complex (the conjugate) binds to the test antibody and, after unbound conjugate is washed away, the attached ligand is visualised by the addition of a chromogen. A chromogen is a colourless substrate acted on by the enzyme portion of the ligand to produce a coloured product. The amount of test antibody is quantified in the same way as an antigen is in the double antibody sandwich method.

(Both types of ELISA methods are illustrated in the following diagrams Fig. 4.01)
The ELISA test

(a) Double antibody sandwich method

1. Antibody is absorbed to well.
2. Antigen is absorbed to well.
3. Test antigen is added; if matched, antigen binds to antibody.
4. Test antiserum is added; if antibody is matched, it binds to antigen.
5. Enzyme-linked antibody specific for test antigen then binds to antigen, forming sandwich.
6. Enzyme-linked anti-gamma globulin (anti-antibody) binds to bound antibody.
7. Enzyme’s substrate (□) is added, and reaction produces a visible colour change (●).

(b) Indirect immunosorbent assay

Enzyme’s substrate (□) is added, and reaction produces a visible colour change (●).

Fig. 4.01: Illustration of both types of ELISA methods
4.1.3 The development of Immunological methods for the analysis of DSP toxins

The development of a monoclonal anti-OA antibody for DSP stemmed from work by Hokama et al. during research into the development of anti-ciguatoxin antibodies. A stick test for the detection of ciguatoxin and related polyethers was subsequently developed (3).

A modification of this stick test has led to the development of a test kit, for the screening of ciguatera and DSP toxins, called 'Ciguatech test kit'. The presence/absence of toxins is determined by the binding of toxins to a membrane attached to a plastic strip and exposing the toxin-laden membrane to an antibody-coloured-latex-bead complex that has high specificity for the toxins of interest. 1 ng of OA can be detected on the test strip and 50 pg OA/g of fish flesh can be determined using a rapid extraction procedure. Colour intensity on the test strip is assigned a value between 0-5, where 0 corresponds to non-detectable OA and 5 is equivalent to 5 ng OA (4). The 'test kit' has recently been incorporated into the French seafood safety monitoring program by detecting the presence of OA and related DSP toxins in mussels during dinoflagellate blooms and depuration operations in France (5).

Levine et al. reported the production of antibodies and the development of a radioimmunoassay for OA. An OA immunogen, was prepared by conjugation of OA to bovine albumin with carbodiimide and this was used to immunise two rabbits. The rabbits responded by producing antibodies that neutralised the simulation of arachidonic acid metabolism by OA. Inhibition of the $[^{3}]$H-OA binding to anti-OA, by unlabelled OA, occurred at concentrations as low as 0.2 pmoles. When the percentage inhibition was plotted against the log of the OA concentration, a linear graph resulted in the approximate range 0.2-9.0 pmoles. Maitotoxin, teleocid, 12-O-tetradecanoylphorbol-13-acetate, aplysia toxin, palytoxin and brevetoxin B when tested at 29, 228, 168, 169, 3.7 and 112 pmole levels, respectively, did not inhibit binding. However no data was presented for the cross-reactivity of the test to the other DSP toxins (6).

Usagawa et al. prepared three monoclonal anti-OA antibodies, OA-1, OA-2, and OA-3, from hybridoma clones obtained by the fusion of mouse 653 myeloma cells with mouse immune spleen cells sensitised to OA-ovalbumin conjugate (OVA). A competitive inhibition enzyme linked immunosorbent assay (ELISA) was also developed, patented
and marketed as a kit under the name 'DSP Check' by Sceti Ltd., Tokyo, Japan. Of the three antibodies produced, the OA-3 antibody was found to have the highest sensitivity to OA and as a result was used in the ELISA test. Both standards and samples can be analysed in 20 min. and the assay is sensitive to 10 ppb for OA. The antibodies show ca. 70% cross-reactivity to DTX-1 but do not react with the 7-O-acyl derivatives of OA and DTX-1, pectenotoxins or yessotoxins (7,8).

The cross-reactivity of this kit to DTX-2 was found to be ca 40%, in our laboratories, using a purified DTX-2 standard (9). 7-O-acyl derivatives of DSP toxins can be determined by converting them into the free acid using a hydrolysis step (using sodium hydroxide) prior to analysis.

This assay requires the use of OA for coating the plates and consequently makes the 'DSP Check' kits very expensive. To overcome this, Shestowsky et al. have developed an alternative immunoassay in which they use two mouse monoclonal antibodies to OA. The method is based on a competitive indirect enzyme-linked immunosorbent assay. One of these antibodies is an anti-OA monoclonal antibody (mAb) called 6/50 (idiotype (Id)) and the other is a syngenic anti-anti-OA mAb called 1/59 (anti-Id). The 1/59 anti-Id mAb is an internal image of OA and can be substituted for the OA coating on the solid-phase. The assay permits reliable measurement of OA in the 9-81 ppb range and is marketed as an 'ELISA kit for quantitation of Okadaic acid detection' kit by Rougier Bio-Tech Ltd., Montreal, Canada.

Anti-OA 6/50 Id antibodies are prepared by immunising Balb/c mice with OA-bovine serum albumin conjugate (BSA). 1/59 anti-Id antibodies are prepared by injection of Bacillus Calmette-Guerin (BCG)vaccine into Balb/c mice followed by several inoculations with 6/50 antibody-tuberculin purified protein derivative conjugate (10).

Application of this kit, for the detection of DTX-2 in our laboratory, showed it to be more difficult to use than the Sceti kit and yielding a low cross-reactivity to DTX-2 (11). DTX-3, calyculin A and brevetoxin-1 cannot be detected by this ELISA kit. DTX-2 and DTX-1 are recognised by the 6/50 anti-OA mAb but they have a 10 and 20 fold lower affinity for anti-OA mAb, respectively, than the affinity between anti-OA and OA (12). The least effectively bound derivatives are the tetra-acetate, where all the free hydroxyl groups of OA are esterified, and DTX-3, which contains the DTX-1 skeleton linked to fatty acid moieties at the C-7 hydroxyl group. These findings suggest that the 6/50
antibody recognises the portion of okadaic acid most distant from the carboxyl group \(^{(12)}\). Derivatives of OA such as the methyl ester, diol ester and OA-alcohol react with the 6/50 antibody almost as well as OA \(^{(13)}\).

More recently, Matsuura \textit{et. al.} reported the development of an ELISA method for OA and its analogs using immobilised mouse monoclonal antibodies to OA, which are resistant to organic solvents. One antibody which binds only OA is used to detect OA selectively. Another antibody which equally binds OA, DTX-1 and 7-O-palmitoyl DTX-1 in methanol, was used to determine the total toxin \(^{(14)}\).

Yasumoto \textit{et al.} have prepared an antibody for the pectenotoxins. The antibody cross reacts equally with PTX-6 and PTX-1 and it is 1,000 fold less sensitive to yessotoxin \(^{(15)}\).

An ELISA method with electrochemical detection has been developed for OA. The method involves a competitive heterogeneous immunoassay in which antigen in solution competes with immobilised antigen for soluble antibody. The excess sample and antibody is removed and a secondary antibody labelled with alkaline phosphatase is added. Phenyl phosphate is hydrolysed by the enzyme label to yield phenol which is then oxidised at +870 mV vs. Ag/AgCl. The dynamic response is inversely proportional to the soluble antigen \(^{(16)}\).

### 4.2 PRINCIPLE OF THE 'DSP CHECK' ELISA TEST (KIT #1)

The procedure of one step ELISA is described as follows.

A constant quantity of OA-bovine serum albumin (OA-BSA) is fixed antigen on an immunoplate in advance. Both the sample and the enzyme labelled monoclonal antibody are simultaneously placed and reacted on the plate. In this step, the enzyme labelled monoclonal-antibody competitively reacts with the fixed antigen and the free antigen from the sample. The binding of the antibody to the fixed antigen is inversely proportional to the concentration of free antigen. After washing the plate, the quantity of the enzyme, which is proportional to that of fixed antibody, is determined. Enzyme activity is colourimetrically measured through the substrate reaction. In this assay, the absorbance decreases with increased concentration of DSP toxins. The time required for the assay is 20 minutes.
4.3 PRINCIPLE OF THE ‘ROUGIER BIO-TECH’ ELISA TEST (KIT #2)

The method employed in the Canadian kit is summarised as follows.

ELISA for quantification of OA is a 3-step procedure based on the competitive inhibition principle. First, diluted samples along with reference standards and negative controls are incubated simultaneously with the stock antibody solution in the appropriate test wells pre-coated with the capture antibody. OA present in the unknown sample and the reference standards will bind to the antibody in the solution and thus reduce the amount of antibody available for binding to the capture antibody. In the next stage, horseradish peroxidase-conjugated anti-mouse Ig is added to the wells as the detecting antibody.

In the final step, enzyme substrate is added to the wells. The substrate will react with the enzyme bound to the secondary antibody and will result in a coloured end product. The reaction is terminated by the addition of acid to the wells containing the substrate. The colour intensity is measured on a microtitre ELISA plate reader and is inversely proportional to the amount of bound conjugate and, therefore, to the amount of OA present in the unknown or the reference standards. Hence, samples containing high concentrations of the OA will not produce a colour reaction, whereas those containing low concentrations will. At this point it is important to note that the manufacturers make the following statement

"This ELISA system is designed to measure okadaic acid present in marine samples (e.g. mussels) and freshwater plankton samples when prepared according to instructions. The kit is not intended for quantitation of other marine toxins (e.g. Dinophysis toxin-1)."
4.4 SAMPLE / TEST SOLUTIONS USED

Samples used in this study included:

1. naturally toxic mussels (Bantry, Co. Cork, Ireland),
2. MUS-2 (NRC, Canada). The detection limit was 0.1 ng,
3. purified DTX-2, isolated from mussels,
4. non toxic mussels spiked with standard DTX-2

The following sets of test solutions were prepared for both ELISA kits and subsequent HPLC analysis (see Map (Fig. 3.02) for details of site location).

- **Table 4.01** Chloroform extracts used

<table>
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<th>Sample No.</th>
<th>Location</th>
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<th>OA content (%)</th>
<th>DTX-2 content (%)</th>
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<td>37</td>
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**Additional Solutions**

- "MUS-2" (No. 383) a certified mussel standard purchased from NRC Canada which contained both OA (2.29 μg/ml) and DTX-1 (0.21 μg/ml).

- A series of 5 OA standards ranging from 0 ppb to 100 ppb
  (OA standard solutions of 10, 20, 33.3, 50 and 100 ppb were used.)

- Dilutions of DTX-2 large extract from site # 4 and #12.
4.5 RESULTS AND DISCUSSION

4.5.1 ELISA testing for DSP toxins

Both kits had been developed for the detection of OA and there was no information available on cross-reactivity with DTX-2. This study is summarised graphically (Fig. 4.02) and in tabulated form (Table 4.01). This preliminary study showed that both methods showed excellent correlation with HPLC when OA was the predominant or only toxin present. However, kit #1 (DSP-Check) showed a much better correlation than kit #2 (Rougier) if DTX-2 was predominant. Also, with an increase in the percentage DTX-2 in the samples, the accuracy of both ELISA kits decreased (Fig. 4.02). Kit #2 was found to give the greatest deviation from the HPLC data when DTX-2 was the predominant toxin and this has recently been confirmed by Chin et al. when it was reported that kit #2 could only detect DTX-2 in concentration ranges 10-fold higher than that for OA (12).

![Fig. 4.02 DSP correlation study - ELISA and HPLC](image)
<table>
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<th>Sample</th>
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<td>(t=γ)</td>
<td>3.1+0.2</td>
<td>(t=γ)</td>
<td>3.9+0.4</td>
</tr>
<tr>
<td>78</td>
<td>(t=γ)</td>
<td>3.5+0.7</td>
<td>(t=γ)</td>
<td>2.9+0.6</td>
<td>(t=γ)</td>
<td>4.2+0.2</td>
</tr>
<tr>
<td>76</td>
<td>(t=γ)</td>
<td>1.6+0.5</td>
<td>(t=γ)</td>
<td>1.0+0.3</td>
<td>(t=γ)</td>
<td>1.6+0.3</td>
</tr>
<tr>
<td>75</td>
<td>(t=γ)</td>
<td>2.5+0.2</td>
<td>(t=γ)</td>
<td>0.5+0.3</td>
<td>(t=γ)</td>
<td>1.1+0.3</td>
</tr>
<tr>
<td>76</td>
<td>(t=γ)</td>
<td>1+1</td>
<td>(t=γ)</td>
<td>10</td>
<td>(t=γ)</td>
<td>12+1</td>
</tr>
</tbody>
</table>

Mean toxin concentration ±SD (μg/hepatopancreas)

For the assay of diarrhetic shellfish toxins.

Comparison of an LC method and two ELISA methods

Table 4.02.
Fig. 4.03

*Chromatograms of BAP derivatives of the chloroform extract from site #13.*

Dated 15/9/91 The sample contained both DTX-2 and OA (DTX-2: 37% and OA: 63%) DCA was used as an internal standard.

(Data file 11-4-94.402)

On a practical basis, the procedure for kit #2 was more complex than kit #1, in that a number of incubation steps were required, making the kit unsuitable for on-site analysis. In view of this and its poor cross reactivity with DTX-2, kit #2 was deemed unsuitable for application in the screening of cultivated mussels in Ireland. However, kit #1 gave excellent linear correlations when concentration data for the sequential dilution of sample #2 (containing 94% DTX-2 by HPLC) were plotted against the dilution factors (Fig.4.04).
4.5.2 Cross-reactivity study with standard DTX-2

Further studies were undertaken to establish the cross-reactivity of DTX-2 using kit #1. Standard calibration plots for OA and DTX-2 are shown in Fig. 4.06. The lower level of response of this ELISA test to DTX-2 compared with OA is clearly demonstrated in this figure. Each point represents the mean of six measurements and from this data (Table 4.02), the average cross-reactivity, calculated from 5 points on the graph was 40 ± 5% for DTX-2.

<table>
<thead>
<tr>
<th>DTX-2 Concentration(ng/g) (DTX-2 Calibration graph)</th>
<th>DTX-2 Concentration(ng/g) (OA Calibration graph)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.67</td>
<td>4.99</td>
</tr>
<tr>
<td>21.53</td>
<td>9.06</td>
</tr>
<tr>
<td>29.80</td>
<td>11.93</td>
</tr>
<tr>
<td>56.06</td>
<td>20.38</td>
</tr>
<tr>
<td>105.71</td>
<td>34.90</td>
</tr>
</tbody>
</table>

Table 4.02  Comparison of OA and DTX-2 calibration data using ELISA Kit #1, showing the reduced cross-reactivity for DTX-2.
Fig. 4.05  
Chromatograms of BAP derivatives of (a) OA standard (14.4 min, 12.5 ng) and (b) DTX-2 purified from toxic shellfish (16.2 min, 12.5 ng)

Fig. 4.06  
Calibration plots for OA and DTX-2 standards using ELISA kit #1  
(Each point represents the mean of six measurements)
Finally, blank mussel samples from Clew Bay Galway were spiked with DTX-2 standard and measurements (N=6) of five samples showed that there was a good linear correlation (r=0.998; Fig. 4.07) with toxin levels in the range 0.03-0.3 μg/g mussel meat. This range is appropriate for regulatory control of DSP in shellfish.

![Graph showing absorbance vs. DTX-2 concentration](image)

Fig. 4.07  Blank mussel samples, spiked with DTX-2 standards, analysed using ELISA kit #1

When OA is the predominant or only toxin detected, the ELISA method is very accurate. When DTX-2 is the predominant or only toxin detected, accurate results can be obtained by ELISA, provided a cross-reactivity correction factor is used. If standards of this toxin are available, the concentration of DTX-2 in samples can be determined directly by the construction of a DTX-2 rather than an OA calibration plot. When OA and DTX-2 are simultaneously present in shellfish then the cross-reactivity correction factor becomes more complicated, but can be worked out from the ratio of OA to DTX-2 in a representative sample, determined by HPLC. If the toxin profile of mussels in a cultivation area was determined to be \( a:b \) OA:DTX-2 by HPLC and if the concentration of a mussel sample was determined to be \( C \) μg total toxin per g HP by
ELISA. Then the individual toxin concentration of this sample can be estimated using the following formulas (assuming a 40% cross-reactivity for DTX-2).

\[ \text{OA concentration, \( \mu g \) per g HP} = \frac{ca}{a + 0.4b} = d \]

\[ \text{DTX-2 concentration, \( \mu g \) per g HP} = 2.5(c - d) = e \]

Summary:
The conclusion of this study is that the ELISA kit 'DSP-Check' should prove useful for the screening of shellfish for the diarrhetic toxins currently found in Ireland and Europe. With respect to the Irish situation, it would be necessary to establish the toxic profile of the mussels at the time of analysis. This can readily be determined by the HPLC analysis of representative mussel samples from a cultivation area. With these provisions, more comprehensive screening can then be carried out using ELISA.

4.6 MATERIALS AND METHODS

Equipment
Microtitre plate reader (DYNATECH MR7000), incubator (Gallenkamp model 1H-100), pH meter (Corning) and the HPLC equipment as detailed in chapter 2.

Reagents
9-Anthryldiazomethane (ADAM), (Serva Feinbiochemica GmbH., Heidleberg),
1-Bromoacetylpyrene (BAP), (Aldrich Chemical Co., U.K.).

ELISA kit #1 : DSP-Check®, Sceti Co Ltd., Tokyo, Japan.
ELISA kit #2 : Okadaic Acid kit, Rougier Bio-Tech Ltd., Montreal, Canada.
KIT #1

Details:
ELISA kit #1:
DSP-Check®,
Sceti Co. Ltd.,
Tokyo, Japan.

KIT Components (20 tests)

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reaction plate (1 x 8 wells)</td>
<td>3 plates</td>
</tr>
<tr>
<td>2</td>
<td>Enzyme conjugated monoclonal anti-OA</td>
<td>1 vial</td>
</tr>
<tr>
<td>3</td>
<td>Substrate solution</td>
<td>1 vial</td>
</tr>
<tr>
<td>4</td>
<td>Chromogen</td>
<td>1 tablet</td>
</tr>
<tr>
<td>5</td>
<td>Stopping reagent</td>
<td>1 Vial</td>
</tr>
<tr>
<td>6</td>
<td>Washing solution</td>
<td>1 vial</td>
</tr>
<tr>
<td>7</td>
<td>OA standard 1 (10ppb)</td>
<td>1 vial</td>
</tr>
<tr>
<td>8</td>
<td>OA standard 1 (100ppb)</td>
<td>1 vial</td>
</tr>
<tr>
<td>9</td>
<td>Disposable micropipette</td>
<td>26 pipettes</td>
</tr>
</tbody>
</table>

(Note: The OA standards were replaced by own standards and a GILSON ® Pipette-man was used to give greater precision when measuring out the desired volumes.)

4.6.1 Analysis protocol Kit #1

A) Preparation of Samples (As outlined in chapter 2)

B) ELISA STEP
Preparation

The OA standards were prepared as described in Appendix No.5. The chromogen [4] was dissolved with the whole substrate solution [3] and the resulting yellow/orange solution was stored in the dark until required. The washing solution [6] was added to the enzyme conjugated monoclonal anti-OA [2] up to the indicated line on the vial. The
vial was shaken several times by hand to ensure that the contents of the vial were properly mixed

(Note: these mixed solutions cannot be stored and have to be used on a same day basis)

**Assay procedure**

All volumes were dispensed using ‘GILSON’ pipette man. The assay procedure is divided up into the following five steps.

1) **Antigen-antibody reaction:**

   50µl of the OA standard and sample solutions were placed into their designated wells. Immediately afterwards, 50µl of the enzyme conjugated antibody solution was added to each well and then allowed to stand for 10 min., at room temperature. (Mixing of the well contents was attained by moving the plates to and fro on the bench.)

2) **Washing the wells:**

   The solution from each well was removed and the wells were washed four times with the washing solution [6]. Small quantities of liquid left at the bottom of each well was eliminated by flipping the plate by hand.

3) **Colour development:**

   50µl of the chromogen solution ([3]+[4]) was then added and the plate was allowed to stand for 6 min. at room temperature in the dark.

4) **Stopping the reaction:**

   50µl of the stopping reagent [5] was added to each well, and again mixing of the well contents was achieved by moving the plates to and fro on the bench.

5) **Judgement:**

   For the quantitative analysis of each test the absorbance of each well was measured using a DYNATECH MR7000 ELISA microtitre plate reader. The absorbance of all solutions were taken at a wavelength of 490nm.
6) **Evaluation of Results:**

The mean absorbance for each triplicate (standards & unknowns) was calculated. OA concentration versus mean absorbance was plotted on two cycle semi-logarithmic graph paper to obtain a standard curve. The OA content of the unknown samples assayed together with the reference standards was determined from the standard curve by means of interpolation.

---

**KIT #2**

Details:

ELISA kit #2:

Okadaic Acid kit,

Rougier Bio-Tech Ltd.,

Montreal, Canada

**KIT Components (96 tests)**

<table>
<thead>
<tr>
<th></th>
<th>Holder containing $8 \times 12$ well precoated strips.</th>
<th>1 holder</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>96-well plate to be used for blanking.</td>
<td>1 plate</td>
</tr>
<tr>
<td>3</td>
<td>Bottle of 150mls, 10-fold concentration Tris-buffered saline (TS).</td>
<td>1 bottle</td>
</tr>
<tr>
<td>4</td>
<td>Fat-free powered-milk.</td>
<td>5 grams</td>
</tr>
<tr>
<td>5</td>
<td>Bottle of 1ml Tween-20. More than enough to make 1500mls of 0.05% Tween-20/TS to be used as a washing buffer</td>
<td>1 bottle</td>
</tr>
<tr>
<td>6</td>
<td>Okadaic acid standards, supplied dry:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Standard I (4.5 ng/vial)</td>
<td>1 vial</td>
</tr>
<tr>
<td></td>
<td>Standard II (13.5 ng/vial)</td>
<td>1 vial</td>
</tr>
<tr>
<td></td>
<td>Standard III (40.5 ng/vial)</td>
<td>1 vial</td>
</tr>
<tr>
<td>7</td>
<td>Vial of 300μl of an anti-okadaic acid (OA) antibody solution.</td>
<td>1 vial</td>
</tr>
<tr>
<td>8</td>
<td>Vial of 300μl of horseradish peroxidase (HRP)-conjugated anti-mouse Ig.</td>
<td>1 vial</td>
</tr>
<tr>
<td>9</td>
<td>Bottle of substrate solution containing 10mls.</td>
<td>2 bottle</td>
</tr>
</tbody>
</table>
Pouch containing 4 x 5mg Ortho-Phenyl diamine (OPD) tablets. 1 pouch
Vial of 500μl of 30% H₂O₂. 1 vial
10ml bottle of 3N sulphuric acid (H₂SO₄). 1 bottle

Reagents and Equipment required but not provided:

- Distilled water
- Methanol (HPLC grade)
- pH meter
- Homogeniser or blender
- Stirring plate
- Vortex mixer
- Cellophane paper/ 'Parafilm'
- 37°C incubator
- Precision pipettes and tips
- Concentrated HCl
- ELISA reader with 490 nm wavelength filter
- 2 cycle semi-logarithmic graph paper

4.6.2 Analysis protocol Kit #2

A) Preparation of Samples
All samples were prepared as previously outlined for the Japanese kit

B) ELISA STEP

Preparation of test reagents
The working solution of TS was prepared by diluting 1:10 the provided concentrated TS and adjusting to pH 7.4 with concentrated HCl. The 1% milk/TS (w/v) was prepared by dissolving 1g of powdered-milk in every 100mls of TS. Approximately 100ml was needed for processing 1 plate. A 0.05% Tween/TS (v/v) was prepared by adding 0.5ml of Tween-20 to one litre of TS. Approximately 500 ml was needed for washing one plate. The OA standard were prepared as described in Appendix No. 5. Note that standard solutions were used shortly after reconstitution although they may be stable for up to 2 weeks when stored at 4°C. The antibody solution was prepared just before use by diluting 100 times the anti-OA stock solution provided. (i.e. Add 100μl of the Anti-OA to 10ml of 1% milk/TS. Approximately 5mls is required per plate)
Note: Only the amount that was needed for the assay was prepared as diluted antibody cannot be stored for further use.

Assay procedure

1) OA- Capture -Antibody reaction:

The required number of strips needed for the assay were removed from the aluminium pouch. Using a precision pipette, 50μl/well of the standards and test samples were applied into designated precoated wells (always in triplicate). Care was taken not to touch the sides of the well with the tip, and all tips were discarded after each sample to avoid cross-contamination. 50μl/well of anti-OA antibody solution was added immediately to the same wells. Care was taken not to splash up onto the rim. The plates were tapped gently to ensure that each well was covered with the sample/antibody solution and that all air bubbles had been removed. The plates were wrapped in Parafilm and incubated while shaking for 1 hr in a 37°C incubator.

2) Washing the wells (first):

After incubation the contents of the reaction wells were removed by flicking the plates and washing with 0.05% Tween/TS 4 times. Between each wash the Tween/TS was removed by gently flicking the plates. The remainder was removed by gently tapping the plate on a dry, soft, clean absorbent paper towel.

3) Addition of detecting antibody:

The HRP-conjugated anti-mouse Ig was prepared just before use by diluting the stock a 100 times with 1% milk/TS. 100μl of the HRP-conjugated anti-mouse Ig solution was added to each well. The plates were sealed and incubated, while shaking for 1 hr, in a 37°C incubator.
4) *Washing the wells (second):*

After incubation the contents of the reaction wells were removed by flicking the plates and washing with 0.05% Tween/TS 4 times as above.

5) *Colour development reaction:*

The final substrate buffer was prepared 15 min before use to ensure that all reagents were at room temperature. 10ml of the final substrate buffer was prepared as follows: 10μl of the 30% H₂O₂ and 2 OPD tablets (5mg OPD/tablet) were added to 10ml of substrate and the mixture was stirred until the OPD tablets were completely dissolved. *(note: OPD is a suspected carcinogen and gloves and mask are used when handling it. Solution must be used within 60 minutes of preparation.)*

100μl of the freshly prepared final substrate buffer was added to each designated well and incubated while shaking for 30 minutes in the 15-30°C incubator. Blanks were prepared by simultaneously adding 100μl of the freshly prepared final substrate buffer to the uncoated wells which were incubated as described above.

6) *Stopping the reaction:*

The reaction was stopped by the addition of 50μl of 3N H₂SO₄ to all wells including the row of blanks.

7) *Judgement / Evaluation of Results:*

For the quantitative analysis of each test the absorbance of each well was measured using a DYNATECH MR7000 ELISA microtitre plate reader. The absorbance of all solutions were taken at a wavelength of 490nm. The mean absorbance for each triplicate (standards & unknowns) was calculated. OA concentration versus mean absorbance was plotted on 2 cycle semi-logarithmic graph paper to obtain a standard curve. The OA content of the unknown samples assayed together with the reference
standards was determined from the standard curve by means of interpolation.

**HPLC procedures**

The analysis of mussel extract samples and standards by HPLC was carried out using the modified ADAM or BAP precolumn derivatisation methods outlined in chapter 2. The concentration of toxin in the original mussel hepatopancreas was calculated as outlined in Appendix No.5.

**Evaluation of ELISA Results**

The concentration of toxin in the original mussel hepatopancreas was determined by correcting for any dilutions made in the pre-treatment procedure (See Appendix No.4)

### 4.7 BIBLIOGRAPHY


After all science is essentially international, and it is only through lack of the historical sense that national qualities have been attributed to it.

Marie Curie (1867-1934)
5.1 INTRODUCTION

The polyether carboxylic acids, OA and DTX-1 have been identified as the toxins responsible for most outbreaks of DSP (1). These compounds exert their bioactivity by the potent inhibition of protein phosphatases, PP1 and PP2A (2). It is this activity that has led to the use of OA as a valuable reagent for biochemical cellular studies and it is now commercially available. However, DTX-2, a new isomer of OA, was recently identified (3) in mussels (Mytilus edulis), cultivated in Ireland, where it continues to be the predominant toxin (4-6). DTX-2 was isolated after exhaustive extraction with methanol and acetone, followed by a combination of adsorption, size exclusion and reverse phase chromatography. The structure of DTX-2 was then determined by nuclear magnetic resonance (NMR) spectroscopic analysis of the isolated product.

Bioassays, with live rodents, are used by most regulatory authorities to screen for diarrhetic toxicity in shellfish but internationally agreed limits for individual toxins will depend on toxicity evaluations. The new toxin, DTX-2, was recently detected in dinoflagellates in Spain (7) and in shellfish from Portugal (8).

The fact that DTX-2 standard is not commercially available has hampered scientific investigations into its bioactivity and toxicological properties. To overcome this problem the 'Ecotoxicology Research Unit' at Cork RTC has successfully isolated and prepared standards of DTX-2 and another novel DSP toxin (DTX-2B).

A methodology for the preparation of pure DSP toxin standards from toxic shellfish, was developed based on a combination of chromatographic techniques successfully used in the isolation of other compounds belonging to the OA class of DSP toxins (9-11). This involved the solvent extraction of toxins from the hepatopancreas of toxic mussels, followed by repeated flash chromatography (silica), gel permeation (Sephadex LH-20), solid phase extraction (octadecsilane) and repeated semi-preparative reversed-phase liquid chromatography (Fig. 5.01).
5.1.1 Isolation of DTX-2 from shellfish

Mussels (*Mytilus edulis*) were collected from south-west Ireland following a bloom of phytoplankton (*Dinophysis acuta*) in the late summer/early autumn months of 1991 and 1994.

Only the hepatopancreas of mussels (20-25% of total meat) was used for toxin isolation (550 g). After homogenisation with methanol (80% v/v, 500 ml), the mixture was centrifuged at 3,000 rpm for 15 min. The supernatant was washed with light petroleum (40-60°C, 2 x 500 ml) and extracted with chloroform (2 x 500 ml). The chloroform extracts were combined, dried (MgSO₄), and evaporated. In the following chromatographic steps, the fractions containing DTX-2 were identified by the analysis of aliquots using HPLC following derivatisation with ADAM or BAP.\(^{(12)}\)
1. The residue was chromatographed on silica, 100g (E. Merck GmbH, Darmstadt, Germany) and eluted with ether followed by a step gradient of methanol/ether. DTX-2 eluted in fractions with 5-20% methanol/ether and this chromatography was repeated.

2. Fractions containing DTX-2 were transferred, using methanol, to a column containing Sephadex LH-20, 7 g (Pharmacia LKB Biotechnology, Uppsala, Sweden). DTX-2 eluted in methanol (20ml), which was evaporated.

3. An SPE cartridge, Mega Bond-Elut, C-18, 10 g (Varian, Harbour City, CA, USA), was conditioned with acetonitrile:water (40:60 v/v, 30ml) and the DTX-2 fraction, in the same solvent, was applied to the cartridge. The SPE column was washed with acetonitrile:water (40:60 v/v, 30ml and 60:40 v/v, 30ml) and DTX-2 eluted using acetonitrile:water (80:20 v/v, 30 ml).

4. Semi-preparative HPLC was carried out using an Ultremex C-18 column (10 x 250 mm, 5μm, Phenomenex). The mobile phase used was initially acetonitrile:water (50:50 v/v) for 5 min, increasing stepwise to 100% acetonitrile over 40 min and a solvent flow of 4 ml/min. DTX-2 eluted mainly in acetonitrile:water (70:30 v/v) and other fractions containing less pure DTX-2 were re-chromatographed.

An extensive programme involving the weekly examination of cultivated mussels (Mytilus edulis) from south-west Ireland for diarrhetic toxins allowed the acquisition of sample material containing DTX-2. HPLC analysis, following derivatisation with ADAM, revealed DTX-2 as the predominant toxin, with small amounts of OA. The isolation of diarrhetic toxins from shellfish is difficult due to the low natural abundance of these compounds, typically less than 1 μg/g shellfish meat. Also, the lack of a suitable chromophore requires the extensive analysis of eluates collected, using either of the HPLC methods previously described.
Standard DTX-2 isolated by the above procedure was subdivided into vials, each containing $2.6 \pm 0.14 \mu g$ of 95% pure DTX-2 per vial. The application of this methodology for the preparation of pure DSP toxin standards from toxic shellfish, has also resulted in the isolation of small quantities of a new DSP toxin, now named DTX-2B, which possesses similar chromatographic properties to that of DTX-2 (Fig. 5.02).

![Chromatogram showing the prenacyl derivatives of a standard mixture of four DSP toxins: OA (tR. = 14.6 min), DTX-2 (tR. = 16.4 min), DTX-2B (tR. = 17.6 min) and DTX-1 (tR. = 21.5 min)](image)

Fig. 5.02
5.1.2 Research Collaboration

Samples of DTX-2 have been supplied, by our laboratory, to a number of shellfish research groups in Europe for use as an analytical standard. The collaboration with these research groups was initiated to examine the standard DTX-2 and other novel DSP toxins in a variety of new tests/methods.

- Dr. Carol MacKintosh, Medical Research Council (MRC), Protein Phosphorylation Unit, University of Dundee, to determine the corresponding specific inhibitors of protein phosphatases 1 and 2A (PP1 and PP2A) by DTX-2 and DTX-2B.

- Dr. Y.F. Pouchus, Faculté de Pharmacie, Université de Nantes, Nantes, France, to determine if DTX-2 can be successfully analysed by a cytotoxicity assay. A positive result would indicate that this test would be suitable for the screening of Irish shellfish for DSP toxicity.

- Dr. Rosa Draisci, Instituto Superiore di Sanità, Roma, Italy, to determine both the full scan positive-ion ISP mass spectra and the \([M+H]^+\) daughter ion mass spectra of DTX-2 and DTX-2B, by LC-MS/MS.

- Dr. Hideo Naoki, Suntory Institute for Bio-organic Research, Osaka, Japan and Prof T. Yasumoto, University of Sendai, Osaka, Japan, to determine the Negative-ion FAB MS/MS spectrum of DTX-2 and DTX-2B.

- A recent pilot plant study was commissioned by Bord Iascaigh Mhara (B.I.M.) to investigate the feasibility of ozone treatment for the reduction of DSP toxin levels in shellfish.
5.2 ASSAYS INVOLVING PROTEIN PHOSPHATASE INHIBITION

5.2.1 Introduction

OA, DTX-1 and DTX-3 exert their toxic effects, because they are extremely potent and specific inhibitors of protein phosphatases 1 and 2A (PP1 and PP2A). This inhibition of the protein phosphatases is also responsible for the tumour promoting properties of these toxins.

The mode of action of these toxins can be illustrated by the following simple analogy:

Imagine that all cells in your body have little internal control circuits (detectors, fuses, switches) that constantly monitor and regulate their rate of growth, energy supplies and specialised functions. Now, a toxin enters the system and completely blocks one set of these control switches. The result is chaos. This is exactly how the OA class of DSP toxins acts in your gut. Of course the "switches" blocked by these toxins are not electrical: they are two regulatory enzymes called protein phosphatases 1 and 2A which are found in all animals and plant cells.

The PP1 and PP2A inhibition properties of the acidic DSP toxins has allowed for the development of a sensitive DSP protein phosphatase bioassay. Standard curves for the percentage inhibition of PP1 and PP2A by OA and DTX-1, were found to be linear between 40-55% inhibition, in this assay.

![Diagram showing the principle behind the protein phosphatase inhibition assay](image)

Fig. 5.03

*Diagram showing the principle behind the protein phosphatase inhibition assay*
Protein phosphatase activity is determined by measuring the release of acid-soluble $^{32}\text{P}$-radioactivity from $^{32}\text{P}$-labelled substrate in a fixed time period. The reaction is stopped by adding trichloroacetic acid (TCA) to inactivate the protein phosphate, and precipitate the unused $^{32}\text{P}$-labelled protein. The acid-soluble fraction is extracted into acid molybdate, which specifically extracts the inorganic phosphate, and $^{32}\text{P}$-phosphate is determined in a scintillation counter. Phosphorylase $a$ is the recommended $[^{32}\text{P}]$ phosphoprotein substrate as it is a good substrate for both PP1 and PP2A and is easy to prepare and standardise. Compared with other DSP toxin assays, the protein phosphatase assay is simple, quick, extremely sensitive, quantitative and versatile.

There are several reports in the literature on the quantities of DSP toxins required to cause acute toxic effects in humans. However, the consequences of chronic doses are poorly understood. MacKintosh et al. have determined that concentrations (IC$_{50}$) of OA needed, to cause a 50% inhibition of the activity of 0.2 mU/ml solutions of the catalytic subunits of PP1 and PP2A (isolated from rabbit skeletal muscle), are 10-15 nM and 0.1 nM, respectively. Holmes et al. has reported IC$_{50}$ values for PP1 and PP2A inhibition by OA of 19 nM and 0.2 nM, respectively, in the same assay. The IC$_{50}$ values for the inhibition of PP1 and PP2A have been shown to increase with increasing concentration of OA.

However, at present there is no information in the literature on the toxicity of DTX-2. For this reason collaboration with Dr. Carol MacKintosh has been established to determine the corresponding inhibition data for DTX-2 and DTX-2B.

5.2.2 Results and discussion

The IC$_{50}$ values for inhibition of PP2A were as follows:

\[
\text{DTX-2} = 0.18 \text{ ng/ml} = 0.22 \text{ nM} \\
\text{DTX-2B} = 0.19 \text{ ng/ml} = 0.24 \text{ nM}
\]

The IC$_{50}$ values for inhibition of PP1 were $> 330$ ng/ml ($> 410$ nM the highest concentration tested).

These results indicate that both DTX-2 and DTX-2B inhibit PP2A with a potency of the same order as OA. The OA IC$_{50}$ values for PP1 and PP2A (isolated from rabbit skeletal...
muscle), are 10-19 nM and 0.1-0.2 nM, respectively, in the standard phosphorylase $a$ phosphatase assay.\(^{17,18}\)

Although PP2A comes from different mammalian tissues, with the same amino acid sequence, it has been found that the purified catalytic subunits of PP2A from bovine heart muscle have a specific activity, using $^{32}$P-labelled phosphorylase phosphatase, which is 3-4 fold higher than those from rabbit skeletal muscle. Therefore, when tested at the same phosphorylase phosphatase activities, the heart enzyme has a lower IC$_{50}$ value for inhibition of OA than the skeletal muscle enzyme (because the molar concentration of PP2A catalytic subunits is lower in the assays using the heart PP2A).\(^{18}\)

The PP1 (human isoform expressed *E. coli*) used in this study has a lower apparent affinity for DSP toxins than its counterpart purified from skeletal muscle. Hence, the apparently high IC$_{50}$ values obtained for PP1 inhibition of DTX-2 and DTX-2B.

5.2.3 Conclusion

Initial results revealed that DTX-2 and DTX-2B have a toxic potential similar to that of OA and should therefore be subjected to similar regulatory control.

*Experimental*: (See Appendix No.5)
5.3 CYTOTOXICITY ASSAYS

5.3.1 Introduction

The cytotoxic effect of OA has been recognised since it was first isolated by Tachibana et al. (19). It was noticed that when rat hepatocytes were exposed to toxic shellfish extracts, a rapid leakage of lactate dehydrogenase occurred, while non-toxic extracts had no disintegrating effect on the cell membranes (20).

A DSP toxin cytotoxicity test has been reported, based on morphological changes of rat hepatocytes when exposed to toxins. The three classes of DSP toxins can be determined and they all produce different changes in the cell’s morphology. OA and DTX-1 have been shown to induce irregular-shaped cells with surface blebs, PTX-1 promotes dose dependant vacuolisation and yessotoxin leaves cellular shape unaffected but induces blebs on the surface. The detection limits for OA, PTX-1 and YTX were 0.5 µg/ml, 5 µg/ml and 20 µg/ml, respectively. The major disadvantages of this cytotoxicity test was the analysis time (up to 72 hrs) and confusing results were obtained when mixtures of toxins were involved (21).

More recently Amzil et al. have developed a rapid cytotoxicity bioassay for OA detection (22). The minimal active concentration (MAC) of shellfish extracts on KB cultured cells is determined after three hours incubation. A high correlation was found between the logarithms of the MAC of tested extracts and the OA concentrations in mussel hepatopancreas measured by liquid chromatography. Approximately half the cells treated with an equivalent of the MAC showed epithelial features, and the other half showed round features, as a result of toxin modification. High doses of toxin results in all the cells becoming round (Fig. 5.04). The MAC of mussel extract is measured by the incubation of 50 µl of serially diluted mussel extracts with 50 µl of a 200,000 cell/ml suspension. The minimal active quantity of OA in the test was 0.125 µg (22,23).

5.3.2 Cytotoxicity studies on standard DTX-2

This section discusses the findings of Dr. Y.F. Pouchus in determining the response of our standard DTX-2, to the rapid cytotoxicity bioassay developed by Amzil et al. (22).
Photographs taken from the cytotoxicity studies of DTX-2

a) Cells treated with the MAC equivalent concentration. Half of the cells show normal epithelial features while the remainder which were modified by the toxin show round features.

b) Untreated control cells have epithelial features with inter-cellular spaces.

c) Cells treated with high toxin doses with all cells showing round features.
5.3.3 Results and discussion

The MAC for DTX-2 in this cytotoxicity test was found to be between 0.125 and 0.0625 μg/ml (0.155-0.078 μM). Therefore, DTX-2 gives a similar response to OA (0.125 μg/ml (0.155 μM) ) in this bioassay. This provides further evidence that DTX-2 has a similar toxic potential to that of OA. Initial findings of this study suggest that this cytotoxicity test could be suitable for the rapid screening of DSP toxicity in Irish shellfish.

*Experimental*: (See Appendix No.6)
5.4 IDENTIFICATION OF OA, DTX-2, DTX-2B AND DTX-1 IN IRISH MUSSELS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY DETECTION (POSITIVE ION)

5.4.1 Introduction

Mass spectroscopy (MS) is a powerful tool for the analysis of marine toxins. In addition to high sensitivity and selectivity, MS can provide structural information useful for the confirmation of toxin identity and also for the identification of new toxins. The greatest potential of MS for routine analysis of 'real samples' lies in its combination with liquid chromatography (LC).

Initial research in mass spectral characterisation of DSP toxins was carried out using fast atom bombardment (FAB) ionisation. Pleasance et al. developed a LC-MS method, using an ion-spray (ISP) interface and atmospheric pressure ionisation (API), for the determination of OA and DTX-1. The LC column was connected to the ISP interface with a fused silica capillary. The positive ion ISP mass spectra of OA and DTX-1 were simple, with abundant peaks due to the protonated toxins [M+H]^+ occurring at m/z 805 and 819 respectively. Selected ion monitoring (SIM) at these m/z values was found to give the best sensitivity and selectivity for the analysis of OA and DTX-1. The method was not as sensitive as the HPLC-FLD method reported by Lee et al., but had the advantage that no derivatisation or complex clean-up stages were required. The detection limit was found to be 2 ng of toxin injected and linearity was observed for up to 50 ng of injected toxin.

The combination of LC with atmospheric pressure ionisation (API) mass spectrometry has been shown to provide the capability of both confirming known compounds and identifying unknown substances in complex matrices, without any sample pretreatments. Detection of intact, underivatised marine toxins and related compounds is possible in relatively crude extracts of both plankton and shellfish. The LC-MS approach using an API and an ISP interface was used in this research in order to confirm the known DSP toxins as well as to obtain structural information for an unknown DSP toxins in mussel samples from the West of Ireland.

The API LC-MS approach was then implemented in order to obtain structural information on the investigated compounds.
5.4.2 Results and discussion

Using a PE-SCIEX API III plus triple-quadrupole instrument (PE-Sciex, Thornhill, Ontario, Canada), initial MS experiments used flow injection analysis (FIA) on separated solutions of the toxins isolated from the Irish mussels: DTX-2 (1 µg/ml) and DTX-2B (1 µg/ml). The same experiments were also performed on separated standard solutions of OA (1 µg/ml) and DTX-1 (1 µg/ml).

Under the mild ionisation conditions of API fragment ions are not normally generated inside the ion source, thus allowing the determination of the molecular weight for unknown compounds. On the other hand, it is quite easy to generate fragment ions once sample ions have been drawn into the vacuum system. Collision induced dissociation (CID) can also be obtained by applying of suitable accelerating voltages in the free-jet expansion region. Although this so called CID up front is not true tandem mass spectrometry, it is easily controlled reproducible and particularly useful in obtaining a low-energy fragmentation of protonated molecules.

In order to determine the molecular weight of DTX-2 and DTX-2B a suitable orifice potential (OR=50 V) was applied, which had previously been found to favour the selective formation of the protonated molecule for marine polyether toxins belonging to the OA group.

Fig. 5.05 shows the FIA full-scan ISP mass spectra of OA, DTX-2, DTX-2B and DTX-1, as obtained with the mass spectrometer operating in the single MS positive-ion mode (full-scan m/z 700-900). The spectra of OA, DTX-2 and DTX-2B, were dominated by the ion at m/z 805, corresponding to the protonated molecule, [M+H]^+, of each compound; likewise, the spectrum of DTX-1 showed a prominent signal at m/z 819, corresponding to the [M+H]^+, of the analyte, thus indicating the molecular weight of 804 for the new DSP toxins, DTX-2 and DTX-2B, which were identical to that of OA.
Fig. 5.05

Effect of orifice potential on the ion current signals in the full scan ionspray mass spectrometry (ISP-MS) flow injection analysis (FIA) of OA, DTX-2, DTX-2B and DTX-1. Orifice potential set to 50 V. Conditions: FIA on solution containing 1 μg/ml of each analyte; mobile phase: CH₃CN:H₂O (80:20) with 0.1% TFA, flow rate = 40 μl/min, loop = 10 μl; scan range m/z = 700-900; dwell time = 2 msec, The mass spectrometry was operated in the single MS positive ion mode.

Research by Draisci et al. concerning some DSP acidic toxins demonstrated that a low-energy up front CID usually produces fragment ions corresponding to successive losses of water from the protonated molecules. This phenomenon has also been observed by other researchers which carried out ionspray mass spectrometry analyses of OA and DTX-1 (32), as well on several marine polyether toxins (36). The FIA experiments were then repeated after a variation of the OR (i.e. OR=80V), in order to achieve CID up-
front of the protonated molecule of DSP toxins (Fig. 5.06). The ISP mass spectrum of OA and its analogue, DTX-1 showed fragment ions corresponding to successive losses of water molecules from the protonated molecule. Interestingly, the same fragment ions corresponding to [M+H-H2O]^+, [M+H-2H2O]^+ and [M+H-3H2O]^+ were noticed in the up-front CID ionspray mass spectrum of DTX-2 and DTX-2B, thus positively confirming these new compounds to be polyether toxins. A comparison of the up-front CID ionspray mass spectrum of OA, DTX-2 and DTX-6 also showed significant differences in the relative intensities of the same fragment ions and this could be useful for confirmation of peak identity in regulatory work.

Fig. 5.06

Effect of orifice potential on the ion current signals in the full scan ionspray mass spectrometry (ISP-MS) flow injection analysis (FIA) of OA, DTX-2, DTX-2B and DTX-1. Orifice potential set to 100 V (up-front CID). Conditions as described as in Fig. 5.05.
The presence of two compounds having the same molecular weight of OA, required a chromatographic separation, in order to achieve the detailed toxin composition of the mussel extracts. Slight modifications were made to the chromatographic conditions previously optimised for LC-ISP-MS analysis of OA and DTX-1 in shellfish tissues \(^{(31)}\). In order to obtain targeted analysis and maximum sensitivity, data acquisition was performed by SIM mode on the \([M+H]^+\), at \(m/z\) 805 for OA, DTX-2 and DTX-2B, and at \(m/z\) 819 for DTX-1. Under these conditions an excellent separation signal was obtained for OA, DTX-2, DTX-2B and DTX-1 by the LC-ISP-MS analysis (Fig. 5.07).

**Fig. 5.07:**

LC-ISP-MS analysis of a mixture of a) OA, b) DTX-2, c) DTX-2B and d) DTX-1. Total selected-ion-monitoring (SIM) chromatogram of ions \(m/z\) 805, corresponding to \([M+H]^+\) of OA, DTX-2, DTX-2B, and \(m/z\) 819, corresponding \([M+H]^+\) of DTX-1. Solution injected contained 0.5 \(\mu g/ml\) of each toxin. Conditions: isocratic HPLC analysis; column Supelcosil LC18-DB, 5\(\mu m\), 4.6 x 250 mm; mobilephase: \(CH_3CN- H_2O \) (80:20) with 0.1% TFA; loop 10 \(\mu l\); flow rate 1 \(ml/min\) (40 \(\mu l/min\) split to MS). LC-ISP-MS in positive ion mode.
The chromatographic behaviour of DTX-2B was found to be very similar to that of OA and DTX-2. Research with LC-ISP-MS reversed-phase chromatography by Quilliam et al. also reported that some unidentified OA isomers eluted after OA, with a retention time very close to OA but different from that of DTX-2\(^{(32)}\). The LC-ISP-MS method was finally used for separation and detection of DSP toxins in crude underivatized extracts of toxic mussel hepatopancreas samples as well as on the toxic fractions isolated from the shellfish. The total ion current profile (full-scan \(m/z\) 700-1700) obtained by LC-ISP-MS analysis of the toxic samples showed three interesting peaks at \(m/z\) 805, which were the OA peak and the two OA analogues DTX-2 and DTX-2B. The \([M+H]^+\) ions for OA, DTX-2, DTX-2B and DTX-1 were then monitored under SIM conditions in the LC-MS. Fig 5.08 and Fig. 5.09 show the SIM LC-ISP-MS chromatograms of toxic mussels collected from site # 4 on 1-9-94 (MUS-DTX-2) and the toxic fraction isolated from mussels collected from site #5 on 20-10-’91 respectively.

**Fig. 5.08**

Chromatogram of LC-ISP-MS analysis of DSP toxins extracted from site #4 on 1-9-94 (MUS-DTX-2) by SIM of ions \(m/z\) 805, corresponding to \([M+H]^+\) of OA, DTX-2, DTX-2B, and \(m/z\) 819, corresponding to \([M+H]^+\) of DTX-1, Conditions as described in Fig. 5.07
Fig. 5.09

LC-ISP-MS analysis of a toxic fraction isolated from Irish mussels (Mytilus edulis) total selected-ion-monitoring (SIM) chromatogram of ions m/z 805, corresponding to [M + H]$^+$ of OA, DTX-2, DTX-2B, and m/z 819, corresponding to [M + H]$^+$ of DTX-1 respectively. Conditions as described in Fig. 5.07.

The LC-MS data confirmed the presence of DTX-2, DTX-2B and OA in the mussel extracts. DTX-1 was not detected in the analysed shellfish, despite the high sensitivity of the LC-ISP-MS method which has a detection limit of 0.4 ng of injected toxin (signal to noise 3:1), corresponding to 0.04 μg of toxin per g of hepatopancreas.\(^\text{31}\)
5.4.3 Conclusion

LC-MS using an API source and ISP interface was a successful approach in this research to define the detailed toxin composition of toxic mussels. It allowed the confirmation of known DSP toxins, such as OA and DTX-2 as well as obtaining structural information of the new biotoxin, DTX-2B. It also provided clear evidence that this compound is an OA isomer, and further structural information by tandem-mass spectrometry (MS-MS) analysis should confirm this. Work is continuing on the LC-MS-MS characterisation of DTX-2B.

Materials and Methods

Samples:
* Fraction no. 7 from isolation work contains 3 DSP peaks.
  add 1ml of Methanol to the Vial and a 50µl portion was derivatised using the Lee et al. method
* Chloroform extract of mussel digestive gland from site #5 on 20-10-'91
* Homogenised mussel digestive gland from site # 4 on 1-9-94 (MUS-DTX-2) x 2 vials (2.5g approx. in each)

Samples were prepared as outlined in chapter 2. For the LC-MS analysis, the residue was dissolved with 1 ml of methanol and 10 µl was injected.

LC-MS: Analyses were performed on a Perkin Elmer model 410 LC pump (Perkin Elmer Ltd., Beaconsfield, England) liquid chromatograph. A Rheodyne 7125 (Cotati, CA, USA) injection valve equipped with a 10 µl internal loop was used for the injection of samples. Separation of DSP toxins was carried out on an analytical column packed with Supelcosil LC18-DB (Bellefonte, PA, USA) (5 µm, 250 x 4.6 mm) at room temperature, under isocratic conditions, with a mobile phase of CH₃CN-H₂O (80:20) containing 0.1% TFA and a flow rate of 1 ml/min. A split of the column effluent was used to obtain a flow rate of 40 µl/min to the mass spectrometer. Mass spectral analysis was performed on a PE-SCIEX API III plus triple-quadrupole (PE-Sciex, Thornhill, Ontario, Canada). The mass spectrometer was equipped with an
API source and an ISP interface set at a voltage of 5500 V. Full-scan mass spectra were acquired in single MS positive-ion mode (LC-MS) over the mass range $m/z$ 700-900 in the LC-MS analyses of isolated toxins and standard toxins and $m/z$ 700-1700 in the LC-MS sample analyses. The potential difference between the ion sampling orifice and the mass analysis entry region (RF-only quadrupole) controlled the extent of fragmentation through CID up front. Specific potentials used were 50 V and 80 V. Data acquisition was performed in selected-ion-monitoring (SIM) mode on the protonated molecules $[\text{M+H}]^+$ of the analytes (i.e. $m/z$ 805 for OA, DTX-2 and DTX-2B; $m/z$ 819 for DTX-1) in the LC-MS analysis of Irish mussels. The $m/z$ values indicated both in text and in figures are in all cases the truncated values of the more accurate experimental values (e.g. $m/z$ 805 is reported instead of 805.5).
5.5 ANALYSIS OF PURIFIED DTX-2 AND DTX-2B FROM IRISH MUSSELS BY NEGATIVE-ION FAB MS/MS

5.5.1 Introduction
During an ionisation process negatively as well as positively charged ions are formed. Normally, the negative ions remain undetected because ion source and focusing potentials allow only the extraction of positive ions from the source. Changes are often necessary since magnetic analysers do not transmit negative ions unless the magnet current is reversed, and a standard electron multiplier does not usually detect negative ions without some modifications. An ability to form negative ions is useful in any analytical technique since the molecular anion may have greater stability than the corresponding positively charged ion and may also provide structural information through different fragmentation processes.

Negative ion techniques provide further ion-molecular reactions which complement those available in positive ionisation and which may be selected so as to provide the information required.

5.5.2 Results and discussion
A molecular ion was observed at 803 (M-H) for all three toxins, OA (Fig. 5.10), DTX-2 and DTX-2B. Both DTX-2 and DTX-2B showed identical mass spectra with (M-H)=803 and these toxins are stereochemical isomers at the position arrowed (C-35) (Fig. 5.11).

![Fig. 5.10](image.png)  
*Fig. 5.10*  Shows the assigned fragmentation patterns for negative-ion FAB MS/MS of OA. (Differences from DTX-2/DTX-2B are shown by asterisks).
Fig. 5.11  Shows the assigned fragmentation patterns for negative-ion FAB MS/MS of DTX-2/DTX-2B the arrow indicates the position where DTX-2 and DTX-2B are stereochemical isomers. (Differences from OA are shown by asterisks).

<table>
<thead>
<tr>
<th>DTX-2/DTX-2B Fragmentation Pattern</th>
<th>OA Fragmentation Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>803-733*  [M-H]  -C₅H₁₀</td>
<td>803-747*  [M-H]  -C₄H₈</td>
</tr>
<tr>
<td>733-689*  [M-H]  -CO₂</td>
<td>747-703*  [M-H]  -CO₂</td>
</tr>
<tr>
<td>733-661*  [M-H]  -C₃H₄O₂</td>
<td>747-663   [M-H]  -C₄H₆O</td>
</tr>
<tr>
<td>733-663*  [M-H]  -C₄H₆O</td>
<td>703-675*  [M-H]  -C₂H₄</td>
</tr>
</tbody>
</table>

Negative-ion FAB MS/MS showed that DTX-2B is probably a stereoisomer of DTX-2 and structurally different from OA. However, this was the only mass spectral technique to distinguish OA and DTX-2/2B.
5.6 ACCUMULATION OF DSP TOXINS BY SHELLFISH AND SUBSEQUENT DEPURATION

5.6.1 Introduction

5.6.1.1 Accumulation of toxins and local variability in toxicity

Bivalve shellfish accumulate toxins, predominantly in their hepatopancreas, after feeding on toxic algae. Edebo et al. found that mussels monitored in Swedish waters had the ability to accumulate toxins very rapidly during toxic periods. The OA level in mussels from one site increased from 0.4 to 5.4 μg/g hepatopancreas in a single week. This revealed that the weekly monitoring of shellfish toxicity, which is practised in many countries, is not adequate to ensure shellfish quality.

Pillet et al. carried out laboratory scale studies on the accumulation of OA and DTX-1 in mussels by feeding daily rations of P. lima. A slow build up of toxin in the hepatopancreas occurred until day 14 of the experiment (1.2 to 2.0 μg/g hepatopancreas), followed by a peak of 3.8 μg/g on day 16 with a rapid decrease thereafter. The experiment showed that, under these experimental conditions, the two toxins studied did not accumulate as a progressive long term build up. The changes of OA and DTX-1 content in the mussels corresponded in both time and ratio to that of the P. lima cells. It has been suggested that these toxins took part in the same metabolic reactions and that no bioconversion occurred between them.

Toxin concentrations in mussels, from different sites in the same area, have also been found to vary considerably. Irish mussels have been shown to vary between 6.3 and 0.0 μg total toxin /g of hepatopancreas when sampled from neighbouring sites on the same day.

5.6.1.2 Factors effecting shellfish depuration rates

Mussels have been shown to cleanse/depurate themselves in the absence of toxic dinoflagellates. The DSP toxin concentration in samples from an Irish mussel site decreased from 2.08 to 0.28 μg / g hepatopancreas in a week. These findings are different from those observed in The Netherlands, where it took up to four weeks for mussels in water temperatures of 14-15°C to depurate in the absence of toxic dinoflagellates. However, in the Dutch case a rat feeding test was used which is only
a semi-quantitative toxicity test and, therefore, these results represent the time required for the total depuration of highly toxic mussels.

Mussels in the Gulf of Trieste underwent a two phase decontamination phase in 1989. There was initially, a rapid fall in mussel toxicity, followed by a levelling off of toxin content. The patterns of contamination and decontamination are specific for shellfish and do not seem to depend on the type of dinoflagellate toxin\(^{(41)}\).

It has been reported that low water temperatures and low nutrient levels slow down both the metabolism and the rate of depuration of mussels. Mussels have shown effective cleansing rates, even at low temperatures, when the sea contains an abundance of non-toxic phytoplankton such as certain diatoms\(^{(37,42)}\). Another important fact is that low cell numbers of toxic species in the water of shellfish growing areas coupled with low populations of non-toxic species, can result in appreciable toxicity\(^{(43)}\).

French researchers estimated that about 30 days are necessary to eliminate any detectable trace of toxins in relatively toxic mussels (3 MU/g hepatopancreas), transferred to a site without *Dinophysis* spp. and a good supply of nutrients. About 20 days were needed for mussels to reach the regulatory limit for DSP toxicity in Japan (0.5 MU/g hepatopancreas). This interval decreases when toxicity is lower\(^{(44)}\).

Also, mussels from a site near the mouth of a river in Sweden showed little toxicity, compared to other mussel sites, during a toxic period. The highest toxicity occurred in an area in Sweden which generally had the highest salinity\(^{(37)}\). Findings by Sedmak *et al.* suggest that a deep closed bay and abundant freshwater inflow are the main conditions for low toxicity in mussels and for a shorter period of danger\(^{(41)}\). Shellfish from an almost closed inlet and with an abundant fresh water inflow at Lim fjord, near the Gulf of Trieste, showed no toxicity although samples from the neighbouring open sea were highly toxic. This supports the theory that *Dinophysis* spp. originate in the sea and dissipate as they encounter freshwater in the coastal area.

More recently Pillet *et al.* studied the effects of DSP-toxin producers, *P. lima* on clearance rates in blue mussels (*Mytilus edulis*)\(^{(45)}\). For this purpose, *P. lima* cells were included in a diet consisting of a suspension of *Isochrysis galbana*. A similar set of experiments with non-toxic *P. micans* cells was used as a control. After 1 hr of filtration (a period which corresponds to the minimal gut passage time) the filtration rates of mussels exposed to 10\(^6\) cells/l of *P. lima* became significantly lower than those measured
in the control suspension of *P. micans*. It has been suggested that such reductions of clearance rate could be the consequence of a direct influence of DSP toxins on mussel gills or could express a physiological stress resulting from the newly assimilated toxic effects \(^{46}\). This may account for the long periods of time observed for natural depuration of mussels in the west of Ireland following the appearance of large blooms of *D. acuta* in 1991 and 1994 \(^{6,39}\).

The economic losses caused by the presence of DSP toxins has led to the evaluation of a mussel depuration method. A water disinfection process involving ozone (O\(_3\)) was found effective in reducing PSP toxins in soft-shell clam within 48 hours \(^{47}\). This disinfection process involving O\(_3\) was later applied to DSP contaminated mussels with samples exhibiting a clear reduction of OA after 5 days, with samples showing between 28 - 78% reduction in toxin levels \(^{48}\). As a result of these findings a pilot plant study was commissioned by Bord Iascaigh Mhara (B I.M.) to investigate the feasibility of O\(_3\) treatment for the reduction of diarrhetic toxin levels in toxic Irish mussels.

### 5.6.2 Examination of ozonolysis for the removal of DSP toxins from toxic Irish mussels

![Diagram of a Shellfish Depuration System similar to the one used in the Ozonolysis trials](image)

*Fig. 5.12 Diagram of a Shellfish Depuration System similar to the one used in the Ozonolysis trials* \(^{49}\)
5.6.2.1 Results and discussion

The results of this study are summarised in the following Tables (5.01a and 5.01b).

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>OA (μg/g of hepatopancreas)</th>
<th>DTX-2 (μg/g of hepatopancreas)</th>
<th>Chromatographic Data files</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀ Ft</td>
<td>0.38</td>
<td>4.71</td>
<td>25-9-94.401</td>
</tr>
<tr>
<td>T₁ Ft</td>
<td>0.58</td>
<td>4.33</td>
<td>28-9-94.151</td>
</tr>
<tr>
<td>T₂ Ft</td>
<td>0.36</td>
<td>3.17</td>
<td>28-9-94.801</td>
</tr>
<tr>
<td>T₃ Ft</td>
<td>0.39</td>
<td>4.43</td>
<td>28-9-94.111</td>
</tr>
<tr>
<td>T₄ Ft</td>
<td>0.30</td>
<td>4.21</td>
<td>25-9-94.801</td>
</tr>
</tbody>
</table>

Tₙ Ft = Control sample subjected to n days Flush through ozone

<table>
<thead>
<tr>
<th>Sample I.D.</th>
<th>OA (μg/g of hepatopancreas)</th>
<th>DTX-2 (μg/g of hepatopancreas)</th>
<th>Chromatographic Data files</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀ ozone</td>
<td>0.86</td>
<td>6.48</td>
<td>25-9-94.501</td>
</tr>
<tr>
<td>T₁ ozone</td>
<td>0.49</td>
<td>5.31</td>
<td>28-9-94.701</td>
</tr>
<tr>
<td>T₂ ozone</td>
<td>0.38</td>
<td>4.24</td>
<td>28-9-94.901</td>
</tr>
<tr>
<td>T₃ ozone</td>
<td>0.25</td>
<td>2.57</td>
<td>28-9-94.121</td>
</tr>
<tr>
<td>T₄ ozone</td>
<td>0.25</td>
<td>3.62</td>
<td>25-9-94.901</td>
</tr>
<tr>
<td>T₅ ozone(B)</td>
<td>0.42</td>
<td>2.2</td>
<td>28-9-94.141</td>
</tr>
</tbody>
</table>

Tₙ ozone = Sample subjected to n days ozonolysis treatment

Table. 5.01a and 5.01b  Summary of ozone results

The mussels treated with O₃ showed a 70% decrease in the level of diarrhetic toxins while the control mussels, without O₃ treatment displayed only a 10% reduction in toxicity (Figs. 5.13 )
These preliminary results indicate that ozonolysis is an effective method for increasing the rate of detoxification of shellfish. A detailed examination of large scale detoxification using $O_3$ and water replenishment will be undertaken in future research work. However, whether or not this process will be economically viable on a commercial scale remains to be seen.

5.6.2.2 Experimental

500 kg of mussels were held in a tank with circulating sea water and treated with ozone for five eight-hour daily periods. The daily DSP toxin analysis using fluorescence HPLC, was carried out on samples which were subjected to $O_3$ treatment as well as untreated control samples.
5.7 CONCLUSION

In the past decade, important breakthroughs have been made in DSP research. However, this research is still in its infancy and more research on the DSP problem is required. Future research to be performed in our laboratories will involve collaboration with other international research and industrial institutions and will have the following objectives-

I. To limit the contamination of shellfish by DSP toxins by implementing surveillance programmes in production areas and exploiting natural decontamination mechanisms.

II. To develop commercial processes to remove toxins from shellfish.

III. To isolate diarrhetic toxins in sufficient quantities to enable toxicity evaluations to establish toxin limits in shellfish and for use as reference standards in new analytical procedures.

IV. To limit the current heavy reliance on bioassays for toxin detection that involve the use of live animals.

V. To develop rapid procedures for toxin detection, suitable for use in both regulatory laboratories and industrial shellfish production facilities.

VI. To improve the analytical methods for detecting diarrhetic shellfish toxins with the aim to producing sensitive, robust and reliable reference procedures.

VII. To implement inter-laboratory calibration studies to ensure harmonisation of methods for the determination of diarrhetic toxins in shellfish.

The examination of the full toxin profile of shellfish areas and the validation of new methods for the detection of DSP toxins is dependant on the availability of standard materials. Therefore, larger scale isolations of DTX-2 and DTX-2B should be undertaken to permit sufficient quantities of these toxins to be supplied to research institutions world-wide. Also, larger quantities of DTX-2B is required for its complete structural determination using NMR.
A primary objective of future research should be to develop measures to reduce toxicity and to investigate methods for commercial detoxification of shellfish contaminated with diarrhetic toxins. This research should focus on the examination of the effect of high temperatures and ozone treatment on contaminated shellfish. Shellfish toxicity in most regions of Europe tends to be sporadic, with certain areas remaining unaffected even though they are geographically close to contaminated regions. Therefore it may be feasible to transfer rope cultured mussels from a contaminated region to holding bins and to use high-powered pumps to aid natural flushing processes.

An understanding of oceanographic features is also important in the study of toxic episodes. There is evidence that a correlation exists between changes of water temperature and the subsequent appearance of high cell counts of toxic algae in the surface water column (Chapter 4). The study and monitoring of local oceanographic profiles may be useful in the prediction of future toxic events.

Compared with other primary screening assays that are commonly used to detect DSP toxins (mouse and rat bioassays, and HPLC methods), the protein phosphatase assay is simple, quick, extremely sensitive, quantitative, versatile and does not involve animals. However, existing methods for determining PP1 and PP2A inhibition centre on the use of radiochemical methods which are clearly unacceptable for regulatory or industrial applications. Therefore, an important part of future research would be the development of an assay procedure using fluorescence and luminescence instrumentation.

The complete validation of the rapid cytotoxicity assay and novel ELISA assays, for the rapid screening of Irish shellfish for DSP toxins, is required.

There is also an urgent need for the development of a better method for the fluorescence HPLC detection of acidic DSP toxins. The standard method involves the derivatisation of these toxins with 9-anthryldiazomethane (ADAM). While this reagent is very reactive and has a good sensitivity, its instability has caused problems for some laboratories and has led to inconsistent results (Chapter 3).
5.8 BIBLIOGRAPHY


[43.] Kat, M. Red Tides: Biology, Environmental Science, and Toxicology. (1989), 73-76.


Chapter 6

The Synthesis and Application of MBQ-PAH

1-Methyl-2(1H)-benzo[6,7-d] quinoxaline-3-propionyl-carboxylic Acid Hydrazide.

A New Fluorescence Derivatisation Reagent for the HPLC Analysis of Carboxylic Acids.

Science is nothing but trained and organised common sense, differing from the latter only as a veteran may differ from a raw recruit....

T.H. Huxley (1825-1895)
6.1 INTRODUCTION

Liquid chromatography suffers as an analytical technique from relatively poor detection capabilities, which are often non-selective, non-specific, and insensitive. These deficiencies have brought about the use of chemical, thermal, photochemical and other physical methods to convert an analyte into one or more derivatives that have enhanced chromatographic and/or detector response properties. Derivatisation is basically the use of chemical reagents/reactions and/or physical methods to convert the original structure of the analyte into another molecule or mixture of reaction products. In some cases, a simple photochemical, acid/base, or thermal reaction will convert the original analyte structure into a product or derivative that has improved or different chromatographic and/or detector response properties. In other cases, or in other chemical reactions/derivatisations, the analyte will have its structure altered by a rearrangement of bonds and atoms, and/or by the addition (tagging) of another molecule to provide the final derivative(s). In some cases, a single reaction product will be formed with vastly improved chromatographic and/or detector response properties, in other cases, it may be preferable to have several such products formed at the same time and in the same reaction sample (1,2).

Generally chemical derivatisation is used to improve detection sensitivity by converting a compound with poor detector response into a highly detectable product. Apart from an increase in detectability, the derivatisation step also improves the selectivity of the overall analytical method through the inherent selective reaction of the derivatisation chemistry employed.

6.1.1 Fluorescence detection

Of the detection methods available in HPLC, fluorescence detection is both sensitive and selective and, as a result, has found wide application in the analysis of trace levels of bioactive compounds in complicated matrices such as blood, serum, urine and tissue. Its increasing use is due not only to improvements in the fluorescence detection systems but also to the development of various fluorogenic reactions (3,4).

Fluorogenic reactions can be classified into two groups by reaction types: 'fluorescence generation' and 'fluorescence tagging' or 'labelling'. In the former, the fluorogenic reagents are generally non-fluorescent but they react with target compounds to form
conjugated-ring molecules, resulting in the production of fluorescence. In the latter, the reagents contain a highly fluorescent aromatic moiety with a reactive functional group which attaches to an analyte to form a fluorescence-tagged derivative. Fluorogenic reactions are further categorised in HPLC as precolumn derivatisation (before separation) and postcolumn derivatisation (after separation). Fluorescence-tagging reagents are used only for precolumn derivatisation.

In selecting a reagent, sensitivity is usually one of the most important aspect of fluorescence methods. A high fluorescence efficiency of the derivatives is therefore necessary.

6.1.2 Analysis of carboxylic acids

Many substances that exhibit bioactivity at low concentrations occur in biological fluids and tissues. The determination of carboxylic acids, such as prostaglandins and bile acids, at trace levels in complex matrices, is important in many biomedical and environmental studies. Thus, derivatisation using a suitable labelling reagent, followed by HPLC analysis, is frequently used for the sensitive determination of carboxylic acids. To date, the use of fluorescent tags seems to be one of the most popular ways for derivatisation. Most carboxylic acids produce no fluorescence or strong absorption since the carboxyl group produces only a weak ultraviolet absorption. In view of the low reactivity of the carboxyl group, pre-chromatographic labelling is preferred because of the greater flexibility in the selection of optimum reaction conditions. Fluorescence detection has been generally accepted as the most useful, considering its versatility, sensitivity, selectivity and ease of use.

Most recent studies on the development of new labelling reagents have focused on compounds with highly sensitive fluorophores, particularly those showing a high reactivity under mild reaction conditions and the fluorimetric derivatisation of carboxylic acids has been the subject of a recent review.\(^{(5)}\)
Fig. 6.01 
Six types of fluorogenic reactions used for the HPLC analysis of carboxylic acids. A = Bromomethyl, B = Bromoacetyl, C = Sulphonate, D = Diazoniethyl, E = Amidation, and F = Hydrazine.

'Type A': Reagents containing bromomethyl groups.
The structures of the most popular bromomethyl group bearing reagents are shown in Fig. 6.02.

Fig. 6.02 
Fluorogenic reagents belonging to type A compounds containing the bromomethyl group.

4-bromo-methyl-7-methoxycoumarin (BrMMC) is one of the most frequently used labelling reagents. The fatty acid-MMC derivatives have reportedly been determined at the fmol level in fish oil dietary supplements with laser-induced fluorescence detection. Br-MMC labelled derivatives have been analysed at $\lambda_{ex}$ of...
318, 325 and 330 nm, with subsequent detection at $\lambda_{ex}$ of 378, 395, 396, 398, 410 nm \(^{(5)}\). Later, it was reported that use of 3-bromomethyl-6,7-dimethoxy-1-methylquinoxalin-2(1H)-one (Br-DMEQ) gave a sensitivity 100 times that of Br-MMC with a detection limit for fatty acids of 0.3-1 fmol (signal-to-noise ratio of 2) \(^{(12-14)}\). Br-DMEQ labelled derivatives have been analysed at $\lambda_{ex}$ of 370 and 380 nm, with subsequent detection at $\lambda_{em}$ of 450, 455 and 460 nm \(^{(5)}\).

More recently, it was found that the use of 3-bromomethyl-6,7-methylenedioxy-1-methyl-quinoxalin-2(1H)-one (Br-MMEQ) gave a 1.6 times higher sensitivity than Br-DMEQ \(^{(15)}\). Reverse phase HPLC analysis of fatty acids-MMEQ derivatives using an aqueous methanol gradient elution was reported. Br-MMEQ labelled derivatives have been analysed at $\lambda_{ex}$ 363 nm, with subsequent detection at $\lambda_{em}$, 437 nm \(^{(5)}\).

**‘Type B’: Reagents containing bromoacetyl groups.**

The structures of the bromoacetyl group bearing reagents are shown in Fig. 6.03.

\[
\text{COCH}_2\text{Br} \quad \text{9-APB} \quad \text{CH}_3\text{O} \quad \text{COCH}_2\text{Br} \quad \text{Br-AMC} \quad \text{COCH}_2\text{Br} \quad \text{BAP}
\]

**Fig. 6.03** *Fluorogenic reagents belonging to type B compounds containing the bromoacetyl group.*

A bromoacetyl group shows a higher reactivity with fatty acids than a bromomethyl group. Recently, 3-bromoacetyl-7-methoxycoumarin (Br-AMC) has been synthesised as a highly reactive fluorescent labeling reagent. Br-AMC readily reacted with carboxylic acids at room temperature in the presence of potassium bicarbonate and 18-crown-6 as catalysts. The detection limit was found to be 0.4 pmol (signal-to-noise ratio of 3). Br-AMC labelled derivatives have been analysed at $\lambda_{ex}$ 365 nm, with subsequent detection at $\lambda_{em}$ 417 nm \(^{(16)}\).

*p-(9-Anthroyloxy)phenacyl bromide (9-APB)* has found application in the analysis of prostaglandins in human plasma, with a detection limit of 4 dmol (signal-to-noise ratio of
5) \(^{(17)}\). 9-APB labelled derivatives have been analysed at \( \lambda_{ex} \) 325 and 375 nm, with subsequent detection at \( \lambda_{em} \) 470 nm \(^{(5)}\). Kamada et al. first used 1-bromoacetylpyrene (BAP) as a derivatising agent for the analysis of free and conjugated bile acids in serum \(^{(18)}\). Subsequently Asukabe et al. applied BAP to the analysis of polyether antibiotics. This method differed from that of Br-AMC in that Kryptofix 222 was used in place of dicyclohexyl-18-crown-6, as a catalyst for the formation of the fluorescent derivatives. Asukabe et al. studied the reactivity of salinomycin with BAP using dicyclohexyl-18-crown-6 as a catalyst and found that the reaction yield was very low (10%). Kryptofix 222 was found to be more effective at converting salinomycin into its 1-pyreneacetyl ester (yield > 70%) \(^{(19)}\). The application of BAP to the analysis of the DSP toxins was first described by Dickey et al. Diisopropylethylamine which acted as the catalyst in converting the DSP toxins into their 1-pyreneacetyl esters, was found to be a more effective catalyst than Kryptofix 222 \(^{(20)}\). The fact that BAP requires heating in aprotic solvents to achieve derivatisation may therefore prove unsuitable for thermally labile carboxylic acids. BAP labelled derivatives of DSP toxins have been analysed at \( \lambda_{ex} \) 365 nm, with subsequent detection at \( \lambda_{em} \) 418 nm \(^{(21-23)}\).

'Type C': **Reagents containing sulphonate groups.**

The structures of sulphonate group bearing reagents are shown in Fig. 6.04.

![Fig. 6.04](image)

Fig. 6.04 **Fluorogenic reagents belonging to type C compounds containing the sulphonate group.**

The powerful alklyating abilities of trifluoromethanesulphonates are well known. Highly reactive labelling reagents, 2-(phthalimido)ethyl trifluoromethanesulphonate (PE-OTf) \(^{(24)}\) and 2-(2,3-naphthalimido)-ethyltrifluoromethanesulphonate (NE-OTf) \(^{(25)}\) were
synthesised for both UV and FD applications. Both PE-OTf and NE-OTf were successfully applied to the determination of carboxylic acids in mouse brain. The detection limits for the method using NE-OTf with UV and FD were 100 fmol and 4 pmol respectively (signal-to-noise ratio of 3). NE-OTf labelled derivatives have been analysed at \( \lambda_{ex} \) 259 nm, with subsequent detection at \( \lambda_{em} \) 394 nm\(^{(25)}\).

Ohrui et al. recently reported another highly sensitive HPLC analysis method for the determination of acid DSP toxins. The extraction procedure was a slightly modified version of the Pereira et al. method. Toxic samples were derivatised with 100 µl 2.25 mM 2,3-(anthracenedicarboximido)ethyltrifluoromethanesulphonate (AE-OTf) in acetonitrile, in the presence of 75 µl of 1.5 mM tetraethylammonium carbonate (TEAC) in acetonitrile. The mixture was allowed react for 15 min. at room temperature.\(^{(26)}\)

'Type D': Reagents containing diazomethyl groups.

The structures of diazomethyl group bearing reagents are shown in Fig. 6.05.

![Fig. 6.05](image)

Fluorogenic reagents belonging to type D compounds containing the diazomethyl group.

The advantage of diazomethane-type reagents is that they can react with carboxylic acids at room temperature without the presence of a catalyst and even in the presence of water. Nimura et al. first reported the use of 9-anthryldiazomethane (ADAM) as a fluorogenic reagent in the HPLC analysis of pmole levels of short chain fatty acids.\(^{(27)}\) Subsequently, ADAM was used for the analysis of prostaglandins,\(^{(28)}\) the fatty acid composition of human serum lipids,\(^{(29)}\) and the microanalysis of free fatty acids in plasma of experimental animals and humans.\(^{(30)}\) Martinez et al. applied ADAM to the analysis of monensin and related antibiotics in beef and chicken livers.\(^{(31,32)}\) Lee et al. developed a fluorimetric HPLC method for the analysis of DSP toxins derivatised with ADAM.
An additional clean-up step using solid phase extraction (SPE) was employed to remove the excess ADAM reagent. HPLC analysis was carried out on a Develosil® ODS column with mobile phase, acetonitrile:methanol:water (8:1:1) \(^{(33)}\). ADAM labelled derivatives have been analysed at \(\lambda_{\text{ex}}\) 254, 255 and 365 nm, with subsequent detection at \(\lambda_{\text{em}}\) 412 and 415 nm \(^{(27,28,32,33)}\).

However, ADAM is unstable and decomposes on storage. In order to overcome this drawback, a simple method for the preparation of ADAM \textit{in situ} was developed \(^{(34)}\). In general, the stability of aryldiazoalkanes generally depends mainly on the aromaticity of the aromatic ring and the electron-withdrawing activity of the ring substituents. 1-Pyrenyldiazomethane (PDAM) was developed as a more stable diazomethane reagent than ADAM. Nimura \textit{et al.} first described the synthesis and application of PDAM for the HPLC-FLD analysis of both short and long chained fatty acids \(^{(35)}\). The fluorescent intensity of fatty acids labelled with PDAM, was 5 times stronger than those labelled with ADAM. The detection limit for PDAM derivatives was found to be 15-30 fmol (signal-to-noise ratio of 3). More recently the use of PDAM as a fluorometric derivatising agent for DSP toxins has been reported \(^{(36,37)}\). PDAM labelled derivatives have been analysed at \(\lambda_{\text{ex}}\) 340 nm, with subsequent detection at \(\lambda_{\text{em}}\) 376 and 395 nm \(^{(35,37)}\).

Also, 4-diazomethyl-7-methoxycoumarin (DAM-MC) has been used for He-Cd laser-induced fluorescence detection, though the reactivity of DAM-MC is less than that of ADAM or PDAM. DAM-MC labelled derivatives have been analysed at \(\lambda_{\text{ex}}\) 325 nm, with subsequent detection at \(\lambda_{\text{em}}\) 386nm \(^{(38)}\).

\textbf{‘Type E’: Amidation reagents}

The structures of amidation reagents are shown in Fig.6.06.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig606.png}
\caption{Fluorogenic reagents belonging to type E compounds.}
\end{figure}
Fluorescent amines can also be utilised for the labelling of carboxyl groups. In general, activation of the carboxyl group is required prior to reaction with amines. For this purpose 1,3-dicyclohexylcarbodiimide or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) have been widely used as activating agents. 2-[p-(5,6-Methylenedioxy-2H-benzotriazol-2-yl)]phenethylamine (MBPA) was synthesised as a sensitive fluorogenic labelling reagent for carboxylic acids. It was applied to the determination of ibuprofen in human serum. MBPA reacted with ibuprofen in the presence of 2-bromo-1-ethylpyridinium tetrafluoroborate to give the corresponding fluorescent amide at room temperature. Linear relationships were found between the peak areas and the concentrations of ibuprofen in the range of 5-100 ng/ml and 0.5-5 ng/ml. The detection limit for ibuprofen in serum was 1.5 pg per injection (20 μl). MBPA labelled derivatives have been analysed at λ<sub>ex</sub> 333 nm, with subsequent detection at λ<sub>em</sub> 372 nm. Ibuprofen in saliva was also reacted with N-(4-aminobutyli)-N-ethylisoluminol (ABEI) in the presence of 1-hydroxybenzotriazole as a pre-activator and EDCA. The labeled derivative was detected by chemiluminescence at λ 389 nm and its detection limit was 0.7 ng (signal-to-noise ratio of 3 per 0.5 ml of saliva). Monodansyl cadaverine (MDC) was proposed as a promising fluorogenic reagent for labelling of fatty acids in the presence of diethylphosphorocanide (DEPC) as an effective activation agent. The labelling reaction of arachidic acid with MDC and DEPC proceeded rapidly in DMF, at room temperature. The detection limit of fatty acids were below 100 fmol (signal-to-noise ratio of 3). MDC labelled derivatives have been analysed at λ<sub>ex</sub> 340 nm, with subsequent detection at λ<sub>em</sub> 518 nm.

"Type F": Reagents containing hydrazine groups.

Most of the previously mentioned labelling reagents require the extraction of carboxylic acids from an aqueous matrix into a suitable aprotic solvent, prior to labelling to attain high reaction yields. The lack of reactivity of the carboxyl group in aqueous media, due to solvation by water molecules, makes efficient derivatisation problematic. An exception are reagents containing a hydrazine moiety which react readily with carboxylic acids in aqueous solution, using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDAC) as a coupling
agent and give the corresponding acid hydrazides. The structures of the discussed hydrazino group bearing reagents are shown in Fig. 6.07.

Yamaguchi et al. synthesised a highly sensitive fluorescent reagent 6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone-3-propionyl-carboxylic acid hydrazide (DMEQ-PAH) which reacted readily in an aqueous solution of fatty acids in the presence of pyridine and EDAC at room temperature. The reagent was applied to the analysis of metabolites of arachidonic acids with detection limits of 3-6 fmol (signal-to-noise ratio of 3) \(^{(42)}\) and to the determination of free fatty acids in human serum with detection limits of 2.5-5 fmol \(^{(43)}\). DMEQ-PAH labelled derivatives have been analysed at \(\lambda_{\text{ex}}\) 360 and 365 nm, with subsequent detection at \(\lambda_{\text{em}}\) 435 and 447 nm. 4-(5,6-Dimethoxy-2-benzimidazoyl)-benzohydrazide (DMBI-BH) was subsequently developed as a more sensitive reagent than DMEQ-PAH with detection limits of 1-3 fmol (signal-to-noise ratio of 3). DMBI-BH labelled derivatives have been analysed at \(\lambda_{\text{ex}}\) 360 nm, with subsequent detection at \(\lambda_{\text{em}}\) 460 nm \(^{(44)}\).

![Figure 6.07](image)

**Fig. 6.07**  *Fluorogenic reagents belonging to type F compounds containing the hydrazines group.*
2-(4-Hydrazinocarbonylphenyl)-4, 5-diphenylimidazole (HCPI) was also synthesised and applied to the analysis of saturated free fatty acids in human serum with a detection limit of 7-57 fmol (signal-to-noise ratio of 3). HCPI-labelled derivatives have been analysed at $\lambda_{\text{ex}}$ 335 nm, with subsequent detection at $\lambda_{\text{em}}$ 455 nm \(^{(45)}\).

More recently, Iwata et al. have synthesised 4-(1-methyl-phenanthro(9,10-d)imidazol-2-yl)-benzohydrazide (MPI-BH) for the analysis of fatty acids by reverse-phase HPLC with a detection limit of 2-12 fmol (signal-to-noise ratio of 3). MPI-BH labelled derivatives have been analysed at $\lambda_{\text{ex}}$ 325 nm, with subsequent detection at $\lambda_{\text{em}}$ 460 nm \(^{(46)}\).

It is interesting to note that methylation of N-H group of HCPI (Fig. 6.07) increased the sensitivity of the reagent with the detection limit reduced from 7-57 fmol to 2-12 fmol for a mixture of fatty acids.

In this chapter, the synthesis of a new fluorogenic reagent, 1-methyl-2(1H)-benzo[6,7-d]quinoxaline-3-propionylcarboxylic acid hydrazide (MBQ-PAH), is described (Fig. 6.08). This reagent reacts readily with carboxylic acids in aqueous solution, under mild conditions, to produce highly fluorescent derivatives \(^{(47)}\).

\[\text{MBQ-PAH}\]

Fig. 6.08 Structure of 1-methyl-2(1H)-benzo[6,7-d]quinoxaline-3-propionylcarboxylic acid hydrazide a new type F fluorogenic reagent for the analysis of fatty acids.
6.2 SYNTHESIS OF 1-METHYL-2(1H)-BENZO[6,7-D]-QUINOXALINONE-3-PROPIONYL CARBOXYLIC ACID HYDRAZIDE.

The synthesis of 1-methyl-2(1H)-benzo[6,7-d]quinoxalinone-3-propionylcarboxylic acid hydrazone (MBQ-PAH) from the starting material, 2,3-diaminonaphthalene, is outlined in Fig. 6.09.

Fig. 6.09 *Synthesis scheme for the preparation of MBQ-PAH.*

**Experimental**

**Materials**

Chemicals were guaranteed reagent grade and solvents for chromatography were of special grade for HPLC (Labscan Ltd.), Ireland. The starting materials were α-ketoglutaric acid (Aldrich Chemical Co., UK) and 2,3-diaminonaphthalene (Lancaster Synthesis Ltd, UK). Lauric (C_{12}), myristic (C_{14}), palmitic (C_{16}), stearic (C_{18}), arachidic (C_{20}) and behenic (C_{22}) acids were purchased from Sigma (Dorset, UK). Stock solutions
of the $\text{C}_{12}$-$\text{C}_{22}$ acids ($1\times10^{-4}$ M) were prepared in $N,N$-dimethylformamide and diluted with water to the required concentrations.

**Instrumentation used in structural characterisation**

Proton nuclear magnetic resonance ($^1\text{H}$ NMR) spectra and carbon 13 nuclear magnetic resonance ($^{13}\text{C}$ NMR) spectra were measured using a model JEOL GFX spectrometer (Japan) at 270 MHz and chemical shift values were expressed in ppm downfield from tetramethylsilane (internal standard). Infrared spectra data was determined using a Nicolet FT-IR model 205 and a ATI Mattson FT-IR. Ultraviolet-visible spectra were recorded on a Shimadzu double beam instrument model UV-240 (Duisberg, Germany). Both nominal and accurate mass spectra data were measured with a Profile HV-4 Kratos (Manchester, UK) double focusing high resolution GC-MS, operating with a direct insertion probe using electron impact mode and perfluorokerosene as a reference. Fluorescent data was acquired using a Shimadzu RF-5001PC spectrofluorophotometer. All reactions were monitored using thin layer chromatography on silica 60F$_{254}$ plates (Merck, Germany) with a mobile phase of Pentane: Dichloromethane: Methanol and visualised using a Mineralight® UV SL-58 developer.
6.2.1 STEP 1

\[
\begin{align*}
\text{NH}_2 & + \text{COOH} \\
\text{NH}_2 & \quad \text{O=C} \\
\end{align*}
\]

\[
\begin{align*}
\text{CH}_2\text{CH}_2\text{COOH} \\
\text{CH}_2\text{CH}_2\text{COOH} \\
\end{align*}
\]

Fig. 6.10  *Synthesis of 2(1H)-benzo[6,7-d]quinoxalinone-3-propionylcarboxylic acid methyl ester. (BQPA = compound II)*

The method employed in this step was similar to that used by Hara *et al.*

2,3-Diaminonaphthalene was (1.50g, 9.48mmol) weighed transferred to a 250ml round-bottomed flask to which hydrochloric acid (114ml, 0.5 mole) was added. The contents of the flask were ultrasonicated for 3 min to aid dissolution, before heating in a boiling water bath. After the 2,3-diaminonaphthalene was fully dissolved, α-ketoglutaric acid (1.32g, 9.102mmol) was added and the mixture was heated for 2 hrs. A colour change from brown to dark-yellow was observed and the resulting precipitates that separated on cooling of the mixture in ice water, were filtered by suction and washed with a portion of cold water (20 ml) to remove any excess of HCl. The residue gave two spots by TLC which corresponded to unreacted 2,3-diaminonaphthalene and 2(1H)-benzo[6,7-d]quinoxalinone-3-propionylcarboxylic acid methyl ester (compound II). Excess starting material was removed by a further washing with a portion of cold methanol (5 ml) and the product was dried *in vacuo* (yield 2.29g, 90%).
6.2.1.1 Structural characterisation of compound II: 2(1H)-benzo[6,7-\textit{d}]quinoxalinone-3-propionylcarboxylic acid methyl ester (BQPA)

Physical Properties:
Dark yellow powder, slightly soluble in hot methanol. m.p. 256-258°C

FT-IR:
1578-1757 cm\(^{-1}\) N-H and carboxylic acid group, 2724-3255 cm\(^{-1}\) and absence of the NH\(_2\) peaks 3263-3450 cm\(^{-1}\) present in starting material 2,3-diaminonaphthalene.

UV:
Absorption bands \(\lambda_{\text{max}}\) 236nm, 283nm and 336nm (methanol), characteristic of naphthalene containing compounds.
$^1$H NMR: $\delta$(ppm) 2.48 (q), 2.70 (2H, t), 3.00 (2H, t), 3.39 (s) 7.340-7.486 (2H, m), 7.56 (1H, s), 7.84 (1H, d), 7.96 (1H, d), 8.23 (1H, s), 12.10 (1H, s).

1H. SAMPLE.. B.

Peak Assignment:  

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<tr>
<td></td>
<td>(2H, t)</td>
</tr>
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</tr>
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</table>

**Solvent peak:** Dimethyl sulfoxide (DMSO) 2.49 ppm quintet (5)

**Note:** The aromatic signals are different from the Monomethyl and Dimethyl Products
Mass Spectral Data:

Fragmentation Pattern

Compound II (Code 13)

\[
\begin{align*}
\text{268-250} & \quad \text{[} \begin{array}{c}
\text{N} \\
\text{O} \\
\text{CH}_2\text{CH}_2\text{COOH} \\
\end{array} \text{]}^+ = -\text{H}_2\text{O} \\
\text{250-222} & \quad \text{LOSS OF 28= CO} \\
\text{[} \begin{array}{c}
\text{N} \\
\text{O} \\
\text{CH}_2\text{CH}_2 \end{array} \text{]}^+ = -\text{CO}
\end{align*}
\]

Elemental analysis

*Elemental analysis by accurate mass*: $\text{C}_{15}\text{H}_{12}\text{N}_2\text{O}_3$

Theoretical 268.08479; Experimental 268.08488 (Dev. ppm 0.32)
6.2.2 STEP 2

Fig. 6.11 Synthesis of 2(1H)-benzo[6,7-d]quinoxalinone-3-propionylcarboxylic acid methyl ester. (BQ-P-ME = compound IIIa) and 1-methyl-2(1H)-benzo[6,7-d]quinoxalinone-3-propylcarboxylic acid methyl ester (MBQ-PAM = compound IIIb)

This step involved the use of diazomethane to methylate both the carboxylic acid and amine groups in compound II. It is the most common methylating reagent for carboxylic acids, and has found wide application in the methylation of phenols, alcohols, enols, and hetero-atoms such as nitrogen and sulphur.

Safety considerations: Although quite safe when handled as a dilute solution in an inert solvent, diazomethane presents several safety hazards. It is both extremely toxic and highly irritating, causing pulmonary edema when inhaled in high concentrations. Long-term, low-level exposure may lead to sensitisation, resulting in asthma-like symptoms. Also, diazomethane and several of its chemical precursors have been cited as carcinogens. Diazomethane has been known to explode quite unaccountably, both as a gas and a liquid, rough surfaces are proven initiators of detonations. Thus, ground-glass joints and any glassware which have not been carefully fire-polished must never be allowed to come in contact with diazomethane or its solution.

Its preparation was carried out in a fume cupboard (hood) behind a screen of safety glass.

The following procedures were used for the preparation of ethereal solutions of diazomethane. p-Tolylsulphonyl-methyl-nitrosamide (2·14g) was dissolved in ether (30
ml), cooled in ice, and a solution of potassium hydroxide in 96 % ethanol (0.4 g in 10 ml) was added. If a precipitate formed, more ethanol was added until it just dissolved. After 6 minutes, the ethereal diazomethane solution was distilled from a water bath. The ethereal solution contained 0.32-0.35g of diazomethane. Receiver flasks were cooled in an ice - salt mixture. Methylation with diazomethane was carried out by dissolving the carboxylic acid, compound II (1.0g ), in 50:50 acetonitrile: methanol (110 ml) and treating with the freshly prepared ethereal diazomethane solution (stored in an ice-bath). The diazomethane solution was added in small portions until gas evolution ceased and the solution acquired a pale yellow colour. The solution was tested for the presence of excess of diazomethane by removing a few drops into a test-tube and introducing a glass-rod moistened with glacial acetic acid, and this resulted in the immediate evolution of gas (indicating the presence of diazomethane).

The reaction mixture was evaporated to dryness in vacuo and gave two additional spots by TLC. NMR analysis showed the presence of both mono and dimethyl products. The residue, dissolved in dichloromethane (40 ml), was purified by wet-flash column chromatography (25 x 3 cm i.d. column) on silica gel 60 (ca. 100g, 60-120 mesh: BDH Chemicals, UK) using a step gradient of pentane:dichloromethane: methanol. Compound IIIa (monomethyl product) eluted with 100% dichloromethane and compound IIIb (dimethyl product) eluted with 0.5% methanol in dichloromethane. (yield Compound IIIa 0.531g, 50.4% : compound IIIb 0.335g, 30%).

The acidity of amines is normally not great enough for the reaction to proceed without a catalyst, and this may explain the relatively low yield of compound IIIb. Alternative methylation methods using (i) BF₃ as a catalyst, which converts the amine to the F₃B-NHR₂ complex (53), and enabling similar reactions to take place, and (ii) the use of iodomethane as a methylating reagent were investigated. However, no net increase in dimethyl product yield was observed.
6.2.2.1 Structural characterisation of compound IIIa: 2(1H)-benzo[6,7-d]-quinoxalinone-3-propionylcarboxylic acid methyl ester (BQP-ME)

Physical properties:
Pale yellow needles, soluble in methanol. m.p. 130-132°C

FT-IR:
1604-1687 cm\(^{-1}\), 1700-1770 cm\(^{-1}\) and 2685-3184 cm\(^{-1}\)

UV:
Absorption bands \(\lambda_{\text{max}}\) 236nm, 283nm and 336nm (methanol), characteristic of naphthalene containing compounds.
**$^1$H NMR:** $\delta$(ppm) 2.49 (q), 2.86 (2H, t), 3.14 (2H, t), 3.38 (s), 3.66 (3H, s), 7.45-7.60 (2H, m), 7.68 (1H, s), 7.95-8.10 (2H, dd), 8.34 (s).

Peak Assignment: Type (ppm)

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<th>Solvent peak: Dimethyl sulphoxide (DMSO) at 2.49ppm quintet (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b and c</td>
<td>(2H, m),</td>
<td>7.45-7.60</td>
<td>The aromatic signals are different from the Mono-methyl and Di-methyl Products</td>
</tr>
<tr>
<td>e and f</td>
<td>(1H, s)</td>
<td>8.34</td>
<td></td>
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<tr>
<td></td>
<td>(1H, s)</td>
<td>7.68</td>
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<td>j</td>
<td>(3H, s)</td>
<td>3.66</td>
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<td>h and i</td>
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<td>(2H, t)</td>
<td>2.86</td>
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Mass Spectral Data:

Fragmentation Pattern:

**Compound IIIa (Code F3)**

\[
\begin{align*}
& N \quad O \\
& H \quad \text{CH}_2\text{CH}_2\text{COOCH}_3 \\
& 282-223 \quad \begin{bmatrix} O \\ C \cdot \text{OCH}_3 \end{bmatrix} \\
& 282-195 \quad \begin{bmatrix} O \\
& \text{CH}_2\text{CH}_2\text{C} \cdot \text{OCH}_3 \end{bmatrix} \\
& 282-250 \quad \left[ \begin{array}{c}
N \\
O \\
\text{C} \\
\text{CH}_2 \\
\text{CH}_2 \\
\end{array} \right]^+ = \text{CH}_3\text{OH}
\end{align*}
\]

Elemental analysis

*Elemental analysis by accurate mass:* \( \text{C}_{16}\text{H}_{14}\text{N}_2\text{O}_3 \)

Theoretical 282.1004; Experimental 282.1005 (Dev ppm 0.29)
6.2.2.2 Structural characterisation of compound IIIb: 1-methyl-2(1H)-benzo[6,7-d] quinoxalinone-3-propylcarboxylic acid methyl ester. (MBQ-PAM)

Physical properties:
Yellow/orange needles, soluble in methanol. m.p. 81-82 °C

FT-IR:
1732 cm⁻¹ and 2775-3101 cm⁻¹. Absence of peak at 1604-1687 cm⁻¹

UV.:
Absorption bands \( \lambda_{\text{max}} \) 236nm, 283nm and 336nm (Methanol), characteristic of naphthalene containing compounds \(^{49}\).
\(^1\)H NMR: \(\delta(ppm)\) 2.91 (2H, t), 3.21 (2H, t), 3.68 (3H, s), 4.08 (3H, s), 5.31 (t), 7.43-7.52 (2H, m), 7.98 (2H, t), 8.21 (1H, s), 8.38 (1H, s).

Peak Assignment: Type (ppm)

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<th>(ppm)</th>
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<td>(2H, t)</td>
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</tr>
<tr>
<td>b and c</td>
<td>(2H, m)</td>
<td>7.43-7.52</td>
</tr>
<tr>
<td>e and f</td>
<td>(1H, s)</td>
<td>8.38</td>
</tr>
<tr>
<td></td>
<td>(1H, s)</td>
<td>8.21</td>
</tr>
<tr>
<td>g</td>
<td>(3H, s)</td>
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<td>j</td>
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</tr>
<tr>
<td></td>
<td>(2H, t)</td>
<td>2.91</td>
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Solvent peak:
Dichloromethane-d\(_2\) (CD\(_2\)Cl\(_2\)) at 5.31 ppm triplet (3)

Note:
Pattern for aromatic different from that of Mono-methyl product
$^{13}$C NMR: δ (ppm) 28.5, 30.3, 51.9, 54.1, 124.2-128.7, 132.1, 133.9, 136.8, 137.6, 152, 156, 174.

Peak Assignment:

<table>
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<th>Assignments</th>
<th>ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1, C2, C3, C4, C7 and C10</td>
<td>(Aromatic C-H)</td>
<td>124.2, 125.7, 126.7, 127.3, 128.0, and 128.7</td>
</tr>
<tr>
<td>C5 and C6</td>
<td></td>
<td>132.1 and 133.9</td>
</tr>
<tr>
<td>C8 and C9</td>
<td></td>
<td>136.8 and 137.6</td>
</tr>
<tr>
<td>C11</td>
<td>(N-CH$_3$)</td>
<td>54.1</td>
</tr>
<tr>
<td>C12</td>
<td>(N-C=O)</td>
<td>156</td>
</tr>
<tr>
<td>C13</td>
<td></td>
<td>152</td>
</tr>
<tr>
<td>C14 and C15</td>
<td>(CH$_2$)</td>
<td>28.5 and 30.3</td>
</tr>
<tr>
<td>C16</td>
<td>(O-C=O)</td>
<td>174</td>
</tr>
</tbody>
</table>

**Solvent peak:**

Dichloromethane-d$_2$ (CD$_2$Cl$_2$)

Quintet at 53.1 ppm 1:2:3:2:1
The $^{13}$C NMR technique of distortionless enhancement by polarisation transfer (DEPT) is a sub-spectrum method for the identification and separation of X-H$_n$ signals. As the acronym DEPT implies, the increased sensitivity derives from the 'polarisation transfer' from attached protons. A number of different pulse sequences effectively break up the $^{13}$C spectrum into sub-spectra of nuclei attached to one, two or three protons, which allows for the identification of CH$_3$, CH$_2$ and CH from a spectrum. Appropriate addition and subtracting of the three spectra leads to the generation of three sub-spectra, each containing only one type of carbon. Quaternary carbon atoms give no signal in the DEPT procedure but are obvious in the standard $^{13}$C spectrum.

![Diagram of DEPT NMR spectrum](image)
Mass Spectral Data:

Fragmentation Pattern:

Compound IIIb (Code D3)

\[
\begin{align*}
\text{OCH}_3 \\
\text{C - OCH}_3
\end{align*}
\]

296-265

296-237

Elemental analysis

Elemental analysis by accurate mass: \( \text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_3 \)

Theoretical 296.11609; Experimental 296.11690 (Dev. ppm 2.73)
6.2.3 STEP 3

Fig. 6.12 Synthesis of (2(1H)-benzo[6,7-d]quinoxalinone-3-propionylcarboxylic acid hydrazide (BQ-PAH = compound IVa) and 1-methyl-2(1H)-benzo[6,7-d] quinoxalinone-3-propionylcarboxylic acid hydrazide (MBQ-PAH = compound IVb)

Compounds IIIa and IIIb were then reacted separately with hydrazine hydrate. The methylester compound (0.5g) was dissolved in 45% aqueous hydrazine hydrate (10 ml) and the mixture was heated at 60 °C for 1 hr. The resulting precipitates were collected by filtration and recrystallized from hot ethanol (yield Compound IVa 0.27g, 50.4% ; compound IVb 0.12g, 30%).
6.2.3.1 Structural characterisation of compound IVa: (2(1H)-benzo[6,7-d]quinoxalinone-3-propionylcarboxylic acid hydrazide (BQ-PAH)

Physical Properties:

Pale yellow / white powder, soluble in methanol. m.p. 287-288°C

Ft-IR:

1587-1692 cm⁻¹, 2674-3142 cm⁻¹ and 3184-3383 cm⁻¹.

UV:

Absorption bands λ_max 236nm, 283nm and 336nm (methanol), characteristic of naphthalene containing compounds.
$^1$H NMR: δ(ppm) 2.49 (q), 2.71 (2H, t), 3.24 (2H, t), 3.42 (3H, 2Hs(broad)), 4.17(s),
7.45-7.60 (2H, m), 7.68 (1H, s) 8.02 (2H, dd), 8.34 (1H, s) 9.17 (1H, s).

Peak Assignment:

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<th></th>
<th>Type</th>
<th>(ppm)</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>b and c</td>
<td>(2H, m)</td>
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<td>e and f</td>
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<td></td>
<td>(1H, s)</td>
<td>7.68</td>
</tr>
<tr>
<td>j</td>
<td>(1H, s)</td>
<td>9.17</td>
</tr>
<tr>
<td>h and i</td>
<td>(2H, t)</td>
<td>3.24</td>
</tr>
<tr>
<td></td>
<td>(2H, t)</td>
<td>2.71</td>
</tr>
</tbody>
</table>

Solvent peak:
Dimethyl sulphoxide (DMSO) at 2.49ppm quintet (5)

Note:
Pattern for aromatic similar to that Di-methyl product
Mass Spectral Data:

![Graph of mass spectral data]

Fragmentation Pattern:

**Compound IVa (Code G3)**

![Structure of Compound IVa]

282-223

\[
\begin{align*}
\text{O} \\
\text{C - NHNH}_2
\end{align*}
\]

282-195

\[
\begin{align*}
\text{O} \\
\text{CH}_2\text{CH}_2\text{C - NHNH}_2
\end{align*}
\]

Elemental analysis

*Elemental analysis by accurate mass*: \( \text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_3 \)

Theoretical 282.11167; Experimental 282.11208 (Dev. ppm 1.42)
6.2.3.2 Structural characterisation of compound IVb: 1-methyl-2(1H)-benzo[6,7-d]quinoxalinone-3-propionylcarboxylic acid hydrazide (MBQ-PAH)

Physical Properties:

Pale pink / light-brown powder, soluble in methanol. m.p. 208-209°C

FT-IR:

1623 cm⁻¹, 2674-3142 cm⁻¹, 3293 cm⁻¹ and 3184-3383 cm⁻¹

UV:

Absorption bands \( \lambda_{\text{max}} \) 236nm, 283nm and 336nm (methanol), characteristic of naphthalene containing compounds.
^1H NMR: \( \delta(ppm) \) 2.49 (q), 2.71 (2H, t), 3.24 (2H, t), 3.42 (3H, s(broad)), 4.17 (s), 7.63 (2H, m), 8.2 (2H, t), 8.45 (1H, s), 8.61 (1H, s), 9.17 (1H, s).

Solvent peak: Dimethyl sulfoxide (DMSO) at 2.49ppm quintet (5)

Peak Assignment: Type (ppm)

| a and d | (2H, t) | 8.2 |
| b and c | (2H, m) | 7.63 |
| e and f | (1H, s) | 8.61 |
| g? | (broad) | 4.17 |
| j | (1H, s) | 9.17 |
| h and i | (2H, t) | 3.24 |
| h and i | (2H, t) | 2.71 |

Note:
Pattern for aromatic Similar to that of Mono-methyl Product
Mass Spectral Data:

Fragmentation Pattern:

Compound IVb (Code J3)

\[
\begin{align*}
\text{CH}_3 & \quad \text{O} \\
\text{N} & \quad \text{CH}_2\text{CH}_2\text{CONHNH}_2 \\
\text{N} & \\
\end{align*}
\]

296-282 \[
\left[ \text{NH}_2 \right]
\]

296-265 \[
\left[ \text{NHNH}_2 \right]
\]

296-237 \[
\left[ \text{C \cdot NHNH}_2 \right]
\]

296-223 \[
\left[ \text{CH}_2\text{C \cdot NHNH}_2 \right]
\]

Elemental analysis

*Elemental analysis by accurate mass: \text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_3*

Theoretical 296.12732; Experimental 296.12718 (Dev. ppm -0.51)
6.3 FLUORESCENT PROPERTIES OF COMPOUND IVa AND IVb

The fluorescent properties of compounds IVa (BQ-PAH) and IVb (MBQ-PAH) in methanol and acetonitrile were examined to assess the potential of these compounds as fluorometric derivatisation reagents. These solvents have been widely used as mobile phases in reverse-phase chromatography. Maximum signal was obtained in methanol with the fluorescence excitation (maximum, 395 nm) and emission (maximum, 483 nm) spectra of both reagents differing in respect to fluorescent intensity (peak height), with MBQ-PAH giving a signal 10 times that of compound 4 (Fig. 6.13).

Fig. 6.13

Fluorescence excitation spectra a (IVb), c (IVa) and emission spectra b (IVb), d (IVa) 1.0 mmol ml⁻¹ in methanol.

Similar fluorescent properties were observed when methylation of the N-H group in the reagent HCPI and its analogues (Fig. 6.14) which gave increased sensitivity (detection limit improving from 7-57 fmol to 2-12 fmol for a mixture of fatty acids) (45,46).
Fig. 6.14  Structure of HCPI and MPI-BH, where the addition of a methyl-group at the N-H site increased sensitivity

Therefore MBQ-PAH was selected for further investigations aimed at developing suitable reaction conditions for quantifying fatty acids. The fluorescent properties of MBQ-PAH in methanol, acetonitrile and water, which are commonly used as mobile phases in reverse-phase chromatography were examined (Fig. 6.15), with the view to selecting solvents for the HPLC separation of MBQ-PAH derivatives of saturated C_{12}-C_{22} fatty acids. The fluorescent intensity was found to be a maximum in methanol with water giving the least intense signal. However, the poor solubility of MBQ-PAH in water makes accurate measurement problematic.

Fig. 6.15
A. Fluorescence excitation and B. emission spectra of compound IVb (1.0 mmol ml\(^{-1}\)) in (1) methanol, (2) acetonitrile and (3) water.
6.4 DERIVATISATION CONDITIONS

Initial conditions were based on those used for DMEQ-hydrazide \(^{(42)}\). Optimisation was carried out on a mixture of myristic, palmitic and stearic acid standards. A solution of MBQ-PAH (0.1%) in \(N,N\)-dimethylformamide (DMF) was used in the procedure. 1-Ethyl-3-(3-dimethylaminopropyl) carbo-diimide (EDC) and pyridine were used to facilitate the dervatisation of the fatty acids with MBQ-PAH.

\[
\begin{align*}
\text{CH}_3 & \text{N} \text{ N} \\
\text{O} & \text{CH}_2\text{CH}_2\text{CONHNH}_2
\end{align*}
\]

\[\text{[IVb]} = \text{MBQ-PAH}\]

\[
\begin{align*}
\text{10\% pyridine in 25mM HCl} & \text{ EDC} \\
\text{CH}_3 & \text{N} \\
\text{O} & \text{CH}_2\text{CH}_2\text{CONHNHCOR}
\end{align*}
\]

Fig. 6.16: Derivatisation of fatty acids with MBQ-PAH.

The peak areas for the fatty acids were maximum at a concentrations of 2.5M EDC. Maximum peak heights were attained with pyridine concentrations in the range of 5-15% with 10% selected as optimum.

The reaction time selected was 40 min which was based on data (Fig. 6.17) that showed longer reaction times did not significantly increase peak area.
Fig. 6.17  Effect of reaction time on the peak area of myristic (C₁₄), palmitic (C₁₆) and stearic acids (C₁₈). A mixture of the three fatty acids (10 nmol ml⁻¹ each) was treated according to the procedure outlined in the experimental section for reaction times up to 180 min.

The derivatisation reaction was carried out over a temperature range of 20-70°C (Fig. 6.18). Peak areas were maximum for myristic acid at 50°C, with palmitic and stearic acid giving peak maximum at 60°C.

Fig. 6.18  Effect of reaction temperature on the peak area of myristic (C₁₄), palmitic (C₁₆) and stearic acids (C₁₈). A mixture of the three fatty acids (10 nmol ml⁻¹ each) was treated according to the procedure outlined in the experimental section over a temperature range of 20-70°C.
However, peaks due to breakdown products increased at temperatures above 45°C. In view of these findings, 45°C was chosen as the optimum reaction temperature (Fig 6.19).

**Fig. 6.19**

Effect of reaction temperature on the peak area of 1 myristic (C₁₄), 2 palmitic (C₁₆), and 3 stearic acids (C₁₈). A mixture of the three fatty acids (10 nmol ml⁻¹ each) was treated according to the procedure outlined in the experimental section. Chromatograms show MBQ-PAH derivatives at reaction temperatures of a) 20°C and b) 60°C. Column Ultracarb™ ODS(20) column 30°C; mobile phase, gradient elution, with aqueous methanol (0-10 min, 10%, 10-18 min, 10-100%, 18-40 min, 100% MeOH), using a flow rate of 0.75 ml min⁻¹.

(Data files a) 459520c and b) 459560c.)

The presence of hydrochloric acid in pyridine was found to increase the peak areas of DMEQ-hydrazide derivatives (43). Similar observations were made using MBQ-PAH. Thus, using hydrochloric acid concentrations in pyridine between 10-35mM, a concentration of 25mM gave maximum peak areas 3 times than that of a pyridine solution without hydrochloric acid (Fig. 6.20).
Fig. 6.20

Chromatograms showing the effect of HCl concentration in pyridine on peak height area of myristic acid (C\textsubscript{14}) MBQ-PAH derivatives. a) Without HCl and b) with 25mM HCl in pyridine solution. Column Ultracarb\textsuperscript{TM} ODS(20) column 30 °C; mobile phase, gradient elution, with aqueous methanol (0-15 min, 20%, 15-20 min, 20-100%, 20-40 min, 100% MeOH), using a flow rate of 0.75 ml min\textsuperscript{-1}. (Data files a) inj7161095 and b) inj3161095.)
6.5 CHROMATOGRAPHIC ANALYSIS OF C_{12}-C_{22} MBQ-PAH FATTY ACID DERIVATIVES

6.5.1 Derivatisation procedure

To the test solution of fatty acids in water (100 µl, equivalent to 40ng each on-column) were added each of EDC in water (50 µl, 2M) and pyridine (50µl, 10% in 25mM HCl) and MBQ-PAH in DMF (100 µl, 0.1%). The mixture was maintained at 45°C for 40 min and a 20 µl portion was injected into the chromatograph. The blank was prepared in the same manner except that the test solution was replaced with water.

6.5.2 Separation of MBQ-PAH derivatives of fatty acids

The simultaneous separation of MBQ-PAH derivatives of C_{12}-C_{22} fatty acids was achieved on a Ultracarb ODS(20) column (5 µm, 3.2 x 250 mm, Phenomenex) by gradient elution with aqueous methanol (0-10 min, 10%, 10-20 min, 10-100%, 20-40 min, 100% MeOH) using a flow rate of 0.75 ml min^{-1} at 30°C. All peaks were completely separated within 36 min. (Fig. 6.21). It was noted that each fatty acids had a different peak response, which may be due to the differences in the yields of fluorescent derivatives from fatty acids and/or variation in the quantum yields of the derivatives (42).

Liquid chromatographic analysis

The HPLC system consisted of a Waters 600 Multi-solvent Quaternary pump (Waters, USA), a column oven (HPLC-technology). Separation was achieved using a gradient elution of aqueous MeOH using an Ultracarb™ ODS(20) column 5 µm, 3.2 x 250 mm, (Phenomenex, Macclesfield, UK), with a precolumn (Ultracarb™ ODS(20) 5 µm, 2 x 30mm.) and an in-line filter (0.5µm x 3mm, Rheodyne, Cotati, CA, USA) with 20µl of sample injected (Rheodyne model 7125) and detection using a Shimadzu RF-551 fluorimetric detector (λ_{ex} 395nm, λ_{em} 483nm)

Software

Chromatographic data handling was performed using an Axxi-Chrom™ 717 chromatographic data station (Axxiom chromatography, UK). Data were transferred to Microsoft Excel for further graphical manipulation.
Fig. 6.21

Chromatogram of MBQ-PAH derivatives of saturated fatty acids and reagent blank (insert). 100 μl of a standard mixture of the acids (equivalent to 40 ng on-column) was treated according to the described procedure. 1, Lauric (C<sub>12</sub>); 2, myristic (C<sub>14</sub>); 3, palmitic (C<sub>16</sub>); 4, stearic (C<sub>18</sub>); 5, arachidic (C<sub>20</sub>); and 6, behenic (C<sub>22</sub>) acids. Column Ultracarb<sup>™</sup> ODS(20) column 30 °C; mobile phase, gradient elution, with aqueous methanol (0-15 min, 20%, 15-20 min, 20-100%, 20-40 min, 100% MeOH), using a flow rate of 0.75 ml min<sup>-1</sup>.
6.5.3 Precision, calibration curve and detection limits

The precision was established by repeated determinations using a myristic acid standard of 20μg/ml which represented 20ng on-column. The relative standard deviation did not exceed 1.3% (n=8). Calibration curves for myristic acid (Fig. 6.22) were linear for 0.10-0.40 μg/ml which represents 10-40ng on-column: (r=0.999). The limit of detection based on peak height of myristic acid was found to be 0.10ng on column at a signal-to-noise ratio of 3 (0.44 pmol) (Fig. 6.23.).

Calibration Curve of Myristic -MBQ-PAH Derivative

![Calibration Curve of Myristic -MBQ-PAH Derivative](image)

**Linear Regression Data:**

\[ Y = A + B \times X \]

<table>
<thead>
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<th>Param</th>
<th>Value</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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<td>2582.2</td>
</tr>
<tr>
<td>B</td>
<td>5183.4</td>
<td>95.9</td>
</tr>
</tbody>
</table>

R= 0.99915  
SD=2537.3, N=7

Fig. 6.22 *Calibration curve of MBQ-PAH derivatives of myristic acid (C\textsubscript{14}) with fluorescent detection at \( \lambda_{ex} \) 395 nm and \( \lambda_{em} \) 483 nm.*
6.6 CONCLUSION

1-Methyl-2(1H)-benzo[6,7-d] quinoxalinone-3-propionylcarboxylic acid hydrazide (MBQ-PAH) was found to be a sensitive fluorescence derivatisation reagent for the analysis of carboxylic acids in high-performance liquid chromatography. The reaction conditions were optimised for various C$_{12}$-C$_{22}$ saturated fatty acids. The reagent readily reacted with the fatty acids in aqueous solution in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) at 40°C to produce the corresponding fluorescent derivatives, which were separated on a reverse-phase column, Ultracarb.
ODS(20), column 30° C with an aqueous:methanol gradient elution, (0-15 min, 20%, 15-20 min, 20-100%, 20-40 min, 100% MeOH), using a flow rate of 0.75 ml min⁻¹. The fluorescent derivatives were detected spectrofluorimetrically at 483 nm with excitation at 395 nm. The detection limit (signal to noise ratio = 3) for the myristic acid was 0.44 pmol for an injection volume of 20 μl. Further work needs to be carried out on the reagent with the view of developing a HPLC method for the analysis of DSP toxins.

6.7 BIBLIOGRAPHY


Appendixes
Appendix No. 1

Recent Publications and Conference Papers


APiii
Appendix No. 2

The determination of % Ethanol stabiliser in commercially available Chloroform by Gas Chromatographic analysis

Varian 3400 Gas Chromatograph (Instrument Settings)

**Carrier gas:** 60ml per min. of 'oxygen-free' nitrogen (OFN)

**Detector:** Flame ionisation detector
- Hydrogen 30ml
- Air 300ml
- Detector temp. 180°C

**Column:** Specifications
- 80/120 ‘Carbopack B’ 3%
- SP 1500 (Packed)
- Dimensions 2M x 4mm ID
- Max Temp. 220°C

**Temperature program:**
- Initial column temp. 70°C
- Ramp rate 10°C per min.

**Injection:** Manual Hamilton 1 μl syringe
- Injection volume 0.5 μl
- Injection port temp. 150°C

**Internal Standard:** Iso-propanol (HPLC grade)
Appendix No. 3

Calculations for the Modified Analytical Methods

The toxins were initially extracted from 6 g of hepatopancreas (HP). Assuming that 6 g of hepatopancreas gives approximately 6 ml of water on homogenisation, then the calculations are as follows-

Toxin content of 6 g HP was extracted with 18 ml solvent
(12 mL 80% methanol and 6 mL water)

The toxin content of \( \frac{6 \times 2.5}{18} \) g HP was contained in 2.5 ml supernatant
The toxin content of \( \frac{6 \times 2.5}{18} \) g HP was contained in 10 ml chloroform
The toxin content of \( \frac{6 \times 2.5 \times 0.5}{18 \times 10} \) g HP was contained in 0.5 ml chloroform
The toxin content of \( \frac{6 \times 2.5 \times 0.5}{18 \times 10} \) g HP was derivatised
The toxin content of \( \frac{6 \times 2.5 \times 0.5}{18 \times 10} \) g HP was contained in 200 µl methanol
(prior to injection into the HPLC system)

If a 20 µl injection volume was used then-

The toxin content of \( \frac{6 \times 2.5 \times 0.5}{18 \times 10 \times 10} \) g HP was injected on column = x (g)

But also, the mass of toxin injected on column was calculated from an okadaic acid standard calibration graph (ng) = y (ng)

The mass of toxin in ng per g of HP = \( \frac{y}{x} \)

Usually the toxin content is expressed as µg per g of HP and thus the formula becomes

\[ \frac{y}{1000x} \]

The mass of toxin in µg per g of HP = 0.24y
Appendix No.4

Conversion from 'Calibration Plot Data' (ppb) to 'Toxin Concentration in Mussel Hepatopancreas' (μg/g)

The following are the calculations for converting toxin concentration in ppm to toxin concentration in μg/g of hepatopancreas for the two ELISA kits, and the HPLC methods.

**DTX-2 SITE No.8 (ELISA)**

\[ X_{\text{ppb}} = \frac{x}{1000} \text{ ppm} \]

\[ \Rightarrow \frac{x}{1000} \text{ μg/ml} \]  \( (x = \text{Calibration reading}) \)

\[ \Rightarrow \frac{4x}{1000} \text{ μg/4ml of Methanol in water (45%)} \]

\[ \Rightarrow \frac{4x}{1000} \text{ μg/0.1ml of Chloroform} \]

\[ \Rightarrow \frac{400x}{1000} \text{ μg/10ml of Chloroform} \]

\[ \Rightarrow \frac{4x}{10} \text{ μg/2.5ml of Chloroform} \]

\[ \Rightarrow 40x \text{ μg/25ml of Chloroform} \]

\[ \Rightarrow 40x \text{ μg/1200g of Hepatopancreas (original weight of extract)} \]

\[ \Rightarrow \frac{40x}{1200} \text{ μg/g of Hepatopancreas} \]

**Conversion Factor** \[ \Rightarrow \frac{x}{30} \text{ μg/g of Hepatopancreas} \]

**DTX-2 SITE No.8 (HPLC)**

\[ x \text{ ng per 20μl (0.02ml) Oncolumn i.e injection volume.} \]

\[ 10x \text{ ng per 200μl of Methanol} \]

\[ 10x \text{ ng per 40μl of Chloroform extract.} \]

\[ \Rightarrow \frac{10x}{0.04} \text{ ng/1ml of Chloroform} \]
\[ \Rightarrow \frac{10x}{0.04} \text{ ng} / 0.25 \text{ ml of Chloroform extract} \]
\[ \Rightarrow \frac{10,000x}{0.04} \text{ ng} / 25 \text{ ml of Chloroform extract} \]
\[ \Rightarrow \frac{10,000x}{0.04} \text{ ng} / 1200 \text{ g of Hepatopancreas} \]
\[ \Rightarrow \frac{10,000x}{(0.04)(1200)} \text{ ng} / 1 \text{ g of Hepatopancreas} \]
\[ \Rightarrow \frac{10x}{48} \text{ \(\mu\)g} / 1 \text{ g of Hepatopancreas} \]
\[ \Rightarrow \frac{x}{4.8} \text{ \(\mu\)g} / 1 \text{ g of Hepatopancreas} \]

**Conversion Factor** \[ \Rightarrow \frac{x}{4.8} \text{ \(\mu\)g/g of Hepatopancreas} \]

**DTX-2 SITE No.5 (ELISA)**

\[ x_{\text{ppb}} = \frac{x}{1000} (\text{ppm}) \Rightarrow \frac{x}{1000} (\mu\text{g/ml}) \quad (x = \text{Calibration reading}) \]
\[ \Rightarrow \frac{4x}{1000} \mu\text{g}/4 \text{ ml of Methanol in water (45\%)} \]
\[ \Rightarrow \frac{4x}{1000} \mu\text{g}/0.1 \text{ ml of Chloroform} \]
\[ \Rightarrow \frac{4x}{10} \mu\text{g}/10 \text{ ml of Chloroform} \]
\[ \Rightarrow \frac{4x}{10} \mu\text{g}/0.5 \text{ ml of Chloroform} \]
\[ \Rightarrow \frac{(4x)(50)}{10} \mu\text{g}/25 \text{ ml of Chloroform} \]
\[ \Rightarrow 20x \mu\text{g}/561.66 \text{ g of Hepatopancreas (original weight of extract)} \]
\[ \Rightarrow \frac{20x}{561.66} \mu\text{g}/\text{g of Hepatopancreas} \]

**Conversion Factor** \[ \Rightarrow 0.0356 x \mu\text{g}/\text{g of Hepatopancreas} \]
DTX-2 SITE No.5 (HPLC)

\[ x \text{ ng per 20} \mu\text{l (0.02ml) On column i.e injection volume.} \]

\[ \frac{100x}{0.04} \text{ ng / 0.5ml of Methanol extract} \]

\[ \frac{5000x}{0.04} \text{ ng / 25ml of Methanol extract} \]

\[ \frac{5000x}{0.04} \text{ ng / 561.66g of Hepatopancreas} \]

\[ \Rightarrow \frac{5000x}{(0.04)(561.66)} \text{ ng / 1g of Hepatopancreas} \]

\[ \Rightarrow \frac{5000x}{(0.04)(561.66)(1000)} \mu\text{g / g of Hepatopancreas} \]

\[ \Rightarrow \frac{5x}{(0.04)(561.66)} \mu\text{g / 1g of Hepatopancreas} \]

**Conversion Factor**

\[ \Rightarrow \frac{x}{4.4928} \mu\text{g / g of Hepatopancreas} \]

MUS-2 (ELISA)

\[ X_{\text{ppb}} = \frac{x}{1000} \text{ (ppm)} \Rightarrow \frac{x}{1000} \text{ (} \mu\text{g/ml) (} x = \text{Calibration reading)} \]

\[ \Rightarrow \frac{3x}{1000} \mu\text{g / 3ml of Methanol in water (45%)} \]

\[ \Rightarrow \frac{3x}{1000} \mu\text{g / 40} \mu\text{l of Methanol} \]

\[ \Rightarrow \frac{3x}{(1000)(0.040)} \mu\text{g / 4.354 ml of Methanol} \]

\[ \Rightarrow \frac{(4.354)(3x)}{(1000)(0.040)} \mu\text{g / 1 ml of Methanol} \]

\[ \Rightarrow \frac{(4.354)(3x)}{40} \mu\text{g / 5 ml of Methanol Extract (subdivided from 20mls)} \]
**Conversion Factor** \( \Rightarrow 0.360 \, \mu g / g \) of MUS - 2

\[
\Rightarrow \frac{(4.354)(4)(3x)}{40} \mu g / 20 \text{ ml of original Methanol Extract}
\]

\[
\Rightarrow \frac{(4.354)(4)(3x)}{40} \mu g / 3.628 \text{ g of Mussel Meat}
\]

\[
\Rightarrow \frac{(4.354)(4)(3x)}{(40)(3.628)} \mu g / 1 \text{ g of Mussel Meat}
\]

**MUS-2(HPLC)**

\( x \, \text{ng per} \ 20 \mu l \) (0.02 ml) on-column i.e. injection volume.

12.5 \( x \) ng /250 \( \mu l \) of Methanol \( \Rightarrow 12.5x \) ng /50 \( \mu l \) of Methanol Sample

\[
\frac{(4.354)(12.5x)}{0.05} \text{ ng / } 4.354 \text{ ml of Methanol extract}
\]

\[
\frac{(4.354)(12.5x)}{0.05} \text{ ng / } 5 \text{ ml of Extract}
\]

\[
\frac{(4.354)(4)(12.5x)}{0.05} \text{ ng / } 20 \text{ ml of Extract (original extract)}
\]

\( \Rightarrow 4354x \, \text{ng / } 3.628 \text{ g of MUS - 2}
\]

\[
\Rightarrow \frac{4354x}{3.628} \, \text{ng / } 1 \text{ g of MUS - 2}
\]

**Conversion Factor** \( \Rightarrow 1.2001 \, \mu g / g \) of MUS - 2

**Site No’s 10 and 11 (ELISA)**

\[
X_{ppb} = \frac{x}{1000} \, (ppm) \Rightarrow \frac{x}{1000} \, (\mu g / ml) \quad (x = \text{Calibration reading})
\]

APix
**Note:**
Assume 2g of hepatopancreas initially

\[
\frac{x}{1000} \text{ g / 25mls of Chloroform}
\]

\[
\frac{(2)(2.5)}{6} \text{ g / 2.5mls of Chloroform}
\]

\[
\frac{(2)(2.5)}{60} \text{ g / 1mls of Chloroform}
\]

\[
= \frac{1}{12} \text{ g / 1ml of Chloroform}
\]

**Site No's 10 and 11 (HPLC)**

\[x \text{ ng per } 20\mu l (0.02ml) \text{ Oncolumn i.e injection volume.}\]

10 ng / 200\mu l of Methanol

10 ng / 0.5ml of Chloroform

\[(20)(12x) \mu g / g \text{ of Hepatopancreas}\]

**Conversion Factor**
\[\Rightarrow \frac{x}{12.5} \mu g / g \text{ of Hepatopancreas}\]

**SITE No. 4 (ELISA)**

\[
\frac{x}{1000} \mu g / 1ml \text{ of Methanol: Water(45%)}
\]

\[
\frac{x}{1000} \mu g / 0.3ml \text{ of Chloroform (300\mu l)}
\]

\[
\frac{x}{300} \mu g / 1ml \text{ of Chloroform}
\]
\[ \frac{12x}{300} \mu g / \text{lg of Hepatopancreas} \]

\[ \frac{x}{25} \mu g / \text{lg of Hepatopancreas} \]

**Conversion Factor** \( \Rightarrow \frac{x}{25} \mu g / \text{g of Hepatopancreas} \)

**SITE No. 4 (HPLC)**

\( x \text{ ng per } 20\mu l \ (0.02ml) \) Oncolumn i.e injection volume

\( 5x / \text{100}\mu l \) (Change in Volume)

\( 5x \text{ ng / 0.25ml} \)

\( \Rightarrow (5)(4)(12) x \text{ ng / g of Hepatopancreas} \)

**Conversion Factor** \( \Rightarrow 0.24 x \mu g / \text{g of Hepatopancreas} \)

**Eastpoint Whiddy (Bantry) and Kilmakologue 1993 (ELISA)**

\( \frac{x}{1000} \mu g / \text{1ml of Methanol : Water (45\%)} \)

\[ \frac{x}{4000} \mu g / \text{0.25ml of Methanol : Water (250\mu l)} \]

\[ \frac{x}{4000} \mu g / \text{1ml of Chloroform (four times initial extract volume)} \]

\[ \frac{x}{4000} \mu g / \frac{1}{12} \text{g of Hepatopancreas} \]

\[ \frac{12x}{4000} \mu g / \text{1g of Hepatopancreas} \]

**Conversion Factor** \( \Rightarrow \frac{x}{333.3} \mu g / \text{g of Hepatopancreas} \)
Eastpoint Whiddy (Bantry) and Kilmakalogue 1993 (HPLC)

Calculations as for Site No.4, 10 and 11.

*Conversion Factor* ⇒ $0.24 \times \mu g / g$ of Hepatopancreas
Calculations of sample concentration to fit linear range of both the DSP Check® (Japan) and Rougier bio-tech® (Canada) ELISA kits

Note: OA standard solutions were made up from our own reference stocks in order to maintain uniformity between the different analytical methods being employed. (Standards obtained from SIGMA cell-culture™'Okadaic acid’ 95% pure) On the basis of the literature received with each brand of kit, a series of OA standard were prepared within a linear working range of 10 →100 ppb.

**Standards**

- **100 ppb** = 0.1 ppm  
  = 0.1 μg per ml  
  Amount of Oa in sample well  
  = 0.1 μg × 0.05 ml  
  (as only 50 μl added)  
  = 0.005 μg  
  = 5.0 ng per 50 μl of the 100 ppb standard

- **10 ppb** = 0.01 μg per 1 ml  
  = 0.5 ng per 50 μl of the 100 ppb standard

**ELISA RANGE 0.5 → 5 ng**

Stock OA standard which was 95% pure = 2.5 μg per 1 ml = 0.1 μg per 40 μl  
Therefore 40 μl of OA standard was taken, blown down under nitrogen and made up to the following volumes with 45% methanol in water to give the desired working standard concentration.

<table>
<thead>
<tr>
<th>OA conc/ppb</th>
<th>Log(Std Conc.)</th>
<th>Final volume ml (45% methanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.00</td>
<td>1.000</td>
<td>10</td>
</tr>
<tr>
<td>20.00</td>
<td>1.300</td>
<td>5</td>
</tr>
<tr>
<td>33.33</td>
<td>1.523</td>
<td>3</td>
</tr>
<tr>
<td>50.00</td>
<td>1.699</td>
<td>2</td>
</tr>
<tr>
<td>100.00</td>
<td>2.000</td>
<td>1</td>
</tr>
</tbody>
</table>
TEST SAMPLES

DTX-2 LARGE MUSSEL EXTRACT Site No.5

This extract was part of a bulk sample of 561.66g of hepatopancreas sampled on 20th October 1991. The extraction procedure was carried out between the 11th and 13th of January 1993.

From previous HPLC analysis it was estimated that the total toxin concentration in the sample was approximately 1.2 mg

i.e.

10ng OA std. gave a peak area of 821282

For the sample: DTX-2 and OA peak areas were 4883000 and 196987 respectively

nanograms of total toxin on column = (4883000 + 196987) \times 10

\[
\begin{align*}
821282 \\
= 62 \text{ ng} \\
62\text{ng per 20µl} \\
620 \text{ ng of Toxin were derivatised}
\end{align*}
\]

But as only 1/2000th of the sample was actually derivatised on the day of analysis the total toxin content of the sample was approximately 1.2 mg.

The ELISA kits required a toxin concentration between 10 and 100ppb. Therefore it was necessary to carry out a 1/5000th dilution of the sample, and this was then made up to 4ml with 45% methanol in water to give a toxin concentration within the range of the kits.

i.e. Approx. Conc. = 1.24/5000 mg per 4mls

\[
\begin{align*}
= 2.48 \times 10^{-4} \text{ mg per 4 mls} \\
= 6.20 \times 10^{-5} \text{ mg per 1 ml} \\
= 0.062 \mu\text{g per ml} \\
= 620 \text{ ppb}
\end{align*}
\]

This was carried out as follows:

- The DTX-2 extract was made up to 25mls with Chloroform.
  (10ml of this solution was kept as a stock solution)
- 0.5ml (1/50 th) of this extract was taken and made up to 10ml with Chloroform

APxiv
0.1mls (1/5000 th) of this solution was then taken and blown down under nitrogen.
The residue remaining was then made up to 4ml with 45% methanol in water.

**DTX-2 LARGE MUSSEL EXTRACT Site No.8**
This extract was part of a bulk sample of 1200g of hepatopancreas sampled on 30th October 1991. The extraction procedure was carried out between the 18th and 22nd of January 1993. Therefore a 1/10000th of the sample was taken and made up to 4mls with 45% methanol in water (Assuming that Site No.8 and Site No.5 roughly contained the same level of toxins). This was carried out as follows:

- The DTX-2 extract was made up to 25ml with Chloroform. (10mls of this solution was kept as a stock solution)
- 0.25ml (1/100 th) of this extract was taken and made up to 10ml with Chloroform.
- 0.1ml (1/10000 th) of this solution was then taken and blown down under nitrogen.
- The residue remaining was then made up to 4ml with 45% methanol in water.

**Mussel Standard (MUS-2) from CANADA**
MUS-2 is a certified reference material for DSP toxins. It is a self-stable homogenised blend of mussel (*Mytilus edulis L.*) digestive gland tissue and the microalgae *Prorocentrum lima*. MUS-2 is available as a set of four polypropylene bottles, each containing four grams of homogenate. The bottles were heat sealed, thermally sterilised and individually packed in tri-laminate pouches.

The MUS-2 standard was purchased from Canada and was prepared as laid down by manufactures guidelines. The sample was coded 'MUS-2 Sample No.383', and contained approximately 11μg OA and 1μg of DTX-1 per gram of mussel meat. 3.628g of the standard mussel meat was extracted into 20 ml of solvent and this was divided into four 5ml portions (0.907g per 5mls).

A toxin concentration equivalent to that of OA standard concentration was desirable.

The MUS-2 std contained 12 μg of toxin per gram of Mussel meat.
12 \times 0.907\mu g per 0.907g of MUS-2.

10.884\mu g of toxin per 0.907g of MUS-2.

Therefore one of the 5ml MUS-2 portions was blown down under nitrogen and made up to 4.354mls with HPLC grade methanol.

i.e. 10.884\mu g of toxin per 4.354ml.

2.5\mu g of toxin per ml of Methanol.

This 2.5\mu g of toxin per ml MUS-2 standard was brought into the ELISA concentration range by taking 40\mu l of the above solution ,blowing down under nitrogen gas and making up to 3ml with 45% methanol in water.

**SITE No. 11 15/9/91 (CHLOROFORM EXTRACT)**

From previous HPLC analysis it was estimated that the total toxin concentration in the sample was approximately 2.3\mu g /g of the original Mussel hepatopancreas.

\[ = 2.3 \times 2.5 \times 2 \mu g \text{ per } 10\text{ml of Chloroform extract} \]

\[ = 2.3 \times 2.5 \times 2 \mu g \text{ per } 1\text{ml of Chloroform extract} \]

\[ = 0.1916 \mu g \text{ per } 1\text{ml of Chloroform extract} \]

\[ =0.02875 \mu g \text{ per } 0.15\text{ml of Chloroform extract} \]

Therefore the test sample was prepared by taking 0.15ml of the chloroform extract, blowing down under nitrogen and making it up to 1ml with 45% methanol in water.

**SITE No. 10 23/9/91 (CHLOROFORM EXTRACT)**

From previous HPLC analysis it was estimated that the total toxin concentration in the sample was approximately 3.43\mu g /g of the original Mussel hepatopancreas.

\[ = 3.43 \times 2.5 \times 2 \mu g \text{ per } 1\text{ml of Chloroform extract} \]

\[ =0.2858 \mu g \text{ per } 1\text{ml of Chloroform extract} \]

\[ =0.0429 \mu g \text{ per } 0.15\text{ml of Chloroform extract} \]
Therefore the test sample was prepared by taking 0.15ml of the chloroform extract, blowing down under nitrogen and making it up to 1ml with 45% methanol in water.

SITE No. 4 28/10/'91 (CHLOROFORM EXTRACT)
From previous HPLC analysis it was estimated that the total toxin concentration in the sample was approximately 1μg /g of the original Mussel hepatopancreas.

\[= 1 \times 2.5 \times 2 \mu g \text{ per 1ml of Chloroform extract}\]
\[= \frac{0.08333}{60} \mu g \text{ per 1ml of Chloroform extract}\]
\[= 0.025 \mu g \text{ per 0.3ml of Chloroform extract}\]

Therefore the test sample was prepared by taking 0.3mls of the chloroform extract, blowing down under nitrogen and making it up to 1ml with 45% methanol in water.

BANTRY (EAST POINT WHIDDY) 29/8/'93 and KILMAKILLOGE 22/10/'93
These test samples were prepared by taking 1mls of the chloroform extract, blowing down under nitrogen and making them up to 250μl with 45% methanol in water.

Note: The test samples were prepared in sets of three in order to maintain uniformity between the two ELISA kits and the HPLC validation
Appendix No. 5

Experimental Procedure for Assays involving protein phosphatase inhibition

Preparation of [γ-32P] Adenosine-5'-Triphosphate (ATP)

Reagents
1 mCi (0.1 ml) pack of [γ-32P] Adenosine-5'-triophosphate (ATP) (Amersham)
Non-radioactive ATP (100 mmol/l, pH adjusted to 7.0 with NaOH)
Plexiglas shields and remote handling devices

Experimental
1. The [γ-32P] ATP was thawed to room temperature and transferred to a microcentrifuge tube, shielded in a Plexiglas box.
2. The original vial was washed with part of a 0.89 ml aliquot of water and added to the tube.
3. The remainder of the water aliquot plus 10 μL of 100 mmol/l ATP were added to the tube, which was then carefully mixed.
4. The content of the tube was diluted 1:100 in water and the subsequent sample divided into aliquots and stored at -20 °C.

Preparation of 32P-Labelled Phosphorylase a

Reagents
Buffer A (50 mmol/l Na 2-glycerophosphate pH 7.5, 10% (v/v) glycerol, 0.1 mmol/l
Buffer B (125 mmol/l Na 2-glycerophosphate, pH 8.6)
Buffer C (50 mmol/l Tris-HCl pH 7.0 (20 °C), 0.1% (v/v) 2-mercaptoethanol)
Buffer D (10 mmol/l Tris-HCl pH 7.0 (20 °C), 0.1% (v/v) 2-mercaptoethanol)
Buffer E (50 mmol/l Tris-HCl pH 7.0 (20 °C) )
EGTA, 0.1% (v/v) 2-mercaptoethanol
Phosphorylase kinase (20 mg/ml in buffer A)
Phosphorylase b (100 mg/ml in buffer A)
[\gamma^{32}P] ATP (Section 4.3.1.1)

90% saturated ammonium sulphate (475 g/l, pH adjusted to 7.0 with NH4OH)

NaF/EDTA (500 mmol/NaF, 100 mmol/l EDTA, pH adjusted to 7.0 with NaOH)

Experimental

1. *The removal of protein phosphatase contamination.* Phosphorylase b and phosphorylase kinase was incubated overnight on ice in buffer A containing 50 mmol/L NaF plus 5 mmol/l sodium pyrophosphate and then re-equilibrated into buffer A without NaF by gel filtration.

2. Into a 50-100ml centrifuge tube was mixed 80 μl of phosphorylase kinase, 160 μl Mg acetate (100 mmol/l), 10 μl of CaCl₂, 1 ml phosphorylase b, 4.25 ml buffer B and finally 2.5 mL [\gamma^{32}P]ATP.

3. The mixture was incubated at 30 °C for 1 hour.

4. An equal volume of ice-cold 90% saturated ammonium sulphate and 0.5 ml of NaF/EDTA was added to the mixture.

5. The tube was placed on ice for 30 minutes to precipitate protein, and then centrifuged at 12,000 g for 10 minutes at 4 °C.

6. The pellet was resuspended in buffer C (not more than 5 ml per 100 mg of phosphorylase) using a pipette.

7. The suspension was dialysed for 24 hours at 4 °C against 2 x2 litres of buffer D to remove residual [\gamma^{32}P]ATP and NaF. The dialysis buffer was monitored for radioactivity at each change. Crystallisation of phosphorylase a during dialysis yielded a cream-coloured suspension.

8. This suspension was transferred to a centrifuge tube, placed on ice for 30 minutes and centrifuged at 12,000 g for 10 minutes at 4 °C.

9. The crystals were resuspended in ice-cold buffer E (no more than 5 ml for each 100 mg of phosphorylase). The protein content and the radioactivity of a sample was determined.

10. This phosphorylase a solution was divided into aliquots and stored at 4 °C.
Standard Assay of Phosphorylase Phosphatase (PP1 and PP2A) Activity

Reagents

Caffeine (75 mmol/l, pH 7.0, stored in the dark at room temperature)
Buffer A (50 mmol/l Tris-HCl pH 7.5 (20 °C), 0.1 mmol/l EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/mL bovine serum albumin (BSA))
Buffer B (50 mmol/L Tris-HCl pH 7.5 (20 °C), 0.1 mmol/l EGTA, plus inhibitor/activator as required)
Buffer C (as for buffer A, but without BSA)
20% Trichloroacetic acid (TCA)
And the $^{32}$P-labelled phosphorylase $\alpha$ as prepared earlier.

Experimental

1. 0.1 mL of 75 mmol/l caffeine and 0.4 ml of buffer C was added to a vial $[^{32}$P] phosphorylase $\alpha$ to give a 3 mg/ml solution.
2. The protein phosphatase sample was diluted in buffer A.
3. A set of 1.5 ml microcentrifuge tubes were labelled, which included duplicates for each sample and two more for blanks.
4. 10 µl of diluted protein phosphatase was added into each sample tube and 10 µl of buffer A was added into the blank tubes. All tubes were placed on ice.
5. 10 µl of buffer B (containing inhibitor/activator as required) was added to each tube.
6. 10 µl of $[^{32}$P] phosphorylase $\alpha$ was added to a microcentrifuge tube and labelled "total".
7. The sample tubes and blank tubes were removed from the ice and placed in a water bath at 30 °C.
8. The phosphatases assays were started by adding 10 µl of $[^{32}$P] phosphorylase $\alpha$ to the tubes, followed by vortex mixing the contents and incubation at 30 °C for 10 minutes.
9. After 10 minutes the reactions were stopped by adding 100 µl of 20% TCA to each tube and vortex mixing the suspensions.
10. Precipitated proteins were sedimented by centrifuging the tubes at 12,000 g for 2 minutes at room temperature in a microcentrifuge.
11. 100 μl of each clear TCA supernatant was transferred to clean tubes. 1 ml of aqueous-compatible scintillation fluid was added to these tubes and to the 'total' tube from step 6. Scintillation counts were obtained using a $^{32}$P-program in a liquid scintillation counter.

12. Calculation: one unit (U) of protein phosphatase activity releases 1 μmol phosphatase from phosphorylase $a$ per min. in the standard assay. Therefore the calculation for a 10 minute assay is as follows-

\[
c_{.p.m. \text{ released}} = \text{Sample c.p.m.} - \text{Blank c.p.m.} \\
\text{(c.p.m. = counts per minute)}
\]

\[
\text{Activity (mU/ml)} = \frac{c_{.p.m. \text{ released}}}{c_{.p.m." \text{Total}"}} \times \frac{0.3}{10} \times \frac{130}{100}
\]

Note:
- The blank value should be < 1% of the total counts, if the blank value rises above 5% of the total then the batch of substrate in use is discarded and made up fresh.
- 0.3 is the number of nanomoles of phosphorylase in the assay.
- 10 is the incubation time in minutes.
- 100 is to convert the results for 1 ml rather than 10 μL of enzyme, and 130/100 is the fraction of the TCA supernatant that is counted.

**Procedure for PP1 And PP2A Inhibition Studies on DTX-2 and DTX-2B**

**Experimental**

The inhibition of PP1 and PP2A in the standard phosphorylase phosphatase assay was measured when the DSP toxin (Under analysis) was incorporated in step 5. The DSP toxin standards were diluted to a concentration of 100 μg/ml in DMSO. Further dilutions (1:300 to 1:3,000,000) were made with buffer B and were tested for inhibition of 0.2 mU/mL pure PP2A (isolated from cardiac muscle) and 0.2 mU/ml pure PP1γ (human isoform expressed in *Escherichia coli*).

One slight modification made to the standard phosphorylase assay was that after the addition of TCA the acid-soluble fraction was extracted into acid molybdate, which was used to specifically extract the inorganic phosphate.
Appendix No. 6

Cytotoxicity assays for the analysis of DSP toxins

Experimental

1. The KB cell line was established from a human epidermoid carcinoma of the mouth.
2. Cells were maintained at 37°C in 95% air, 5% CO₂ atmosphere in microplates and supplemented with 10% (v/v) fetal calf serum to which 100 IU penicillin/ml and 100 µg streptomycin/ml were added. 50 µl of a 200,000 cell/ml were placed in each well of the microplate.
3. A set of toxin standard solutions were prepared with a geometrical progression of concentrations by dilution (by a factor of 2) of a concentrated solution. 50 µl of each solution was added into 50 µl of the cell suspension in each well. Four replicates were tested for each concentration.
4. The cell cultures in the wells were examined directly under a microscope after 4 hours to find the concentration of toxin which produced the minimal active concentration (MAC) i.e. this is the lowest concentration of toxin needed to produce round features in > 30% of the cells.